The Mangalica Pig is a Novel Biomedical Model for Human Obesity and its Metabolic Complications

by

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Abstract

The Mangalica pig is a genetic model of excessive adiposity; therefore, this European heritage breed may serve as an applied model to improve pork quality in modern commercial lean breeds and as a biomedical model to study obesity-related disease in humans. First growth performance and phenotypic differences were compared between Mangalica breeds and the Yorkshire. Red Mangalica pigs exhibited the most intramuscular fat; moreover, the York x Red Mangalica progeny displayed significantly increased marbling score and lower cook loss compared to the purebred Yorkshire, suggesting Mangalica pigs exhibit superior meat quality and Red Mangalica could serve as a model for studying marbling. Since Mangalica pigs displayed an obese phenotype that was driven by excessive voluntary energy intake, the second study aimed to establish the Mangalica pig as a model for obesity-induced metabolic syndrome. Mangalica pigs spontaneously developed risk factors associated with metabolic syndrome: obesity, hyperglycemia, hyperinsulinemia, dyslipidemia, and low-grade chronic inflammation. Together, these data suggest the Mangalica pig represents a model that enhances biomedical and meat science research.

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List of Abbreviations

AgRP	Agouti-related Protein
αMSH	Alpha Melanocortin Stimulating Hormone
AP	Area Postrema
APOP	Association for Pet Obesity Prevention
ARC	Arcuate Nucleus
BMI	Body Mass Index
BRFSS	Behavioral Risk Factor Surveillance System
BW	Body Weight
CCK	Cholecystokinin
CCL2	Chemokine Ligand 2
C/EBPα	CCAAT/Enhancer-Binding Protein Alpha
C/EBPβ	CCAAT/Enhancer-Binding Protein Beta
CDC	Center for Disease Control and Prevention
CNS	Central Nervous System
CXCL8	Chemokine Ligand 8 (aka Interleukin 8)
CXCL10	Chemokine Ligand 10
DMX	Dorsal Motor Nucleus of the Vagus Nerve
ELISA	Enzyme-Linked Immunosorbent Assay
GIP	Glucose-dependent Insulinotropic Polypeptide
GLP-1	Glucagon-Like Peptide
HDL	High Density Lipoprotein
IL-6	Interleukin 6
IM	Intramuscular
IV	Intravenous
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemoattractant Protein-1
MCP-2	Monocyte Chemoattractant Protein-2
MCP-3	Monocyte Chemoattractant Protein-3
MCP-4	Monocyte Chemoattractant Protein-4
NFκB	Nuclear Factor Kappa B
NIH	National Institute of Health
NPY	Neuropeptide Y
NRC	National Research Council
OGTT	Oral Glucose Tolerance Test
PCR	Polymerase Chain Reaction

POMC	Pro-opiomelanocortin
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PRRS	Porcine Reproduction and Respiratory Syndrome
PYY	Peptide YY
RANTES	Regulated on Activation Normal T cell Expressed and Secreted
SC	Subcutaneous
SCFA	Short Chain Fatty Acids
SNP	Single Nucleotide Polymorphism
TLR	Toll-Like Receptor
TLR-4	Toll-like Receptor 4
TNFα	Tumor Necrosis Factor Alpha
US	United States
USDA	United States Department of Agriculture
WHO	World Health Organization

Introduction

An obesity epidemic has emerged in America during the last 30 years. According to the Centers for Disease Control and Prevention (CDC), approximately 34.9% of today's US adult population are clinically classified as obese, having a body mass index (BMI) equal to or greater than 30 kg/m2 (CDC, 2015). This emerging epidemic is particularly alarming because the accumulation of excess body fat increases the risk of health problems including metabolic syndrome, diabetes mellitus, heart disease, stroke, sleep apnea, osteoarthritis, certain types of cancers, and ultimately reduces life expectancy (CDC, 2015). In 2012, an estimated $190.2 billion was spent treating obesity related illness (Institute of Medicine, 2012). Unfortunately, Alabama is at the epicenter of this obesity epidemic placing a large burden on the state budget and causing an alarming increased incidence of type 2 diabetes and heart failure among Alabamians, many of whom do not have adequate access to healthcare. Despite the magnitude of this problem and great effort by physicians, scientists, and public policy makers to address it, no effective strategies exist for intervention or long-term prevention of obesity in susceptible individuals.

This review and the novel data reported in subsequent chapters will demonstrate that the Mangalica pig is a promising obese porcine breed whose use as a model of overeating-induced obesity and metabolic syndrome may speed discovery of new therapies to combat human obesity and obesity-linked disease. This conclusion will be shown by placing the Mangalica model in context by first focusing upon what is known concerning the development of obesity and obesity-linked disease while highlighting the existing limitations in our understanding that currently prevent the development of

effective therapies. The use of the pig as a biomedical model to address these deficiencies will also be discussed, and finally, the Mangalica pig will be compared to existing porcine models by discussing how their phenotypes allow study of various aspects of the current model of obesity induced disease.

Specifically, this study was conducted to establish the Mangalica pig as a novel, more faithful translational model for obesity-induced metabolic syndrome. These lard-type hogs are genetically inclined to deposit excessive amounts of adipose tissue; therefore, this breed displays great potential to serve as a relevant animal model of obesity induced metabolic syndrome. We hypothesized that if the Mangalica pig is a suitable translational model, then its growth performance would significantly differ from the Yorkshire and it would develop obesity-induced metabolic syndrome based upon exhibiting at least 3 of 6 clinical markers for metabolic syndrome, while feeding a high fat diet would fail to suppress their feed intake. Therefore, the objectives of this study were to 1) determine differences in the growth performance of Mangalica breeds compared to the Yorkshire hog, 2) determine if the Mangalica pig develops obesity-induced metabolic syndrome and 3) characterize the impact of feeding high fat diets to obese Mangalica pigs on their daily feed intake and body composition.

Chapter 1

Literature Review

The worldwide incidence of obesity has risen dramatically during the last three decades. This has become a public health crisis in industrialized countries due to the link between obesity and increased risk for developing metabolic and cardiovascular diseases. Currently, no effective strategies exist to prevent obesity or obesity-associated disease. The lack of therapeutic options to stem the tide of the current obesity epidemic points to a great need for more faithful mammalian research models of obesity to be developed that allow these issues to be better addressed in both humans and their companion animals. The following review will detail what is currently known about the prevalence and causes of obesity, links between obesity and disease, and the potential for pigs to serve as useful translational models of obesity and metabolic disease that allow gaps in our understanding of these issues to be filled.

1.1 The obesity epidemic: a public health crisis without a cure

The formal definition of obesity indicates that obesity is a physical state of excessive adiposity arising when an individual is in a persistent, positive energy balance. The Centers for Disease Control and Prevention (CDC) has tracked the prevalence of obesity since the early 1980's as part of the Behavioral Risk Factor Surveillance System because increased adiposity confers greater risk for developing multiple chronic degenerative diseases (CDC, 2015). As shown in Figure 1.1, the CDC data has revealed an alarming increase in both juvenile and adult obesity during the last 30 years (CDC,

2015). This obesity epidemic has spurred great interest in developing strategies to prevent the accumulation of body fat.

Behavioral modification has been the traditional therapeutic option of preference since intuitively, adiposity can increase through either chronic over-nutrition, a lack of sufficient physical exercise or both. However, formal weight loss studies in humans and the failure of a burgeoning weight loss industry to prevent the obesity epidemic each suggest a similar conclusion. The etiology of obesity is more complex than the formal definition above implies, e.g. that obesity arises from the intuitive, simple act of consuming more calories than are burned (Swinburn et al., 2011). Indeed, multiple factors, many of which may not be a matter of conscious choice, impact energy balance, and thus, influence an individual's body weight. For instance, genome-wide allelic scanning studies suggest most single nucleotide polymorphisms (SNPs) that associate with obesity reside in genes that regulate the drive to eat (Loos, 2012). Such studies suggest the sensation of hunger may be stronger in some individuals than others. Also, it is clear from multiple longitudinal studies in humans that individuals who achieve significant weight loss through dieting have a high rate of recidivism, often ultimately gaining more weight back than was originally lost (Wadden et al., 2004). This implies that physiological mechanisms exist to defend body weight. Such mechanisms would make long-term weight loss a goal that may not be achievable through behavior modification alone. So while overeating can promote obesity, preventing obesity is not as straightforward as counseling afflicted individuals to eat less or exercise more. In fact, the functional cause of obesity is currently subject to great debate in the scientific literature (Qi, 2014; Chen, 2015). Furthermore, while an association between obesity and

metabolic disease is well established epidemiologically, the underlying links between the two are poorly understood mechanistically (Virtue and Vidal-Puig, 2008). Such gaps in our knowledge limit our ability to develop new therapies to prevent obesity and obesity-related disease.

1.1.1 Obesity is clinically defined by body mass index (BMI)

Body Mass Index (BMI) is a practical proxy for degree of adiposity because, being based upon an individual's weight relative to their height, it is very simple to calculate and multiple studies have shown a high correlation between BMI and total body fat in individuals possessing typical body types (NIH, 1998). Epidemiological studies have established the associated risk of developing diseases such as cardiovascular disease, hypertension, diabetes, cancers etc. with increasing BMI (NIH, 1998). Once links between BMI and disease risk became clear, the National Institutes of Health (NIH) advocated that obesity be clinically defined in humans based upon breaks in BMI that are associated with significantly increased risk factors (NIH 1998). This practice is now the widely adopted standard used to screen for obesity and to track its prevalence in adults. In growing juveniles who have yet to reach their mature height, the estimate of obesity is not strictly based upon weight status but rather is determined by considering BMI on a percentile basis (Ogden et al., 2014).

Weight status classification by BMI is summarized in Table 1.1. Based upon this classification system, healthy, or ideal (generally risk-free) in the adult, is defined by having a BMI falling between 18.5 and 24.9. Individuals with a BMI falling between 25.0 and 29.9 are considered overweight and those with a BMI equal to or greater than 30 kg/m2 are clinically considered obese. The associated risk for developing multiple

chronic degenerative diseases jumps substantially for individuals whose BMI surpasses each upper categorical threshold. For instance, epidemiological studies have shown that individuals who are obese have a 50 to 100 percent greater risk of premature death from all causes compared to individuals with a BMI in the normal range of 18.5 to 24.9 (NIH, 1998). Individuals with a BMI greater than 40 are considered candidates for invasive intervention strategies, such as bariatric surgery, since the obesity-associated risk factors are considered greater than the risk associated with the intervention.

This clinical definition, while useful, is not without limitations. The correlation between BMI and body fatness varies by sex, race, and age. For instance, when BMI is held constant, women tend to have more body fat than men while older people tend to have more body fat than younger adults. In addition, highly trained athletes may have a high BMI because of increased muscularity rather than increased body fatness. Therefore, BMI is only used as a screening tool, not a diagnostic tool, to identify possible weight problems for adults. In recognition of these limitations, the National Heart, Lung, and Blood Institute also recommends looking at waist circumference and other risk factors, such as high blood pressure and physical inactivity, for assessing an individual's likelihood of developing overweight or obesity-related diseases (NIH, 1998).

1.1.2 The alarming worldwide increase in the prevalence of obesity

Since 1984, the CDC has tracked the prevalence of obesity as part of its Behavioral Risk Factor Surveillance System (BRFSS). This system has been used to collect prevalence data among adult U.S. residents regarding risk behaviors and preventive health practices that can affect health status (CDC, 2015). These data are collected nationwide via a cross-sectional telephone survey conducted monthly by state

health departments via a standardized questionnaire as directed by the CDC (CDC, 2015). According to the latest data, more than one third (34.9% or 78.6 million) of today's US adult population is obese based upon BMI (CDC, 2015). Approximately 17% (or 12.7 million) of children and adolescents ages 2-19 years are obese (CDC, 2015). This is triple the rate from just one generation ago. According to the latest BRFSS survey from 2013, no state in the US had a prevalence of obesity of less than 20%. Mississippi and West Virginia had a prevalence of obesity of 35% or greater (CDC, 2015). Regionally, the South had the highest prevalence of obesity (30.2%), followed by the Midwest (30.1%), the Northeast (26.5%), and the West (24.9%). The state of Alabama sits at the epicenter of the obesity epidemic in the U.S. with over 34% of its adult population being clinically identified as obese based upon their BMI.

Not only is obesity a significant human health concern but our pets are also weighing in on the matter as well. According to the 2014 National Pet Obesity Awareness Day Survey of practicing veterinarians conducted by the Association for Pet Obesity Prevention (APOP), 53% of US dogs and 58% of US cats are overweight, while 17.6% of dogs are clinically obese (defined as having greater than 30% normal or ideal body weight) and 28.1 % of cats our nation's cats are obese.

1.1.3 The negative consequences of obesity

Obesity is not simply an issue that impacts lifestyle, leads to lower self-esteem, promotes depression and discomfort in social situations, all issues that in and of themselves suggest preventative strategies would be desirable (The Hormone Foundation, 2004). Obesity also increases a person's risk for developing life threatening health conditions such as cardiovascular disease, hypertension and diabetes. These issues are

dramatically impacting both the quality of life and the financial security of those afflicted (NIH, 2004).

1.1.3.1 Obesity associates with risk factors for disease

Importantly, the dramatic increase in obesity during the last several decades has led to an increase in national mortality rates in many industrialized countries (Calle et al, 2003; Reeves et al., 2007; Emerging Risk Factors Collaboration, 2009; Prospective Studies Collaboration, 2009). This increased mortality is largely driven by the association of obesity with chronic degenerative disease. For instance, a substantial body of evidence from prospective studies of generally healthy people has demonstrated that higher BMI is associated with higher incidence of cardiovascular disease (Manson et.al., 1990; Ni Mhurchu et al., 2004; Jee et.al., 2005). Likewise both ischemic and hemorrhagic stroke associate positively with BMI in both Asian and Western populations (Rexrode et al., 1997; Jood et al., 2004; Song et al., 2004; Park et al., 2008). Cancer represents another large category of diseases that link obesity and higher mortality. Being overweight or obese raises one's risk for colon, breast, endometrial, and gallbladder cancers (Whitlock and Huxley, 2014). Perhaps most important is the link between obesity, diabetes and metabolic disease. Diabetes is a leading cause of early death through promoting cardiovascular disease, stroke, kidney disease, and blindness and most people who have type 2 diabetes (non-insulin dependent diabetes mellitus) are overweight (Cederberg and Laakso, 2014). Other obesity-associated diseases include hypertension, dyslipidemia, osteoarthritis, fatty liver disease, Gout, sleep apnea, and fertility complications (CDC, 2015). Increased BMI is estimated to account for 60% of the risk of developing type 2 diabetes, 30-40% of the risk for hypertension and endometrial carcinoma, 20-25% of the

risk for coronary-heart disease and stroke and about 10% of carcinoma of the breast and colon (Wolf, 1998; Wolf and Colditz, 1998). Increased BMI alone has been estimated to account for 2.5 million deaths annually while the combined total for physical inactivity was estimated to be 4.5 million annually (WHO, 2004). These numbers are projected to grow substantially as the incidence of obesity increases worldwide.

Our four-legged companions face some of the same obesity-related health problems as humans do, such as insulin resistance and type 2 diabetes, high blood pressure, heart and respiratory disease, osteoarthritis, cranial cruciate ligament injury, kidney disease, many forms of cancer, and decreased life expectancy of up to 2.5 years (Slimdoggy, 2014; Slimkitty, 2014).

1.1.3.2 Obesity associates with a large societal cost

Given obesity confers greater risk for the development of multiple chronic degenerative diseases, it is not surprising that the increased prevalence of obesity has coincided with significantly increased health care costs. Such rapidly increasing costs represent a significant financial burden on both the individual and the state while placing greater stress on health care delivery systems. For instance, Finkelstein et al. (2003) estimated annual health care spending in the US related to obesity to be $78.5 billion at end of the 1990's. More recently, Finkelstein et al. (2008) showed that the financial burden of obesity leads to a 10% increase in overall medical spending and estimated annual obesity-related healthcare costs alone increased to $147 billion in 2008. Moreover, the medical costs for individuals who are clinically obese were estimated to be $1,429 higher annually than the costs incurred by individuals of clinically ideal weight (CDC, 2015). In 2012, an estimated $190.2 billion was spent nationwide on treating

obesity-related illnesses (CDC, 2015). If US trends based upon historical data for 1988-2008 continue, the prevalence of obesity in US adults will increase from 32% currently, to approximately 50% by 2030, with estimated associated healthcare costs increasing $48-66 billion per year in the US due to expected increases in obesity-induced diabetes, heart disease, stroke, and cancers (Wang et al., 2011). By 2050, 60% of men and 50% of women could be clinically obese worldwide (King, 2011). These increases in caseloads and the associated costs of treatment will greatly stress the health care delivery system, and without the implementation of successful strategies to prevent obesity and control the associated risk factors, health systems are in danger of being overwhelmed (Wang et al., 2011).

The financial burden of obesity is also affecting veterinary medicine. The cost of treating a dog or cat with any one of the obesity-related disorders disorders is obviously very expensive. As illustrated in Table 1.2 and Table 1.3, which depicts estimated treatment costs for obesity-related problems in companion animals, the total treatment costs incurred by the owner over a lifetime of an obese pet could easily exceed $10,000 depending upon the disease and age at diagnosis (Slimdoggy, 2014). According to Pet Plan USA, in 2011 alone, pet insurance claims for diabetes increased by 253%, while claims for heart disease rose 32% and arthritis claims increased 348% (Slimdoggy, 2014; Slimkitty, 2014). Such data likely understates the magnitude of the crisis as pet insurance is largely only purchased by affluent owners. These financial burdens might be expected to be prohibitive to many owners, leaving the afflicted pet without necessary treatment and thus leading to a significantly lower quality of life for the animal due to health concerns. Additionally this would be expected to place a greater burden upon individual

veterinary practices, as it is likely that veterinarians will provide treatment at greatly reduced prices for the sake of an animal's quality of life. Regardless, these concerns in veterinary medicine raise the question, "What does the rising trend in pet obesity tell us about our own health, our environment, and the etiology of obesity?"

1.1.4 The cause of obesity is unclear

The cause of obesity is a subject of great controversy in the scientific literature and it is our poor understanding of the underlying mechanisms that represents the primary limitation to the development of effective therapies (King, 2011; Wang, 2011; Swinburn et al., 2011; Chandaria 2014). The traditional view of how best to combat obesity is derived from a naïve interpretation of energy balance, i.e. weight gain is simply a function of eating more calories than the body burns (Edwards, 2014). Thus, strategies to mitigate weight gain have focused upon limiting caloric intake and primarily involve behavior modification and an emphasis on choice. However, there is little evidence that this view has been effective, at least on the population level. Some clinicians now argue that dieting might exacerbate the problem due to the high degree of recidivism associated with rapid weight loss that often results in individuals gaining back more weight than was lost during the diet (Swinburn et al., 2011; Capehorn, 2014; Chandaria et al., 2014; Edwards, 2014). A study by Sarlio-Lähteenkorva et al. (2000) exemplifies this issue. Of a cohort of 911 subjects who achieved significant weight loss through dieting, only 6% maintained weight loss of at least 5% of their body weight after 6 years. Such recidivism has been consistently observed across weight loss studies and it is expected that nearly 33% of the weight loss achieved by dieting will be regained by the individual within the first year alone following a weight loss intervention (Wadden et al., 2004).

Modern industrialized societies represent "obesogenic" environments characterized by a rapidly decreasing need for physical exertion that is driven by technological advances. This trend is coupled with an overabundance of cheap, nutrient-dense, easily obtainable food that is often sold to the masses with sophisticated marketing strategies meant to drive consumption. The emerging view of the etiology of obesity suggests that the current weight gain such societies are experiencing is the result of a complex interaction between genetics and the obesogenic environment. This view maintains that within an obesogenic environment, our properly working biology in and of itself will lead to weight gain. Meanwhile genetically susceptible individuals are much more prone to develop severe obesity and subsequent metabolic consequences. However, little is known about the functional defects in genetically susceptible individuals that cause them to be more susceptible to an environment of largess.

Given this paradigm, a philosophical argument has emerged concerning the best way to mitigate the obesity epidemic with one position maintaining the most effective course of action is to regulate or modify the obesogenic environment itself while the other position argues a better understanding of the biology of energy balance will reveal therapeutic targets that can be leveraged as strategies to prevent obesity (Swinburn et al. 2011; King, 2011; Chandaria, 2014; Chen, 2015). Each position has compelling merit and it seems likely that to effectively address the obesity epidemic some combination of both approaches will be useful. However, state-led efforts to regulate the obesogenic environment through regulation of the food industry have met with significant political resistance (Swinburn et. al. 2011). Some advancement is being made through federal initiatives that attempt to modify the obesogenic environment by funding education

programs to be incorporated into schools, hospitals, and worksites to increase obesity awareness in the hopes of altering demand for energy-dense foods that promote unhealthy weight gain. Political resistance notwithstanding, our current lack of understanding of the physiological basis underlying the etiology of obesity makes any approach to preventing obesity less likely to be effective.

1.1.4.1 Obesity is a disease of excessive adipose tissue development

Adipose tissue was once thought of as a metabolically passive form of connective tissue that functioned mainly to cushion organs and insulate the body, its mention in biochemistry texts almost an afterthought. This view was further propagated by the ubiquitous distribution of adipose tissue throughout the body in anatomical locations termed "depots" which discouraged the impression of adipose tissue as a discreet entity. However, the obesity epidemic motivated newfound interest in adipose tissue research and this has spawned the realization that adipose is a dynamic tissue that profoundly regulates energy balance, reproduction, and developmental processes through the hormones, termed adipokines, and metabolites it secretes. Now the dispersed depots of adipose tissue are collectively referred to as the "adipose organ" in recognition of their coordinated function (Cinti, 2012; Smorlesi et al., 2012; Giordano et al., 2014). While the studies reported in subsequent chapters did not examine mechanisms of adipose tissue growth per se, adipose tissue development, metabolism, and endocrine activity are central to the development of obesity and an overview of these subjects is below. The role that adipose tissue dysfunction plays in obesity and metabolic disease will then be treated in greater depth in subsequent sections of this review where appropriate.

1.1.4.2 Overview of adipose tissue

Adipose tissue is an active organ that plays a pivotal role in metabolic, physiological and endocrine homeostasis. This highly specialized form of connective tissue of mesenchymal origin is comprised of multiple cell types held loosely together in a collagen matrix (Ailhaud et al., 1992*)*. The major cell type is the terminally-differentiated adipocyte, with a smaller number of proliferation-competent preadipocytes also residing in the tissue of adults. The latter are fibroblast-like, unipotent cells that are 'committed' to the adipocyte lineage. The stromal component of adipose tissue consists of endothelial cells, pericytes, mast cells, fibroblasts, leukocytes such as activated macrophages, and hematopoietic cells (Rink et al., 1996).

Adipose tissue development is first appreciable in the fetus during mid to late gestation. A second peak of accelerated fat expansion occurs just before puberty. Adult adipocyte number, and therefore fat mass, is generally determined during fetal development though it has been hypothesized that a chronic positive energy balance can stimulate further rounds of preadipocyte recruitment and proliferation in adults (Budge et al. 2009). In a newborn human, fat comprises 16-20% of total body weight while the porcine neonate is born with only about 1% body fat (McGlone and Pond, 2003; Williams and Fruhbeck, 2009).

The adipose organ is comprised of two distinct types characterized as brown and white adipose tissue. Brown adipose tissue is primarily thermogenic and functions to increase heat production through metabolizing fatty acids in a process that uncouples oxidative phosphorylation in the mitochondria so that energy is released as heat rather than used to generate ATP. Brown adipose tissue is most abundant in neonates and

progressively disappears with age so it does not appear to play a major role in the development of obesity or diabetes in humans.

Increases in adiposity, therefore, are the result of expanded white adipose tissue mass. Energy is stored in white fat cells as triglycerides, making white adipose tissue the largest energy reserve in the body. White adipose tissue also functions as an endocrine organ through the release of adipokines which contribute to the regulation of muscle and liver metabolism and the control of food intake by the brain. Therefore, factors that alter the storage or mobilization of triglycerides in white adipocytes and/or influence adipokine release should have a significant impact on metabolic homeostasis in health and disease.

1.1.4.1.2 Cellular development of adipose tissue.

Obesity results from increased white adipocyte cell size (hypertrophy) and number (hyperplasia). Fat cell number is determined by adipogenesis, the process which generates mature adipocytes from preadipocytes (reviewed by Ailhaud, 1997; Gregoire, 2001). The cells destined to become mature adipocytes first specify to the preadipocyte lineage from an as yet uncharacterized cell type that resides within the mesenchyme and is thus simply identified as a mesenchymal stem cell. Preadipocytes then proliferate and differentiate into mature adipocytes.

Adipogenesis is regulated by a nuclear cascade of adipogenic transcription factors that has been largely characterized using murine cell culture models. In this cascade, CCAAT/enhancer-binding protein (C/EBP) β is first expressed which triggers the expression of peroxisome proliferator-activated receptor gamma (PPARγ). Then, PPARγ turns on the expression of CEBPα, and together, these two transcription factors

upregulate enzymes, such as fatty acid synthase, lipoprotein lipase, and steroyl-CoA desatuase, which are necessary for lipid metabolism and the adipocyte phenotype (Yan et al. 2013). Mature adipocytes carry out the bulk of adipose tissue functions, given their ability to metabolize glucose, store and release free fatty acids, and secrete adipokines. However, other cell types, especially macrophages, contribute to the overall ability of adipose tissue to regulate energy homeostasis by releasing cytokines such as tumor necrosis factor α (TNFα) and interleukin 6 (IL-6).

The mature adipocyte is unilocular, with about 90% of the cell volume occupied by the lipid droplet. Fat cells have a thin cytoplasm and a flattened, semilunar nucleus. The central lipid droplet mostly contains triglycerides, but small amounts of diacylglycerols, free fatty acids, phospholipids, and cholesterol may also be present (Williams and Fruhbeck, 2009). Individual adipocytes can range from 20 to 200 μm in diameter and can increase their volume by a thousand-fold in order to accommodate lipid storage during times of excess energy (Williams and Fruhbeck, 2009). When existing adipocytes reach their maximum lipid capacity, new adipocytes are recruited from the precursor pool and begin to uptake lipids (Budge et al. 2009). The extent to which adipocytes reach their maximum size depends upon the balance between lipogenesis (lipid filling due to the formation of triglycerides) and lipolysis (the breakdown of triglycerides into glycerol and free fatty acids). These processes are highly regulated by hormones such as insulin, catecholamines, cytokines and growth hormone. Dysregulation of the lipogenic and lipolytic pathways contributes to the development of insulin resistance and vascular dysfunctions (Hausman et al., 2009). Discussion of the exquisite endocrine regulation of lipid metabolism is beyond the scope of this literature review and

the reader is referred to several excellent reviews which cover these issues in greater detail (Hausman et al., 2009; Fruhbeck et al., 2014; Nielsen et al., 2014).

1.1.4.1.3 Adipose tissue is an important endocrine organ.

Adipose tissue is the largest endocrine organ in the body and secretes over 600 adipokines (Bluher and Mantzoros, 2015). Adipokines are major players in the control of energy balance, insulin resistance, and inflammatory responses, with leptin and adiponectin being two that are commonly measured in the literature in relation to obesity studies. Adipose tissue also secretes many proinflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6). Leptin plays an important role in regulating satiety, appetite, and food intake; it influences reproductive performance and puberty, and may also play a role in obesity mediated inflammation (Bluher and Mantzoros 2015). Adiponectin enhances insulin sensitivity in the liver and skeletal muscle thus potentiating the ability of insulin to regulate glucose homeostasis and fatty acid oxidation (Bai and Sun 2015; Bluher and Mantzoros, 2015). Furthermore, adiponectin acts on the brain to increase energy expenditure (Bluher and Mantzoros, 2015). Adiponectin is easily detectable in blood, and decreased circulating levels negatively correlate with multiple metabolic disorders including obesity, type 2 diabetes, coronary artery disease, and heart failure (Bluher and Mantzoros 2015). Expression of pro-inflammatory cytokines TNFα and IL-6, have been associated with hyperinsulinemia, and systemic inflammation (Bai and Sun 2015). The role of adipose tissue-derived cytokines in mediating inflammation during obesity will be discussed in greater detail in later sections.

1.1.4.1.4 Adipose tissue depots and metabolic syndrome

White adipose tissue in humans develops in discreet anatomical depots which differ in morphology and function and are categorized as abdominal visceral and subcutaneous fat (Wajchenberg et al., 2002). The relative distribution of these depots determines body shape. There is a well-recognized sexual dimorphism in the regional distribution of adipose tissue, with visceral fat representing about 6% of total body fat in women but 20% in men, reflecting the greater propensity of men to accumulate excess abdominal fat (Wajchenberg et al., 2002). The cellular composition of the different depots varies, with visceral fat having more macrophages and fewer preadipocytes than subcutaneous fat, whereas subcutaneous adipocytes in obese subjects are larger than their visceral counterparts (Lafontan et al., 2003).

Increased abdominal visceral fat is a critical factor in the development and manifestation of the metabolic syndrome, i.e., glucose intolerance, hyperinsulinemia, hypertriglyceremia, altered circulating lipoprotein levels and hypertension (Arner, 2001; Lafontan et al., 2003). Visceral fat has lower responsiveness to insulin than subcutaneous fat, making it more lipolytically active (Wajchenberg et al., 2002; Lafontan et al., 2003). Since the output of visceral fat drains into the hepatic portal blood, an increased influx of free fatty acids leads to altered hepatic metabolism. Chronic elevation of free fatty acids impairs glucose metabolism and insulin sensitivity in both liver and muscle and reduces pancreatic β cell function (Lewis et al., 2002). There are also significant differences between the depots in the secretion of adipokines that are associated with insulin resistance, with visceral fat secreting more IL-6 but less leptin and adiponectin than

subcutaneous fat (Bouloumie et al., 2005). Proinflammatory adipokines secreted from visceral fat act directly on the liver to oppose the action of insulin.

1.1.4.2 Obesity as a disease of energy imbalance

That energy balance is tightly regulated in humans is illustrated by epidemiological data that has characterized average weight gain for populations in Europe and the US (Lewis, et al 2000; Resnicka, et al., 2000). Such studies indicate that the average change in a person's body weight over a decade is less than 10 kg (22 lbs) with the median weight change being closer to only 5 kg. Viewing this in terms of calories illustrates just how tightly energy balance is controlled. For instance, 1 lb of fat is equivalent to 3500 calories, so 22 lbs of fat represents 77,000 calories. If we assume an adult requires 2500 calories/day to achieve a neutral energy balance (e.g. satisfy maintenance requirements without weight gain), that adult would consume 9,125,000 calories over the course of a decade (365*2500*10). Thus a weight gain of 22 lbs of fat represents a mere .84% of the total calories consumed by that individual (77,000/9125000= .0084 or .84%). In other words, someone who gained 22 lbs over a decade literally had an energy balance that was regulated within 99.2% of maintenance. To illustrate this in more practical terms, an excess of 77,000 calories/decade translates into 20.5 calories a day, which is literally equal to the consumption of just 1-2 potato chips per day. Given this math, it is not surprising that weight mitigation strategies that depend solely upon behavior modification/choice have proven ineffective to prevent the rising prevalence of obesity.

If epidemiological data implies that energy balance is in fact tightly regulated, why then do people become obese? Could defects in the systems regulating energy

balance explain this apparent paradox?

1.1.4.2.1 Functional definition of obesity based upon energy balance

The formal definition of obesity states that obesity is a physical state of excessive adiposity arising when an individual is in a persistent, positive energy balance. However, application of this definition has not allowed obesity to be effectively prevented. The clinical definition of obesity relates the degree of adiposity to risk factors based upon BMI in a fashion that allows the screening of obesity and its prevalence to be tracked. A third definition that more easily allows mechanistic scientific inquiry might be coined "the working" definition and is stated:

Weight gain = Energy intake – Energy expenditure

This working definition of obesity implies weight gain is the difference between two distinct terms-energy intake and energy expenditure-such that weight gain occurs when energy intake is greater than energy expenditure. While the implied relationship is intuitive, this is a useful approach. It has become clear that energy intake and expenditure are functions of multiple underlying components and that distinct homeostatic mechanisms exist to coordinate these physiological circuits within a regulatory network spanning the gut, pancreas, adipose tissue, and the brain in a way that defends or maintains body weight within relatively narrow ranges (Chandaria, 2014).

Broadly, energy intake is a function of two separate behaviors that are controlled by distinct feedback mechanisms, e.g. how often one eats per day and how long one eats per meal. On the other hand, energy expenditure is a multifactorial term being influenced by many factors both inside and outside of the body such as an animal's resting energy

expenditure, level of physical activity, the animal's physiological state (growth, gestation, and lactation etc), and types of nutrients consumed, the gut microbiome, and even the environment. Therefore obesity is a disease driven by factors that either increase energy intake, decrease energy expenditure or both and there are many physiological targets that could affect such changes.

1.1.4.2.2 Energy intake: eating behavior in monogastric mammals.

Humans and pigs are omnivores. Unlike herbivores or carnivores, whose food sources are usually quite restricted, omnivores have extended opportunities to eat a huge variety of materials. Additionally, omnivores characteristically display episodic patterns of eating such that regular bouts of eating have a discreet size and duration with feeding behavior stopping at the feeling of fullness (satiation) and starting again at the sensation of hunger (de Graaf, et al., 2004). The pattern of eating behavior in humans and pigs is heavily influenced by culture or management practices (in swine) but the episodic nature of eating is conserved across cultures and between these species (Bellisle and Blundell, 2013). Eating behavior has been extensively characterized for the pig with the frequency of meals ranging from 9 to 10 meals per day just after weaning, to three meals daily in the adult pig (Auffray and Marcilloux, 1980; Houpt 1984). Pigs are aroused to eat when other pigs nearby are eating, suggesting social or visual cues play important roles in eating behavior as well as intrinsic factors (Hansen et al., 1982). Feed consumption is diurnal (less than one-third of feeding activity occurs during the night) and this diurnal pattern becomes more pronounced in heavier pigs which primarily exhibit two main consumption peaks-one in the morning and one in the late afternoon (Quiniou et al.,

1999; Quiniou et al., 2000; Collin et al., 2001; Renaudeau et al., 2002). Thus in pigs, voluntary feed intake increases in a curvilinear relationship with body weight.

Interestingly, while humans are omnivores that are prone to becoming obese, pigs appear to be resistant to the development of obesity as pigs are well known to "regulate their energy". Classic studies have demonstrated that pigs will increase their energy intake as the caloric value of their diet falls apparently responding to the caloric content rather than to the bulk of the diet (Owen and Ridgman, 1968; Houpt et al., 1979). Likewise, an increase in energy density of the ration is associated with a reduction in voluntary feed intake, though the overall effect often is that total energy intake is increased in such circumstances (NRC, 2012; Noblet and Van Milgen, 2013). It is now generally accepted that pigs eat to their energy requirements and that pigs vary intake to match nutrient need. While high energy rations will promote weight gain, the corresponding depression in feed intake limits this impact. It is important to note that there currently are no established porcine models of hyperphagia.

1.1.4.2.2.1 Eating behavior is controlled by both chronic and acute mechanisms.

Daily feed intake is the sum of each individual meal and is determined by the number and duration of feeding bouts. The size of each meal is determined by a rapidly-acting, negative feedback control initiated by the presence of food in the alimentary canal (Houpt, 1984). This negative feedback is initiated well before complete absorption of nutrients. Representing the acute regulation of energy intake, this is primarily orchestrated by feedback signals originating from the gut. Meanwhile, the number of feeding bouts is controlled by hormonal signals from peripheral organs acting on nuclei within the hypothalamus and these circuits represent the chronic regulation of energy

intake (Ellacott and Cone, 2004; Heisler *et al.*, 2006). Presumably in pigs, it is these chronic circuits that primarily dictate their ability to tightly coordinate energy intake with energy needs.

1.1.4.2.2.2 Chronic regulation: neurons in the brain and hormones from peripheral tissues determine the number of eating bouts.

The arcuate nucleus (ARC) of the hypothalamus is located at the base of the third ventricle immediately above the median eminence and is often described as the "feeding center of the brain," as it contains two neuronal populations crucial for controlling food intake and regulating body weight (Berthoud, 2002; Hillebrand et al., 2002; Cowley *et al.*, 2003; Ellacott and Cone, 2004; Elmquist *et al.*, 2005; Cota *et al.*, 2006; Heisler *et al.*, 2006). Orexigenic neurons express the neuropeptides, neuropeptide Y (NPY) and Agouti-related protein (AgRP), which stimulate feeding when expressed. The opposing anorexigenic neurons express neuropeptides such as pro-opiomelanocortin (POMC)-derived α-MSH that signal satiety by inhibiting feeding behavior. The specialized blood-brain barrier of the ARC allows circulating hormones such as insulin, adipose tissue-derived leptin, and gut-derived ghrelin to serve as peripheral signals, which reciprocally regulate the NPY/AgRP and α-MSH neurons (Arnold *et al.*, 2006).

Leptin and ghrelin are two hormones that play an important role in regulation of energy intake and body weight through controlling the timing of eating or the chronic component of energy intake. Leptin is released by adipose tissue, with circulating leptin levels increasing with increasing adiposity. Thus, leptin serves as a putative chronic indicator of energy balance by signaling status of the body's energy stores to the brain. Leptin influences the activity of neurons in the hypothalamus to effectively lower energy

intake. By activating the POMC neuron, leptin acts as a foot on the brake pedal to signal satiety to the brain (Cowley *et al.*, 2003; Ellacott and Cone, 2004; Elmquist *et al.*, 2005). Ghrelin on the other hand, represents a more responsive chronic regulator of satiety. When the gut is empty, it shrinks and enteroendocrine cells of the intestinal mucosa secrete ghrelin into the circulation. Ghrelin then stimulates hunger by acting on receptors in the arcuate nucleus thus activating NPY and AgRP and suppressing POMC neurons (Cota *et al.*, 2006; Heisler *et al.*, 2006). The overall effect is to motivate the animal to seek food. Thus leptin represents a long term chronic signal that limits energy intake in response to chronic positive energy balance by relaying information about energy reserves in the body, while ghrelin is a short term chronic signal relaying information about immediate availability of energy.

1.1.4.2.2.3 Acute regulation: Gut-derived hormones regulate length of eating bout.

There are a number of gut hormones that convey a "full" or "hungry" signal to the brain. In mammals, cholecystokinin (CCK) is secreted by enteroendocrine I cells. When released from the intestinal mucosa during a meal, CCK acts as a powerful negative feedback signal by acting on the central nervous system (CNS) via the blood stream, whereby it activates vagal nervous input into the brain stem (Klok et al., 2007). This is also conserved in the pig as CCK rises in the blood of pigs after feeding to levels double or greater than the fasted pig (Go et al., 1971; Englert, 1973, Houpt, 1984). Meanwhile, injection of either porcine CCK or the synthetic octapeptide (CCK-8) at levels approximating postprandial levels, significantly depress meal size (Anika et al., 1981). Other molecules such as glucagon-like peptide 1 (GLP-1) secreted by enteroendocrine L cells, peptide YY (PYY) secreted by enteroendocrine L cells, and glucose-dependent

insulinotropic polypeptide (GIP) secreted by enteroendocrine K cells represent additional gut-derived peptide hormones that also signal satiety. These signals are released during or immediately after a meal from the gut mucosa where they travel through the circulation to act on receptors expressed by neurons residing in the area postrema (AP) of the caudal brain stem. These neurons then project onto the dorsal motor nucleus of the vagus nerve (DMX), which then transmits the satiety signal to the hypothalamus via projections onto nuclei within the arcuate nucleus.

1.1.4.2.2.4 Ambient temperature alters eating behavior to allow homeotherms to defend body temperature.

Environmental temperature can also control energy intake and energy expenditure. Cold temperatures stimulate food intake and energy is used for heat production to help defend body temperature. Hot temperatures tend to decrease food intake so that less heat is produced. Rodent studies have revealed a general pathway for the homeothermic coordination of ambient temperature and feeding behavior. In this paradigm, peripheral temperature-sensing neurons of the somatosensory system match energy intake and expenditure to body temperature through their modulatory actions on the feeding center of the brain. Neuronal bodies of peripheral temperature sensing neurons congregate in the dorsal root ganglia and allow the transmission of signals from peripheral tissues such as the skin through the spinal cord to the hindbrain and ultimately the hypothalamus. Thus information concerning ambient temperature can be relayed directly to regions of the brain that both regulate length of feeding bout (e.g. the nucleus tractus solitarri of the caudal brainstem) and the number of feeding bouts (e.g. arcuate nucleus of the hypothalamus). Since neuropeptides within the ARC that

regulate energy balance often have reciprocal actions on feeding behavior and metabolic rate, such that a factor that signals hunger will also lower basal metabolic rate, it is possible that afferent signaling during chronic exposure to high ambient temperature may uncouple the dual action of the ARC on feeding and energy expenditure such that elevation of an orexigenic gene may only serve to lower metabolic rate under conditions of heat stress (Boston et al., 1997; Gao and Horvath, 2007; Abizaid and Horvath, 2008).

1.1.4.2.3 Energy expenditure.

Energy expenditure is dictated by exercise and resting energy expenditure, which includes basal metabolic rate, diet induced thermogenesis, and non-exercise associated thermogenesis. Diet induced thermogenesis describes the energy generated by muscle contraction from the gastrointestinal tract upon digestion and absorption of a meal; in other words, it is the energetic tax associated with eating. Non-exercise associated thermogenesis, generated through involuntary muscle shivering, describes energy used to generate body heat to defend body temperature. The primary nutrients available to the pig for energy are glucose, fatty acids and amino acids. These can be used in varying degrees to support oxidative metabolism and the physiological functions of tissues and organs. The amount of energy used to support these functions defines the basal metabolic rate, or the maintenance energy requirement needed to maintain stable body weight. Fasting heat production represents the greatest portion of maintenance energy.

1.1.4.2.3.1 The biological basis for maintenance energy requirements.

The maintenance energy requirement varies depending upon environmental temperature, body composition, level of nutrition, and activity level of the individual. Some of the major cellular and physiological functions that contribute to overall energy

needs include maintenance of cellular Na^+, K^+, and Ca^{2+} ion gradients, protein synthesis and degradation, muscle contraction, protein phosphorylation, substrate cycles, and membrane phospholipid turnover (Burrin, 2001). Variations in basal metabolic rate associated with the level of nutrition, physiological state, age, and species appear due in part to changes in the splanchnic organ size relative to body weight (Koong et al., 1985). Estimates of energy expenditure are generally made based upon measurements of O_2 consumption. The estimated relative contributions of various tissues to whole body O_2 consumption in animals and humans are 18-20% for brain, 8-10% for kidney, 10-12% for heart, 18-22% for muscle, and 35-53% for splanchnic organs (Smith and Baldwin, 1974; Yen et al., 1991; Ebner et al.,1994; Ten Have et al., 1996).

1.1.4.3 Obesity as an inflammatory disease

Prolonged or excessive obesity promotes a state of systemic low-grade chronic inflammation, which is thought to play a major role in the development of obesity-associated metabolic diseases (Huh et al. 2014). For instance, excessive adiposity is correlated with marked increases in circulating inflammatory signals (TNFα, IL-6, C-reactive protein) the elevation of which contributes to the associated insulin resistance (Zeyda and Stulnig, 2009). While inflammatory changes in obesity have been recognized for many years, the primary casual mechanisms by which obesity results in activation of immune pathways are not yet fully understood. However, it is commonly accepted that obesity-induced inflammation is driven by activation of innate immunity involving both the systemic activation and adipose-specific activation.

1.1.4.3.1 Obesity and innate immunity

Innate immunity is a receptor-driven, non-specific, short term, immediate defense against infection. In this system, members of the toll-like receptor (TLR) superfamily recognize various bacterial moieties triggering activation of the nuclear factor kappa B (NFκB) signaling pathway ultimately resulting in the production and release of proinflammatory cytokines such as TNFα and IL6 from the activated cell. This results in the recruitment of immune cells, such as macrophages, to the site of infection which phagocytize microbes and serve as a powerful source of additional proinflammatory cytokines that further recruit other cells that participate in both innate and adaptive immunity (i.e. additional macrophages or lymphocytes).

Currently it is thought that it is changes in both the intestinal microbiota and the endocrine function of growing adipose tissue give rise to the stimuli that triggers innate immunity in obese individuals. These models are described below.

1.1.4.3.2 Systemic activation of inflammatory pathways in obesity.

The systemic activation of inflammatory pathways during obesity is believed to be mediated by TLR4 across multiple tissues and organ systems. Morbid obesity is commonly associated with a state of metabolic endotoxemia whereby a high fat diet promotes changes in the intestinal microbiota to a proinflammatory, gram-negative profile. Such changes ultimately increase translocation of bacterial lipopolysaccharride (LPS) from the intestinal lumen to the circulation as intestinal LPS can be absorbed by chylomicrons (lipoprotein particles that transport dietary lipids) via the same mechanism that dietary fats are absorbed (Cani et al., 2007). Once in the circulation, LPS can interact with TL4R expressed on any target cell type leading to the systemic activation of

TLR4-receptor mediated innate immunity. In support of this model, Shah et al., (2009) characterized adipose tissue mRNA changes before and after endotoxemia and revealed marked upregulation of adipose tissue inflammatory genes, including many related to macrophage activation, recruitment, and retention. Consistent with such findings, obesity-induced endotoxemia was shown to activate innate immunity in organs of the hypothalamic-pituitary-gondal axis and modulated adipose tissue inflammation and insulin signaling pathways (Mehta et al., 2010).

1.1.4.3.3 Adipose-specific activation of immune pathways.

A key phenomenon causing elevated adipose tissue inflammation is macrophage infiltration, as most of the proinflammatory cytokines produced by adipose tissue come from resident macrophages (Gabler and Spurlock, 2008; Bai and Sun, 2015). Recent studies indicate that adipocytes themselves can induce macrophage recruitment as these cells in humans, rodents, and pigs express the TLR4 and possess a functional innate immunity pathway that can be stimulated by exposure to inflammatory signals (Ajuwon et al., 2004 a,b). Treatment with LPS initiates the secretion of TNFα and IL6 from adipose tissue within 1-2 hours of exposure *in vitro* and importantly, these secreted factors affect adipocyte metabolism indicating that they are bioactive (Ajuwon et al., 2004 a,b). Inflamed adipocytes also secrete chemokines that attract monocytes. Finally, as adipocytes become enlarged, free fatty acids begin to leak out into circulation and these also can also activate TLR4. Thus, adipose-derived cytokines, chemokines, and metabolites can serve as powerful signals stimulating macrophage invasion into adipose tissue.

Once attracted to adipose tissue, invading macrophages can then release monocyte-derived TNFα and IL-6 which amplifies the inflammatory signal. These feedback and further activate TL4R expressed on resident macrophages causing even greater release of pro-inflammatory cytokines from adipose tissue which leads to the recruitment of even more monocytes from circulation to the site of inflammation (Bai and Sun, 2015). Since macrophage accumulation is directly proportional to adiposity in an animal, this crosstalk between adipocytes and macrophages appears responsible for the chronic inflammatory state of adipose tissue in obese individuals (Weisberg et al., 2003).

1.1.4.3.4 Activation of macrophages/macrophage infiltration

Upon sensing infiltration signals such as free fatty acids, proinflammatory cytokines, and chemokines, macrophages polarize from the M2 (anti-inflammatory) to the M1 (pro-inflammatory) phenotype. Consistent with this, M1 macrophages are increased in models of high-fat induced obesity and insulin resistance (Lumeng et al., 2007; Fujisaka et al., 2009). Thus, an obese state is associated with not only greatly increased numbers of macrophages within adipose tissue but also an increased ratio of M1 to M2 macrophages (Dalmas et. al., 2011). This is an important switch as M2 macrophages protect against insulin resistance while M1 macrophages confer sensitivity to TNFα-induced insulin resistance (Dalmas et. al., 2011).

Currently the thiaziolidienes, a class of insulin sensitizing drugs, and the statins, a class of cholesterol lowering drugs, are being investigated as therapeutic agents that might potentially block the activation of adipose tissue infiltrating macrophages (Kolak et al., 2007, Shah and Reilly, 2011). While the mechanism underlying macrophage

activation is beyond the scope of this review, it's important to point out that this step represents a potentially important therapeutic target.

1.1.4.3.5 Adipose tissue inflammation impairs insulin signaling.

Proinflammatory cytokines are well known to induce catabolic effects on cellular metabolism resulting in the breakdown of lipids, proteins and glycogen while reducing glucose uptake in insulin sensitive tissues such as the liver, adipose and skeletal muscle. Such effects are in direct opposition to the anabolic action of insulin which generally induces glucose uptake in peripheral tissues, lipid storage in adipose and glycogen and protein synthesis in skeletal muscle. Thus, adipose tissue inflammation, through the metabolic action of cytokines, induces insulin resistance by opposing the biological action of insulin on target tissues. Cytokines orchestrate these shifts in metabolism by directly effecting biochemical pathways that promote catabolism and also via crosstalking with insulin signaling pathways in ways that impair multiple steps between the insulin receptor and its downstream signaling molecules.

1.1.4.3.6 The Adipose tissue inflammatory secretome.

As stated above**,** cytokines and chemokines produced by adipocytes, macrophages and stromal cells in adipose tissue leads to exacerbation of the adipose tissue inflammation during obesity. This suggests that changes in the endocrine function of adipose tissue drive the inflammatory state that develops in this tissue. Thus, there has been great interest in characterizing changes in the secretory products of adipose tissue in lean and obese animals. Consistent with a proinflammatory shift in endocrine output, increased adipose tissue secretion of TNF and IL-6 is well documented in obese

individuals in all mammalian species studied, including the pig (Gabler and Spurlock, 2008; Carroll, 2008).

Chemokines, a class of hormones that function as signals which attract monocytes, are also critical to the recruitment of monocytes and macrophages to adipose tissue. For instance, adipocyte-derived MCP-1 (CCL2) is a major contributor of macrophage recruitment and adipose tissue remodeling (Dahlman et al., 2005). In mice, MCP-1 deficiency leads to greater insulin sensitivity and fewer adipose tissue resident macrophages whereas knocking out the gene protected against high fat-induced inflammation and overexpressing MCP-1 resulted in the opposite action (Weisberg et al., 2006). Circulating MCP-1 is elevated in obesity in humans and rodents. Other chemokines known to attract macrophages are upregulated in the adipose tissue of obese rodents and humans include MCP-2, -3, and -4, RANTES, CXCL8 (IL-8) and CXCL 10 (Straczkowski et al., 2002; Wu et al., 2007; Sell and Eckel, 2009).

1.1.4.4 The Microbiome's influence on energy balance and inflammation

All higher organisms carry an intestinal microbiota which serves a number of important physiological functions critical to the health and development of the host. These functions include the extraction of nutrients-especially volatile fatty acids-from foodstuffs, the recycling of bile salts, production of vitamin K, and the enhanced digestion of otherwise indigestible substances such as cellulose. In addition, the microbiota also function as an intestinal immune shield, or "biological buffer," due to an ability to out compete invading pathogenic microorganisms for colonization of the intestinal ecosystem of their host (Flint et al., 2012; Nicholson, 2012; Kim and Isaacson 2015). The intestinal microbiota also plays an important role in maintaining immune

homeostasis beyond a niche-buffering capacity in higher mammals, such as humans and pigs, because gut microbes can directly stimulate innate immunity pathways in the host (Flint et al., 2012; Nicholson, 2012). Thus, intestinal microbiota may impact both the energy balance and inflammatory status of the host, two critical components that influence the etiology of obesity and obesity-linked metabolic diseases.

The literature indicates the gut microbiome influences obesity due to the ability of intestinal microbes to alter 1) the efficiency of energy extraction during digestion, 2) the secretion of gut-derived hormones that regulate appetite, and 3) the systemic inflammatory status within the host. This symbiosis between gut microbes and host is the result of the complex and dynamic three-way interaction between the host, the microbiome, and the diet. For instance, dietary components are processed in the gut by mammalian digestive processes and microbial action. The types of metabolic products generated are dependent upon host genetics, host physiology, and the composition of the gut microbiome. This unique profile of metabolic products and bacterial constituents enter the host circulation, thus impacting host metabolism and immune function by altering metabolism or cellular behavior of target tissues. This complex system is not unidirectional. For instance, modification in the diet could alter the gut microbiota, while alterations in the gut microbiota could in turn alter how efficiently the diet is utilized by the host. Having a novel porcine model that allows these factors to be studied, especially in relationship to the etiology of obesity and its spontaneous metabolic complications, should greatly increase our understanding of these complex interactions thereby potentially enhancing the development of more effective therapies to prevent obesity and metabolic disease.

There appears to be a characteristic composition of the gut microbiota in humans and pigs that, if maintained, will promote optimal health and metabolic homeostasis. The human gastrointestinal tract on average consists of approximately $1x10^{14}$ bacteria (Parekh et al., 2014). The majority (64%) of the intestinal bacteria belong to the phyla Firmicutes, followed by Bacteroidetes (23%), Probacteria (8%), and Actinobacteria (3%), with the remaining 2% comprised from Fusobacteria, Verrucomicrobia, and TM7 based upon the taxonomic classification of intestinal microorganisms using 16S rRNA pyrosequencing to identify specific types (Cardinelli et al., 2015). The intestinal microbiota of pubertal pigs (5-6 months of age) appears to be very similar to that of humans (Kim et al., 2011; Kim and Isaacson 2015). External factors such as genetics, dietary modifications, and metabolic surgery all can influence the gut microbiota and its effect on body weight (Cardinelli et al., 2015). Importantly, the pig appears to represent a faithful model for the human gut microbiota allowing study of the influence of these parameters on the microbiome and host health status.

The gut microbiota appears to represent an important aspect in the etiology of obesity and its complications. Several studies have demonstrated that the composition of the gut microbiota in obese individuals is indeed distinct from that of lean individuals (Backhed et al., 2004; Backhead et al., 2005; Backhead et al., 2007; Turnbaugh et al., 2010). The classification of intestinal microorganisms using 16S rRNA pyrosequencing has consistently shown that both obese animals and humans exhibit higher Firmicutes and lower Bacteroidetes populations than their normal weight counterparts (Tai and Wen, 2015). Furthermore, the obese gut microbiota is less diverse than that of lean animals (Remely et al., 2015). Finally, transplantation studies have demonstrated that implanting

microbiota from healthy donors into hosts exhibiting metabolic syndrome can improve insulin sensitivity in the recipient (Turnbaugh et al., 2009; Sanz et al., 2010; Kootte et al., 2012; Liou et al., 2013; Ridaura et al., 2013).

So how do gut microbes regulate energy balance and inflammation in the host? Generally it is thought that the gut microbiota enhances short chain fatty acid (SCFA) absorption and signaling and serves as a significant source of bacterial lipopolysaccharide (LPS). Differences in the microbiome may alter energy extraction from the diet through influencing the bioavailability of monosaccharides and SCFA residing in otherwise indigestible dietary plant polysaccharides. The absorption of SCFA from the colon is very efficient, being almost 100%, and SCFA produced by bacterial fermentation of dietary carbohydrates typically account for 5-10% of human energy needs (McNeil, 1984). Epidemiological data indicates typical weight gain in western societies is driven by chronic energy intakes representing as few as 20 calories above daily maintenance requirements suggesting small changes in the efficiency of SCFA fermentation within the colon would be sufficient to explain this weight gain. Also, it appears the SCFAs themselves can indirectly regulate energy intake by the host. For instance, the infusion of propionate and butyrate into the colon of both rats and humans increased secretion of the appetite reducing gut-derived hormones, PYY and GLP-1, in a dose-dependent manner and was shown to protect against high-fat diet-induced obesity in rodent models (Cherbut C et al., 1998; Freeland and Wolever 2010; Duca et. al., 2012; Lin et al., 2012). A shift to an inflammatory microbiota can result in metabolic endotoxemia, a condition associated with 2 to 3-fold increases in plasma LPS concentrations due to the translocation of LPS from the intestinal lumen to the

circulation. Metabolic endotoxemia has been implicated in the pathology of several chronic diseases such as obesity, insulin resistance, diabetes and atherosclerosis (Cani et al., 2007). Finally, high fat diets have been shown to exacerbate such inflammation by promoting translocation of whole bacterial cells across the intestinal wall into blood and tissues (Amar, J et al., 2011).

1.1.5 Current working model of obesity as a progressive disease promoting inflammation, metabolic syndrome, and death.

While the actual causes of obesity are still poorly understood, the link between obesity and associated risk factors has become much clearer as illustrated by the "slippery slope" model of obesity-induced metabolic disease depicted in Figure 1.2. In this model it is assumed that obesity and its metabolic complications is a progressive disease state. Obesity results from an interaction of genetics with an obesogenic environment whereby many contributing factors including gene alleles, metabolism, behavior, environment, culture, and socioeconomic status contribute to increasing adiposity. Adipose tissue expansion promotes a state of low grade chronic inflammation as inflammatory signals derive from both adipose tissue and the gut. Chronic inflammation then alters homeostatic mechanisms that regulate energy intake and metabolism. This promotes hyperphagia and the development of insulin resistance leading to further weight gain and a more pronounced inflammatory state. If unchecked, this progresses to a host of metabolic dysfunctions that lead to the development of metabolic syndrome, diabetes, and chronic degenerative disease such as cardiovascular disease, atherosclerosis, stroke, arthritis, and many types of cancers. While obesity is not

lethal, the associated risk factors can dramatically decrease the quality of life and ultimately shorten one's life span.

Virtue and Vidal-Puig (2008) summarized three theories that attempt to explain how obesity causes insulin resistance. The adipokine hypothesis implies an endocrine defect centering on fat-derived hormones whereby obesity leads to an alteration in the profile of adipokines such as adiponectin, leptin and proinflammatory cytokines. In the obese state, adipose tissue secretes proportionally more adipokines (cytokines) that cause insulin resistance and fewer that promote insulin sensitivity (i.e. adiponectin). Furthermore, leptin resistance develops in target tissues, likely in response to the altered endocrinology associated with obesity, and contributes to the maintenance of a positive energy balance. The lipotoxicity hypothesis implies a chronic positive energy balance overwhelms the ability of adipose to expand and thus buffer against the accumulation of excess lipids. When an individual reaches their adipose tissue expansion limit, according to this model, then lipid is deposited in non-adipose tissue organs such as liver and muscle resulting in systemic insulin resistance by a lipotoxic mechanism. The inflammation hypothesis indicates that obesity is associated with an increase in adipocyte secretion of factors such as proinflammatory cytokines and chemokines which initiate macrophage infiltration into adipose and their subsequent activation promote a low grade chronic inflammatory state that leads to insulin resistance. It is likely that all three proposed mechanisms contribute to obesity-induced insulin resistance. Furthermore, all three are consistent with the development of obesity-induced inflammation.

Regardless of the cause of obesity or the specific mechanisms in which obesity induces insulin resistance, there is now a consensus that the development of inflammation

represents the causative link between obesity and its associated risk factors. Thus, obesity is now best thought of as an inflammatory disease as illustrated by the "slippery slope" model.

There is a great need for animal models that mimic human obesity and the progressive nature of obesity-induced metabolic dysfunction as depicted by the "slippery slope" model in order to allow research aimed at better understanding the emergence of obesity and to develop new treatments and therapies to combat obesity.

1.1.6 Lack of a faithful biomedical model for human obesity and metabolic disease limits development of a cure

Whereas humans are the primary species we wish to know more about, humans are the species least accessible to experimental manipulations. Therefore, by necessity, animal models serve as vital tools in biomedical research. Such models allow us to investigate the underlying mechanisms of disease, increase our understanding of human health, and ultimately allow improvements in the treatment or prevention of health problems. Given the emerging obesity epidemic and resultant increase in prevalence of metabolic disease, great effort has been devoted to obesity and diabetes research using animal models. Cats, dogs, nonhuman primates, and rodents have comprised the most commonly adopted species for this purpose (Cefula, 2006; Henson and O'Brien, 2006; Islam and Loots, 2009; Chatzigeorgiou et al., 2009). These models can be broadly classified as spontaneous/congenital, diet-induced, chemical-induced, surgical, and transgenic depending upon the experimental manipulation that drives the obesity-associated metabolic problems characteristic of each (Srinivasan and Ramarao, 2007).

Despite the use of multiple animal models, our lack of understanding concerning the etiology of obesity and its secondary health effects persists. This is primarily due to inherent limitations in these models that prevent extrapolation to the human condition (Wang et al., 2014). These limitations point to a great need to develop more faithful mammalian models of obesity-induced metabolic disease in order to better study these aspects and to greatly speed the rate that effective therapies can be developed. The Mangalica pig is a fat porcine breed whose juvenile onset of excessive adiposity is driven by hyperphagia. This unique phenotype makes it an attractive potential model to address these bottlenecks.

1.1.6.1 Limitations of current *in vivo*, non-porcine models

Rodent models have been by far the predominant models used to study obesity in the literature to date. Traditionally, rat models have been a favored model to study obesity because they exhibit impressive reproductive fecundity, short generation time, relatively large body size (facilitating biological sampling), and have relatively low associated housing costs (Ben-Jonathan et al., 2008). Mouse models became more prevalent as transgenic technology developed because the ability to selectively manipulate the murine genome facilitates experiments that could not be conducted with rats alone (Ben-Jonathan et al., 2008). The characterization of several genetic strains of obese rats and mice has further allowed these species to predominate the obesity literature during the last two decades.

While offering advantages due to housing and ease of manipulation etc, rodent models generally require multiple genetic manipulations/anomalies in order to mimic some of the changes that occur in humans (Bellinger et al. 2006). For instance, the most

popular genetic rodent models become obese due to well-characterized, monoallelic gene defects. The *ob/ob* or *obese* mouse is the most prevalent murine congenital obesity model. These mice display a mutation that causes leptin deficiency so this strain has been used as a model of hyperphagic obesity and diabetes. The hyperphagia, reduced energy expenditure, and diabetic complications displayed by this strain are corrected by leptin replacement therapy. However, human obesity does not associate with leptin deficiency and unfortunately, leptin therapy does not correct human obesity. Importantly, following leptin replacement therapy, the diabetic complications displayed by *ob/ob* mice correct before the obese phenotype normalizes indicating that obesity plays a secondary role to leptin signaling in causing the metabolic problems exhibited by this model (Pelleymounter et al., 1995; Haalas et al., 1995; Lindstrom, 2007). Finally, obese humans exhibit increased levels of circulating leptin compared to lean individuals and the metabolic dysfunction associated with human obesity appears to be caused by the obese state itself, making the relevance of the *ob/ob* mouse model for human patients very questionable.

The Zucker rat or *fa/fa* rat model has also been used extensively as an alternative to mouse models. The fatty Zucker rat is homozygous recessive (*fa/fa*) for the leptin receptor, resulting in a dysfunctional leptin receptor in the feeding center of the brain that drives hyperphagic obesity in these rats. The lean Zucker rat is either homozygous dominant (*Fa/Fa*) or heterozygous (*Fa/fa*) and represents useful control for the Zucker genetic background making this strain especially attractive for reasons of experimental design. However, obesity in the *fa/fa* rat is associated with hyperphagia, defective non-shivering thermogenesis, increased feed efficiency, and a nutrient repartitioning profile

that are all due specifically to downstream leptin signaling defects and like congenital mouse models, despite the animal becoming severely obese, these aspects do not faithfully model the majority of human obesity (Bray, 1977; Chua et. Al., 1996; de Artinano et al., 2009).

When diabetic manifestations in either wild-type or congenital rodent models do not develop spontaneously, dietary manipulations and genetic manipulation through transgenic technologies have been combined to "improve" the models. For instance, rodents have been used to study insulin resistance, but such models generally require the deletion of certain proteins and receptors in order to examine the effects of obesity on the development of insulin resistance and additional conditions such as hyperlipidemia (Bellinger et al. 2006). Despite the problems in interpretation, these approaches have persisted because they allow study of gene defects in isolation and often facilitate mechanistic studies that would not be feasible in outbred genetic backgrounds. Such approaches provide experimental advantages as well as largely mitigating the need for time-consuming feeding schemes or invasive procedures that risk confounding side effects to induce the diabetic symptoms. However, since such rodent models require multiple or severe genetic manipulations, the use of such knockout strategies creates similar problems as the use of congenital models concerning the extrapolation of data to the human condition. By definition, transgenic manipulation creates substantial differences between factors driving the obesity between rodents and humans that may confound the detection of subtle changes of physiological importance to the etiology of obesity and metabolic disease (Bellinger et al. 2006). So while such rodent models have

yielded insight into obesity and metabolic disease, a higher mammal model is needed to better study obesity and its related conditions in humans.

1.2 The pig as a biomedical model for obesity and metabolic disease that could speed development of a cure

1.2.1 Taxonomy and Nomenclature of the Pig

Pigs are a type of animal that display presence of a notochord in the fetus giving rise to vertebrae, and give live birth; therefore, classifying them in the kingdom Animalia, phylum Chordata, and class Mammalia. Being even toed ungulates, or hooved animals, pigs are thus further placed in the order Artiodactyla. Since pigs are omnivorous non-ruminants they are also included in the suborder Suina, along with hippopotamuses and peccaries, and are members of the Suidae family. All domestic pigs are descended from the wild boar and belong to the genus *Sus*. Pigs used in modern agricultural production and research belong to the species *scrofa* and subspecies *domestica* (McGlone and Pond 2003; Swindle, 2007).

1.2.1.1 Derivation of the modern, contemporary Yorkshire breed

All pigs are thought to have descended from the African wild boar about 40 million years ago and later developed into European and Asian lineages. While the literature is somewhat controversial, the Yorkshire breed, although developed in Yorkshire, England, is believed to be of Asian influence. Yorkshires were imported into the United States around 1830. While the Yorkshire is a dominant breed in the market today, early US Yorks failed to become popular largely because of their slow growth properties. Yorks were later crossbred with British Large Whites, which had

been crossbred with Chinese pigs a century before in order to reduce time to market. The improved Yorkshire displayed good mothering ability, larger litters, more length, more scale and frame and farmers started to accept Yorkshire breeding stock. This breed has been very successful in the market ever since (National Swine Registry). A naïve herd of purebred Yorkshire pigs resides in the biosecure Swine Education and Research Center on campus at Auburn University.

1.2.1.2 Derivation of the unimproved, heritage Mangalica breed

The Mangalica pig is a very unique European heritage breed exhibiting a distinct phenotype with a short, deep body, dense, curly hair coat, and large ears. The Mangalica breed lineage has been well documented. In 1833, Archduke József of Hungary received a stock of Serbian Sumadia pigs as a gift from the Serbian Prince Milos. Archduke József crossbred the Sumadia pig with Hungarian aboriginal breeds, the Alföldi and the Bakony, resulting in the Blonde and Black Mangalica. The Blonde and Black were crossbred to form the Swallow-Belly Mangalica, and the Blonde was also crossbred with another aboriginal breed, the Szalonta, to generate the Red Mangalica. The Black Mangalica became extinct during the last century. The Blonde coat color can range from shades of light gray to yellow, while the Swallow-belly is black with a yellow/blonde throat and underbelly. The Red Mangalica is ginger in color with a slightly thinner and slicker hair coat (Egerszegi et al. 2003).

This new hog was larger, heavier, and fattened better than the breeds before it. This hardy pig was found to be highly adaptable to extreme environments, disease resistant, and displayed great motherliness. This “wooly” pig quickly gained popularity in Europe for its excellent meat quality, and farming Mangalicas became a way of life for

many people of Hungary. By the 1920s, Hungary was processing half a million Mangalica pigs a year to meet the European demands for lard, bacon, sausage, and salami (Rátky et al 2013).

Beginning in 1950, diet habits changed as butter and vegetable oils began replacing lard in the market and the demand for leaner pork increased as well (Rátky et al 2013). With the Mangalica carcass only yielding about 30-35% lean and 65-70% fat (Egerszegi et al. 2003), the breed quickly became unfavorable to consumers. Consequently, the Mangalica was on the brink of extinction by the 1970s, with only 34 breeding sows on record in 1975 (Rátky et al 2013). In 1990, governmental and scientific research programs were established to preserve the Mangalica breed for its desired hardy characteristics and excellent meat quality (Rátky et al 2013).

1.2.1.3 Common terminology of swine production

There is a common agriculture terminology used in the literature to describe pigs. "Swine" refers to the species as a whole or more than one animal. The word "pig" can be used interchangeably with the word "swine." A "shoat" is a weaned pig. The term "gilt" refers to a sexually immature female or a female that has not yet produced offspring. A "sow" describes a sexually mature female. A "boar" is a sexually mature male, while a "barrow" refers to a castrated male. The term "porcine" is an adjective used to describe all things pertaining to swine. In terms of body condition, boars are leaner than sows/gilts, which are leaner than barrows, as castrated male pigs demonstrate the highest propensity to fatten (Swindle, 2007).

1.2.2 Reproductive characteristics of the female pig

The female reproductive tract is composed of paired ovaries and oviducts, the uterus, cervix, vagina, and vulva. The female pig is a polytocous mammal, or capable of producing many offspring in a single birth. The female pig is a polyestrus species with an average estrous cycle of 21 days. Gilts typically reach puberty around 6 months of age, although the onset of puberty can be influenced by breed, nutrition, social environment, and exposure to mature boars (Senegar, 2003).

The estrous cycle can be divided into four phases: proestrus, estrus, metestrus, and diestrus. During proestrus, preovulatory follicles undergo rapid growth and estrogen concentrations increase. Estrus is the period of sexual receptivity and lasts about 48-72 hours in swine (Berghardt et al. 2004). Estrus is defined by a female's behavior. A female pig will become "rigid," or "stand for mating", when exposed to androgens in boar urine. Other signs of estrus include mating of other sows, restlessness, swelling of the vulva, discharge from the vulva, and frequent urination (Gillespie 1997). Ovulation in the pig occurs between 30-40 hours from the onset of estrus (Berghardt et al. 2004). Metestrus describes the transitional period between estrus and the full development of corpora lutea where estrogen concentrations decrease and progesterone concentrations increase. During diestrus 10-25 corpra lutea are fully developed and the highest levels of progesterone are present (Berghardt et al. 2004). In the absence of a pregnancy progesterone levels begin declining at day 15 of the estrous cycle, signaling luteolysis. The average gestation period for swine is approximately 114 days with average litter sizes of 10-12 piglets (Berghardt et al. 2004).

1.2.3 The pig life cycle

Pork production is a significant aspect of American agriculture and an important part of the American diet. In 2012 there were 60,200 hog and pig operations in the Unites States (USDA Farms, Land in Farms, and Livestock Operations 2012 Summary) with production being concentrated in the Midwest and in North Carolina (2007 USDA Census of Agriculture). Based upon the prominence of swine in the food supply chain, it is useful to view the pig life cycle in term of a production context. Pork production can be divided into four phases: breeding/gestation, farrowing, nursery pigs, and grow-finishing. Each phase of production requires certain management practices in order to successfully grow and develop the animal from birth to market.

1.2.3.1 Production-related growth phases in the pig

The average gestation period for swine is about 114 days (Swindle, 2007). The average birth weight for domestic swine piglets is about 3 to 3.5 lbs (Campbell, Kenealy, and Campbell, 2003). Newborn piglets are born with only about 1 % body fat (McGlone and Pond, 2003); therefore, they have difficulty defending their body temperature and require an external heat source such as a heat lamp to prevent cold stress (Swindle, 2007) and maintain an environmental temperature of 86 to 93° F (30-34°C) (Merck, 2010). It is important that the neonate nurses shortly after birth to obtain colostrum for energy and immunity (Merck, 2010). Piglets are born with low glycogen stores and need colostrum to replenish their energy reserves and prevent hypoglycemia, the most common cause of piglet death. Colostrum also provides antibodies to protect the piglet against infectious agents such as intestinal colibacillosis and porcine reproduction and respiratory syndrome (PRRS) (Merck, 2010).

Several critical management procedures are typically performed on neonatal pigs to enhance survival rates or manage animal welfare issues related to necessary management practices. After birth, the umbilicus should be cleaned with iodine solution to prevent infection. Three days postpartum, piglets should receive iron dextran injection to protect against anemia. During the first week, needle teeth should be clipped to prevent mammary irritation to the sow, tails docked to avert tail biting, and males may be castrated (Swindle, 2007).

In the U.S., weaning typically occurs around 3-4 weeks of age. Prior to weaning, piglets may be provided with creep feeds to aid in the transition from a liquid milk diet to a dry matter carbohydrate diet. Upon weaning, piglets are removed from the sow and relocated to an isolated facility to improve biological security. Weaning is a stressful time period for the animal; often times a decrease in feed intake is observed and the animals are more susceptible to disease during the weaning transition (Campbell, Kenealy, and Campbell, 2003).

Upon weaning, all hogs intended for slaughter; i.e. gilts not intended for replacement use and barrows, enter the grower-finishing phase. The greatest amount of growth and changes in body composition occur during this phase in pigs (McGlone and Pond, 2003). Grower-finisher pigs are usually fed *ad libitum* typically being given access to a commercially formulated diet that contains a balance of protein, energy, vitamins, and minerals to allow for maximum growth of muscle and bone. The sexes may be separated in order to formulate a more uniform feeding (Campbell, Kenealy, and Campbell, 2003).

1.2.3.2 Body composition and developmental trajectory

A pig's energy balance is tightly coordinated with its developmental trajectory, or growth curve. A growth curve describes the rate of increase in weight with time (Kyriazakis and Whittemore, 2006). The rate of growth can be influenced by many factors including nutrition, genetics, sex, and environment (McGlone and Pond, 2003). At the steepest slope of the growth curve, the pig will require the highest energy intake in order for the pig to reach its full genetic potential. From birth to puberty is known as the self-accelerating phase, in which rapid growth is occurring due to increase in muscle, bone, and organ size. If a pig is not allowed to eat to its energy during this time period, growth may be retarded and puberty may be delayed, shifting the growth curve to the right and increasing time to market. The point of inflection is generally associated with puberty and marks the end of exponential growth and where the curve begins to reflect slowed growth. The self-retarding phase describes the period from puberty to mature body weight/slaughter. During this phase growth starts to slow as animal approaches mature body weight and energy intake begins to decline as the maximum amount of lean is reached and excess energy starts to be stored as fat (Kyriazakis and Whittemore, 2006). Modern market hogs are typically slaughtered between 240-280 lbs, or at 6 months of age or less, with less than 1 inch backfat (Campbell, Kenealy, and Campbell, 2003; Stender, 2012).

1.2.3.3 Development of porcine adipose tissue depots

In pigs, the major depots or anatomical locations of the carcass are subcutaneous (under the skin and overlying superficial muscles; i.e. backfat), intermuscular (between muscles; i.e. seam fat), and intramuscular (within muscles; i.e. marbling) (Wood, 1990).

At market weight, the subcutaneous depot represents 75% of the total extractable lipid in the pork carcass and contains roughly 45% of the adipose cells found in the porcine body (Lee et al., 1973 a, b). Moody and Zobrisky (1966) first characterized three distinct layers within backfat and these individual layers are measurable both in the live animal (via real-time ultrasound) and the carcass. Furthermore, each layer is metabolically distinct (Allen et al., 1976). Backfat layer thickness is typically measured as the ¾ fat depth (3/4 distance across the loin from the midline) at the 10^{th}-11^{th} rib interface. Back fat and intramuscular fat represent the two most economically important depots in the pork carcass. Backfat is the largest depot but it is undesirable and often trimmed and discarded, while marbling, on the other hand, is considered a primary determinant of pork quality by consumers at the point of sale.

Adipose tissue develops in a unique pattern as the pig grows and approaches maturity. Although pigs represent a relatively low fetal load to the dam, newborn pigs are born with extremely low lipid stores (Reed et al., 1993). At birth, the amount of intramuscular fat is greater than the subcutaneous depot and represents greater than 50% of the extractable lipid of the carcass (Kauffman et al., 1986). However, piglets ingest a high fat diet during suckling and there is a resultant rapid increase in fat stores during the immediate postnatal period of growth. This slows temporarily in association with weaning and accelerates once again as the pig approaches sexual maturity (Reeds et al., 1993). This latter acceleration occurs presumably because the animal reaches its potential for lean gain, and the slower rate of muscle growth allows dietary energy to support the growth of adipose tissue.

At market weight, the majority of carcass fat is contained in the subcutaneous depot but as the pig approaches sexual maturity, the rate of intramuscular fat deposition surpasses that of the subcutaneous depot (Carr et al., 1978; Gu et al., 1992). At birth all adipocytes are multilocular (contain multiple fat droplets) but by as early as day 3 postpartum, many have become unilocular (containing one central lipid droplet). Marked increase in adipocytes size is accompanied by an increase in the size of the central droplet with age (Mersmann et al., 1975). It is important to note that small cells are observed at all ages yielding biphasic cell size distributions. Fortin (1986) monitored the development of backfat in pigs from 14.5-137 kg live weight using a serial slaughter procedure. It was observed that the middle subcutaneous layer developed more rapidly than the outer layer, with the rate of development of the inner subcutaneous fat layer being intermediate. Compared to subcutaneous fat, adipocytes of the latter depots are physiologically less mature and exhibit a delayed growth pattern characteristic of late maturing tissues (Lee and Kauffman, 1974). These authors concluded that intramuscular fat behaves differently from subcutaneous fat both in terms of cellularity and metabolic activity. The depot sequence of adipose tissue development in pigs occurs as follows: subcutaneous fat matures earliest (middle>inner>outer layer), followed by intermuscular fat, and finally, intramuscular fat develops the latest (Allen et al., 1976).

The swine industry is faced with the problem of adopting a strategy to limit subcutaneous fat without negatively affecting the amount of marbling that is present. Currently it is not understood how the temporal development of adipose tissue is regulated in growing animals, but differences in the development of fat depots suggests that the growth of adipose tissue is regulated in a depot specific manner. Interestingly, a

number of studies have reported moderate to low phenotypic and genotypic correlations between percent intramuscular fat and subcutaneous fat depth (Wood et al., 1986; Cameron, 1990; Lo et al., 1992). The lack of a correlation between these traits suggests that selection to increase intramuscular fat without increasing subcutaneous fat may be possible. Thus, it is possible that technologies may be developed which could effectively target adipose tissue development in a depot specific manner allowing producers to finely manage the carcass composition of their pigs. Uncovering the mechanisms responsible for depot-specific fat development would also be very useful for uncoupling obesity from metabolic disease. Visceral obesity is thought to most closely associate with metabolic syndrome in humans, thus being able to selectively limit visceral adipose tissue development might lead to the prevention of obesity-related mortality in humans.

1.2.3.4 Comparison of growth performance and carcass characteristics between Yorkshire and Mangalica breeds

Body weight, typical growth parameters, and carcass measurements are compared in Table 1.4 for Yorkshire, Ossabaw, and Mangalica pig breeds. The Yorkshire is a typical modern market hog. The Yorkshire is fast growing with lean genetics resulting in 65-70% carcass lean. Moreover, the Yorkshire has a higher average daily gain, daily feed intake, and a greater feed efficiency compared to the lard-type hogs because lean genetics are more efficient in converting feed than breeds of lower lean gains (Stender, 2012). For these reasons, the Yorkshire is the most prized breed for pork production in the United States. Due to costs, poor growth parameters, and time to market, pork from lard-type breeds like the Mangalica is only economically viable in niche markets today. While the Ossabaw and Mangalica will likely never be mass-produced in the pork

industry, they serve as great models to study human diseases. The Ossabaw has already proved as an excellent model for atherosclerosis and the Mangalica may serve as a novel porcine model of hyperphagic-induced juvenile obesity.

1.2.3.5 The effects of supplementing dietary fat in swine ration

Fat use in swine diets has greatly increased in recent years. In the past, adding fat to feed was not economically practical because the oils were too costly and solid fat required a heating system. Today, companies can install heated feed tanks on farms and feed mills to deliver specific types of fat or blended fat to producers and feed manufactures. There are several advantages and disadvantages to adding fat to swine diets.

Research has shown that incorporating fat into typical grain-soybean mill diets will improve performance and carcass value of grower finisher pigs by increasing daily gain, decreasing feed intake, and decreasing feed to gain ratio though this also increases backfat thickness. Moreover, supplementing with fat increases the caloric density of the diet because fat contains 2.25 times more energy than carbohydrate or protein. Therefore supplementing fat also reduces feed intake since pigs tend to "eat to their energy". On average, feed intake is reduced by 3-5% for every 5% fat added to the diet. Pigs fed a diet supplemented with fat will gain weight more rapidly, thus possibly decreasing time to market. Every 5% fat added to the diet will increase weight gain by 3-5%. Also, a 5% addition of fat to the diet increases backfat by about 0.1 inch. Furthermore, fat improves feed efficiency by greatly improving feed:gain. The amount of feed per pound of gain is reduced by 8-10% when 5% fat is added to the diet. This means that less feed has to be mixed and handled per each pig that is marketed.

Fat supplementation also improves feed quality, the environment, and reproductive performance. Fat helps to prevent segregation of fine particles of feed form sorting away from larger particles. This results in decreased loss of feed. In addition, air quality is improved with reduced feed dust in swine confinement buildings, resulting in fewer respiratory problems for pigs and the people working in the buildings. Another benefit of incorporating fat in swine diets is that it has been shown to reduce heat stress in animals. Since fat does not produce as much heat when metabolized by animals as other energy sources, it creates less of a heat burden on animals during periods of potential heat stress. Therefore, adding fat to the diet during hot weather could be very beneficial to the animal. Additionally, high fat diets fed during late gestation and lactation have shown to increase the survivability of newborn pigs by 4%. A high fat diet may result in slightly heavier birth weights of piglets and increased milk fat and milk production in the sow. Also, sows on a high fat diet have been shown to return to estrus sooner after weaning (Cromwell, 1997).

1.2.4 Swine faithfully model several important aspects of human physiology

The pig is well positioned as a biomedical model that can be used to overcome the limitations associated with using rodent models for the study of metabolic syndrome and obesity (Gabler and Spurlock, 2008). Pigs are phylogenetically more closely related to humans. Humans and pigs are both omnivores, and like in humans, anatomically discreet depots of brown fat are largely absent in the pig. Additionally, the vasculature, the proportion of skeletal muscle and adipose tissue to total body mass, and circulating levels of glucose are all very similar in the pig and humans (Lunney, 2007; Gabler and Spurlock, 2008). Pigs also have nutritional requirements similar to that of humans and

have a tendency toward sedentary behavior (Boullion et al 2003; Larsen and Rolin 2004). Pigs and humans have similar cardiovascular systems, pancreas morphology, and pharmacokinetics (Bellinger et al. 2006). Given this, there is a well-developed literature concerning the use of swine to study atherosclerosis, cardiovascular disease, and diabetes (Skold et al., 1966; Gerrity et al., 2001; Brambilla and Cantafora, 2004; Dyson et al., 2006). Furthermore, pigs and humans exhibit similar mature body weights (Boullion et al. 2002). A pig's larger body size makes it possible to obtain greater volumes of blood opposed to rodents, making pigs an easier experimental model to utilize (Larsen and Rolin 2004). Because of their anatomical, physiological, and metabolic similarities to humans, it is reasonable to use the human criteria for swine in modeling diabetes (Bellinger et al., 2006). However, there currently is no established porcine model of hyperphagic obesity and frank metabolic dysregulation.

1.2.5 Existing swine breeds used exclusively as models of obesity and metabolic disease.

The pig has a long history of serving as a biomedical model with reports of such use dating back in the literature as early as the 1930's. The pig began being heavily utilized for cardiovascular research beginning in the 1950's and this application continues through the present day. The first reports of the pig for use specifically as a model of obesity began appearing during the early 1970's. These efforts centered upon a novel, small-framed lard type hog, the Ossabaw pig. This breed became isolated on the Ossabaw Islands off the coast of Georgia and is believed to be derived from a feral population of pigs that was brought to the Americas during the 16th century by the Spanish (Bellinger et al. 2006). It is thought that scarce food resources on the island in winter may have selected for a "thrifty genotype", which promotes an obese phenotype when these pigs

are maintained on a nutrient dense diet (Bellinger et al. 2006). Such a genotype would facilitate very efficient utilization of energy, thus driving weight gain.

Several experimental paradigms have been applied to this breed to model obesity. Traditionally, in work conducted from the early 1970's through the turn of the century, Ossabaw pigs were largely maintained on high grain diets with genetically lean breeds often serving as a control group, or in the case of USDA experiment stations, fat Ossabaw pigs were compared to ones that had undergone selection for leanness. Generally this literature revealed that Ossabaw and contemporary pigs had similar plasma concentrations of glucose and free fatty acids, but insulin levels were slightly higher in Ossabaw pigs suggesting these pigs develop mild insulin sensitivity (Kasser et al., 1981; Wangness et al., 1981). Moreover, insulin binding was lower in liver microsomes from Ossabaw versus lean York pigs, consistent with Ossabaw pigs being moderately insensitive to insulin. Insulin binding also decreased as Ossabaw pigs approached market weight (grew fatter). Meanwhile, Ossabaw pigs had greater plasma TG, cholesterol, and HDL than contemporary pigs (Etherton and Kris-Etherton, 1980; Meserole and Etherton, 1984). However, these older studies comparing Ossabaw (obese) to Yorkshire (lean) controls represent a confounding design because of the potential for significant breed differences and the fact that there was a large difference in body weight between when age was held constant and these differences in live weight were not solely due to differences in adiposity.

A newfound interest in developing the Ossabaw breed as a biomedical model for obesity-linked cardiovascular disease emerged in the 2000's. The Ossabaw literature has since been largely characterized by studies in which pubertal pigs that are fed a high-fat

diet for periods spanning at least 12 weeks to create obese pigs are compared to littermates fed a normal diet (eliminating the need to maintain multiple breeding herds). When fed a high fat diet, this breed has been known to develop indices of metabolic syndrome. This literature has firmly established the Ossabaw pig as a porcine model of obesity that develops a prediabetic state, where glucose levels are relatively normal and the pigs display mild hyperinsulinemia and dyslipidemia (Dyson et al., 2006). Thus they model genetically susceptible, diet-induced obesity and begin showing a progression of metabolic symptoms that places this model near the top of the "slippery slope" paradigm.

Given new interest in using swine as biomedical models, the Göttingen minipig was developed at the Institute of Animal Breeding and Genetics of the University of Göttingen, Germany specifically for biomedical research (Bellinger et al. 2006). The Göttingen minipig only weighs approximately 20 kg as a mature adult; therefore, its small size and ease of handling make it ideal for research settings on medical campuses (Swindle, 2007). However, these pigs require pancreatic insult in order to manifest diabetes, making the Göttingen minipig a less attractive model than the Ossabaw.

1.2.6 Limitations of swine models of obesity and metabolic disease.

There are three primary ways researchers have attempted to establish a swine model for conducting obesity- and diabetes-related research in the literature; however, each are not without limitations. Since swine do not spontaneously develop diabetes (Larsen and Rolin 2004), metabolic disease must be promoted by the 1) genetic selection for susceptibility, 2) dietary manipulation usually involving the feeding of high fat containing rations, or 3) insult through either surgical or chemical means. Selecting animals for an obese phenotype is one way to establish a pig model to study obesity

related conditions; however, this method is rather impractical because it requires multiple breeding herds which can become costly to maintain. In addition, pigs typically only develop one or two of the risk factors associated with metabolic disease. For instance, lean and obese Ossabaw pigs have been created by genetic selection for divergent body composition over multiple generations (Hetzer and Harvey, 1967). Adult, obese Ossabaw pigs exhibit mild insulin resistance, which is exacerbated when fed a high fat diet for 5 months (Dyson et al., 2006). However, these pigs do not appear to manifest metabolic disturbances during prepubescence and their divergent body composition appears to be driven by lower fasting heat production in the obese lines while genetic differences between the two cohorts potentially confound mechanistic studies (Davey and Berskin, 1977; Wangness et al., 1977; Mersmann et al., 1982; Mersmann, 1986).

Another method for establishing swine as a model is to use pigs with lean genetics and then feed a high fat diet to induce obesity. This method more closely mimics hyperphagic-induced obesity seen in humans. However, pigs typically only eat to their energy which makes it difficult to develop a pig that models morbid obesity. Generally feeding energy dense rations will result in a significant depression in voluntary feed intake which limits adiposity and confounds interpretation of metabolic status and studies into the etiology of obesity. Thus, it is a challenge to induce true metabolic syndrome through ration manipulation alone.

Pancreatectomy has been used as another method to induce diabetes in pigs (Larsen and Rolin, 2004). Lohr et al. (1989) reported that a 40% pancreatectomy resulted in mild impairment of glucose intolerance, 80% pancreatectomy resulted in significant hyperglycemia, and total pancreatectomy resulted in severe hyperglycemia. Due to

animal welfare considerations associated with such an invasive surgery, this method should only be considered when other methods are not feasible (Larsen and Rolin 2004). Another disadvantage of surgical induction of diabetes is that it requires the removal of both exocrine and endocrine tissue, which is not characteristic of diabetes in humans (Larsen and Rolin 2004). Streptozotocin is a compound that damages pancreatic beta cells so it has been used to chemically induce diabetes in pigs. A disadvantage of this method however centers upon dosing considerations. It is reported that low doses (30-40 mg/kg) of Streptozotocin have no effect on glucose tolerance in pigs, but a 100-150 mg/kg dose has been shown to induce insulin-dependent diabetes in pigs (Larsen and Rolin 2004). However, there is concern that higher doses begin impacting other tissues as well and thus the affect may not be strictly due to a selective targeting of the pancreas. In addition, no inflammatory response has been observed in pigs induced with Streptozotocin (Larsen and Rolin 2004). Furthermore, diabetes is a progressive disease in humans; therefore, another limitation of this method is that it only allows study of the final stages of the disease (Larsen and Rolin 2004).

Despite a well-developed literature concerning the use of pigs to model human obesity and metabolic disease, there is not currently a swine model available that faithfully manifests all indices of obesity-induced metabolic syndrome nor are there reports in the literature of a porcine model that spontaneously develops diabetes (Larsen and Rolin 2004; Bellinger et al., 2006). In 2006, Gerstein and Walton proposed the theory that pigs do not spontaneously develop diabetes because they have been bred to efficiently convert ample amounts of food. These authors argued that domesticated pigs have been bred for thousands of years to grow efficiently whereas dogs and cats have

been selected to maximize work. Pigs should therefore be protected against the toxic effects of a diabetogenic environment whereas dogs and cats should have no such protection. Intuitively, this appears to be the case given an obesity epidemic in companion animals mirrors that being experienced by their owners.

It is well known that pigs regulate their voluntary intake to match their energetic needs, which largely precludes the manifestation of hyperphagia in the porcine. Also, modern breeds have undergone intense selection for rapid growth, and in some cases, it is unlikely that such improved, high lean gain breeds actually eat enough to maximize this heightened genetic potential for rapid growth. Interestingly, consistent with the theory of Gerstein and Walton, wild boars have more red (slow twitch/smaller diameter/oxidative) than white (fast twitch/larger diameter/glycolytic) muscle fibers, whereas domesticated pigs have predominantly white fibers. Thus, skeletal muscle of domesticated pigs displays an overall metabolic phenotype that might be expected to predispose them to utilizing glucose better than their feral counterparts. However, obesity and diabetes correlates to a higher white fiber distribution in skeletal muscles, whereas endurance and resistance training promotes higher red fibers in humans, suggesting that exercise promotes a "wild boar" muscle fiber profile whereas obesity promotes a "domesticated boar" profile (Baskin et al., 2015).

Nonetheless, consistent with pigs and humans displaying different susceptibilities to diabetes, some notable differences exist in glucose and lipid metabolism between humans and pigs. For instance, pigs have been reported to have greater glucose tolerance and an ability to dispose of intravenous glucose more efficiently than humans (Ferrannini et al., 1985; Kruszynska et al., 1993; Ahren and Pacini, 1998; Larsen et al., 2002a). Pigs

have lower plasma free fatty acid and ketone values than humans (Barth, 1990). During fasting there is a similar increase in FFA, glycerol and ketone bodies, but during fasting or anesthesia, pigs exhibit greater hepatic glucose production than humans (Lauritsen, et al., 2002). Finally, adipose is the primary site of fatty acid synthesis in pigs, whereas the liver is the primary site in humans (Nafikov and Beitz, 2007). These differences in metabolism may underlie why the literature currently lacks a suitable porcine model for spontaneous diabetes. Development of such a porcine model would represent an exciting advance.

1.3 Clinical considerations of obesity and metabolic disease research with special reference to the pig model

1.3.1 Definition of metabolic syndrome in the pig

Metabolic syndrome was initially referred to as Syndrome X or the insulin resistance syndrome (Eckel et al. 2005). Kylin, a Swedish physician, first described metabolic syndrome in the 1920s as a clustering of hypertension, hyperglycemia, and gout (Kylin, 1923). In 1947, Vague reported that the obesity phenotype was associated with diabetes and cardiovascular disease (Vague, 1947). In 1988, Reaven classified metabolic syndrome as a condition in which obesity coincides with hypertension, diabetes, dyslipidaemia and cardiovascular disease (Reaven, 1988). Ten years after Reaven described metabolic syndrome, the World Health Organization (WHO) sought to develop the first formalized universal definition of the disease (Shin et al. 2013). The diagnostic criteria set by WHO included markers of abnormal glucose metabolism such as diabetes, impaired fasting glycaemia, impaired glucose tolerance, or insulin resistance. In addition to abnormal glucose metabolism, an individual must also portray at least two

of the following four risk factors: obesity, hypertension, hypercholesterolemia, or elevated triglyceride levels (Shin et al. 2013).

From a pathophysiological standpoint, metabolic syndrome is formally defined as a constellation of atherothrombotic-inflammatory abnormalities for which insulin resistance is the central component (Grundy et al., 2004). Thus the clinical criteria proposed by WHO should not be referred to as the definition of metabolic syndrome but rather such criteria represent a simple screening tool allowing diagnosis in clinical practice (Despres et al., 1990; Despres, 2006). Spontaneous metabolic syndrome is rare in pigs with almost no case reports in the literature and there are no spontaneous pig models available (Bellinger et al., 2006). Likewise, there is no standard criterion applied to pigs in the literature relative to their use as a biomedical model for metabolic disease. Because of their anatomical and metabolic similarities to humans including omnivorous diet, it is reasonable to use the human criteria for swine in modeling diabetes (Bellinger et al., 2006). Those criterions have been adopted for the studies reported in this thesis.

1.3.1.1 Hyperglycemia and Insulin Resistance

Typically, the first clinical symptoms of metabolic syndrome are glucose intolerance and insulin resistance. Glucose is a six-carbon monosaccharide. Certain cell types in the body, such as red blood cells, brain cells, and kidney cells rely exclusively on glucose for energy thus proper maintenance of glucose homeostasis is essential in pigs and humans. Consumption of a meal causes a rise in blood glucose levels, which stimulates insulin to be secreted from pancreatic beta cells in order to bring blood glucose levels back to normal. Insulin stimulates the uptake of glucose through facilitative transport of glucose into skeletal muscle, in which glucose is either burned for energy or

stored as glycogen, or into adipose tissue, which converts glucose to triglycerides for storage. In insulin resistance states, insulin-responsive tissues, such as liver and skeletal muscle, stop responding to normal insulin concentrations. The pancreas compensates by increasing insulin secretion, which leads to hyperinsulinemia as insulin sensitivity becomes increasingly impaired. If the pancreas fails to produce enough insulin, blood glucose levels rise, leading to hyperglycaemia. Diabetes is a state of chronic hyperglycaemia either through a lack of insulin or because of severely impaired insulin sensitivity despite abnormally high circulating levels of insulin.

Elevated basal glucose levels are considered the hallmark of hyperglycemia and diabetes mellitus and are also closely associated with pancreatic beta cell function and insulin sensitivity (Porte, 1999). Normal fasting blood glucose, or euglycaemia, for humans (and pigs) is represented by plasma glucose values falling within the narrow range of 70 to 100 mg/dl (Bellinger et al. 2006). Thus, plasma glucose levels are often used as a diagnostic tool for assessing insulin sensitivity. The American Diabetes Association and WHO have developed guidelines for administering glucose tests. For an oral glucose tolerance test (OGTT), a fasted patient (human) is given a 75 gram oral dose of glucose and blood sugar levels are recorded for two hours post ingestion. Impaired fasting glucose is defined as >100mg/dl (5.6mmol/L) but less than 126 mg/dl (7 mmol/L) with values over 126 considered symptomatic of diabetes. A 2h OGTT glycaemia of <140 mg/dl is considered normal, a glycaemia of 140 mg/dl (7.8 mmol/L) to 197 mg/dl is symptomatic of impaired glucose tolerance, and a glycaemia greater than or equal to 200 mg/dl (11.1 mmol/L) is considered sufficient to support a diagnosis of diabetes mellitus (Bellinger, et al. 2006).

In pigs, an OGTT is usually conducted by dosing the pig with 2g/kg BW glucose along with a small amount of feed and then measuring plasma glucose over 2 hrs. No set standards for interpretation exist in the literature for pigs however. Generally, pigs are considered diabetic if there is an elevated glucose response over controls despite little insulin response. Also, some researchers have compared peak curve values or area under the curve for glucose and insulin values when assessing the potential for metabolic differences to exist between treatment groups. Generally pigs exhibiting elevated glucose and insulin values versus controls are considered to be diabetic.

1.3.1.2 Obesity and Inflammation

Obesity and inflammation are two other risk factors used to characterize metabolic syndrome. WHO has defined obese as having a body mass index greater than or equal to 30 kg/m2 (Shin et al. 2013). Recent findings have demonstrated that obesity is tightly associated with systemic low-grade chronic inflammation, which is thought to play a major role in the development of metabolic diseases (Huh et al. 2014). Inflammation is generally assessed by measuring the expression of proinflammatory genes in adipose tissue via real-time PCR, levels of circulating cytokines in the plasma via ELISA, and by measuring macrophage invasion in target tissues histochemically. Immune function can be further assessed by conducting endotoxin challenges and assessing the febrile response by measuring body temperature and the levels of circulating factors such as TNFα and cortisol which should exhibit a sharp, acute rise in the plasma and blood glucose and insulin which characteristically drop following administration of endotoxin. Few studies have assessed inflammatory status in porcine models of obesity and metabolic disease.

1.3.1.3 Dyslipidemia

Dyslipidemia is another risk factor of metabolic syndrome and refers to abnormal amounts of lipids in the blood, such as free fatty acids, triglycerides, and cholesterol. Diabetes can include various types of dyslipidemia, but one phenotype is particularly common, which is attributed mostly to increased free fatty acid flux secondary to insulin resistance (Mooradian, 2009). This diabetic dyslipidemia phenotype is characterized by high plasma triglyceride concentration, low HDL cholesterol concentration, and increased LDL cholesterol concentration (Mooradian, 2009). In 1999, WHO reported dyslipidemia as: triglycerides greater or equal to 150 mg/dL, and HDL cholesterol less than 35 mg/dL in men and HDL cholesterol less than 39 mg/dL in women. As triglycerides increase, HDL cholesterol decreases due to a decrease in the cholesteryl ester content of lipoprotein. This alteration in the lipoprotein composition leads to clearance of HDL from circulation; consequently, LDL cholesterol levels rise (Eckel et al. 2005).

1.3.2 Methods of blood collection and dosing considerations

Blood sampling can be helpful in diagnosing a disease, monitoring response to treatment, and is also important for health surveillance and certification. Use of the pig as a biomedical model also dictates the need to sample blood, often of large volumes. Common sites for collecting blood samples in swine include the auricular (ear) vein, tail vein, external jugular vein, femoral vein, and anterior/cranial vena cava (Swindle, 2007). The site of blood collection depends on the age of pig and the volume of blood needed for the sample. Recommended maximal blood draw for pigs according to age and weight is summarized in Table 1.5.

1.3.2.1 Venipuncture vs. Cathetherization

Two common methods of blood sampling with pigs include venipuncture and catheterization and the choice of when to use either largely depends upon the number of samples and total blood volume that is required. Venipuncture is commonly used when repeated draws are not necessary because it is relatively noninvasive, inexpensive and generally facilitates suitable volume collection requiring only minimal restraint of the pig. Catheterization is often considered when serial sampling is a necessity. It is substantially more invasive, generally requires anesthesia, and the establishment of indwelling catheters promote infection and vascular complications while also mandating substantial effort be devoted to daily catheter maintenance to keep catheters patent.

1.3.2.2 Common sites for venipuncture in the pig

Venipuncture from the ear veins of pigs is suitable for single and multiple sampling of volumes less than 1 ml. This method is suitable on pigs after weaning age (Hanie, 2006). There are usually three prominent veins in the ear with the lateral or central vein usually being the largest. The ear veins are branches of the auricular vein and the superficial cervical vein. Their pattern and relative sizes vary from pig to pig. A rubber band may be used as a tourniquet around the base of the ear to distend the vein. A 20-gauge needle is suitable for most animals (Hanie, 2006). This technique is suitable for measurement of hematocrit and hemoglobin levels, and for making blood smears (Sjaastad and Aass, 2000).

The coccygeal, or tail, vein is another potential site for drawing blood via venipuncture but it is not often used. The coccygeal vein is located on the ventral midline of the tail. The tail is elevated and a 20-gauge needle is inserted near the middle

of the base of the tail. Only blood volumes of 5 ml or less may be obtained from the tail vein, and this method is only suitable for adult swine (Hanie, 2006).

External jugular venipuncture is the preferred method for blood collection in adult swine (Sjaastad and Aass, 2000). Unlike cattle and horses in which the jugular vein lies close to the surface, the jugular vein of pigs lies deep in the tissues of the neck, making jugular venipuncture a "blind stick" (McGlone and Pond 2003). First, the animal must be restrained. Typically a hog snare is used to place a wire rope behind the canine teeth so that it does not slip off or move rostrally. Blood collection is performed easiest when the neck is stretched well upwards and the pig stands on all four legs (Sjaastad and Aass, 2000). For most pigs, a 20-gauge needle is appropriate (Hanie, 2006). The deepest point of the jugular furrow is located on the neck and a needle is inserted perpendicular to the skin and directed dorsocaudally to obtain the sample (Sjaastad and Aass, 2000). The right jugular vein is preferred to avoid damaging the phrenic nerve, which runs parallel with the left jugular vein (Hanie, 2006). 10-30 ml of blood may be collected from the jugular vein (Sjaastad and Aass, 2000).

The femoral vein is another site for blood collection. This method may be performed on small pigs restrained in dorsal recumbency, or on anesthetized animals in order to access the vein in the rear leg. Unless the animal is already anesthetized, other less stressful methods of venipuncture are preferred.

Sampling from the cranial vena cava is technically more difficult to learn, however it is the most satisfactory location for obtaining large blood samples (e.g. > 20 ml) and can be performed on any size pig (Hanie, 2006). The cranial vena cava is located in the thoracic region between the first set of ribs and gives rise to the jugular veins. The

right side of the animal should be used to access the cranial vena cava in order to avoid damaging the phrenic nerve. Pigs under 50 lbs are placed in dorsal recumbency and the cranial vena cava can be accessed using a 20-gauge needle inserted at the caudal extent of the right jugular furrow. Pigs larger than 50 lbs are restrained standing using a hog snare. The needle is inserted as described previously. A depth of 4 inches may be required to reach the vein. No more than 3 attempts should be made to limit injury and bruising at the sapling site (Sjaastad and Aass, 2000).

1.3.2.3 Special considerations associated with establishing indwelling catheters in pigs

In experiments where it is necessary to take frequent blood samples, it may be desirable to insert an indwelling venous catheter. Venous catheters are commonly placed in the external jugular vein. Under general anesthesia a lateral incision is made at the jugular furrow and the external jugular vein is bluntly dissected. The catheter is inserted into the vein with the aid of a guide wire, known as the Seldinger technique (Seldinger 1953). Once the catheter is in place it is directed dorsocaudally and led out through the skin, where it is placed in protective pouch and sutured to the side of the animal's neck. It is then possible to take blood samples without disturbing or stressing the animal. Catheters should be flushed at a minimum of every 12 hours using heparinized saline to prevent clotting. To reduce the risk of introducing bacteria, the injection cap should be cleaned with isopropyl alcohol before each use and an antibacterial solution should be applied to the catheter exteriorization site (Bollen, Hansen, and Rasmussen, 2000. The Laboratory Swine). A more detailed catheter procedure is described later in the methods section of chapter 3.

The study in Roberts et al., 2015 suggests that refinements to jugular catheterization and catheter maintenance need to be addressed for future experiments with the obese Mangalica pig. First, any invasive surgical procedure should be done in the most sterile environment possible to reduce the risk of post-operative infection. A less invasive alternative would be to insert the catheter using ultrasonography to locate the jugular vein. Secondly, catheters need to be established for as short of amount of time as possible and promptly removed after the last sampling occurs. Therefore, all collaborators should design a protocol prior to performing any procedure to designate a specific date of catheter implantation, length of sampling period needed for data collection, and prompt catheter removal. This protocol should also include a scheduled date, shortly after data collection is complete, to extinguish the animal to prevent any prolonged suffering due to possible unknown and underlying causes; i.e. thrombosis, due to catheterization.

1.3.3 Dosing Considerations in pigs

Drugs may be introduced into the body via several routes of administration. The more common routes of drug administration include giving medication by mouth (orally), or by injection into a vein (intravenously), into a muscle (intramuscularly), or beneath the skin (subcutaneously). Selection of the appropriate route of administration depends on a number of factors such as drug preparation, licensing of the drug, how quickly you want the onset of action to occur, the condition of the patient, patient cooperation, cost constraints, level of convenience, and many other factors (Kahn and Line, 2010).

Drugs can be administered orally in veterinary medicine as liquids, capsules, tablets, pastes, boluses, powders, and medicated premixes/medicated feeds. Drug

absorption of oral medication takes place along the gastrointestinal tract, usually with most absorption occurring in the small intestine. The drug passes through the intestinal wall and enters the portal vein and then travels to the liver before being transported via the bloodstream to its target site. Enzymes in the intestinal wall and liver metabolize many drugs, decreasing the amount of drug reaching the bloodstream. Therefore, orally administered drugs are usually given at a greater concentration than if injected intravenously. The oral route is usually the safest and least expensive method in medicating an animal; however, administering oral medication has its disadvantages. Some orally administered drugs, such as nonsteroidal anti-inflammatories, irritate the digestive tract and can harm the lining of the stomach and small intestine, potentially causing nausea, vomiting, and gastric ulcers. Drugs may be absorbed poorly in the digestive tract or are destroyed by acid and digestive enzymes in the stomach. Oral administration is not the route of choice in emergencies due to the time it takes for the drug to be absorbed in the GI tract (Kahn and Line, 2010).

The intravenous (IV) route can be used for giving single or multiple doses of medicines. Good restraint and skin preparation is necessary. For multiple doses of drugs, an intravenous catheter may be implanted to decrease trauma to the vein and reduce stress for the patient and staff. The intravenous route produces the fastest onset of action, with the drug taking effect usually within 0-3 minutes. A range of drugs can be administered using this route, including antimicrobials, intravenous anesthetics, analgesics and diuretics. It is a suitable route for administering large volumes of drugs and fluids; however, intravenous medications should be administered slowly to reduce the risk of toxic or allergic reactions. The main injection sites used in dogs and cats are the cephalic

vein in the forelimb, the saphenous vein in the hind limb, and the jugular vein in the neck (Shellim, 2011).

The intramuscular (IM) route offers a slower onset of action, usually 20-30 minutes, compared to the IV route, but usually induces a longer duration of action. This is generally the most painful route and should be avoided when administering irritant drugs or large volumes, as these can be very painful. Relevant volumes will vary between species and breeds, but as a guideline a maximum of 2 ml should be given IM in cats and 5 ml for dogs. It is important to remember to always draw back on the syringe before injecting into a muscle, to ensure you are not injecting into a blood vessel. The main muscle groups suitable for intramuscular injection are the triceps, quadriceps, and the lumbodorsal muscles located on either side of the midline (Shellim, 2011).

The subcutaneous (SC) route is frequently used in veterinary practice to administer a wide range of non-irritant medications, including antibiotics, analgesics and vaccines. Also, SC is used for many protein drugs, because such drugs would be destroyed in the digestive tract if orally administered. This route offers a slower onset of action, typically 30-45 minutes, compared to the IM and IV routes and can be particularly slow in patients whose circulation is compromised. This site should be avoided when administering irritant drugs and it is important to draw back on the syringe before injecting, in order to prevent accidental injection into blood vessels. SC injections can be given in the scruff or flank region of the animal. When administering multiple medications or injecting several days in a row, alter the injection site(s) to avoid irritation and thickening of the skin (Shellim, 2011).

Special attention should be paid to the withdrawal time when administering drugs

to food animals and production animals. Drug withdrawal time is the amount of time necessary for an animal to metabolize the administered product to a concentration level that is safe and acceptable for consumption. Every federally approved drug or animal health product has a withdrawal period printed on the product label or package insert. Withdrawal times are not the same for all drugs or for all products. For example, there is a 4-15 days withdrawal period for penicillin products in cattle intended for slaughter, and milk produced from a dairy cow must be disposed of for 72 hours post injection of penicillin. Withdrawal periods may be extended when combinations of drugs are used or when drugs are used in an extra-label manner. A veterinarian should be consulted in any situation where it is uncertain of a specific drug withdrawal period (www.extension.org).

The typical site of injection in a grower/finisher pig is in the neck behind the base of the ear because damage to the ham or loin can result in condemnation of the meat. The appropriate dose of a drug is usually formulated based upon the body weight of the animal. Thus, a drug dosing error is usually due to an inaccurate estimation of body weight. This is not much of a problem in swine because most pig production facilities have access to a scale. However, Roberts et al., 2015 established that there are differences in the pharmacokinetics of lean verses lard-type pigs; therefore, special dosing considerations are necessary for intramuscular injection in lard-type pigs. Since Mangalica pigs are about 2.5 to 3 times fatter than Yorkshires, Mangalica pigs were dosed at greater concentrations in Roberts et al., 2015 than a Yorkshire of equal body weight to achieve the desired effect of the drug. Two hypotheses are suggested for this. One, adipose tissue is known to absorb and store compounds, especially steroid-like compounds. Therefore, the excessive adiposity of the Mangalica may contribute to

absorption and storage of the drug, thus decreasing the effect of the drug. Two, although adipose tissue is vascularized, it does not receive as much blood flow as skeletal muscle; therefore, it is proposed there is a greater time release for a drug injected into fatty tissue verses lean muscle.

1.3.4 Considerations associated with processing blood samples

Blood sampling is a very important laboratory diagnostic tool commonly used in veterinary medicine to evaluate the health status of an individual patient or herd of animals. Blood test may reveal information about organ function, mineral content, drug effectiveness, etc. therefore aiding in diagnosing disease and monitoring the progression of disease. Blood delivers necessary substances such as oxygen, nutrients, and hormones to cells and tissues throughout the body, aids in the removal of wastes, and is an important part of the immune system. Blood is comprised of red blood cells, white blood cells, and platelets. All of these parameters may be altered in a disease state and thus their measurement offers potential diagnostic insight.

Upon centrifugation a blood sample can be separated into two components- serum and plasma. Serum is collected coagulated blood (whole blood without anticoagulant). Plasma is collected from non-coagulated blood (whole blood with anticoagulant). In other words, serum is the fluid portion of whole blood without the clotting factors or blood cells. Plasma is the fluid containing soluble clotting factors and is most often used in medicine for blood transfusions. Serum is used to determine blood type and for various diagnostic tests because serum has more antigens than plasma or blood. Also, anticoagulants in plasma or blood may interfere with testing.

Due to the hematological properties of blood, it is important to know beforehand

if serum or plasma is required for a diagnostic or experimental endpoint so that the sample can be handled and stored properly. There are commercially formulated tubes for blood sample collection with a standardized color system applied to the tube top indicating the intended use for that test tube. Table 1.6 summarizes these commercially available tubes and their uses. For example, red top tubes are used for serum; therefore they do not contain an anticoagulant. Blood may be dispensed into red top tubes to test for certain antibodies, minerals, and proteins. Green top tubes are for plasma and used for sodium and calcium sensitive assays. Gray top tubes are used for determining glucose levels and thus contain sodium fluoride, which is effective in inhibiting the glycolytic enzyme that breaks down glucose in blood.

1.4 Summary and Implications

Obesity is characterized by a multifaceted etiology and has emerged into a global epidemic over the last 30 years. Hyperphagic obesity is the most common form of obesity in humans, which often leads to low-grade chronic inflammation, insulin resistance, metabolic disease, and chronic degenerative diseases such as cardiovascular disease and diabetes. A major limitation to the development of strategies to prevent and treat obesity and its associated complications is due to the lack of a research model, for there is currently no animal model that faithfully exhibits the full progression of disease as seen in obese humans. Therefore, this study aimed to establish the Mangalica pig as a translational model to study human obesity and metabolic syndrome. More specifically, the purpose of this study was to determine if increasing adiposity due to voluntary hyperphagia spontaneously induces altered metabolic and inflammatory function in the Mangalica pig in order to determine it's useful as a biomedical model.

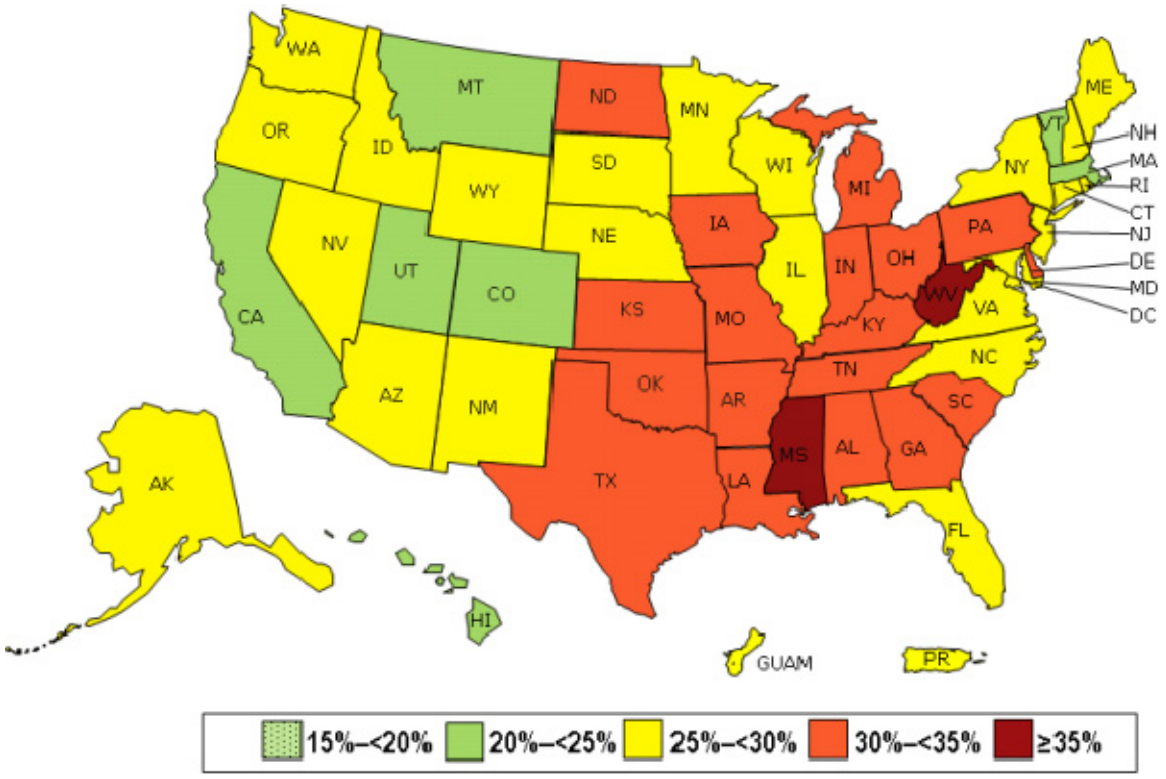

Figure 1.1 Prevalence of Self-Reported Obesity Among U.S. Adults by State and Territory, 2013. Source: Behavioral Risk Factor Surveillance System, CDC

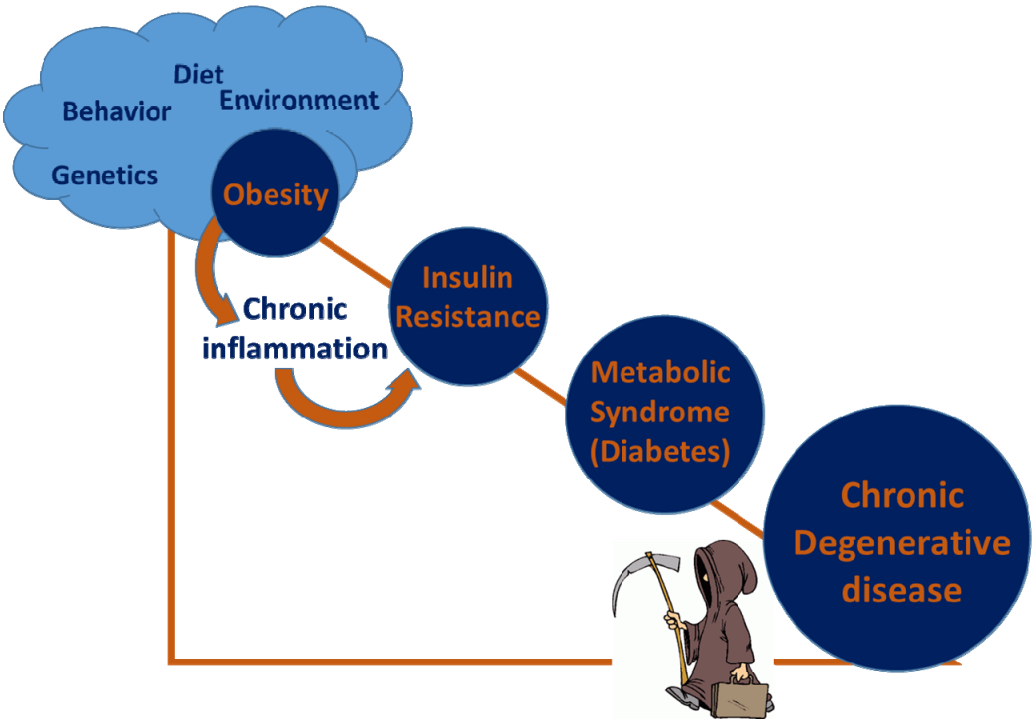

Figure 1.2 The slippery slope model depicting the progressive nature of the obesity-inflammation-metabolic syndrome disease state in humans.

Table 1.1 Classification of weight status by body mass index (BMI)

BMI	Weight Status
Below 18.5	Underweight
18.5 – 24.9	Normal
25.0 – 29.9	Overweight
30.0 - 34.9	Obese
35.0 – 39.9	Morbidly Obese
40.0 and Above	Candidate for drug therapy/bariatric surgery

Table 1.2 Annual veterinary treatment costs, canine diseases (Slimdoggy, 2014).

Disorder/Disease	Average Cost
Heart disease	$1,912
Hypertension	$1,700
Osteoarthritis	$1,656
Cancer	$2,447
Diabetes	$1,108
Pancreatitis	$1,422
Obesity + ruptured ACL	$2,367
Chronic kidney disease	$1,823

Table 1.3 Annual veterinary treatment costs, feline diseases (Slimkitty, 2014).

Disorder/Disease	Average Cost
Heart disease	$1,065
Hypertension	$1,241
Osteoarthritis	$286
Cancer	$994
Diabetes	$860
Pancreatitis	$1,483
Chronic kidney disease	$1065
Hyperthyroidism	$1,401

Table 1.4 Breed comparison of typical body composition and growth parameters

Measurement[1]	Unit	Yorkshire	Ossabaw	Mangalica
Live Body Weight[2]	lbs	250	130	375
% Carcass Lean	%	65-70	N.R.[3]	30-35
Backfat at 10th Rib	in	0.9	2.1	3.2
Rib Loin Eye Area	in^2	6.2	5.3	3.80
Average Daily Gain	lb/d	1.70	.75	.55
Daily Feed Intake	lb/d	5.5	3.5	5.0
Feed Efficiency	G/F	.31	.214	.11
Time to Market[4]	d	147	333	454

[1]Values for Yorkshire adapted from McGlone and Pond, 2003; Stender, 2012. Values for Ossabaw adapted from Martin et al., 1973; Buhlinger et al., 1978; Wangness et al., 1980; Dyson et al., 2006. Values for Mangalica adapted from Egerszegi et al., 2003; Brussow et al., 2005; Ratky et al., 2005.

[2] Live body weight is represented as market weight for Yorkshire and mature body weight for Ossabaw and Mangalica breeds.

[3] N.R. = Not Reported as no citation was available in literature to address this parameter.

[4] Time to market is calculated as market weight set at 250lbs/ADG. Thus it is normalized for Yorkshire market weight. Ossabaw pigs do not reach 250 lbs mature live weight.

Table 1.5 Maximum blood volume draw for swine

Age	Weight	Total Blood Volume (mL)	Maximum Draw (mL)
Newborn	3 lbs	110	10
Nursery pig	35 lbs	1,280	120
Sow	440 lbs	16,000	1,600

Table 1.6 Blood collection tubes (tubes with * are commonly used)

Color of Tube Top	Fluid Type	Anticoagulant	Example Uses
Red*	Serum	None	Antibodies, minerals, proteins
Blue	Either	Na heparin or none	Special blood chemistries
Brown	Plasma	Na heparin	Lead determination
Black/Light Blue	Plasma	Na citrate	Coagulation studies
Gray	Plasma	Glycolytic inhibitors	Glucose determinations
Green*	Plasma	Lithium heparin	Na, Ca sensitive assays
Yellow	Plasma	Sodium citrate	DNA extraction
Purple/Lavender*	Plasma	EDTA	Clotting factors

Chapter 2:

Growth Performance and Body Composition Data Reveals Blonde and Red Mangalica Pigs are Novel Models for Human Obesity and Pork Quality

2.1 Abstract

Blonde, Red, and Swallow-bellied Mangalica pigs were recently imported to Auburn University due to their extreme propensity to fatten and reputation for producing superior quality pork. However, the Mangalica phenotype is poorly described in the scientific literature and needs to be better characterized in order to assess the potential of the Mangalica breed to serve as useful models to study both human obesity and pork quality. Therefore our objectives were 1) to characterize differences in growth performance and carcass merit of purebred Yorkshire, Blonde, Red, and Swallow-bellied Mangalica pigs, and 2) to compare indices of meat quality in purebred Red Mangalica, Yorkshire and crossbred (Red x Yorkshire) pigs. To achieve this, pigs were allowed ad libitum access to water and diets formulated to meet National Research Council recommendations. Feed intake was recorded daily and weight gain was measured every 7 d. Carcass and quality characteristics were recorded 24 hr postmortem according to National Pork Producers Council Guidelines (2000). Data were analyzed using GLM procedure and mixed model analysis (SAS, 2002). Comparison of growth and carcass parameters between Yorkshire, Blonde, Red and Swallow-bellied barrows demonstrated divergent phenotypes. Backfat thickness was 2.9-fold greater in Blonde than Yorkshire and 1.2 fold greater in Blonde than Red and Swallow-bellied pigs ($P < .0001$). Marbling score was greatest in Red pigs being 1.65-fold greater than in Blonde or Swallow-bellied

and 3.5-fold greater than in Yorkshire pigs ($P < 0.01$). In contrast, loin-eye area (LEA) was 2.4-fold greater in Yorkshire versus Blonde, Red or Swallow-bellied pigs ($P < 0.0001$). Daily feed intake, average daily gain (ADG), and feed efficiency was highest in Yorkshire and lowest in Swallow-bellied pigs ($P < .0002$). To determine if the divergent carcass parameters also associated with meaningful physiological differences, leptin mRNA and circulating protein levels were measured. Consistent with the obese phenotype of the Mangalica, leptin mRNA expression was 2.2-fold higher in the subcutaneous adipose tissue of Mangalica versus Yorkshire pigs ($P < 0.01$). Likewise circulating levels of leptin were 1.8-fold higher in Mangalica versus Yorkshire pigs ($P < 0.05$). Next, indices of meat quality were compared in Red, Red x Yorkshire, and Yorkshire pigs given Red Mangalica displayed the highest degrees of marbling. Consistent with growth performance across breeds, backfat thickness was 1.8-fold and 3.4-fold greater in Red than Red x Yorkshire and Yorkshire pigs ($P < 0.0001$), marbling score was 1.5 and 2.8-fold greater in Red than Red x Yorkshire and Yorkshire pigs ($P < 0.005$) and LEA was 1.5-fold and 2.3-fold greater in Yorkshire than Red x Yorkshire and Red pigs ($P < 0.0001$). Loin and ham ultimate pH was significantly greater in Red than Red x Yorkshire or Yorkshire pigs ($P < 0.01$) mirroring color ($P < .005$) and firmness scores ($P < .003$). Cook loss was significantly lesser in Red than Yorkshire pigs ($P < 0.007$) while Warner-Bratzler Shear Force (WBS) was not different in chops between groups ($P < 0.11$). Mangalica breeds contained a greater percentage of monounsaturated and polyunsaturated fatty acids in adipose and muscle compared to Yorkshire. These experiments identify the Blonde Mangalica as the breed with the highest potential to fatten, leading us to conclude that Blonde Mangalicas are best

suited as a model for human obesity. Furthermore, Mangalica and Yorkshire pigs exhibit divergent indices of pork quality supporting the hypothesis that the Mangalica represent a useful model to study pork quality. Collectively, these data indicate that while Mangalica exhibit poorer growth performance, Mangalica pork exhibits superior meat quality attributes suggesting higher price points for Mangalica pork in niche markets are justified.

2.2 Introduction

Recommendations by physicians to limit consumption of animal fat have negatively impacted consumer demand for fatty pork products. In response, pigs have been genetically selected over the past three to four decades to exhibit greater feed efficiency and leanness and this approach has successfully improved the cutability of pork carcasses. However, important pork quality traits such as flavor, juiciness, tenderness, color, and water-holding capacity are often adversely affected in heavier muscled, leaner carcasses (NPPC, 2010). Such decreased pork quality threatens to negatively impact consumer demand, results in significant economic losses, and poses problems for producers that are subject to carcass merit marketing systems (Carr et al., 1997; Brewer et al., 1999).

During this same time span, the U.S. has experienced an obesity epidemic spurring a sharp rise in obesity-associated mortality rates amongst consumers (Calle et al, 2003; Reeves et al., 2007; Emerging Risk Factors Collaboration, 2009; Prospective Studies Collaboration, 2009). The land grant mission dictates that research scientists improve both the economic and physical wellbeing of stakeholders, thus it is incumbent

upon researchers to address issues that negatively impact the individual's health and wealth. Better understanding adipose tissue development would address the need to control adiposity on the growing animal. Such an advance would simultaneously improve production efficiency, allow pork products that are both healthier and more enjoyable to consume, and improve the health of humans through insights that might be applied to human obesity.

Fortunately several newly developed technologies promise to rapidly advance our understanding of factors impacting carcass merit and adipose tissue development. Such experimental techniques as Real-time PCR, Next Generation Sequencing technologies like RNAseq, improved annotation of the porcine genome, and more robust bioinformatics approaches are greatly enhancing the ability of scientists to better study development and disease in the pig. However, in conjunction with the emergence of these powerful research tools, there is also a great need for the creation of novel porcine models that uniquely enable the study of pork quality or that can be leveraged as biomedical models for human disease (NRC, 1998). To address this need for novel swine models, Blonde, Red, and Swallow-bellied Mangalica pigs were recently imported to Auburn University due to their extreme propensity to fatten and reputation for producing superior quality pork.

The value of importing the Mangalica genetics to Auburn University centers upon the extreme phenotype represented by this breed compared to modern swine breeds. The modern, genetically improved Yorkshire is prized for its high feed efficiency, rapid growth, and high reproductive performance, making it the most popular breed of pig for pork production in the United States. The Yorkshire carcass is approximately 70% lean

and only 30% fat (McGlone and Pond, 2003). In contrast, the Mangalica pig, an unimproved, European heritage breed domesticated in order to utilize its lard for high quality sausage, is about 30% lean and 70% fat (Egerszegi et al. 2003). In swine, energy balance is tightly coordinated with developmental trajectory so there is currently no modern swine breed that displays hyperphagia nor are there reports in the literature of a porcine model that spontaneously develops diabetes, two important characteristics of the progressive nature of obesity in humans (McGlone and Pond, 2003; Larsen and Rolin 2004; Kyriazakis and Whittemore, 2006; Bellinger et al., 2006). The Ossabaw pig, when fed a high fat diet, develops a pre-diabetic state whereby normal glucose levels are accompanied by mild hyperinsulinemia and dyslipidemia (Dyson et al., 2006). Göttingen minipigs, by nature of severe experimental insult to their pancreas, mimic the final stages of diabetes (Bellinger et al. 2006; Swindle, 2007). While these two breeds fill useful experimental niches, there remains a need for a model that displays the full progressive nature of obesity-induced metabolic disease as exemplified by the current slippery slope paradigm (NRC, 1998; Bellinger et al. 2006; Wang et al., 2014). A breed of pig that spontaneously displays extreme degrees of adiposity represents attractive potential as a model organism that may exhibit such attributes.

Little information exists in the literature concerning growth performance, body composition, and meat quality traits in the Mangalica pig. To assess the potential of the Mangalica breed to serve as a useful model to study pork quality or human obesity and its metabolic complications, these attributes need to be better characterized. Therefore, the objectives of this study were to 1) to characterize differences in growth performance and carcass merit of purebred Yorkshire, Blonde, Red, and Swallow-bellied Mangalica pigs,

and 2) to compare indices of meat quality in purebred Red Mangalica, Yorkshire and crossbred (Red x Yorkshire) pigs. If the Mangalica and Yorkshire differ phenotypically, then they will exhibit differences in growth parameters and meat quality, with the Mangalica pig displaying higher indices of meat quality due to its high fat content.

2.3 Materials and Methods

Animals and Design

All experimental procedures were approved by the Auburn University Institutional Animal Care and Use Committee. The Auburn University College of Agriculture is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AALAC) and this study was conducted in accordance with the Federation of Animal Science Societies' Guide for the Care and Use of Agricultural Animals in Research and Teaching. Purebred Yorkshire pigs (n=6), red (n=6), blonde (n=6), and swallow-bellied (n=6) lines of Mangalica pigs were housed individually in 12.2m^2 pens at the Auburn University Swine Research and Education Center (SREC) for the duration of these experiments. In addition, Yorkshire boars were crossbred to red Mangalica sows to yield 6 York x Red crosses. All pigs were fed a typical grower ration (17% CP) from 40 to 120 lbs body weight (BW) and a finisher ration (15% CP) from 120 to 240 lbs BW. Pigs were provided *ad libitum* access to water. Daily feed intakes and weekly body weights were recorded during a period spanning growth from 40 lbs live weight until reaching harvest weight of 240 lbs to facilitate measurement of average daily gain (ADG), feed efficiency (lbs gained/ lbs feed), and total feed intake.

Carcass Fabrication

Animals were harvested at the Auburn University Lambert- Powell Meats Lab under USDA-FSIS inspection. Hot carcass weight was recorded after harvest, and carcasses were chilled at 2 ± 1 ° C for 24 h. At 24 h postmortem, carcass pH was recorded in the left side round using a pH Spear probe (Oakton Instruments, Vernon Hills, IL). Both live weight and hot carcass weight were recorded to determine dressing percentage. Following the 24 h chill period, carcasses were ribbed between the 10^{th} and 11^{th} rib. Backfat was measured at the 10^{th} and last rib, and loin eye area (LEA) was also measured. Backfat and LEA were adjusted based on the recommended equation of NPPC (2000). The following equations were used: Adjusted Backfat to 250 lb. = Actual Backfat + [(250-actual wt.) x actual backfat/ (actual wt.-b)] and Adjusted LEA to 250 lb. = Actual LEA + [(250-actual wt.) x actual LEA/ (actual wt. + 155)].

Evaluation of subjective scores for marbling, wetness, firmness, and muscle score were determined by a trained observer using published visual standards (NPPC 2000). Additionally, the longissimus muscle at the 10^{th} rib was evaluated for objective color measurements with a Hunter Miniscan XE Plus (Hunter Lab, Reston, VA) to determine Hunter L*, a*, and b* values. The Miniscan was calibrated according to the manufacturer's recommendations and utilized a D65 light source, a 10° viewing angle, and a 35mm viewing area. Following carcass evaluation, a section of the longissimus muscle was removed from the 11^{th} rib to the last lumbar vertebrae from each carcass to use for meat quality analysis.

Warner-Bratzler Shear Force and Cook Loss

Warner Bratzler shear force (WBS) evaluation was performed using pork loin chops cut to 2.54cm thickness were thawed in a vacuum package bag at approximately 4°C for 24 h. Chops were weighed and placed on a Calphalon Removable Plate Grill (Caphalon, Perrysburg, OH) clamshell style contact grill pre-heated to 177°C. Temperature was monitored with copper constantan thermocouple wire inserted into the geometric center of the chop and attached to a hand-held Omega data logger HH309A (Omega, Stamford, CT) temperature recorder. Chops were cooked for 7 min which resulted in a final temperature of 71°C. Cooked chops were removed and reweighed before being placed on non-absorbent wax-coated paper to cool to room temperature then chops were wrapped in aluminum foil and placed in refrigerator at 4°C for approximately 24 h. Six 1.27cm-diameter cores were removed from each chop with a brass cork borer (Model 1601A Series Brass Cork Borer, Boekel Scientific, Feasterville, PA) parallel to the longitudinal orientation of the muscle fibers. Each core was sheared once at its center using a TA-XT2i Texture Analyser (Texture Technologies Corp., Scarsdale, NY). The peak force measurements were averaged from the six cores of each sample and were used for analysis. The probe was programmed to be lowered 30 mm after detection of resistance. The penetration speed was 3.3 mm/s with a post-test speed of 10 mm/s and a pre-test speed of 2.0 mm/s. Cook loss was measured as the percent of pre-cooked weight lost during cooking and calculated as Cook Loss = (Initial weight – Cooked weight) x 100.

Isolation of Pig Preadipocytes and Mature Fat Cells

Two-day-old Mangalica pigs were obtained from the Auburn University Swine Research and Education Center and harvested by CO_2 asphyxiation in a manner approved by the Animal Care and Use Committee at Auburn University. Primary stromal-vascular (**S-V**) cells and mature fat cells were harvested from neonatal pig adipose tissue by a collagenase digestion procedure as previously described according to Brandebourg et al. (2005a,b) Plating medium consisted of DME/F12 (1:1, vol/vol) containing 15 mmol/L of $NaHCO_3$, 15 mmol/L of HEPES buffer, and 50 mg/L of gentamicin sulfate supplemented with 10% FBS. These cell samples were then snap frozen in liquid nitrogen and stored at -80° C until mRNA analysis.

Gene Expression Analysis

Upon exsanguination, subcutaneous adipose tissues were immediately collected, snap frozen in liquid nitrogen, and stored at -80° C until mRNA analysis. Total RNA was extracted from adipose tissue using a two-step purification protocol with total RNA first being extracted from whole tissue using RNAzol® RT (MRC, Inc, Cincinnati, OH) followed by purification using RNAeasy spin columns (QIAGEN, Inc., Valencia, CA) according to the manufacturers' recommendations. RNA was quantified using a BioTek Synergy 4 plate reader utilizing the Take3 system (BioTek U.S., Winooski, VT) with all samples exhibiting an OD 260/280 between 1.8 and 2.0 and an OD 260/230 value between 1.8 and 2.2. Spectral scans ranging from 200 to 400 nm further verified sample purity as all RNA samples produced smooth curves exhibiting one peak at 260 nm. Total RNA integrity was accessed both visually by resolving 2 μg of RNA on a denaturing formaldehyde gel containing ethidium bromide and by determining an RNA Integrity

Number (RIN) using an Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Clara, CA). All samples demonstrated sharp ribosomal bands with a 28S to 18S ratio greater than 1 and RIN values greater than 7.0 and were thus judged intact and non-degraded. Total RNA was then reverse transcribed using Superscript II reverse transcriptase (Promega Inc, Madison, WI) and oligo-dT primers. Real-time PCR was performed on the resultant cDNA using a Roche Lightcycler® 480 Real-time PCR machine and LightCycler® 480 SYBR Green I Master Mix (Roche Applied Science, Indianapolis, IN) according to manufacturer's recommendations. All PCR reactions were performed using intron-spanning primers under optimized conditions with primer efficiencies ranging between 90-101% as verified with standard curves. Product purity was assessed by melting curve analysis and expected amplicon sizes were verified on a 2 % agarose gel stained with ethidium bromide. Values were normalized to Ribosomal Protein S15 (S15) mRNA expression. The S15 mRNA levels represent an appropriate control as the efficiency of the S15 primers was 100% and S15 mRNA expression was not different between any groups tested ($P < 0.93$). Data are expressed as fold change relative to baseline and calculated according to Pfaffl, 2010.

Leptin Protein Determination

Blood samples were collected, allowed to clot, centrifuged (3000 x g, 10 min, 4ºC) and resulting serum was collected and stored at -80ºC until analysis. Serum leptin concentrations were measured by an ELISA kit from R&D Systems (Minneapolis, MN). The lowest level of detection was 62.5 pg/ml.

Statistical analysis

Changes in gene expression were calculated from the cycle threshold, after correction using S15 expression and analyzed using the Pair Wise Fixed Reallocation Randomization Test of REST-MCS v2.0 (http://rest.gene-quantification.info/). Carcass traits and RNA quality data were analyzed as a completely randomized block design using a mixed linear model of SAS v9.2 with individual animal serving as the experimental unit, i.e. individual block (SAS Institute, Inc., Cary, NC).

2.4 Results

Pigs were randomly assigned to individual pens at 40 lbs live weight and allowed *ad libitum* access to typical growth stage-matched rations until pigs reached approximately 240 lbs live weight. Growth performance was monitored throughout this period and body composition compared at harvest for Yorkshire pigs and the three true breeding varieties of Mangalica pigs. As depicted in Figure 2-1, Mangalica and Yorkshire pigs displayed obvious phenotypic differences with Mangalica representing a lard-type body composition and Yorkshire pigs resembling modern, lean and muscled pigs. Typical true-breeding differences between Mangalica varieties are shown in Figure 2-2. As shown in Table 2-1, all indices of growth performance and body composition measured were significantly different between Yorkshire and Mangalica pigs. At market weight, Mangalica pigs were significantly fatter exhibiting both greater backfat at the 10^{th} rib ($P < 0.0001$) and significantly greater amounts of marbling within the longissimus muscle than Yorkshire pigs ($P < 0.01$). However, Mangalica pigs developed significantly less muscle mass at matched weights than Yorkshire pigs as estimated by *Longissimus*

dorsi muscle area which was greater than twice the size in Yorkshire carcasses compared to the three Mangalica breeds ($P < 0.0001$). These differences in adiposity and muscling were so extreme as to be visually appreciable. (Figure 2-3; Figure 2-4). Consistent with these extreme differences in carcass composition, Yorkshire pigs grew faster as evidenced be higher average daily gains ($P < 0.0001$) and exhibited superior feed efficiency ($P < 0.0001$) compared to Mangalica pigs despite Yorkshires displaying slightly higher voluntary feed intake levels ($P < 0.05$).

To determine if the divergent phenotypes were associated with meaningful physiological differences, leptin mRNA was measured in subcutaneous adipose tissue of market weight Yorkshire and Blonde Mangalica pigs using real-time PCR (Figure 2-5). Consistent with the obese phenotype of the Mangalica, leptin mRNA expression was 2.2-fold higher in the subcutaneous adipose tissue of Mangalica versus Yorkshire pigs ($P < 0.01$). Serum levels of leptin were also measured in these pigs using a porcine-specific ELISA (Figure 2-5). Consistent with adipose tissue gene expression data, circulating levels of leptin were 1.8-fold higher in Mangalica versus Yorkshire pigs ($P < 0.05$).

Differences in growth and body composition parameters were also observed between the three Mangalica pig breeds (Table 2-1). Blonde Mangalica were fatter than their Red and Swallow-bellied counterparts based upon subcutaneous fat thickness ($P < 0.05$). Interestingly, Red Mangalica exhibited significantly higher degrees of marbling than other Mangalica types ($P < 0.05$). Swallow-bellied Mangalica exhibited lower average daily gains ($P < 0.05$) likely due to their lower voluntary intake ($P < 0.05$) and lower muscling ($P < 0.05$) compared to their Blonde and Red counterparts.

Indices of meat quality were compared in Red Mangalica, Red Mangalica x

Yorkshire, and Yorkshire pigs and the data is presented in Table 2-2. There were no differences in live weight between groups indicating differences in carcass parameters were not confounded by slaughter weight ($P < 0.98$). Consistent with the body composition data in Table 2-1, back fat thickness was 1.8 fold and 3.4 fold greater in Red Mangalica than Red Mangalica x Yorkshire and Yorkshire pigs ($P < 0.0001$). Marbling score was 1.5 and 2.8-fold greater in Red Mangalica than Red Mangalica x Yorkshire and Yorkshire pigs ($P < 0.005$), and LEA was 1.5-fold and 2.3-fold greater in Yorkshire than Red Mangalica x Yorkshire and Red Mangalica pigs ($P < 0.0001$). Loin and ham ultimate pH was significantly greater in Red Mangalica than Red Mangalica x Yorkshire or Yorkshire pigs ($P < 0.01$) mirroring color ($P < .005$) and firmness scores ($P < .003$). Cook loss was significantly lesser in Red Mangalica than Yorkshire pigs ($P < 0.007$) while WBS was not different in chops between groups ($P < 0.11$).

2.5 Discussion

There is great need to establish novel biomedical models that allow the study of human obesity and its metabolic complications. Likewise, models that would allow investigation of factors affecting marbling development and pork quality are lacking. The growth trials herein were conducted in order to better characterize the phenotypes of Blonde, Red, and Swallow-bellied Mangalica. This was a necessary first step in determining if this breed can be used to fill these critical gaps in available animal models. These data show that Blonde Mangalica exhibited the greatest adiposity suggesting they represent the most promising breed to be developed as a model for obesity. While all Mangalica breeds demonstrated superior pork quality attributes, the Red Mangalica had

3.5-fold greater marbling compared to Yorkshire pigs. This suggests the Red Mangalica pig is a promising model in which to study factors regulating these parameters.

In the current study, Yorkshire pigs exhibited an ADG of 2.14 lbs/day, a voluntary feed intake (FI) of 5.98 lbs/day and a feed efficiency of .357 for the period on test. Expected industry performance for purebred Yorkshire herds over similar growing periods as this trial range between 1.70-1.95 lb/day for ADG ,4.3-5.5 lbs/day for FI, and .31-.45 for feed efficiency (McGlone and Pond, 2003; Sutherland et al., 2005). Yorkshire pigs in the current study exhibited tenth rib back fat thickness of .92 inches and a LEA of 6.52 in^2 at market weight compared to an industry standard of .9 inches backfat and LEA of 6.2 in^2. Thus, Yorkshire pigs in the current study gained faster but also consumed more feed than typical herds while exhibiting similar adiposity and slightly greater muscularity compared to the current industry standard Yorkshire hog. Such differences could be due to differences in breeding stock, management practices and differences in breeding programs. Also, the swine herd at Auburn University is a naïve herd reared within a biosecure facility. The lack of immune stimulation compared to environments typical of industry may in part explain the greater growth and feed intake in these pigs, though overall feed efficiency in this study was typical for Yorkshires. Finally, pigs were individually penned so while they were able to socialize with neighbors, they did not have to contend with a dominance hierarchy concerning feeding. Nonetheless, growth performance exhibited by Yorkshire pigs in the current study was consistent with expected ranges for this breed nationwide indicating the Yorkshire pigs in the current study represent a valid baseline in which to compare the Mangalica.

Growth performance in the Mangalica pig is poorly characterized with few refereed manuscripts existing in the literature addressing this issue. Furthermore, the existing studies largely characterize Mangalica herds that were reared in what would be considered primitive conditions compared to modern production facilities in the U.S, often involving pasture-based systems characteristic of rural Eastern European subsistence farming. Nonetheless, a survey of such studies indicates that Mangalica pigs exhibit an ADG of .55 lbs/day, a daily feed intake of roughly 5 lbs/day and a feed efficiency of .11 (Egerszegi et al., 2003; Brussow et al., 2005; Ratky et al., 2005). In the current study, all Mangalica breeds studied significantly exceeded those performance standards. This was expected as Mangalica on this trial were given ad libitum access to concentrated, balanced rations formulated to match their stage of growth with the express goal of maximizing their growth rate and potential to fatten. This is in sharp contrast with the nutrition of Mangalica in traditional growing systems in which a significant portion of their diet is met through foraging on pasture and in woodlots.

The Mangalica pig is an interesting heritage breed that was once prized in Hungary for its lard production and superior meat quality. Unlike the modern Yorkshire, which has undergone intense, methodical genetic selection for rapid, lean growth, the Mangalica genetics reflect a general lack of selection pressure. Therefore, Mangalica genetics are best described as remaining primitive or unimproved relative to the breed's derivation. There are three breeds of Mangalica pigs that exist today, the Red, Blonde, and Swallow-belly. The Mangalica breed was first established in the 1830s when efforts were undertaken to create a unique lard-type hog which displayed high quality fat. Initially, the Sumadia pig was crossbred with Hungarian aboriginal breeds, the Alföldi

and the Bakony, to create the Blonde and Black Mangalica. The Blonde and Black were then crossbred to form the Swallow-Belly Mangalica. The Blonde was also crossbred with another aboriginal breed, the Szalonta, to generate the Red Mangalica. The Blonde coat color can range from shades of light gray to yellow, while the Swallow-belly is black with a yellow/blonde throat and underbelly. The Red Mangalica is ginger in color with a slightly thinner and slicker hair coat (Egerszegi et al. 2003). All three pigs have been prized for their hardiness and lard.

Due to their extreme propensity to fatten, the Mangalica are an attractive model to study fat development. Since the three breeds are derived from different lineages, we hypothesized that breed differences may exist that could be experimentally useful, e.g. one breed might be revealed as a good model for studying meat quality, while another breed might be best suited as a model for obesity. After conducting a survey of Pub Med, this paper appears to be the first to characterize growth performance, body composition, and meat quality traits in the three Mangalica pig breeds. Thus, this study represents a necessary and important contribution to the literature.

As expected, Yorkshire and Mangalica pigs demonstrated divergent phenotypes based upon the growth and carcass parameters measured. Importantly, our data also revealed unique differences in growth performance and body composition between the three Mangalica breeds. Blonde Mangalica exhibited the highest degree of adiposity based upon subcutaneous fat thickness at the tenth rib. Given this, the Blonde Mangalica represents the most promising breed to further develop as a biomedical model for human obesity. Surprisingly, other phenotypic differences emerged between Mangalica breeds. The Red Mangalica exhibited the most intramuscular fat of any breed examined. In

addition, when Red Mangalica pigs were crossbred with the Yorkshire, the F1 crosses displayed higher marbling scores and significantly lower cook loss versus the purebred Yorkshires. Such crosses provide a potentially powerful new tool for studying the genetic basis for marbling in pigs.

Pork quality continues to be a serious issue in the pork industry. The emphasis on selecting pigs for leanness has resulted in a reduction in pork quality due to a loss of color and intramuscular fat (NPB, 1998). Color is the most important appearance quality trait affecting the visible appeal of pork to consumers (Faustman and Cassens, 1991; Cheftel and Culioli, 1997; Risvik, 1994). Marbling is an important sensory trait that contributes to the juiciness and flavor of the product and is another key criteria impacting consumer choice at the meat counter (NPB, 1998). Unfortunately, selection for leaner pigs has generally reduced the marbling content, contributing to a less satisfying eating experience by the consumer (NPB. 1998). This has led to the creation of niche markets whereby consumers are willing to pay a premium for high quality pork products, especially at high end restaurants (Honeyman et al., 2006). Mangalica pork is currently being marketed by targeting such niche markets given the breed's reputation for exhibiting superior pork quality and due to the higher price point necessitated by the breed's poor growth performance. However, this reputation for superior quality is largely inferred due to the breed's derivation, place in Eastern European cultural history, and the word of mouth of modern day chefs. Little data exists in the literature to verify these claims. In the current study, Red Mangalica pork exhibited significantly higher marbling, firmness, and color scores, while exhibiting lower cook loss consistent with the perception of juicier chops. Collectively, these data indicate that while Mangalica pigs

exhibit poorer growth performance, Mangalica pork displays superior meat quality attributes suggesting higher price points for Mangalica pork in niche markets are justified.

Figure 2-1. Visual comparison of weight-matched Blonde Mangalica (*left*) and Yorkshire (*right*) pigs as they neared harvest weight.

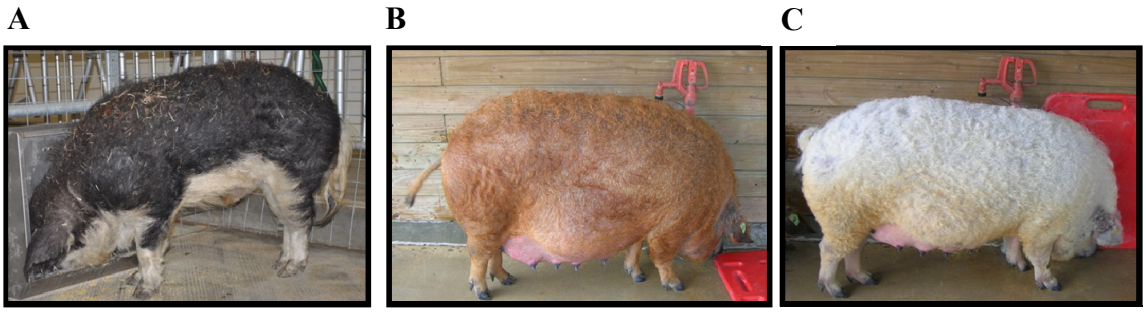

Figure 2-2. True breeding characteristics of the Swallow-bellied Mangalica (***Panel A***), the Red Mangalica (***Panel B***), and the Blonde Mangalica (***Panel C***). Animals depicted are not age-, live weight-, or gender matched.

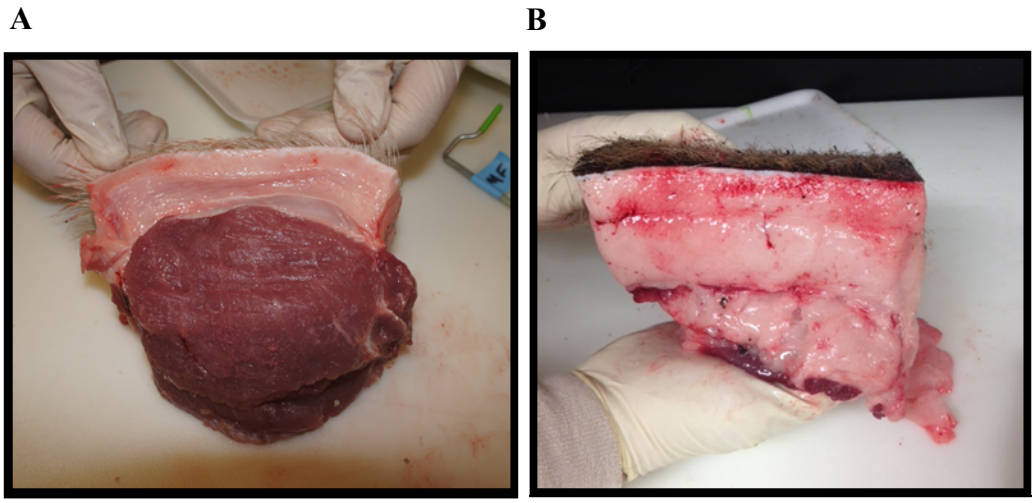

Figure 2-3. Depiction of differences in subcutaneous fat development and thickness at 240 lbs slaughter weight between Yorkshire (***Panel A***) and Blonde Mangalica (***Panel B***) pigs. Tissue was harvested immediately following exsanguination by dissecting a plug of tissue over the tenth rib from the skin to the underlying *longissimus* muscle. Three distinct adipose tissue layers are visible on the Mangalica sample while the much thinner Yorkshire sample is devoid of the inner layer of subcutaneous fat and exhibits much thinner existing layers compared to the Mangalica.

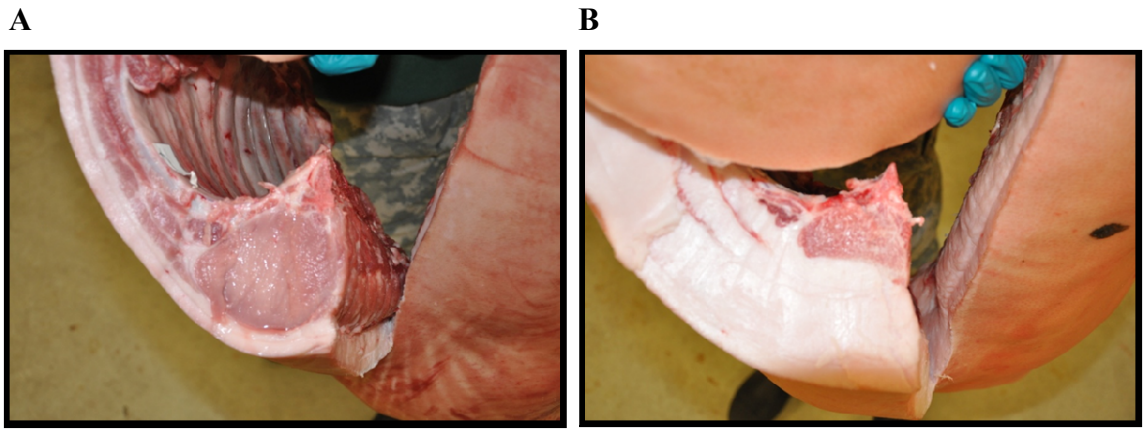

Figure 2-4. Depiction of differences in back fat and loin eye area on carcasses at 240 lbs slaughter weight between Yorkshire (***Panel A)*** and Blonde Mangalica pigs (***Panel B***). Carcasses were split at the tenth rib to measure backfat thickness and loin eye area following storage at 4 °C for 24 h. These representative images are consistent with carcass data reported in Table 2-1.

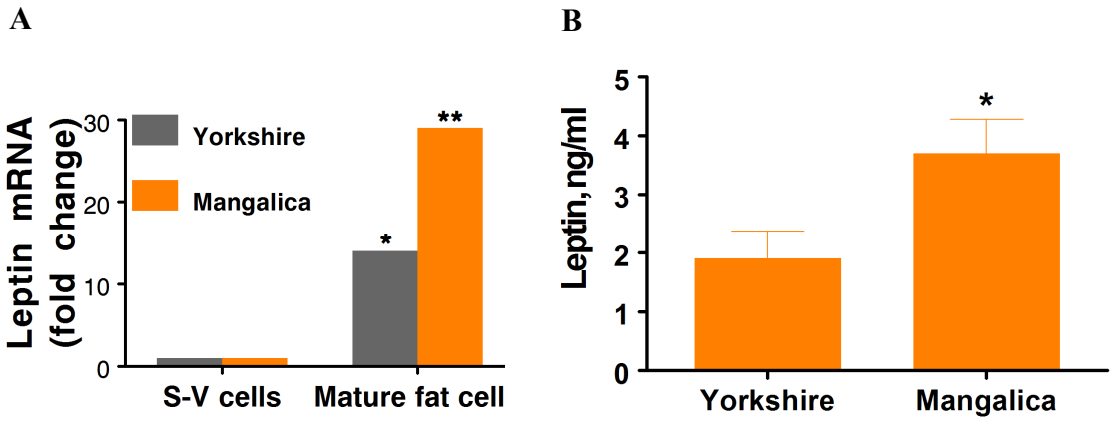

Figure 2-5. The expression of leptin mRNA and protein as markers for adiposity in lean Yorkshire and obese Mangalica pigs. ***Panel A***: *Leptin* mRNA levels in preadipocytes (S-V cells) and adipocytes harvested from the subcutaneous adipose tissue. ***Panel B***: Leptin protein levels measured in the circulation of market weight pigs. These data are consistent with increased adiposity in obese versus lean pigs. The mRNA levels were measured using real-time PCR. Expression levels were normalized relative to the porcine S15 gene and are presented as fold change relative to expression in lean pig values. Protein levels were measured using an ELISA assay.

Table 2-1. Growth performance and body composition of Yorkshire and three Mangalica breeds fed ad libitum[1,2]

Variable	Yorkshire	Blonde	Red	Swallow-belly	SEM	*P*-Value
Subcutaneous fat, in.	0.92[c]	2.63[a]	2.28[b]	2.28[b]	0.08	0.0001
Intramuscular fat	1.20[c]	2.53[b]	4.17[a]	2.67[b]	0.55	0.01
L.dorsi muscle area, in^2	6.52[a]	3.1[b]	3.17[b]	2.72[b]	0.19	0.0001
Average daily gain, lb	2.14[a]	1.24[b]	1.37[b]	0.89[c]	0.07	0.0001
Daily feed intake, lb	5.98[a]	5.06[b]	5.35[b]	4.2[c]	0.21	0.0002
Feed efficiency	0.357[a]	0.245[b]	0.256[b]	0.212[c]	0.006	0.0001

[1]Yorkshire breed has been selected for rapid growth; Mangalica breeds: Blonde, Red, Swallow-belly.

[2]Values are group mean ± SEM, n=6, differing superscripts within a variable denote differences between breeds $P < 0.05$

Table 2-2. Meat quality traits by group[1]

Variable	York	York x Red	Red	*P*-value
Number of pigs	6	6	6	NA[2]
Live weight, kg	243.8 ± 7.3	243.3 ± 7.3	245.8 ± 7.3	0.98
Back fat, in.	0.77 ± 0.11	1.44 ± 0.11	2.64 ± 0.11	0.0001
Marbling score	1.62 ± 0.39	2.93 ± 0.39	4.45 ± 0.39	0.0005
LEA, in^2	8.5 ± 0.47	5.8 ± 0.47	3.8 ± 0.47	0.0001
Loin Ultimate pH[3]	5.36 ± 0.11	5.54 ± 0.11	5.86 ± 0.11	0.01
Ham Ultimate pH[3]	5.43 ± 0.09	5.67 ± 0.09	5.97 ± 0.09	0.003
Color	3.89 ± 0.25	3.53 ± 0.25	4.90 ± 0.25	0.05
L^*, lightness	60.4 ± 1.63	60.8 ± 0.8	51.9 ± 1.63	0.002
a^*, redness	10.2 ± 0.3	9.6 ± 0.3	11.9 ± 0.3	0.0004
b^*, yellowness	17.4 ± 0.5	17.3 ± 0.5	16.0 ± 0.5	0.10
Cook loss, %[5]	15.3 ± 0.98	12.8 ± 0.98	10.2 ± 0.98	0.0071
WBS	3.77 ± 0.16	3.43 ± 0.16	3.85 ± 0.16	0.18

[1]Values are lsmeans ± SEM

[2] NA=not applicable;

[3]Ulimate pH: measured 24 h post-harvest on chilled carcasses

[4] Visual (subjective) color score: five point scale where 1= very light and pale; 5= dark red etc.

[5] Subjective Marbling Score: 1 to 2.4= Devoid; 2.5 to 4= Traces; 4 to 5 = Slight; etc.

Chapter 3:

The Blonde Mangalica Models the Progressive Nature of Obesity Induced Metabolic Disease

3.1 Abstract

Novel animal models of juvenile, hyperphagic obesity and its complications are needed in order to more quickly develop effective strategies for the intervention or long-term prevention of obesity in susceptible individuals. Our objective was to characterize the metabolic phenotype and gut microbiota of genetically obese Mangalica pigs, a unique breed whose excessive adiposity is driven by hyperphagia. Obese or lean groups were created by either allowing *ad libitum* access to feed or restricting energy intake to 40% of *ad libitum* levels. Obese pigs exhibited 2.5-fold greater subcutaneous adipose tissue mass ($P < 0.001$) but no differences in muscle mass ($P < 0.39$) compared to their lean counterparts. Obese pigs displayed impaired glucose tolerance and hyperinsulineamia following oral glucose challenge ($P < 0.001$). Dyslipidemia was also indicated by elevated levels of fasted plasma triglycerides ($P < 0.05$) and total cholesterol ($P < 0.05$). Obese animals displayed a more severe response to acute endotoxin challenge than their lean counterparts, based upon rectal temperatures, and plasma tumor necrosis factor-alpha, cortisol, and glucose values suggesting obesity in these pigs was associated with altered immune function. Consistent with obesity-induced inflammation, the mRNA expression of the proinflammatory cytokines, tumor necrosis factor-alpha and interleukin-6 was higher in the arcuate nucleus ($P < 0.01$) and subcutaneous adipose tissue ($P < 0.01$) of obese versus lean pigs. Consistent with what is observed in human obesity, as

animals aged and increased in adiposity, a general reduction in the overall diversity in the gut bacteria was observed with several other changes in specific bacterial taxa as indicated by denaturing gradient gel electrophoresis. Finally, in order to better understand the hyperphagia displayed by these breeds, a 30% fat diet was fed to assess the ability of energy density to depress voluntary feed intake in obese pigs. The high fat diet failed to alter feed intake compared to the control diet indicating mechanisms regulating satiety are impaired in these pigs ($P > 0.93$). These data provide evidence that obese Mangalica pigs indeed develop a metabolic phenotype consistent with insulin resistance and the development of an inflammatory state. Furthermore, obesity-related changes in the gut microbiota mirror those seen in obese humans. These data are consistent with the hypothesis that obese Mangalica pigs represent a faithful model of obesity-induced metabolic disease in humans.

3.2 Introduction

A worldwide obesity epidemic represents a critical threat to public health by precipitating increased risk to a growing number of individuals for developing diabetes, heart disease, stroke, arthritis, and certain cancers (Jensen, 2011). For instance, approximately 34.9% of today's US adult population has a body mass index (BMI) equal to or greater than 30 kg/m^2, and thus are clinically classified as obese (Ogden et al., 2014). In 2012, an estimated $190.2 billion was spent nationwide in the US on treating obesity related illness (CDC, 2015). Furthermore, there has been an alarming increase in the incidence of obesity and type 2 diabetes (NIDDM) among juveniles since 1980 which suggests the societal impact of obesity-related issues will only intensify (Niehues et al., 2014; Ogden et al., 2014). Unfortunately, no effective

strategies currently exist for the intervention or long-term prevention of obesity in susceptible individuals. To address this limitation, there is great need to develop novel animal models that mimic the etiology of obesity and the subsequent progression of metabolic dysregulation, especially during prepubescence.

The pig is well positioned as a biomedical model that can be used to overcome limitations inherent in using rodent models for the study of metabolic syndrome and obesity (Spurlock and Gabler, 2008). For instance, pigs are closer phylogenetically to humans, both are omnivores, and like in humans, anatomically discreet depots of brown fat are largely absent in the pig while the vasculature, the proportion of skeletal muscle and adipose tissue to total body mass and circulating levels of glucose are very similar in the pig and humans (Lunney, 2007; Spurlock and Gabler, 2008). Likewise, there is a well-developed literature concerning the use of swine to study atherosclerosis, cardiovascular disease, and diabetes (Skold et al., 1966; Gerrity et al., 2001; Brambilla and Cantafora, 2004; Dyson et al., 2006). Furthermore, multiple porcine models for obesity currently exist but each are not without limitations. For instance, adult, obese Ossabaw pigs exhibit mild insulin resistance which is exacerbated when fed a high fat diet for 5 months (Dyson et al., 2005). However, these pigs do not appear to manifest metabolic disturbances during prepubescence and they do not display the full progression of obesity-induced metabolic complications seen in humans (Davey and Berskin, 1977; Wangness et al., 1977; Mersmann et al., 1982; Mersmann, 1986; Dyson et al., 2006). There currently is no established porcine model of hyperphagic obesity and frank metabolic disease (Larsen and Rolin 2004; Bellinger et al., 2006).

The Mangalica pig is a novel, obese native swine breed of Hungary. Within the Mangalica breed, there are three types representing a continuum in propensity to fatten. Blond Mangalica are the fattest and can reach an adult body composition where adipose tissue comprises 70% of their total muscle, bone and fat mass (Rátky J et al., 2005). This extreme, early onset, morbidly obese phenotype is driven by a voluntary energy intake representing three times that required to maximize muscle and skeletal growth in this breed (Roberts et al., 2015). To date no report exists characterizing the metabolic phenotype resulting from this hyperphagic obesity despite the Mangalica pig displaying great potential to serve as a relevant animal model of obesity-induced disease given this unique phenotype.

It is widely accepted that the composition of the gut microbiota in obese individuals is distinct from that of lean individuals wherein obese individuals display a more proinflammatory microbiota that is accompanied by an increase in bacterial genes involved in polysaccharide metabolism, potentially increasing the capacity for energy harvest from foods (Backhed et al., 2004; Backhead et al., 2005; Backhead et al., 2007; Turnbaugh et al., 2010). Furthermore, fecal transplantation from lean donors into patients with metabolic syndrome can ameliorate insulin resistance in recipients while donor body composition is replicated in gnotobiotic mice following fecal transplant from twins discordant for obesity (Turnbaugh et al., 2009; Sanz et al., 2010; Kootte et al., 2012; Ridaura et al., 2013). Thus, the gut microbiota appears to represent an important aspect in the etiology of obesity and obesity-induced changes in the microbiota may in part underlie the development of obesity-induced inflammation.

The aim of this study was to validate the Mangalica pig as a faithful model for hyperphagic obesity and its complications. Our objectives were to 1) determine if obese Mangalica pigs develop markers of inflammation and metabolic dysregulation, 2) evaluate changes in the microbiota as obesity manifests in these animals and 3) determine if high fat diets depress voluntary feed intake in obese pigs. The resulting data are consistent with the hypothesis that obese Mangalica pigs represent a faithful model of obesity-induced metabolic disease and that obesity-related changes in the gut microbiota in these pigs mirror those seen in obese humans.

3.3 Materials and Methods

Animals and Design

All experimental procedures were approved by the Auburn University Institutional Animal Care and Use Committee. The Auburn University College of Agriculture is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AALAC) and this study was conducted in accordance with the Federation of Animal Science Societies' Guide for the Care and Use of Agricultural Animals in Research and Teaching**.** Twenty-two weaned Mangalica pigs were obtained from the Auburn University research herd housed at the Auburn University Swine Research and Education Center. Pigs were individually housed in pens 12.2m^2 in size which provided *ad libitum* access to water. To establish lean and obese groups, some pigs were allowed *ad libitum* access to the basal diet which met or exceeded nutrient recommendations (NRC, 2012) while other pigs were fed the basal diet at levels that were 40% of the voluntary intake of their *ad libitum* fed counterparts. Since Mangalica pigs voluntarily consume 3-fold more

energy than needed to maximize muscle and bone growth, pigs on the restricted diet still achieved healthy, normal growth. Given Mangalica pigs exhibit hyperphagia and that swine in general regulate their energy intake, a subset of obese pigs were allowed *ad libitum* access to the basal diet supplemented with 30% soybean oil for 49 d in order to examine the effect of energy density on voluntary feed intake compared to their obese counterparts provided *ad libitum* access to the basal diet alone. Thus treatment groups for this study included lean pigs fed the basal diet on a calorie restricted basis (n=6), obese pigs allowed *ad libitum* access to the basal diet (n=8) and obese pigs allowed *ad libitum* access to the basal diet supplemented with 30% dietary fat (n=8).

Ultrasound

Real-time ultrasound was performed on all pigs to assess body composition in growing animals by determining on test ultrasound 10^{th} rib subcutaneous fat depth and Longissimus muscle depth according to Perkins et al., (2014a,b). All ultrasound data were collected by the same Ultrasound Guidelines Council certified technician using an Aloka 500 (Aloka America, Wallingford, CT) with a 17 cm transducer using CUP Lab image capture software.

Jugular Catheterization

Access to feed and water was withheld for 12h prior to surgery. An anesthetic drug combination (TKX) containing a final concentration of 50 mg/ml tiletamine, 50 mg/ml zolazepam, 100 mg/ml ketamine, and 100 mg/ml xylazine was prepared by reconstitution of 1 vial of Telazol® (Zoetis Inc., Kalamazoo, MO) with 2.5 ml of

ketamine (100 mg/ml); (Ketacine®, Putney Inc., Portland, ME) and 2.5 ml of xylazine (100 mg/ml); (Anased®, Lloyd Inc., Shenandoah, IA). Pigs were restrained using a sorting board and anesthesia was induced by an intramuscular injection of 1 ml of the TKX combination per 45 kg body weight, delivering 2.2 mg/kg BW tiletamine, 2.2 mg/kg BW zolazepam, 2.2 mg/kg BW ketamine, and 2.2 mg/kg BW xylazine.

Upon torpor, pigs were placed in dorsal recumbency and hair covering the area of the jugular furrow was clipped using electric clippers (Oster Professional Products, Mississauga, Ontario, Canada). The surgical site was scrubbed three times with 10% povidone iodine (Pivodine Solution, MWI, Boise, ID) and rinsed with isopropyl alcohol. Prior to incision, 10 – 15 ml of a 2% lidocaine solution was administered subcutaneously at the proposed incision site. A 10 – 15 centimeter incision was made through the skin and cutaneous trunci muscles over the area of the jugular furrow. Subcutaneous tissues were bluntly dissected to expose the ventral surfaces of the sternocephalicus and brachiocephalicus muscles. Blunt dissection was continued in the tissue plane between these two muscles to locate the external jugular vein. The jugular vein was carefully dissected free from adjacent loose connective tissue and the vein bluntly undermined until it was freely movable and could be elevated to the incision site. A loop of chromic gut (Catgut Chrom, Braun Medical Inc., Bethlehem, PA) was passed around the proximal exposed portion of the vein and the hemostat applied to the suture loop to occlude the vein.

The catheters were inserted by utilizing a commercially available catheter kit (Long Term Catheter, MILA International, Inc.) and the Seldinger technique

(Seldinger, 1953). Briefly, a flexible 4 cm 16 gauge over the needle catheter (20 cm) included with the kit was introduced into the lumen of the jugular vein and advanced proximally as the hemostat securing the previously placed suture loop occluding the proximal portion of the jugular vein was released. The needle was removed from the flexible catheter and a 19-gauge guide introducing wire threaded through the catheter and into the vessel lumen utilizing the wire dispenser provided. When the introducing wire was in the desired depth, the over the needle catheter was drawn over the exposed portion of the guide wire and discarded. A 20 cm, 16-gauge catheter over the wire catheter was then threaded onto the introducing wire and advanced into the vessel. The guide wire was then carefully removed. When fully introduced, catheter patency was checked by aspiration of blood and the catheter flushed with heparinized saline. The catheter port was secured in place near the jugular vein utilizing suture anchors present on the port. A 53 cm extension set (Baxter Healthcare Cooperation, Deerfield, IL) was attached to the catheter and placed into the incision, exiting the skin at the cranial portion of the surgical wound, leaving approximately 12 cm protruding. The surgical wound was then closed routinely. Following closure of the skin, the exposed portion of the catheter extension was directed caudo-dorsally and threaded through a rubber pouch which was subsequently glued to the neck with an adhesive (Kamar®, Kamar Products Inc., Zionsville, IN) and the edges of the pouch sutured to skin for additional support. Proper function of the catheter was again checked by aspiration of venous blood and the catheter again flushed with heparinized saline. Following surgery, animals were placed in sternal recumbency and allowed to recover from anesthesia in individual

pens.

Post operatively, flunixin meglumine (1.1 mg/kg); (Banamine®, Merck Animal Health) was administered intravenously immediately following surgery and for two days post surgery. Oxytetracycline (45 mg/kg BW); (Liquamycin® LA 200®, Zoetis Inc., Kalamazoo, MO) was administered intramuscularly on the day of surgery and for two days post operation at half dosage to minimize the chance of post-operative sepsis. For the duration of the study, catheters were flushed twice daily with 30 ml heparinized saline to maintain patency.

Oral Glucose Tolerance Test (OGTT), Endotoxin Challenge and Clinical Characteristics

Lean and obese pigs were subjected to an OGTT when obese pigs reached an average body weight of 390 lbs. Pigs were fasted for 24 h and then offered a control diet equal to 1% of their body weight that had been supplemented with glucose equivalent to 2 g per kg BW. Blood was obtained 15 min before and 15, 30, 60, 120, and 180 min after consumption of the glucose dose. Blood was directly analyzed for glucose using a glucometer (One Touch, Lifescan, Milpitas, CA). Values obtained by the glucometer were validated against values obtained using a clinical glucose analyzer (YSI 2300 STAT Plus, YSI Inc., Yellow Springs, OH) and found to be consistently parallel. Blood samples were centrifuged (3000 x g, 10 min, 4ºC) and resulting plasma was collected and stored at -80ºC until analysis. Plasma insulin (porcine insulin ELISA kit, ALPCO, Salem, NH), cortisol (Coat-a-Count RIA kit, Diagnostic Products Corporation, Los Angelas, CA), and TNFα (Endogen, Woburn, MA) were determined using commercially available kits according to manufacturer

instructions. Fasted plasma triglycerides, cholesterol and blood metabolites were measured following a 24 h fast at the Clinical Pathology Lab at Auburn University College of Veterinary Medicine.

For endotoxin challenges, each animal served as its own control. Baseline measurements were recorded by conducting a control experiment in which saline was administered intramuscularly. Two days after the control experiment, LPS (25 μg/kg body weight (BW), Escheridia coli serotype 055:B5, Sigma, St Louis, MO) was administered intramuscularly. On both days, blood samples were collected beginning 1 hr prior to injection and hourly for 8 hours following administration of vehicle for determination of hormone (plasma cortisol, tumor necrosis factor alpha, insulin), metabolite (glucose, nonesterified fatty acids), and plasma haptoglobin profiles. Rectal temperatures were recorded hourly as an indication of the clinical response to injection.

Gene expression analysis

Total RNA was extracted from hypothalamic and adipose tissue using a two-step purification protocol with total RNA first being extracted from whole tissue using RNAzol® RT (MRC, Inc, Cincinnati, OH) followed by a second purification using RNAeasy spin columns (QIAGEN, Inc., Valencia, CA) according to the manufacturers' recommendations. RNA was quantified and assessed for purity using a BioTek Synergy 4 plate reader utilizing the Take3 system (BioTek U.S., Winooski, VT). Total RNA integrity was accessed both visually by resolving 2 μg of RNA on a denaturing formaldehyde gel stained with GelRed Nucleic Acid stain and by determining an RNA Integrity Number (RIN) using an Agilent 2100 bioanalyzer

(Agilent Technologies, Inc., Clara, CA). Total RNA was then reverse transcribed using 160 units of Superscript II reverse transcriptase (Promega Inc, Madison, WI) and 0.5 μg Oligo$(dT)_{15}$ primers in a reaction volume of 20 μl also containing 1 μg RNA/reaction, 6 mM $MgCl_2$, 0.5 mM each of dNTP, and 20 units RNasin with the reaction being performed in a single cycle with the following steps: heating for 5m at 65 °C, annealing for 5m at 25 °C, elongation for 50m at 42 °C and heating for 15m at 70 °C. The cDNA was subsequently stored at -80 °C until used in gene expression assays. Real-time PCR was performed on the resultant cDNA using a Roche Lightcycler® 480 Real-time PCR machine and LightCycler® 480 SYBR Green I Master Mix (Roche Applied Science, Indianapolis, IN) according to manufacturer's directions Each sample was run in three separate PCR runs with resulting Cp values averaged across values obtained from three separate plates. All PCR reactions were performed using intron-spanning primers under optimized conditions with primer efficiencies ranging between 90-100 % as verified with standard curves. Product purity was assessed by melting curve analysis and expected amplicon sizes were verified on a 2 % agarose gel stained with GelRed Nucleic Acid stain. Values were normalized to *S15* mRNA expression and *S15* mRNA expression was not different between any groups tested ($P > .05$). Data are expressed as fold change relative to baseline and calculated according to Pfaffl, (2010).

DNA extraction from fecal samples

To analyse total faecal microflora, faecal samples were homogenized in RNAlater extracted according to the method described by Matsuki *et al*. (2004), and

the quality of DNA was determined by electrophoresis in 1% (w/v) agarose gels. Extracted DNA samples were stored at −20°C until use.

Denaturing gradient gel electrophoresis (DGGE)

For DGGE, the V3 variable region of bacterial 16S rDNA was amplified by PCR using the primers described by Muyzer *et al*. (1993). A 'touchdown' PCR was performed following the program described by Lubbs *et al*. (2009). The products were determined by electrophoresis in 2% (w/v) agarose gels. Denaturing gradient gel electrophoresis was performed whereby PCR products were separated on 8% (w/v) polyacrylamide gels (generated from 40% acrylamide–bisacrylamide 37·5:1 stock solution; Amresco, Solon, OH, USA) in 0·5× Tris–acetate–EDTA (TAE) buffer along a 30–60% linear denaturing gradient (Muyzer *et al*. 1993). Electrophoresis was performed in 0·5× TAE at 150 V and 60°C for 7 h. Gels were stained by GelRed (Biotium, Hayward, CA, USA) for 15 min and photographed. In each gel, one control sample was loaded to the outside lanes as the marker, and all gel images were aligned and merged into one image according to the marker (Gafan *et al*. 2005).

DGGE Gel analysis

The DGGE gel image was analysed using Quantity One software (Bio-Rad) according to the user guide. The gauss trace quantity and relative intensity of each band (total intensity of all bands in each lane was defined as 100%) in the lane were calculated. The band type tables with relative intensity values and gauss trace quantities were exported to Excel (Microsoft, Mountain View, CA, USA). To visualize the similarity of the faecal microflora from samples, a dendrogram was constructed based on Dice's coefficient (UPGMA algorithm) implemented in the

Quantity One software (Fuentes *et al*. 2008). The Shannon–Wiener diversity index (Shannon index) of samples was calculated by Shannon calculator (www.changbioscience.com/genetics/shannon.html) based on gauss trace quantities (Gafan *et al*. 2005).

Statistical analysis

Changes in gene expression were calculated from the cycle threshold, after correction using S15 expression and analyzed using the Pair Wise Fixed Reallocation Randomization Test of REST-MCS v2.0 (http://rest.gene-quantification.info/). Growth and clinical characteristics were analyzed as a completely randomized block design using a mixed linear model of SAS v9.2 with individual animal serving as the experimental unit, i.e. individual block (SAS Institute, Inc.,
Cary, NC).

3.4 Results

Growth and Body Composition Characteristics

To create lean and obese cohorts, all animals were fed the same base diet but pigs randomly assigned to the obese group were allowed *ad libitum* access to feed for the duration of the trial while pigs assigned to the lean control group were restricted to a caloric intake representing 40% of voluntary intake of the obese group. Initial body weights were not different between the lean and obese groups ($P > 0.92$), while obese pigs weighed 34% more than their lean counterparts by the end of the trial ($P < 0.001$; Figure 3.1). Body composition was assessed by determining ultrasonic subcutaneous fat depth and *Longissimus* muscle depth. Subcutaneous fat depth was

increased 67% in obese versus lean pigs ($P < 0.01$) while muscle depth between the two groups was not significantly different ($P > 0.56$) suggesting differences in body weight were reflective of differences in adiposity rather than skeletal muscle mass (Figure 3.1). Furthermore, these data indicate that Mangalica pigs express a voluntary feed intake that is 2.5 times that necessary to achieve mature muscle mass and frame size. To assess adiposity molecularly, real-time PCR was used to measure mRNA expression of four marker genes in the subcutaneous adipose tissue of lean and obese pigs (Figure 3.2). Expression of these genes are known to increase with adiposity. Consistent with ultrasonic imaging that indicated obese pigs are significantly fatter, the mRNA for *leptin* ($P < 0.05$), *adiponectin* ($P < 0.05$), *peroxisome proliferater activated receptor gamma* (*PPARγ*), and *steroyl coenzyme A desaturase* (*SCD*) were upregulated 3.44-, 3.96-, 2.90- and 4.30-fold respectively in the adipose tissue of obese pigs relative to their lean counterparts (Figure 3.2).

Because Mangalica pigs represent a model of hyperphagic obesity that displays elevated leptin levels and pigs in general regulate their energy intake to coordinate their energy plane with their developmental trajectory, obese pigs were allowed *ad libitum* access to either the control diet or a high fat diet in which the control diet was supplemented with 30% soybean oil for 49 days and performance parameters were evaluated. As expected, obese pigs fed the high fat diet displayed a 296% higher total gain in body weight ($P < 0.001$), a 306% higher average daily gain ($P < 0.001$), and were 233% more efficient at converting the ration to weight gain ($P < 0.01$) compared to their obese counterparts fed the control diet (Figure 3.3). However, surprisingly, feed intake was not different in the control and high fat diet

groups pointing to severe dysregulation in the mechanism governing feed intake in obese pigs ($P > 0.94$). Daily high and low ambient temperatures are shown in Figure 3.4. There were no extended periods where the pigs on trial would be assumed to be under severe cold stress based upon the live weights of the obese pigs during the 7 weeks of the high fat feeding experiment.

Oral Glucose Tolerance Test

Pigs were fasted for 24 hours and subsequently challenged with an oral dose of glucose (2 grams/kg BW) whereby blood samples were collected at -15, 0, 15, 30, 60, 120, and 180 minutes relative to glucose administration to determine the potential impact of adiposity on blood glucose and insulin values in lean versus obese groups. Importantly, fasted glucose levels were significantly higher in obese versus lean pigs ($P < 0.05$; Figure 3.5). In response to glucose administration, a significant increase in blood glucose levels was observed from baseline by 15 minutes in lean and obese pigs with values being significantly higher in obese versus lean pigs at the peak of the curves (Figure 3.5). Furthermore, blood glucose values returned to baseline levels by 30 minutes in lean pigs after the initial dose, while glucose remained elevated in obese pigs, with values not returning to baseline levels until 180 minutes post dosing (Figure 3.5).

Plasma insulin values were also measured following glucose administration. Consistent with the glucose data, fasted insulin levels were significantly higher in obese versus lean pigs ($P < 0.05$; Figure 3.6). Similar to the glucose response, plasma insulin levels rose significantly from baseline by 15 minutes in both lean and obese pigs with peak insulin values being almost twice as high in obese versus lean pigs

(Figure 3.6). Insulin values returned to baseline levels by 120 minutes in both lean and obese pigs but insulin levels were significantly higher in obese versus lean pigs at every time point measured ($P < 0.01$; Figure 3.6).

Several indexes of insulin sensitivity were then utilized to assess plasma glucose and insulin values from the OGTT to determine if obese pigs developed an insulin resistant state (Table 3.1). Utilizing fasted values, HOMO and QUICKI indexes both indicated that obese pigs displayed impaired insulin sensitivity. Using peak curve values compared to fasted baseline, the Masuda index likewise indicated the development of insulin resistance in obese pigs compared to their lean counterparts.

Clinical Characteristics

To observe indications of dyslipidemia, fasted plasma triglycerides and total cholesterol were measured in lean and obese pigs (Figure 3.7). Consistent with altered lipid metabolism in obesity, plasma triglyceride levels were 76% higher in obese versus lean pigs ($P < 0.001$). Likewise, total cholesterol levels were 33% higher in obese pigs versus their lean counterparts ($P < 0.05$).

Next, to assess the impact of divergent body composition on acute inflammation in these pigs, an endotoxin (LPS) challenge was performed (Figures 3.8 thru 3.11). Each animal was utilized as its own control. Thus two days prior to injection of LPS, animals were injected with saline and plasma samples collected at -60, 0, 60, 120, 180, 240, 300, 360, and 420 minutes relative to injection in order to establish baseline values for rectal temperature and plasma parameters. The baseline mean rectal temperatures were 101.1 ± .17 and 101.0 ± .18 for the lean and obese

groups respectively ($P > 0.99$; Figure 3.8). Saline injection did not affect rectal temperature in either the lean or obese groups ($P > 0.92$; Figure 3.8). Following injection of LPS expected clinical responses were observed in the pigs: Within 60 minutes post-injection, both the lean and obese pigs became lethargic, went off feed, and experienced a febrile response. Lean and obese pigs exhibited a significant rise in rectal temperature by 60 minutes post injection ($P < 0.05$). The lean pigs returned to baseline rectal temperatures by 240 minutes post injection of LPS while, conversely, obese pigs did not return to baseline rectal temperatures during the timeframe measurements were recorded indicating that obese pigs mounted a significantly greater febrile response to LPS injection (Figure 3.8).

The mean baseline plasma tumor necrosis factor alpha (TNFα) values were approximately 2-fold higher for obese versus lean pigs suggesting the development of an inflammatory state in obese pigs ($P < 0.05$; Figure 3.9). Following LPS injection, plasma TNFα levels peaked at 1997 pg/ml by 60 minutes post-injection whereas the peak value in lean pigs was 1278 pg/ml ($P < 0.01$). The circulating levels of TNFα returned to baseline an hour later in obese versus lean pigs ($P < 0.01$; Figure 3.9).

The mean baseline plasma cortisol values were approximately 1.5-fold higher for obese versus lean pigs prior to LPS injection ($P < 0.05$; Figure 3.10). Following injection, plasma cortisol levels rapidly increased by 60 minutes post-injection peaking by 240 minutes at levels nearly 20-fold higher than baseline. The cortisol response was significantly lower in lean answers by comparison with peaked values only reaching 10-fold those of baseline values ($P < 0.01$; Figure 3.10). The circulating levels of cortisol returned to baseline in lean pigs by 420 minutes,

meanwhile plasma cortisol remained elevated at nearly 10-fold baseline values in the obese pigs ($P < 0.01$; Figure 3.10).

Glucose levels were stable in obese pigs in response to saline injection, but were slightly above those of the lean pigs, averaging 110 mg/dL ($P < 0.05$; Figure 3.11). This is consistent with obese pigs displaying hyperglycemia. Following LPS injection, blood glucose levels in obese pigs decreased significantly by 120 minutes ($P < 0.01$) and levels remained below baseline values throughout the duration in which measurements were recorded. Meanwhile, blood glucose levels of lean pigs dropped to a much lesser extent compared to control values and normalized to baseline by 6 hours after LPS was administered (Figure 3.11). Overall, lean pigs displayed a 25% decrease in plasma glucose within 2h post injection as their post-prandial glucose values fell to those associated with an overnight fast ($P < 0.001$). Their levels ranged from 65 mg/dL to 105 mg/dL from time -60 to 420. In obese pigs, glucose concentrations peaked at 125 mg/dL 60 minutes post-injection, and then showed a precipitous drop off from 60 to 180 minutes, bottoming out at 45 mg/dL ($P < 0.001$). Glucose remained below normal, at approximately 60 mg/dL, until 8 hours post-injection in obese pigs with the overall response to LPS being greater in the obese relative the lean pigs.

To further assess the inflammatory status associated with divergent body composition in these pigs, proinflammatory cytokine expression was measured via real-time PCR in the arcuate nucleus (Figure 3.12) and subcutaneous adipose tissue (Figure 3.13) of lean and obese pigs. Consistent with an obesity-induced inflammatory state, mRNA expression of *interleukin 6* (*IL6*) and *tumor necrosis*

factor alpha (*TNFα*) was 2.44- and 3.2-fold higher in the arcuate nucleus of obese versus lean pigs ($P < 0.05$). Likewise mRNA for IL6 was 4.74-fold higher and mRNA for TNFα was 3.4-fold higher in the subcutaneous adipose tissue of obese versus lean pigs ($P < 0.05$).

Metagenomic analysis of Fecal Microbiota

DNA was extracted from the feces of five lean and five obese pigs and next generation shotgun sequencing was performed. The metagenomic data was then analyzed using the MG-RAST server and software. Pigs were weighed and fecal samples taken on the same day. The average weight of the 5 lean and obese pigs (in pounds) is shown in the bar graph (Figure 3.1). DGGE analysis was run on DNA from the fecal samples (Figure 3.14). The lean and obese groups partitioned separately based on similarity of banding with the microbiome of obese pigs demonstrating less diversity than the microbiome of their lean counterparts (Figure 3.14). This decreased diversity the microbiome of obese pigs is consistent with human obesity and rodent models.

3.5 Discussion

There currently are no established porcine models of hyperphagic, juvenile obesity which give rise to frank metabolic disease (Larsen and Rolin 2004; Jensen, 2011; Bellinger et al., 2006). This inability to model the progressive nature of obesity-induced disease is limiting efforts to develop therapies that might prevent obesity-associated mortality in humans. The present study aimed to assess the Mangalica pig's potential to serve as an animal model for human obesity and its

metabolic complications. Lean and obese groups were created by either allowing ad libitum access to feed or by limiting access to 40% of voluntary feed intake over the course of 18 months. These data indicate that the Mangalica pig indeed serves as a novel biomedical model for human obesity and its metabolic complications. The extreme adiposity exhibited by obese Mangalica pigs associates with increased innate immune function and higher tissue expression of proinflammatory cytokines, hyperglycemia, hyperinsulineamia, insulin resistance, and dyslipidemia indicating the spontaneous development of metabolic syndrome and a diabetic state. Collectively, these observations support a role for the Mangalica pig as a model that occupies a place further down the slippery slope paradigm of obesity-induced mortality than any currently existing porcine model. The use of obese Mangalica pigs in this regard thus allows the pursuit of new avenues of research concerning the progressive nature of obesity-induced disease.

The striking metabolic differences that exist between lean and obese pigs makes the Mangalica an exciting potential biomedical model. However, our approach used a strategy whereby lean pigs were created by a chronic limit feeding strategy involving an aggressive level of caloric restriction. Such a feeding paradigm in modern, improved breeds would be expected to shift or delay the growth curve resulting in slowed growth, stunted frame size, and less skeletal muscle compared to non-restricted, age-matched animals. Indeed this was the case as limit-fed pigs grew slower exhibiting an ADG of .86 ± .01 compared to an ADG of 1.24 ± .03 for the obese group. Such an affect could potentially confound the measure of metabolic parameters and the assessment of insulin sensitivity because it is well recognized that,

through shear mass, skeletal muscle accounts for approximately 70-80% of glucose disposal in mammals (Porte, 1999; Bellinger et al., 2006). However, feed-restricted pigs in the current study also exhibited a 5% improvement in gain to feed relative to their obese counterparts indicating this slower growth rate was associated with less fat accumulation, a primary goal of the feeding strategy. Importantly, when body composition was assessed by ultrasound at the mature live weight for this breed, estimated loin area and loin depth were not different between lean and obese pigs suggesting that despite the slower growth seen in lean pigs they were still able to reach their mature muscle mass. Thus the limit feeding regimen did not pose a confounding element. This is an important point because it suggests that any metabolic differences seen between lean and obese groups would most likely be driven by differences in adiposity which is a necessary assumption to be met in this model.

An advantage of the experimental design utilized for the current studies is that through our approach whereby lean and obese groups were created from the same genetic background while all pigs were fed the same ration across groups, albeit at different levels of intake, we were able to avoid several potentially confounding factors. For instance, genetics, diet, or the requisite experimental manipulation required to induce diabetes all represent inherent limitations that complicate data interpretation in established porcine biomedical models of obesity. Early work with Ossabaw pigs is confounded by the tendency to use modern, genetically lean breeds as controls relative to the lard-type Ossabaw pig (Kasser et al., 1981; Wangness et al., 1981; Etherton and Kris-Etherton, 1980; Meserole and Etherton, 1984; Mersmann,

1986). While Ossabaw pigs in such trials consistently displayed higher glucose values than the lean breed controls, it is difficult to assess the degree to which adiposity impacted these values within Ossabaw pigs themselves. This issue has been avoided with the recent trend by researchers using the Ossabaw model to create lean and obese treatment groups by feeding high fat diets to one group of post-pubertal Ossabaw pigs while maintaining other Ossabaw pigs on a low energy diet allowing direct comparison of serology between animals of the same breed (Dyson et al., 2006). However, while obesity is driven by consumption of a high fat diet in this paradigm, it is unclear whether the subsequent metabolic problems arise because of the developing obesity or if they are secondary to the consumption of very high levels of dietary lipids (Bellinger, 2006). Finally, in models like the Gottingen minipig, metabolic complications are caused by chemical or surgical insult (Larsen and Rolin 2004). Administering pancreatic toxins such as streptozotocin may also have confounding, non-specific effects on the brain and other organ systems which make it difficult to understand the changes observed in serology or tissue behavior (Larsen and Rolin 2004).

Given a primary objective of this work was to assess whether obese Mangalica pigs develop metabolic syndrome and faithfully mirror the condition seen in humans, the definition used to assess insulin resistance in our model is not a trivial consideration. In humans, clinical diagnosis of metabolic syndrome is actually contentious (Mittal, 2008). There is consensus about the essential components-obesity, glucose intolerance, hypertension, and dyslipidemia-but opinions differ greatly on other components. This has made it difficult to track prevalence given

there is no consensus definition applied by health organizations worldwide and institutions such as the World Health Organization (WHO), International Diabetes Federation, American Heart Association and the National Heart, Lung, and Blood Institute have established different defining criteria. However, concerning insulin resistance, the diagnostic standard has become much clearer as the American Diabetes Association and WHO have developed guidelines for administering and interpreting glucose tests. For an OGTT, a fasted patient (human) is given a 75 gram oral dose of glucose and blood sugar levels are recorded for two hours post ingestion. Impaired fasting glucose is defined as >100mg/dl (5.6mmol/L) but less than 126 mg/dl (7 mmol/L) with values over 126 considered symptomatic of diabetes. A 2h OGTT glycaemia of <140 mg/dl is considered normal, a glycaemia of 140 mg/dl (7.8 mmol/L) to 197 mg/dl is symptomatic of impaired glucose tolerance, and a glycaemia greater than or equal to 200 mg/dl (11.1 mmol/L) is considered sufficient to support a diagnosis of diabetes mellitus (Bellinger, et al. 2006). Several insulin sensitivity indexes, such as HOMA, QUICKI and the Matsuda Index, have also been developed that consider either baseline or peak values from glucose and insulin response curves to assess whole body insulin sensitivity.

Because of their anatomical, physiological, and metabolic similarities to humans, it is reasonable to use the human criteria for swine in modeling diabetes (Bellinger et al., 2006). However, no set standards for interpretation exist in the literature for clearly defining hyperglycaemia and insulin resistance in pigs. Generally, pigs are considered diabetic if there is an elevated glucose response over controls despite little insulin response. Some researchers have compared peak curve

values or area under the curve for both glucose and insulin values when assessing the potential for metabolic differences to exist between treatment groups. In recent literature, this is becoming a more prevalent standard though this definition is applied regardless of how such values compare to the human standards (Bellinger et al., 2006). Since it was necessary to remain consistent with the pig literature in order to meaningfully compare our results with other studies, we opted to define hyperglycemia and hyperinsulinemia as having significantly higher levels of glucose and insulin than control groups as per the standard in the pig literature. However, we adopted a more rigorous guideline whereby pigs could only be described as diabetic if they displayed both elevated glucose and insulin values relative to controls in both the fasted and challenged states. These values fell within ranges that would be considered symptomatic in humans, and insulin sensitivity indexes indicated impaired insulin sensitivity.

Even using this stricter definition to assess the presence of a diabetic state, it is clear that obese Mangalica pigs spontaneously developed diabetes in the present study based upon OGTT and fasted plasma glucose and insulin values. This is an important observation as to date there is no swine model available that faithfully manifests all indices of obesity-induced metabolic syndrome nor are there reports in the literature of a porcine model that spontaneously develops diabetes (Larsen and Rolin 2004; Bellinger et al., 2006). In the present study, fasting plasma glucose and insulin values were significantly higher in obese versus lean pigs. Likewise both peak curve values and the duration of the elevated response to glucose challenge (i.e. the shape of the response curves) were significantly greater in the obese pigs for glucose

and insulin as well. Finally QUICKI, HOMA and Matsuda analysis of curve values were all consistent with the development of insulin insensitivity. Coupled with the manifestation of insulin resistance, obese Mangalica exhibited lipid profiles consistent with the development of dyslipidemia, as plasma triglyceride levels were 76% higher and total cholesterol levels were 33% higher in obese versus lean pigs. This cluster of three indices alone would be sufficient to clinically diagnose a human as exhibiting metabolic syndrome.

The ranges of glucose and insulin values recorded in the present study are consistent with those reported for modern genetically lean breeds such as the Yorkshire and for heritage breeds such as the Ossabaw while also generally reflecting values that are recorded in humans. For instance, fasted glucose values for lean and obese Mangalica were in the range of 80 mg/dl and 115 mg/dl whereas peak curve values following an OGTT reached 283 and 434 mg/dl respectively in the current study. In lean, modern pigs, fasting glucose levels range around 70-80 mg/dl and peak values generally only climb to 140 mg/dl following an OGTT (Mersmann, 1986; Leininger et al., 1999; Leininger et al., 2000a,b; Fisher et al., 2013). This blunted glucose response is one reason that it is generally accepted that modern swine are resistance to developing diabetes. Ossabaw pigs on the other hand display glucose values in the fasted and challenged states that are more consistent with Mangalica pigs and humans as fasted values in the Ossabaw generally fall between 70-80 mg/dl while peak values have been reported to fall over a wide range often reaching as high as 250 mg/dl in obese Ossabaw pigs (Dyson et al., 2006; Neeb et al., 2010; Bell et al., 2010; Kreutz et al., 2011; Faris et al., 2012; Pederson et al., 2013; Newell-Fugate et

al., 2014). Similar relationships are seen for insulin values across these species. For instance, fasted insulin values were in the range of 13 μU/ml and 23 μU/ml for lean and obese Mangalica respectively. Peak insulin curve values following an OGTT reached 46 and 84 μU/ml respectively in the current study. Meanwhile, insulin values have been reported to fall within 4-20 μU/ml in fasted, modern genetically lean pigs while the insulin response has been reported to be anywhere from nonexistent to peaking at 30 μU/ml following an OGTT (Mersmann, 1986; Leininger et al., 1999; Leininger et al., 2000a,b; Fisher et al., 2013). On the other hand, Ossabaw pigs have displayed fasting insulin levels between 10-20 μU/ml with peak curve values of 100 μU/ml (Dyson et al., 2006; Neeb et al., 2010; Bell et al., 2010; Kreutz et al., 2011; Faris et al., 2012; Pederson et al., 2013; Newell-Fugate et al., 2014). Thus, genetically lean pigs tend to show blunted glucose and insulin responses while being resistant to developing hyperglycemia and hyperinsulinemia. Ossabaw pigs appear to display little difference between the glucose responses of lean and obese treatment groups though obese Ossabaw appear to develop a mild insulin resistance that is evident during a challenge suggesting these pigs are prediabetic. Mangalica on the other hand, appear to develop a spontaneous diabetic state placing them further downstream of Yorkshire and Ossabaw pigs on the "slippery slope" model of obesity-induced mortality suggesting this breed offers a wider utility as a model of obesity-induced metabolic complications.

A critical link between obesity and its metabolic complications is the development of a chronic state of low grade inflammation (Huh et al. 2014). This is believed to be caused by the release of chemokines and cytokines from enlarging

adipocytes which stimulate macrophage invasion into adipose tissue (Bai and Sun 2015). Free fatty acids are thought to activate TL4R which would be expected to stimulate resident macrophages and enlarged adipocytes to release additional cytokines thus magnifying the signal that triggers macrophage invasion into adipose tissue and subsequently causes the tissue to become inflamed. A progressively proinflammatory microbiome is also believed to exacerbate this in obese individuals by promoting metabolic endotoxemia. Such a changing microbiota results in the release of LPS which is subsequently absorbed into the obese host's circulation and can thus further activate adipocytes and macrophages, both being cell types which express the TL4R (Cani et al., 2007). Importantly, proinflammatory cytokines have a metabolic biological activity as well. Cytokines are catabolic and blunt glucose uptake while signaling the breakdown of glycogen, protein, and lipids in the liver, muscle and adipose tissue. Thus, cytokines oppose the action of insulin and promote higher blood glucose and lipid levels in inflamed individuals leading to insulin resistance and the plasma profile that is clinically diagnostic of metabolic syndrome. Thus, inflammation is considered a hallmark characteristic of metabolic syndrome and a prime therapeutic target for its treatment.

We evaluated the inflammatory status in lean and obese pigs by performing an endotoxin challenge and by using real-time PCR to measure expression of proinflammatory cytokine mRNA in adipose tissue and the brain. Obese pigs displayed a low-grade inflammatory state based upon exhibiting higher baseline levels of TNFα and cortisol compared to their lean counterparts. Obese pigs also exhibited a significantly more robust acute response to endotoxin challenge further

suggesting altered immune function. Finally, consistent with circulating parameters, obese pigs also displayed significantly higher levels of TNFα and IL-6 mRNA in both subcutaneous adipose tissue and the arcuate nucleus. Surprisingly, little work has been done to characterize inflammation in porcine models of obesity and only one study exists in the literature that specifically examines the impact of endotoxin in obese pigs (Duburcq et al., 2014). The responses exhibited by lean Mangalica pigs to endotoxin challenge in the current study are consistent with those observed in studies that have performed such challenges in Yorkshire hogs though responses in the present study were more robust (Leininger et al., 1999; Leininger et al., 2000a,b). The heightened response displayed by obese pigs in the current study relative to their lean counterparts represents a very dramatic acute endotoxemia compared to similar studies in Yorkshire pigs. It should be noted that age, weight, breed, and gender of the pigs were not equal in these studies and these factors could have substantial impact on the magnitude of response mounted to endotoxin challenge. Overall though, the results of the present study were within limits that previous studies suggest are expected for pigs thus the difference seen between lean and obese Mangalitsa pigs in the present study were physiologically relevant. Furthermore, that obese pigs displayed a more robust response to endotoxin is consistent with the one study that has examined this issue in the Yucatan pig (Duburcq et al., 2014). One explanation for the more robust response to endotoxin in the obese pigs versus their lean counterparts is that their innate immune system is already primed by a persistent state of low grade chronic inflammation associated with their obesity. This speculation is consistent with elevated mRNA expression of proinflammatory cytokines in the

adipose tissue of obese Mangalitsa pigs and the higher baseline levels of circulating TNFα also observed in these pigs. Collectively, these results indicate that the obese pigs did have altered immune function compared to their lean companions. Importantly, obese Mangalica pigs model the critical link between obesity and the onset of insulin resistance.

It is well known that pigs regulate their voluntary feed intake relative to their energy requirements, i.e. pigs "eat to their energy" (Owen and Ridgman, 1968; Houpt et al., 1979 (Houpt, 1984; Mersmann, 1986; Fisher et al., 2013). This is a major limitation for attempts to create obese cohorts by feeding an energy dense diet to either modern, genetically lean hogs or heritage breeds such as the Ossabaw pigs (Fisher et al., 2013). This approach will lead to heavier, fatter pigs because overall pigs will experience higher energy intakes than those on low-fat rations. However, because the energy-dense diet will also depress voluntary feed intake, the ultimate degree of achievable energy intake and thus the ultimate degree of adiposity is limited by this self-regulating feedback mechanism (NRC, 2013; Noblet and Van Milgen, 2013). Despite being able to become quite fat, the tendency to eat only to their energy demands likely limits the degree to which Ossabaw pigs exhibit the progressive nature of obesity-induced metabolic complications.

Given that pigs are known to "eat to their energy", the observation that high fat rations failed to depress feed intake in Mangalica pigs was both striking and unexpected. Typically, feed intake is reduced by 3-5%, weight gain is increased by 3-5%, backfat is increased by about 0.1 inch, and feed efficiency is increased 8-10% for every 5% fat added to the swine diet (Cromwell, 1997). In the present study, the

30% high fat diet failed to depress feed intake and Mangalica pigs fed the high fat diet showed a 3-fold greater total gain, a 2.5-fold increase in ADG, and 2-fold increase in feed efficiency. These responses were much greater than typically seen in response to fat feeding and likely are due to the fact that feed intake was unchanged.

This lack of self-regulating feedback allowed pigs on the high fat diet to consume significantly more energy than their counterparts eating an equal amount of the control diet. One potential explanation for the failure of the high fat diet to depress feed intake could be induction of cold stress given this experiment was conducted in a setting where environmental temperature was not controlled. In theory, cold stress would increase feed intake in order to help the pig defend its body temperature. Given fat has a lower heat increment, a pig might be expected to consume a greater amount of a high fat diet when experiencing cold stress compared to pigs consuming a ration that has a lower fat content. Such an involuntary response to low ambient temperature might override the depressive effects of energy density on voluntary intake. However, as Figure 3.4 demonstrates, there were no prolonged periods of ambient temperatures that would induce severe cold stress. Also, when examining mean weekly feed intakes for pigs during the high-fat experiment, there were no associations between feed intake and temperature. Thus, more likely, the failure of the energy dense ration to depress voluntary intake in obese Mangalica pigs points to a defect on the neural circuits that control satiety within the arcuate nucleus. This is also consistent with the observation that restricting feed intake to levels that are 2.5-fold below voluntary feed intake still allows mature frame size and muscle mass to be reached in these pigs. Such an observation suggests that these pigs eat

roughly 2-3 times more than they need to eat in order to support normal muscle and bone growth. Importantly, this implicates the Mangalica pig as a unique model of hyperphagic obesity. It is tempting to speculate that the inflammation observed in the hypothalamus, as suggested by increased cytokine expression, could be an underlying mechanism explaining both hyperphagia in these pigs and the failure for of these pigs to adjust their feed intake in response to a very energy dense ration. Such a unifying hypothesis will need to be tested in future experiments.

In conclusion, these data strongly support the hypothesis that the Mangalitsa pig serves as a faithful model for human obesity and metabolic syndrome. The obese pigs in this study exhibited a corpulent phenotype and notable metabolic, immunological, and hormonal differences consistent with the development of metabolic syndrome and a diabetic state. Importantly, the lean and obese pigs showed marked differences in their innate immune response to endotoxin, revealing that adiposity had altered immune function, a critical link between obesity and its downstream metabolic complications. The spontaneous manifestation of metabolic complications indicate that Mangalica pigs model the progressive nature of obesity-induced disease indicating that the Mangalica fills a novel niche downstream of the Ossabaw pig on the slippery slope model of obesity-induced mortality. These findings argue for the use of the Mangalitsa in future biomedical studies. Doing so will hopefully enable scientists to better understand how to deal with our big, fat problem.

Table 3.1. Indexes of Insulin sensitivity[1]

Variable	Lean	Obese	P-value
Number of pigs	6	6	NA[2]
QUICKI[3]	.33	.29	0.05
HOMA-IR[4]	1.65	6.81	0.001
HOMA-B[5]	152	126	0.05
HOMA-S[6]	61	33	0.01
Matsuda Index[7]	4.74	1.60	0.001
Insulinogenic Index[8]	.45	.18	0.001
Disposition Index[9]	2.1	.31	0.001

[1]Values are means

[2] NA=not applicable;

[3]QUICKI= Quantitative Insulin Sensitivity Check Index Visual; normal ranges between .3-.45 and insulin resistance is <.3; lower numbers reflect greater insulin resistance.

[4] HOMA-IR = Homeostatic model assessment- insulin resistant; normal is indicated by values lower than ≤ 1.8.

[5] HOMA-B = Homeostatic model assessment- insulin resistant-β-cell function

[6] HOMA-S = Homeostatic model assessment- insulin resistant-insulin sensitivity

[7] Matsuda Index = whole body insulin resistance is indicated for values ≤ 2.5

[8] Insolinogenic Index = defects in insulin secretion are indicated for values < 0.4

[9] Disposition Index = (Insulinogenic index)*(Matsuda index); normal is indicated for values > 1

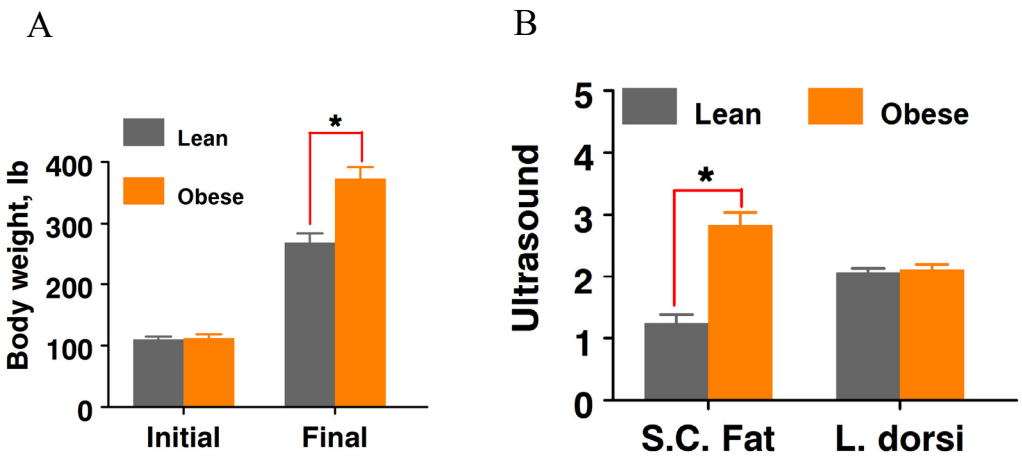

Figure 3.1. Characterization of the phenotype of pigs fed to become either lean or obese. Lean or obese pigs were created by either restricting caloric intake or by allowing voluntary feed intake to be dictated by choice. ***Panel A***: Initial and final body weights (live weight; lbs) indicate that dietary paradigm resulted in divergent body weights with obese pigs being significantly heavier. ***Panel B***: Ultrasound data measured at the 10th rib indicate that pigs allowed free choice accumulated 2.5-fold more subcutaneous back fat while exhibiting no difference in loin muscle suggesting that the differences in body weight were driven by differences in adiposity.

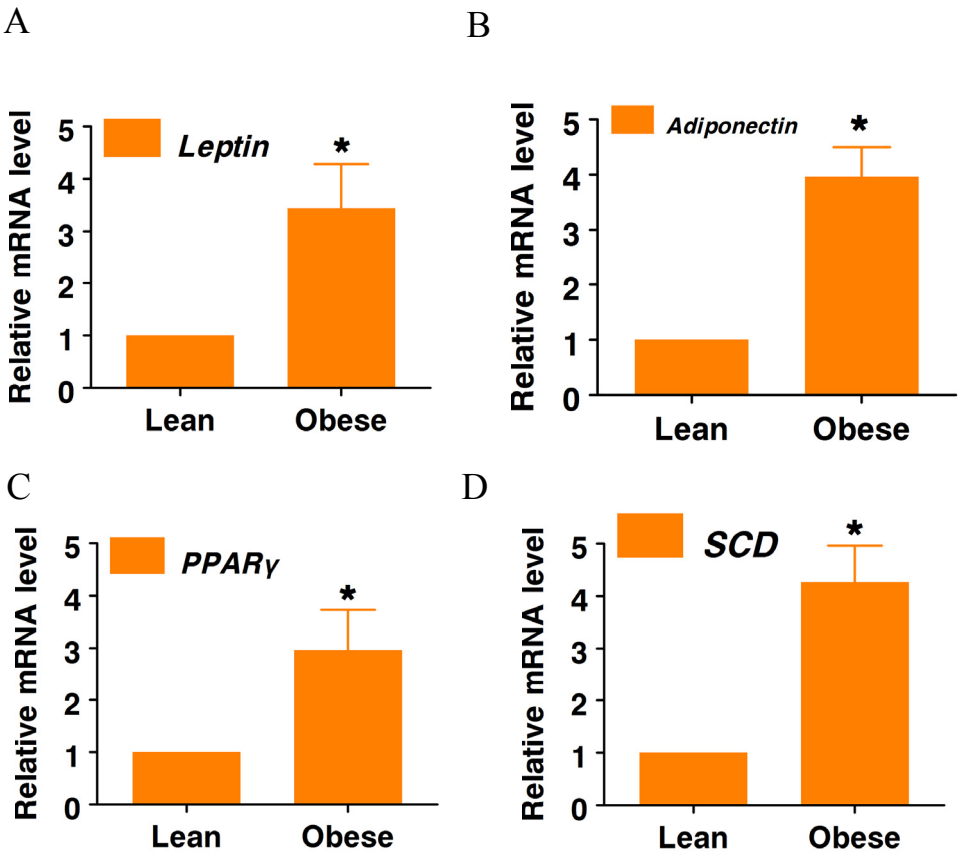

Figure 3.2. The mRNA expression of gene markers for adiposity in subcutaneous adipose tissue of lean and obese pigs. ***Panel A***: *Leptin*, ***Panel B***: *Adiponectin*, ***Panel C***: *Peroxisome proliferater activated receptor gamma (PPARγ)*, ***Panel D***: *Steroyl coenzyme A desaturase (SCD)*. These data are consistent with increased adiposity in obsess versus lean pigs. The mRNA levels were measured using real-time PCR. Expression levels were normalized relative to the porcine S15 gene and are presented as fold change relative to expression in lean pig values.

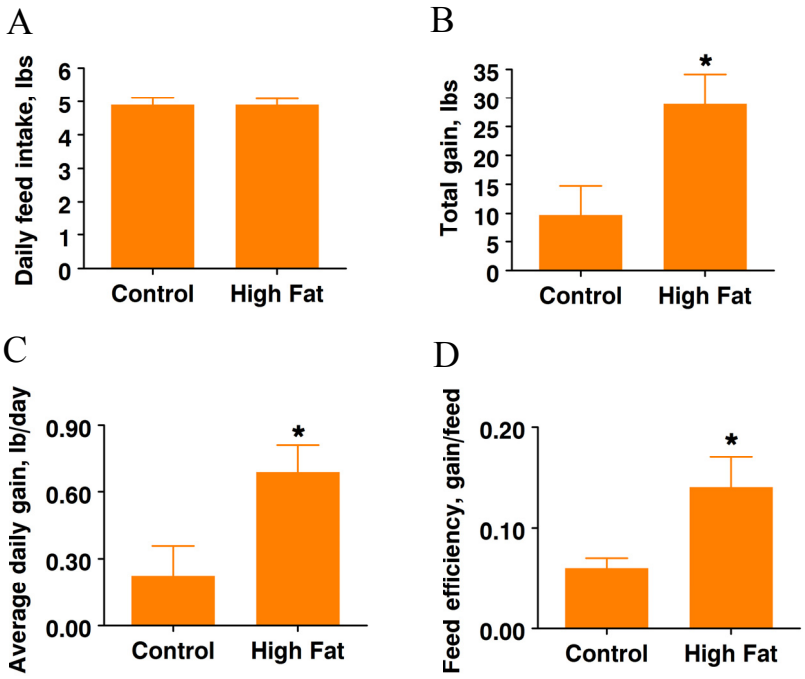

Figure 3.3. The effect of feeding high fat diets on performance parameters in lean and obese pigs. ***Panel A***: Daily feed intake in obese pigs fed the control diet or a diet containing 30% soybean oil for 49 days. ***Panel B***: Total gain (lbs) in obese pigs fed the control diet or a diet containing 30% soybean oil for 49 days. ***Panel C***: Average daily gain in obese pigs fed the control diet or a diet containing 30% soybean oil for 49 days. ***Panel D***: Feed efficiency in obese pigs fed the control diet or a diet containing 30% soybean oil for 49 days. These data point to a lesion in the feeding center of the pig brain as unexpectedly, feeding the extreme high fat diet failed to suppress voluntary feed intake in pigs fed the high fat diet. These data are consistent with Mangalica pigs being a faithful model of hyperphagic obesity.

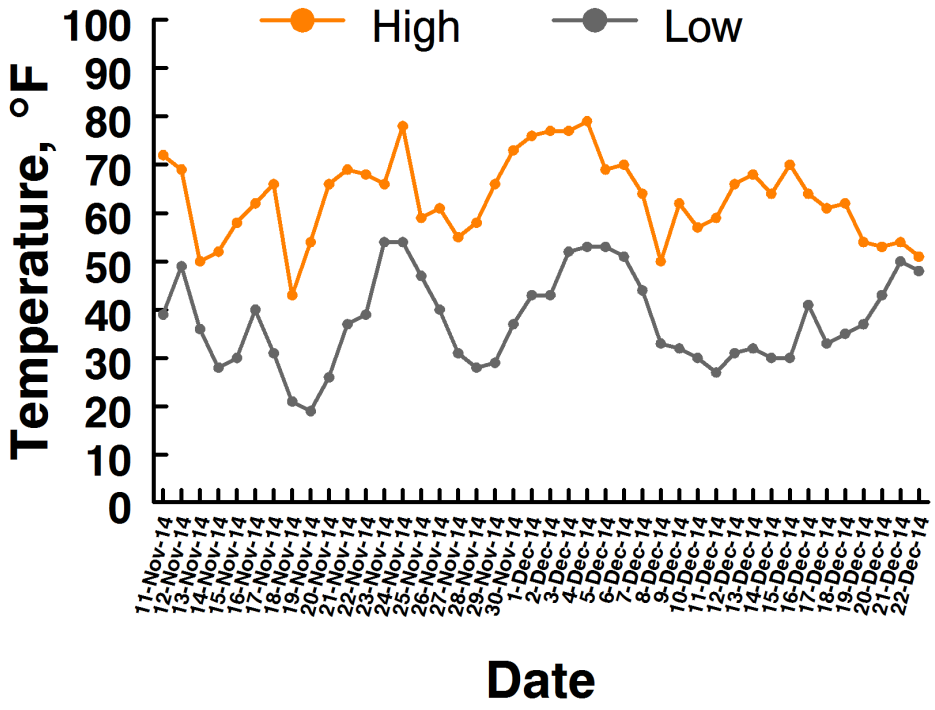

Figure 3.4. Daily high and low ambient temperatures during the period that high fat diets were fed. The thermoneutral zone for finishing hogs of 260 lbs live weight or greater rages between 45-70 ºF.

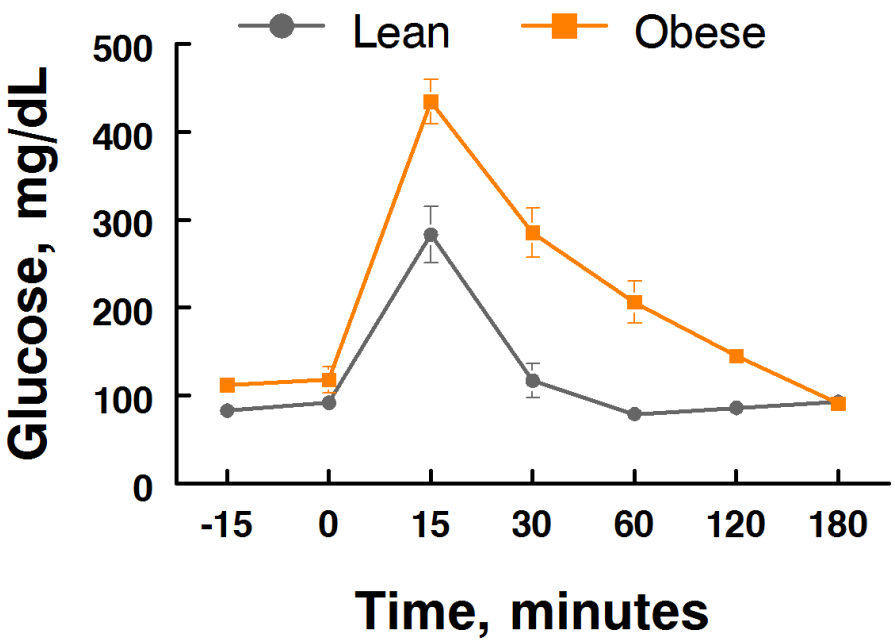

Figure 3.5. Oral glucose tolerance test (OGTT) conducted on lean and obese pigs indicates that obese pigs develop glucose intolerance. This manifests as the result of their increased adiposity. Plasma glucose levels were measured following administration of an oral dose of glucose (2 g/kg BW) to fasted pigs.

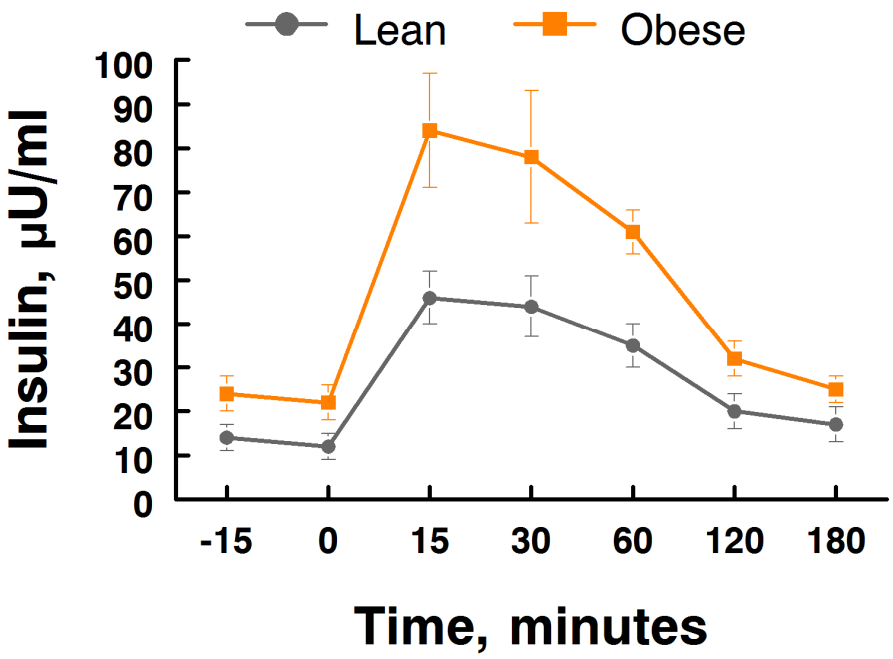

Figure 3.6. Oral glucose tolerance test (OGTT) conducted on lean and obese pigs indicates that obese pigs develop hyperinsulinemia. This manifests as the result of their increased adiposity. Plasma insulin levels were measured following administration of an oral dose of glucose (2 g/kg BW) to fasted pigs.

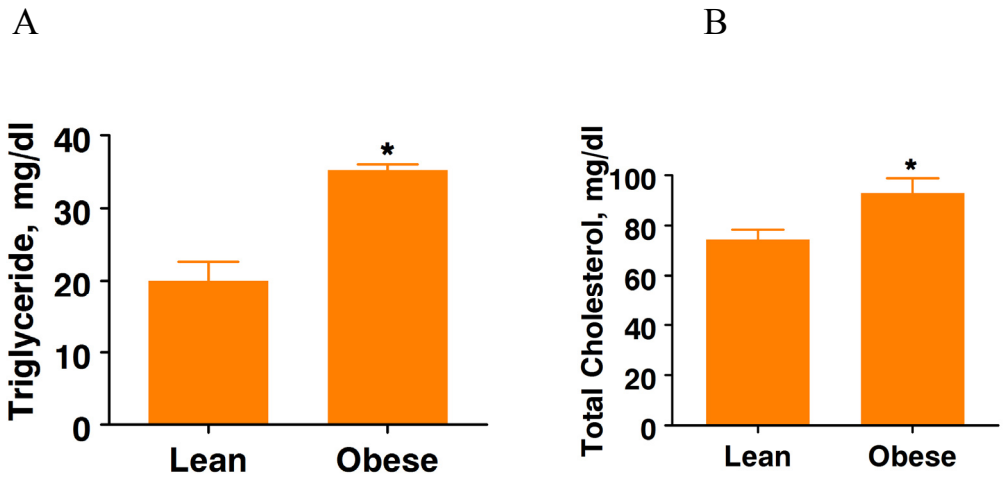

Figure 3.7. Plasma metabolic parameters in lean and obese pigs fasted overnight. ***Panel A***: Plasma triglyceride levels. ***Panel B***: Plasma total cholesterol levels. These data are consistent with the spontaneous development of hyperlipidemia and hypercholesterolemia in obese pigs.

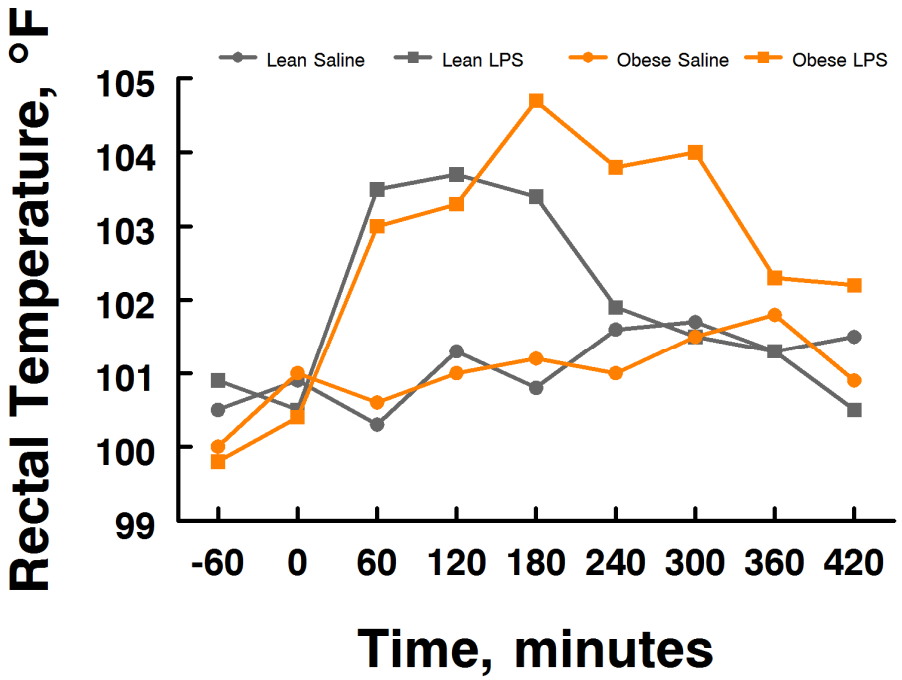

Figure 3.8. Obese pigs have a higher febrile response to endotoxin (LPS) than lean pigs. Endotoxin challenge conducted on lean and obese pigs indicate that obese pigs develop a heightened inflammatory response as the result of their increased adiposity. Rectal temperatures were measured following administration of an Intramuscular (I.M.) dose of LPS (25 µg/kg BW) to lean and obese pigs.

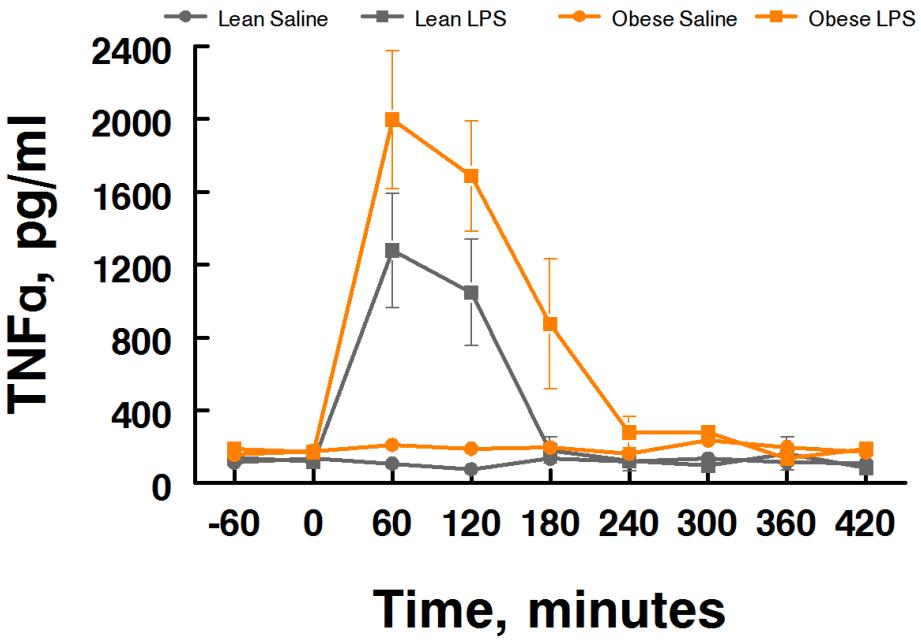

Figure 3.9. Obese pigs have higher circulating tumor necrosis factor-alpha (TNFα) levels in response to endotoxin (LPS) than lean pigs. Endotoxin challenge conducted on lean and obese pigs indicate that obese pigs develop a heightened inflammatory response as the result of their increased adiposity. Plasma TNFα levels were measured following administration of an Intramuscular (I.M.) dose of LPS (25 µg/kg BW) to lean and obese pigs.

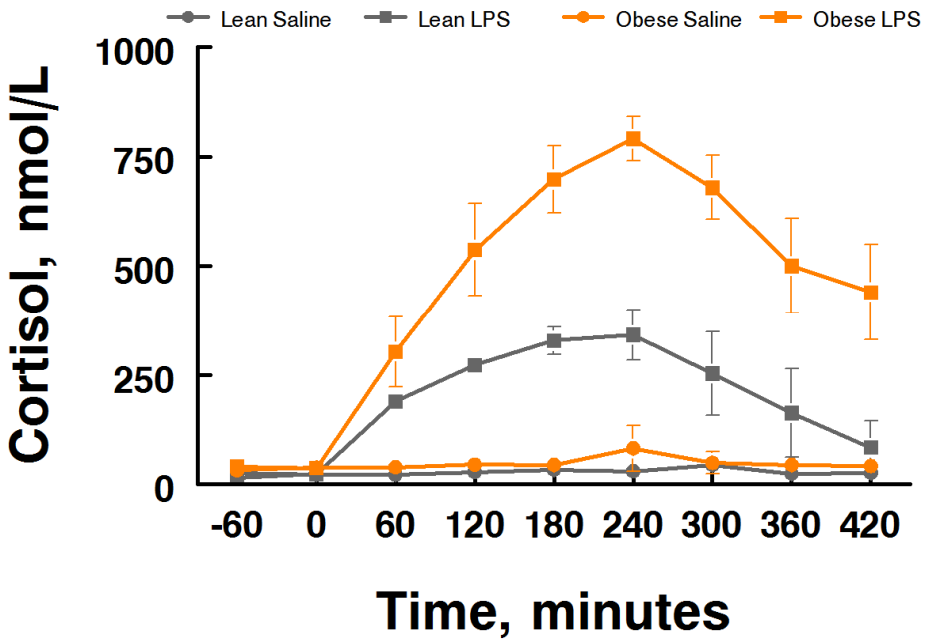

Figure 3.10. Obese pigs have higher circulating cortisol levels in response to endotoxin (LPS) than lean pigs. Endotoxin challenge conducted on lean and obese pigs indicate that obese pigs develop a heightened inflammatory response as the result of their increased adiposity. Plasma cortisol was measured following administration of an Intramuscular (I.M.) dose of LPS (25 µg/kg BW) to lean and obese pigs.

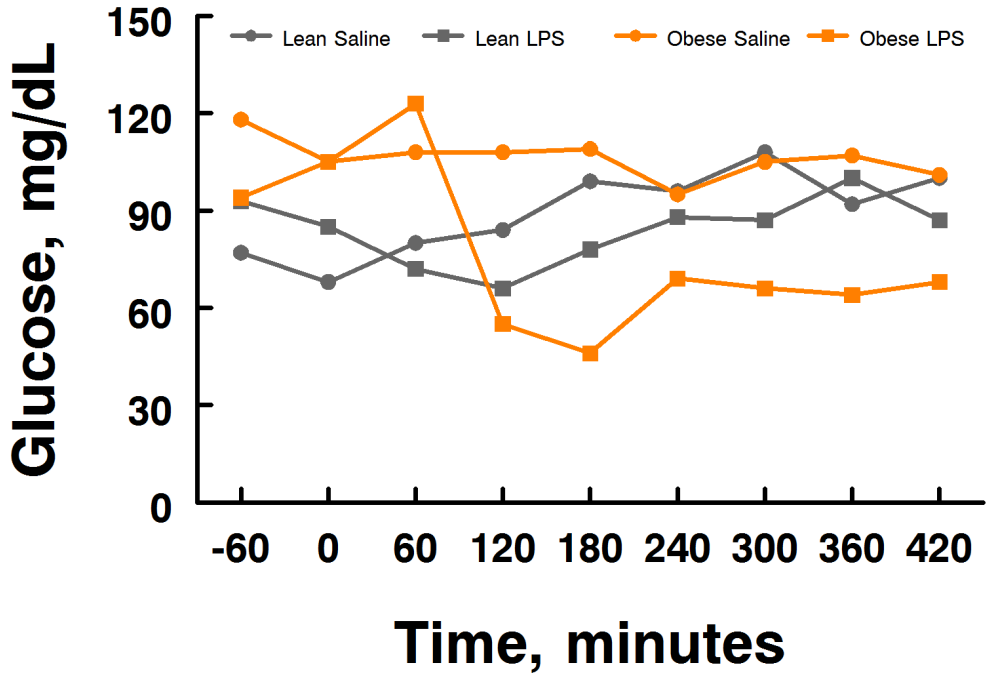

Figure 3.11. Obese pigs have higher lower plasma glucose levels in response to endotoxin (LPS) than lean pigs. Endotoxin challenge conducted on lean and obese pigs indicate that obese pigs develop a heightened inflammatory response as the result of their increased adiposity. Plasma glucose was measured following administration of an Intramuscular (I.M.) dose of LPS (25 µg/kg BW) to lean and obese pigs.

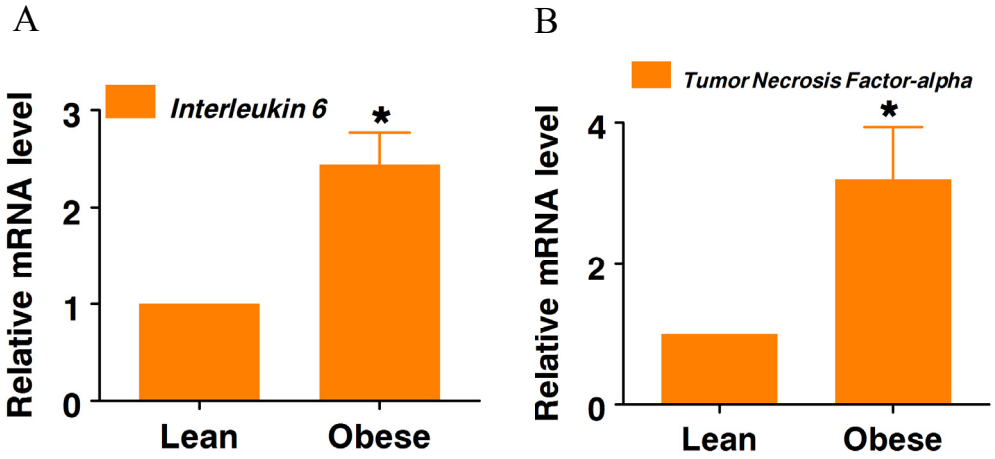

Figure 3.12. The mRNA expression of inflammatory gene markers in the arcuate nucleus of lean and obese pigs. ***Panel A***: *Interleukin 6 (IL-6)*, ***Panel B***: *Tumor necrosis factor-alpha (TNFα)*. These data are consistent with the development of inflammation within the arcuate nucleus with increased adiposity in obese versus lean pigs. The mRNA levels were measured using real-time PCR. Expression levels were normalized relative to the porcine S15 gene and are presented as fold change relative to expression in lean pig values.

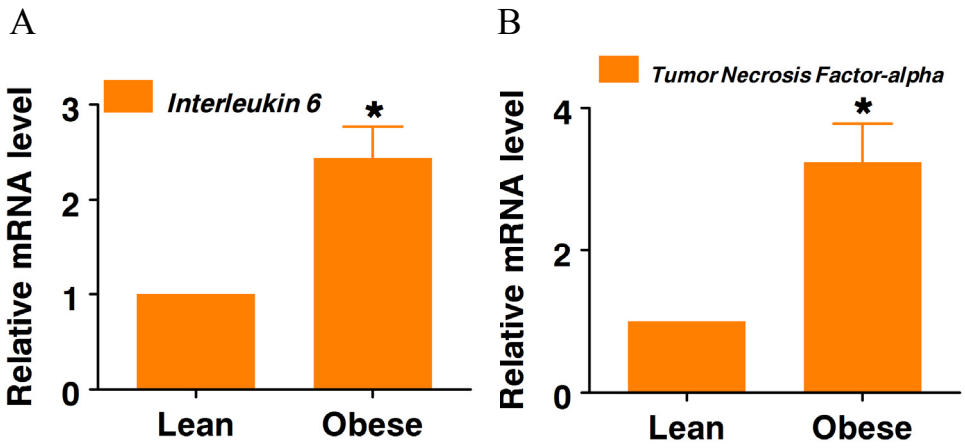

Figure 3.13. The mRNA expression of inflammatory gene markers in the subcutaneous adipose tissue of lean and obese pigs. ***Panel A***: *Interleukin 6 (IL-6)*, ***Panel B***: *Tumor necrosis factor-alpha (TNFα)*. These data are consistent with the development of inflammation within adipose tissue with increasing adiposity in obese versus lean pigs. The mRNA levels were measured using real-time PCR. Expression levels were normalized relative to the porcine S15 gene and are presented as fold change relative to expression in lean pig values.

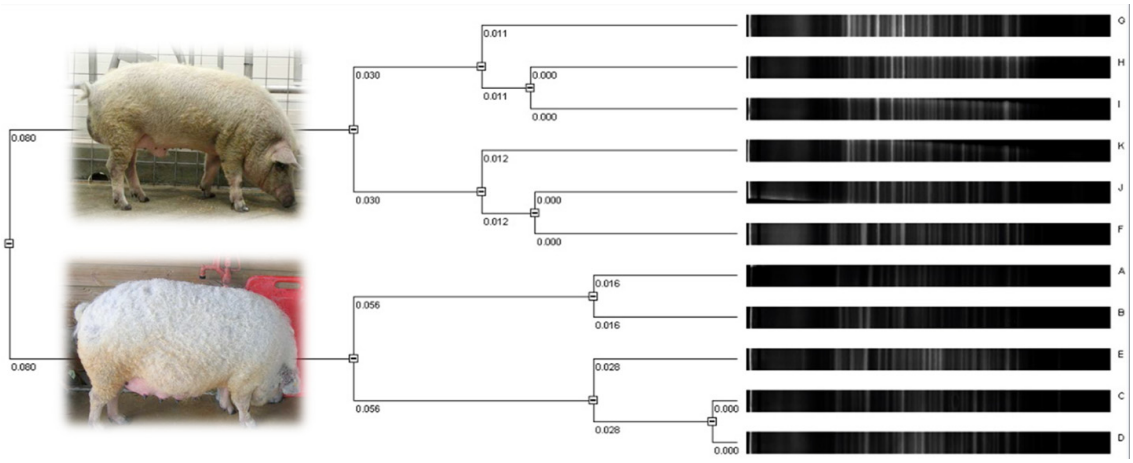

Figure 3.14. Body weight in lean and obese pigs and corresponding diversity of gut microbes. Denaturing gradient gel electrophoresis (DGGE) analysis of gut microbiota.

Chapter 4:

Summary and Future Directions

Data in this paper indicates that the Mangalica pig serves as a novel biomedical model for human obesity and its metabolic complications. The Mangalica pig is very unique in that it appears to be the first swine model to display the progressive nature of obesity-related disease without surgical or chemical manipulation. These pigs exhibit hyperphagic obesity that spontaneously gives rise to an inflammatory state and a diabetic condition. When allowed to be in a chronic positive energy balance, this model exhibits an extreme obese phenotype characterized by excessive amounts of adipose tissue. This extreme adiposity associates with increased innate immune function and higher tissue expression of proinflammatory cytokines, along with hyperglycemia, hyperinsulineamia, insulin resistance, and dyslipidemia, which are characteristic of diabetes and metabolic syndrome.

In addition, the gut microbiota of the Mangalica pig was characterized by a bacteria phylum similar to that of humans. The gut microbiota of the obese Mangalica was found to be less diverse compared to its leaner counterpart. This decrease in gut microbiota is also observed in obese humans. Moreover, this study revealed that a high fat diet failed to depress feed intake in obese Mangalica pigs, suggesting these pigs are experiencing impaired regulation of satiety in the arcuate nucleus possibly secondary to increased hypothalamic inflammation, although further research is needed. This would represent the first porcine model of hyperphagia and a unique

model in which to study the impact of hypothalamic inflammation and innate immune activation on POMC and NYP neurons.

The Mangalica may serve as a useful model in which to study systemic inflammation during obesity. Because the Mangalica displays an obese phenotype and a gut microbiota similar to humans, this model may allow us to ask if inflammation is initiated by the increase in proinflammatory cytokine production and macrophage recruitment in adipose tissue or from the gut microbiota switching to a more gram-negative profile which produces LPS and leads to inflammation. The scientific literature is currently undecided on the specific role of each in causing the underlying low-grade chronic inflammation during obesity. Better understanding the causative link to inflammation would allow potential therapeutic approaches to be applied, which also could be tested in this novel pig model.

This work establishes the Mangalica as a model of hyperphagic obesity that demonstrates the progressive transition to inflammation and then diabetes. Whether these pigs progress toward chronic disease states such as cardiovascular disease or hypertension that underlie obesity-induced mortality remains to be established. High blood pressure is associated with metabolic complications, renal disease, and cardiovascular disease. Therefore, studying hypertension would improve the Mangalica as a model of obesity-induced disease by firmly establishing it as a unique swine model that demonstrates the full progression from development of obesity to its associated mortality. Future studies should specifically address these next steps in the progressive model.

This study also determined that the Mangalica might serve as a good model for studying and improving the quality of pork. The genetics of the Mangalica represent a primitive model, unlike the genetics of commercial Yorkshire pigs. The skeletal muscle of this lard-type hog displays higher marbling and darker color, resulting in a more flavorful and enjoyable cut of pork. The Mangalica displays a divergent meat quality phenotype that is more desirable while serving as an extreme genome that can be leveraged for genetic studies into the underlying drivers of meat quality.

After almost becoming extinct, this study reveals a new purpose for the Mangalica pig other than representing a breed that provides a unique pork product being served in niche markets. The Mangalica pig is an exciting model because it may be useful in improving pork quality and providing new insight about the development and progression of obesity and obesity-related diseases. Because this breed of pig faithfully models each stage of obesity-induced disease, it may allow for better study of the causes of obesity and for development of new treatment options. The Mangalica pig may bring us closer in finding a cure for obesity and obesity-related disease by allowing obesity to be studied in ways that it has not been before.

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