

Dietary manipulation causes childhood obesity-like characteristics in pigs

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ABSTRACT

An animal model to study complications resulting from childhood obesity is lacking. Our objective was to develop a porcine model for studying mechanisms underlying diet-induced childhood obesity. Pre-pubertal female pigs, age 35 d, were fed a high-energy diet (HED; $n = 12$), containing tallow and refined sugars, or a control corn-based diet ($n = 11$) for 16 wk. Initially, HED pigs self-regulated energy intake similar to controls, but, by wk 5, consumed more ($P < 0.001$) energy per kg body weight. At wk 15 and 22, pigs were subjected to an oral glucose tolerance test (OGTT); blood glucose increased ($P < 0.05$) in control pigs and returned to baseline levels within 60 min. HED pigs were hyperglycemic at time 0, and blood glucose did not return to baseline ($P = 0.01$), even 3 h post-challenge. During OGTT, glucose area under the curve was higher and insulin area under the curve was lower in HED pigs compared to controls ($P = 0.001$). Pigs given 6 wk of dietary intervention, consuming a control diet, marginally improved glucose area under the curve and LDL-cholesterol although insulin area under the curve was unaffected. Chronic HED intake increased ($P < 0.05$) subcutaneous, intramuscular, and perirenal fat deposition, and induced hyperglycemia, hypoinsulinemia, and low-density lipoprotein hypercholesterolemia; however, a 6 wk dietary intervention partially recovered a normal physiology. These data suggest pre-pubertal pigs fed HED are a viable animal model for studying childhood obesity.

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LIST OF ABBREVIATIONS

AA: Amino acid

ALK: Alkaline phosphatase

ALT: Alanine aminotransferase

AST: Aspartate aminotransferase

BW: Body weight

CON: Control

d: Day

GGT: γ -glutamyl transferase

HDL: High density lipoprotein

HED: High energy diet

HOMA-B: pancreatic β -cell function estimation by homeostatic model

HOMA-IR: insulin resistance estimation by homeostatic model assessment

LDL: Low density lipoprotein

LMA: Longissimus muscle area

ME: Metabolizable energy

OGTT: Oral glucose tolerance test

QUICKI: quantitative insulin sensitivity check index

SE: Standard error

SubQ: Subcutaneous

TG: Triglyceride

USubQ: Ultrasonic subcutaneous

wk: Week

CHAPTER I
Literature Review

Introduction

The incidence of childhood obesity, simply defined as excess accumulation of fat during the period of life between infancy and puberty, has been increasing during recent decades and has now reached epidemic proportions in many developed countries [1].

The Center for Disease Control and Prevention defines obesity as at, or above, the 95th percentile of body mass index (BMI) for age; overweight is defined as between the 85th and 95th percentile of BMI for age [2, 3]. As many as 70% of obese children grow up to become obese adults [4-6], making this pandemic a serious public health concern while simultaneously increasing cost of health care. Although the highest incidence of childhood obesity has been observed in developed countries, the incidence is increasing in developing countries as highly processed, energy-dense foods become more readily available. Childhood obesity is prevalent in the Middle East, Central and Eastern Europe, and the United States [7]. One review estimated as many as 25% of children in the United States are overweight and 11% are obese [1]. Overweight and obesity are associated with many health maladies including hyperlipidemia, hypoinsulinemia, hyperglycemia, hypertension, as well as potential consequences for reproductive health and capability [1]. Additionally, an increased incidence of psychological disorders, such as depression, have been observed in obese children [8].

Studying causative mechanisms underlying development of childhood obesity in humans is challenging. Certainly there are ethical concerns, but studying childhood obesity is further complicated by normal body growth. Survey based data from humans and epidemiological studies, primarily in rodents, form the basis for current data regarding obesity. Survey based data rely on the subject's ability to recall food

consumed in a given period which may result in under- or over-reporting. Rodent models are invaluable for studying human disease, yet there are limitations. Perhaps the single most limiting factor to using rodents as a childhood obesity model is the time frame available for study. There is limited utility to rodent models of childhood obesity because of the short childhood period. A domestic animal model which closely resembles human physiology is needed to study causative mechanisms underlying childhood obesity.

Pigs as a Biomedical Model

For nearly four decades, pigs have been used as an animal model for studying human diseases including cardiovascular disease, gastric ulcers, alcoholism, dentition, stress physiology, behavior, malnutrition, obesity, infectious diseases, and toxicology [9]. Pigs have become a valuable biomedical model for studying human disease due, in large part, to demonstrated similarities to humans in regards to anatomy of heart and coronary arteries, mature body size, skeletal muscle fiber size and fiber type, physiology, and metabolism [9-12]. Pigs naturally consume omnivorous diets, similar to those consumed by humans, and pigs exhibit a strong taste preference for sucrose [9, 13].

Evolution of the pig has resulted in a diverse variety of size, shape, and general characteristics of modern swine breeds [9]. Minipig varieties have been developed in response to the use of swine as biomedical models. Minipigs, developed from feral herds as well as those selected from naturally occurring miniature swine, offer the advantages of small mature size, lower feed cost, and less space requirements compared to conventional pig breeds [14]. However, age at onset of puberty varies widely and is

highly dependent on the strain of minipig. Minipigs display significant gender differences in the development of obesity, with females having more potential for fat accumulation than male counterparts [15], comparable to the situation in humans, in which females generally have a higher percentage of body fat [16-18]. Generally, male humans have more visceral fat compared to their female counterparts [17]. Thus, female pigs may be more prone to development of metabolic impairments, such as insulin resistance and higher plasma lipid concentrations, associated with central obesity [19-21].

Contemporary swine breeds fulfill an important role in porcine biomedical models. Such pigs are readily available from a host of sources and can be maintained in a variety of research settings. Importantly, in an agricultural setting, commercial pigs are not used in maintaining the breeding herd and thus can go into the food supply, thereby recovering some of the production costs. Pigs as a biomedical model have been vetted by decades of agriculturally-based research. A wealth of nutritional, physiological, and metabolic research from an agricultural perspective further our understanding of the pig and offer insight into precise mechanisms for further developing an animal model of childhood obesity.

The childhood period in pigs can be defined as the period of life from weaning to puberty. Typically, commercial type pigs are weaned at 3 wk and reach puberty at 6-7 months of age, equating to a childhood period of approximately 22 wks. Pigs readily develop atherogenic lesions after only a few weeks of consuming a diet high in cholesterol and fat [14]. A unique feature of pigs is their ability to regulate energy intake. Studies have demonstrated that pigs regulate energy intake based on caloric needs and energy density of the diet, and pigs will regulate intake of other nutrients, including

protein and amino acids, when given a choice between diets [22-24]. The exact mechanism of intake regulation is not known, however, continued insult of this mechanism may result in its breakdown and subsequent loss of intake regulation.

One consideration when using swine as a biomedical model is the common production practice of castration. Pigs are usually castrated within 28 d of birth. The procedure of castration is beneficial in swine production settings because it eliminates boar odor, decreases fighting behavior, and allows mixing of castrated males and intact females [9, 25]. Additionally, castrated males generally exhibit a higher propensity for fat deposition compared to intact pigs. Castrated animals are perhaps a less direct comparison to humans due to gender perception. However, castration does not negate the similarities between humans and pigs. A simple, direct comparison to humans may be made using intact male and female swine as biomedical models.

Development of Obesity

Although the exact mechanisms contributing to development of obesity are not known, two main theories have developed regarding its onset. The first theory suggests development and onset of obesity is thermodynamic in nature [1, 26-28]. When energy intake exceeds expenditure for an individual, the result is fat deposition. This theory is supported by increased energy consumption, without an increase in level of physical activity, and the concomitant increase in the prevalence of obesity. The second theory proposes that type of calorie consumed may be more important than total calorie intake [1, 26-28]. This theory is supported by parallel increases in obesity and consumption of

refined sugars and oils. Metabolic impairments develop over time and arise when readily available food is rich in energy [29].

Childhood obesity is of particular concern due to lasting consequences persisting into adulthood. Excessive weight gain during the period between infancy and puberty is likely the foundation for chronic health maladies which rob citizens of long, healthy, and prosperous lives. Childhood is a period of growth which is accompanied by a gradual decline in the rate of deposition of protein, water, and bone while rate of fat deposition is increased [9]. With maternal influences removed at the end of infancy or at weaning, childhood growth is most susceptible to external manipulation including environment and nutrition [9].

Obese children have been shown to exhibit many of the pro-inflammatory, pro-atherogenic changes associated with vascular disease in adulthood including insulin resistance, hepatic steatosis, elevated LDL cholesterol, decreased HDL cholesterol, and decreased adiponectin levels [30-39]. Perhaps most concerning is that these risk factors are relatively stable in adolescents if BMI is unchanged and persists into adulthood [40, 41]. Although atherosclerosis may not be evident in obese or diabetic children, the predisposition to developing it is likely underway well before adulthood and is likely enhanced by obesity during childhood [42].

Increased energy intake in addition to increased intake of fats and sugars may lead to development and progression of obesity [1, 26-28]. Perhaps more detrimental are the secondary effects of obesity including diabetes mellitus, hypertension, impaired glucose tolerance, and insulin resistance which can manifest in addition to obesity. Of particular concern with childhood obesity is the potential for health maladies to persist into

adulthood, robbing our citizenry of prosperous lives. Studying causative mechanisms underlying development and progression of obesity in an animal model may yield clues useful to developing childhood obesity intervention strategies.

Childhood Eating Behavior and Patterns

Environmental factors strongly influence eating behavior. Such factors include a changing food supply, increased reliance on foods prepared and consumed away from home, food advertising, marketing and promotion, and pricing [43]. Increasingly, both parents in a family work and time at home is limited. With limited time to spend at home and preparing meals, type of food consumed is significantly impacted. Increased reliance on highly processed and convenience foods have resulted in alterations to the glycemic load and nutritional composition of the diet. Increased reliance on foods consumed away from home, which are generally of greater portion size and contain more energy than meals prepared at home, is evidenced by survey studies which show decreased percentage of energy intake from foods consumed at home and an increased percentage of energy intake from foods consumed from restaurants and fast food outlets in adolescents (ages 12-18 y) [44]. Generally, fast food meals are designed to be quickly prepared and consumed, but are also higher in fat and carbohydrates compared to meals prepared at home. With as much as 75% of children reporting eating at a fast food restaurant at least once per week, those who reported eating at a fast food restaurant at least three times per week consume, on average, 38.5% more energy than those who did not eat at a fast food restaurant [45]. Thus, the likelihood of fast food consumption increases with the age of the child [46].

Examination of overall at home and away from home diet quality for American children age 2-19 y revealed that 26% of total meals and snacks were consumed away from home and provide 32% of total daily energy [47]. Away from home meals and snacks are higher in fat and sodium, and lower in iron, fiber, and calcium compared to at home meals [47, 48]. Additionally, portion sizes have increased in recent decades, mirrored by an increase in per capita availability of added sugars and fats [46, 49-51]. Studies from the US, Europe, and Australia have underscored the tendency for adolescents to consume more than recommended intakes of soft drinks, confectionery foods, and fast foods, and lower than recommended intake of fruits, vegetables, and whole grains [52-56] based on a balanced diet. Snacking behavior and increased soft drink consumption are associated with increased fat and sugar consumption [51]. These observations underscore the decreased quality of children's diets in recent decades [46].

Adipose Growth

During early postnatal growth, adipose tissue growth is rapid with adipocytes increasing in size primarily through accretion of triglycerol, stored in the fat droplet. Adipose tissue growth is the result of the storage of energy in the body as fat. Subcutaneous fat is the most prominent fat depot in pigs, regardless of stage of growth [9]. Individual layers of subcutaneous fat are observable, though the layers grow at different rates and respond to nutritional restriction differently [9]. In market pigs, subcutaneous fat may represent as much as 70% of carcass adipose tissue [9]. In addition to the subcutaneous depot, fat can also be deposited in perirenal, and mesentery depots,

although generally less fat is stored in those depots. Nutritional intake and environmental factors strongly influence fat deposition in various depots.

Commercial pigs are typically fed low-fat (5%), high carbohydrate diets [9]. Excess energy intake from commercial swine diets may lead to a state of obesity as pigs can synthesize long chain fatty acids *de novo* from carbohydrates [9]. In humans, fatty acid synthesis occurs in the liver whereas the adipocyte is the major site of synthesis in pigs [9]. Additionally, pigs fed diets low in protein will deposit more fat compared to pigs fed adequate levels of protein [9, 57].

Lipoprotein Profile in Pigs

Lipoproteins, proteins with a lipid attached, are important in transporting lipids in the bloodstream. Of major importance in pigs are very-low-density lipoproteins (VLDL) containing 60% of serum triacylglycerol, low-density-lipoprotein (LDL) containing 40-70% of serum cholesterol, and high-density-lipoprotein (HDL) containing 20-35% of serum cholesterol [9]. The lipoprotein profile is influenced by the lipid and protein composition of the diet [58, 59] and age [60, 61]. Large amounts of triglycerol are transported in a very-low-density particle, the chylomicron, following consumption of a meal with substantial amounts of fat. Insulin stimulates hepatic VLDL production in humans and rats [62-64]. Obese swine have higher plasma insulin concentrations [65, 66], and corresponding elevated VLDL-triglyceride concentrations compared to lean swine [67]. Human and pig lipoprotein profiles are similar with both having more HDL than LDL. Apolipoprotein B-100, a major protein of LDL, is produced exclusively by

the liver in both humans and pigs. Importantly, high concentrations of triglycerides and low concentrations of HDL cholesterol are strong predictors of insulin resistance [68].

Hypercholesterolemia

Cholesterol, a naturally occurring sterol in animal tissues, is synthesized in the liver, adipose tissue, intestine, and central nervous system. It serves many purposes in the body including serving as a protective agent in skin and myelin sheaths of nerve cells, as a detoxifying agent in the bloodstream, and a precursor of many steroids. Deposits of cholesterol form when present in excess, hypercholesterolemia, and in certain pathological conditions. A balance is maintained by adjusting endogenous synthesis to dietary cholesterol intake [69]. Dietary saturated fats produce hypercholesterolemia and polyunsaturated fats have a hypocholesterolemic effect in many species [58]. Saturated fat from the diet increases LDL cholesterol concentrations in most species [58]. Pigs fed tallow have higher concentrations of plasma LDL and HDL cholesterol, as well as more total lipids in the liver compared to pigs fed soybean oil [58]. Saturated fats are known to increase plasma concentration of total cholesterol in a range of laboratory animals, humans, and pigs [70-73].

Cardiovascular Disease

Adult diseases may have origins in childhood and, not surprisingly, excess weight gain is often a precursor to a variety of physiological and psychological aberrations which ultimately predispose individuals to morbidity and mortality [74]. Evidence suggests that foundations of adult vascular disease are laid in childhood and are

accelerated by the co-existence of disease such as diabetes, hypertension, obesity, and hyperlipidemia [75]. Hypertension is more commonly encountered in obese children compared with normal weight children, and elevated blood pressure during adolescence is seemingly a predictor of endothelial dysfunction in adulthood [30]. Hypertension is another indicator of arterial compliance, and adults with hypertension have lower arterial compliance compared to matched controls [76-78]. Carotid elasticity was found to be lower in children considered to be at increased cardiovascular risk due to the presence of elevated blood pressure and total cholesterol compared to a control group [79]. In adults, arterial stiffness is increased with the presence of obesity, diabetes, and hypertension, and although limited, data from children support the same consequence. Increased adiposity leads to increased stiffness of larger arteries, regardless of age.

Early intervention, including weight loss, lifestyle management, and pharmacologic interventions, may reduce many risk factors of vascular disease [75]. By intervening during early stages of obesity onset, negative health effects of obesity may be completely reversed. Weight loss during early onset of obesity will prevent additional metabolic and physiologic dysfunction. Nutritional strategies implemented during childhood may have lasting impacts on food consumed into adulthood. Such intervention strategies may include matching energy intake and expenditure and minimizing refined oil and sugar consumption, thereby decreasing energy intake. Such nutritional intervention strategies decrease incidence of obesity both in childhood and adulthood. Lifestyle management, including exercise and diet management, is another crucial component of controlling the incidence of obesity. A combination of increased physical activity and diet management, to consume healthier meals lower in fat and refined

products, will result in an energy intake which is more closely matched with expenditure. Pharmacologic interventions for obesity are not yet well understood although a variety of options may be available. Possible pharmacologic interventions may include leptin or other appetite suppressants which increases the sensation of satiety. Other possible pharmacologic interventions include use of compounds which inhibit absorption of fat from dietary sources. Although pharmacologic intervention strategies may be a viable option in severely obese individuals, more useful intervention strategies for children include weight loss, lifestyle management, and dietary management. Encouraging better, healthier lifestyle habits at a young age decreases risk of obesity and transcend generations, thus preventing obesity for future generations. Several cardiovascular risk factors are known components of metabolic syndrome [80-85], discussed in a later section.

Body Mass Index

Body mass index (BMI) was created as a measure of muscling, calculated as mass (kg) divided by height squared (m^2) [86, 87]. BMI has evolved to become a convenient calculation of body fat, and is routinely used in clinical settings, along with waist circumference and skin fold thickness, to diagnose obesity and overweight [1]. BMI is advantageous in clinical settings because it is a fast and noninvasive method to estimate body fat. Although BMI is seemingly useful for differentiating obesity in adults, its usefulness in children may be limited because it does not take into account the changes to body shape with the progression of normal growth [1]. As children grow, target BMI for normal weight changes with age and as the population becomes more obese.

Body mass index fails to differentiate between fat, muscle, and bone which may exaggerate obesity in large, muscular children [1]. Further complicating the use of BMI as an indicator of obesity and overweight are the differences in maturation pattern between genders and different ethnic groups [1]. Although BMI is currently the clinical standard for diagnosing obesity, perhaps a surrogate of visceral or central adiposity would be a more reliable indicator in children. Waist circumference may be a suitable alternative to the abstract nature of variables used to calculate BMI. Waist circumference specifically targets central obesity, a risk factor for type II diabetes and coronary heart disease [1, 88]. It is an easily obtained, objective measurement which may allow clinicians to predict obesity and metabolic syndrome in children with increased accuracy.

Glucose Tolerance

Glucose metabolism is essential for supplying energy to cells and is tightly controlled to maintain homeostatic blood glucose concentrations throughout the day. Carbohydrates can either be stored as glycogen or converted to fat, most energy stored in the body is stored as fat [89, 90]. Glycogen stores may later be broken down to liberate glucose, the ultimate energy source for most cells [89, 90]. Only monosaccharides can be absorbed from the gastrointestinal tract (GIT); di-, tri-, and polysaccharides must be hydrolyzed by digestive enzymes for absorption to occur [89, 90]. A variety of digestive enzymes aid in hydrolysis of more complex carbohydrates, examples include amylase and sucrase. Proximal portions of the small intestine, duodenum and jejunum, exhibit the greatest capacity to absorb monosaccharides from the GIT; few monosaccharides are

absorbed in the distal small intestine, ileum, and little, if any, absorption occurs in the stomach [89, 90].

Two families of glucose transporters exist. Sodium-glucose co-transporters (SGLT) actively transport glucose across the intestinal mucosal cell during absorption from the lumen and across the kidney tubule [89, 90]. By coupling sodium with glucose, this family of co-transporters is able to transport glucose against its concentration gradient while transporting sodium down its concentration gradient, maintained by membrane-bound potassium ATPase [89, 90]. Facilitative glucose transporters comprise the second family of glucose transporters. This family actively transports glucose down a concentration gradient and are present in nearly all mammalian cells [89, 90]. Thirteen glucose transporters (GLUT) have been identified, each with different kinetic properties, substrate specificities, and expression profiles [89, 90].

Normal metabolism begins with consumption of a meal which may consist of mono-, di-, tri-, or polysaccharides. Blood glucose levels increase following meal consumption as carbohydrates are broken down into constituent monosaccharides for absorption from the small intestine [89, 90]. Monosaccharides may be converted to glucose in the intestinal mucosa cells and glucose appears unchanged in the portal vein following absorption [89, 90]. GLUT1 is the glucose transporter for erythrocytes and is responsible for the basal glucose uptake which sustains respiration in cells [89, 90]. GLUT1 is also present in endothelial cells of barrier tissues such as the blood-brain barrier [89, 90]. Expression levels of GLUT1 are increased when glucose concentrations in the blood are reduced and expression is decreased by increased concentrations of glucose in the blood [89, 90]. As blood glucose levels increase beyond the normal

physiological range of four to six mmol/L [9], GLUT2 transports glucose into pancreatic β -cells, stimulating insulin release [89, 90]. Insulin then acts to stimulate glucose uptake and utilization in muscle and adipose tissue via the GLUT4 transporter [89, 90]. Glucose can then be utilized by cells for normal physiological function or can be converted to glycogen via glycogenesis and stored in liver and muscle tissues [89, 90]. Glycogen formation requires input of two molecules of adenosine triphosphate (ATP) per glucose molecule, adding one unit of glucose at a time to form a long chain of glycogen [89, 90]. Due to insulin stimulated glucose uptake, blood glucose levels return to homeostatic levels within a few hours following a meal. Conversely, in fasted or food-deprived states, blood glucose levels are diminished and glycogen stores can be broken down to liberate glucose (glycogenolysis) [89, 90]. Glucagon, a hormone produced by pancreatic α -cells, plays an important role in glycogenolysis [89, 90]. Glucagon mobilizes stores of glucose and fatty acids for oxidation [89, 90].

In abnormal metabolic states, glucose metabolism is significantly altered. One metabolic defect, common to diabetes, is inadequate production of insulin, inadequate sensitivity to insulin, or both [89, 90]. In addition to stimulating glucose uptake and utilization by various cells in the body, insulin also stimulates protein synthesis, decreases protein catabolism, and indirectly stimulates lipogenesis and inhibits lipolysis [89, 90]. Insulin is essential for normal glucose metabolism, thus in states of hypoinsulinemia or insulin resistance, hyperglycemia and impaired glucose tolerance result [89, 90]. Chronic hyperglycemia and hypoinsulinemia can lead to additional health maladies, chief among them manifestation of the metabolic disease, diabetes mellitus. In

addition, in hyperglycemic states, the action of glucagon is unnecessary and glycogen stores are not mobilized.

Several factors affect circulating glucose levels including food intake, metabolism, rate of digestion, and level of physical activity. Lack of insulin production or reduced receptor sensitivity to insulin may result in an inability to lower blood glucose concentrations in response to a meal. Insulin resistance, characterized by the inability of the GLUT4 transporter to translocate to the cell membrane for glucose uptake in skeletal muscle and adipose tissue, also results in a state of hyperglycemia. In a study of 55 obese children aged 4-10 y, and 112 obese children aged 11-18 y, 25% and 21%, respectively, had impaired glucose tolerance, with blood glucose concentrations remaining elevated during glucose tolerance testing [91].

Glucose and fructose occur primarily as monosaccharides in fruits, vegetables, high-fructose corn syrup, and honey [92]. Fructose is a hexomer of glucose, both are of the molecular formula $C_6H_{12}O_6$, yet differ in structure [89, 90]. Carbon number 1 is the anomeric carbon, the carbon comprising the carbonyl function, in glucose whereas carbon number two is the anomeric carbon in fructose [89, 90]. An α,β -glycosidic bond joins fructose and glucose in sucrose, a disaccharide [92]. High-fructose corn syrup incorporation into a variety of food products is increasing. In the United States, high fructose corn syrup is produced by enzymatically converting some of the glucose in corn syrup to fructose, yielding a higher concentration of fructose in the resulting syrup. Incorporating high fructose corn syrup into foods offers the advantages of lower cost in addition to flavor enhancement, depression of freezing point and osmotic pressure, and moisture retention [42]. Although there is much ambiguity in studies which have

evaluated the effects of consuming large amounts of fructose [42, 93-95], consumers have expressed both surprise and concern in the common use of high-fructose corn syrup in a variety of products.

Mechanisms of fructose absorption are not yet completely known. Fructose uptake can occur against the concentration gradient suggesting a possible active transport mechanism [89, 90]. Fructose transport occurs independently of the active sodium-dependent glucose transport, and rate of uptake is slower than that of glucose, because dietary fructose cannot pass through the liver [89, 90]. Fructose may also be absorbed via facilitated transport, involving a specific transporter, in the absence of glucose [89, 90]. It has been suggested that GLUT5 may function as a fructose transporter [96]. Facilitative fructose transport can only occur down a concentration gradient. Fructose is efficiently trapped and phosphorylated by the liver, because there is no rate limiting step in its metabolism, resulting in very little circulating fructose, and ensuring a downhill concentration gradient [89, 90]. Simultaneous or co-consumption of fructose and glucose accelerates fructose absorption and increases the threshold for malabsorption symptoms [89, 90].

Although high fructose corn syrup incorporation into food products offers numerous advantages, consequences of its consumption are unclear. No rate limiting steps have been identified in fructose metabolism, thus, fructose is most likely stored as liver glycogen, however, it may also be catabolized for energy or used for *de novo* adipogenesis [89, 90]. However, rate limiting steps in glucose metabolism allow the monosaccharide to enter the portal blood stream for uptake and utilization in skeletal muscle and adipose tissue [89, 90].

Insulin

Discovered in the early twentieth century, insulin is well known for its role in glucose metabolism and in the disease diabetes mellitus. After a carbohydrate-rich meal, blood glucose concentrations increase in response to digestion and absorption of glucose [89]. GLUT2 transports glucose into pancreatic β -cells, stimulating insulin release and increasing circulating insulin [89, 90]. Insulin then stimulates glucose uptake and utilization in liver, muscle, and adipose tissue, thus removing glucose from the blood [89, 90]. The action of insulin allows tight regulation of blood glucose levels throughout the day. Individuals with insufficient insulin production are unable to remove glucose from circulation, resulting in hyperglycemia and loss of glucose in urine [89, 90]. Insufficient insulin production may be a result of pancreatic β -cell failure leading to uncontrolled glucose production, lipolysis, ketogenesis, and, ultimately, death [89, 90]. Failure of insulin secretion, failure of biological action of insulin, or both can lead to a diabetic state [97].

An increase the concentration of insulin necessary to occupy binding sites has been observed in obese swine with increasing age and/or body weight [98]. Obese swine are observed to have approximately two-fold higher plasma insulin concentration as compared to lean swine, suggesting that binding affinity may be influenced by plasma insulin concentration [66]. Porcine studies of induced obesity have underscored the role of insulin in regulating glucose homeostasis. Administering streptozotocin, a naturally occurring compound which is partially toxic to insulin producing β -cells of the pancreas, to pigs results in hyperglycemia, characteristic of a diabetic state [99, 100]. Diabetic pigs

have lowered adipose tissue lipogenic capacity, which indicates insulin is important for fat lipogenesis in adipose [101]. Although reported insulin responses have varied [102-105], at least one study has demonstrated that hyperinsulinemia stimulates glucose incorporation into adipose tissue lipids [106].

Impaired insulin sensitivity or insulin resistance, by decreased production, or failure of GLUT4 transporters to be translocated to the plasma membrane, in skeletal muscle and the liver, is directly responsible for development of type II diabetes [107]. A strong association between insulin resistance, failure of GLUT4 transporters to translocate to the plasma membrane [108], and increased risk for myocardial infarction and stroke has been established in epidemiological studies [109, 110]. Insulin resistance and the compensatory hyperinsulinemia may predispose patients to hypertension, hyperlipidemia, and diabetes, and may be an underlying cause of cardiovascular disease [111]. Detection of porcine insulin resistance is important because the model replicates many features of human insulin resistance such as hyperinsulinemia, hyperglycemia, hypertension, and low plasma adiponectin levels [112]. Thus, early metabolic alterations in large animal models may be reflective of diet-induced obesity in humans [113].

Blood glucose, blood pressure, and BMI measurements may be less sensitive, but more specific, predictors of insulin resistance compared to individual components of metabolic syndrome [114]. An inverse relationship between intra-abdominal fat and β -cell function was recently evaluated in a human cohort study [115]. Whether decreased production, decreased tissue sensitivity to insulin, or a combination of the two is the mechanism, insulin plays a crucial role in development, progression, and treatment of diabetes mellitus. The amino acid sequence of insulin is highly conserved across species,

allowing humans to use insulin from porcine and bovine sources [97]. Porcine-derived insulin has been used to treat diabetes in humans since 1922 and other recombinant forms of insulin are becoming more available [97].

Diabetes Mellitus

Diabetes mellitus is a complex disease manifested by hyperglycemia and has become a world wide epidemic. Data collected in 2007 indicate a prevalence of 7.8% in the population [116]. Diabetes may arise from insulin deficiency, insulin resistance, or some combination of the three [117]. Basal plasma insulin concentration has been shown to positively correlate to degree of obesity [118] and insulin resistance [119]. Diabetes is believed to accelerate atherosclerosis, increase incidence of myocardial infarction, and increase risk of stroke [97]. Two forms predominate, identified as type I and type II diabetes. The defining characteristic of both forms is the inability of insulin to reduce blood glucose concentrations [89, 90].

Type I diabetes, an autoimmune disease affecting the pancreas, is called insulin-dependent or juvenile onset diabetes [89]. In this type of diabetes, insulin is either absent or present in very low concentrations [97] as β -cells fail to produce and secrete insulin [90]. Symptoms include polyuria, polydipsia, weakness, fatigue, and polyphagia accompanied by weight loss [97]. Glucose is poorly utilized in the absence of insulin, creating a state in which energy demands remain yet the body is unable to capture circulating glucose [97]. Thus, energy stores continue to be broken down despite the presence of ample circulating glucose [97]. Treatment for type I diabetes is primarily

accomplished by careful monitoring of glucose, diet, and exogenous insulin therapy to replace missing endogenous insulin [97].

β -cell depletion applies to juvenile diabetics [120, 121] and may also apply to maturity-onset diabetics who have been reported to have reduced islet frequency and volume and reduced pancreas insulin content [122-125]. In such patients, β -cells are degranulated, indicative of a decrease in the number of functional β -cells working near capacity [122]. Some suggest that diabetics experience reduced β -cell sensitivity to glucose [126, 127]; others suggest glucose sensitivity is normal with diminished insulin secretory response [128]. New juvenile diabetics presenting with ketosis or diabetic acidosis have approximately one-third the basal plasma insulin concentrations of normal subjects [129]. Such a reduction of insulin effectiveness may be due to obesity.

Type II diabetes is known as noninsulin-dependent or adult onset diabetes [89], however, this type of diabetes is being observed more frequently in younger patients, including children. Type II diabetes is the most common metabolic disease worldwide and obesity is thought to be a contributing factor [108]. This form of diabetes is characterized by the failure of insulin, although present, to stimulate translocation of GLUT4 transporters to the plasma membrane of cells, so termed insulin resistance [108]. This is generally considered to be a milder form of diabetes in comparison to type I diabetes because it is highly dependent upon the degree of biological failure [97]. Insulin resistance, characteristic of type II diabetes, results in increased glucose output by the liver, even when dietary glucose is present, leading to hyperglycemia [108]. Symptoms of type II diabetes include polyuria, polydipsia, blurred vision, fatigue, and frequent or slow-healing infections as a consequence of hyperglycemia [108]. Potential roles of

obesity in type II diabetes reflects the abundance of insulin receptors in adipose tissue [97]. At the onset of type II diabetes, hypertriglyceridemia, low HDL cholesterol, and moderately high LDL cholesterol commonly accompany insulin resistance and relative failure of β -cells [75]. Hyperinsulinemia is a key feature of type II diabetes [130, 131], in addition to hyperlipidemia [111, 132, 133], obesity [134-137], and hypertension [136-138].

If left untreated, type II diabetes can progress to type I diabetes. Treatment for type II diabetes generally includes weight loss, diet management, and insulin therapy [97]. Insulin therapy is more complex with type II diabetes because therapy is dependent upon the remaining functional β -cells and the degree of insulin insensitivity, or the ability to translocate GLUT4 transporters to the membrane for glucose uptake [97].

Metabolic Syndrome

Near the end of the 20th century, a cluster of risk factors for cardiovascular disease were described, notably the simultaneous presence of obesity, type II diabetes, hyperlipidemia, and hypertension [139, 140]. This clustering of risk factors would later become known as the metabolic syndrome [141-145] which is diagnosed by the presence of three of these characteristics [142, 143]. Metabolic syndrome seemingly contributes to increased intima-media thickness, or thickening of arterial walls, with increasing characteristics of metabolic syndrome present [146]. Prospective studies which followed children for more than three decades have provided further support that childhood obesity and presence of risk factors have negative effects on vascular health in adulthood [147, 148].

The National Cholesterol Education Program's Adult Treatment Panel defined metabolic syndrome in its third report (ATP III). The ATP III defined metabolic syndrome as any three or more of the following criteria: waist circumference (greater than 102 cm in men and 88 cm in women), serum triglycerides greater than or equal to 1.7 mmol/L, blood pressure greater than or equal to 130/85 mmHg, HDL cholesterol (less than 1.0 mmol/L in men and 1.3 mmol/L in women), and serum glucose greater than or equal to 6.1 mmol/L [149, 150]. In comparison, the World Health Organization (WHO) defines metabolic syndrome with slightly different parameters. In addition to one of the following: diabetes, impaired fasting glucose, impaired glucose tolerance, or insulin resistance, a patient must also fulfill two of the following criteria: waist: hip ratio (greater than 0.90 in men and 0.85 in women), serum triglycerides of at least 1.7 mmol/L or elevated HDL cholesterol (greater than 0.9 mmol/L in men and 1.0 mmol/L in women), blood pressure of at least 140/90 mmHg, a urinary albumin excretion rate greater than 20 µg/min or albumin:creatinine ratio of at least 30 mg/g [145].

Some criteria of metabolic syndrome, such as waist circumference and HDL cholesterol, have specific gender-related thresholds, which implies the outcomes and risk factor levels differ between genders. Although some suggest different thresholds should be considered, no evidence has been found to support gender, race, or ethnicity threshold differences as they relate to cardiovascular disease [114]. Irrespective of how defined, individuals with metabolic syndrome have a higher risk of cardiovascular disease than individuals without the syndrome [151], unsurprisingly as individual components of metabolic syndrome are known to be major cardiovascular risk factors [80-85].

A likely shortcoming of both the ATP III and WHO definitions of metabolic syndrome is that each component is of equal weight, yet some components clearly have more predictive power for cardiovascular disease than others [114]. Additionally, neither the WHO or ATP III consider age, level of physical activity, or familial history of cardiovascular disease in defining metabolic syndrome [114]. The definitions of metabolic syndrome by the WHO and ATP III underscore the controversy in defining the syndrome as it is a cluster of factors which increase the risk of cardiovascular disease. Another important consideration in the definition of metabolic syndrome is the potential for additive consequences of the components. This would suggest the possibility for metabolic syndrome to be of more severe consequence than the sum of its component characteristics. Variations in defining metabolic syndrome have been demonstrated to lead to as much as a 24% discrepancy in the prevalence of metabolic syndrome, depending on sex and ethnicity of the population [151].

Hepatobiliary Injury

Bilirubin is a major end product of normal red blood cell turnover and is produced by Kupffer cells lining the lobule sinusoid [9]. From Kupffer cells, bilirubin is transported to the hepatocyte for conjugation, then secreted into bile [9]. Hepatocytes continuously secrete bile, although it is needed only intermittently for fat digestion [9]. Conjugated bilirubin can be converted into other forms and excreted from the body, giving the characteristic colors to feces and urine [9]. Previous studies have demonstrated that secretion rate of bile acids increases dramatically in response to increased dietary fat content [152]. Bile acids in the pig are hydrophilic and, therefore,

spontaneously form cholesterol-supersaturated bile, as is the pattern in humans [9]. Studies suggest that an increased expression of heme oxygenase, the enzyme which breaks hemoglobin down into bilirubin, may be associated with enhanced insulin sensitivity and glucose metabolism [153, 154]. Interestingly, a growing body of literature suggests that higher levels of total bilirubin serve as a powerful antioxidant and anti-inflammatory agent [155-165] and may protect against coronary heart disease [166, 167].

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are commonly measured clinical indicators of liver health [9]. Aspartate aminotransferase catalyzes the reversible transfer of an α -amino group between aspartate and glutamate and is important in amino acid metabolism. Alanine aminotransferase catalyzes two parts of the alanine cycle involving transfer of an amino group. Alkaline phosphatase (ALK) is a dephosphorylation enzyme important in amino acid metabolism and indicative of several types of hepatic injury and bone growth [9]. Biliary obstruction stimulates synthesis of ALK within canalicular membranes proximal to the obstruction [168]. Elevated ALK activity in the serum has been associated with obstructive liver disease for nearly four decades [169]. Two theories have evolved regarding elevated ALK levels. The “retention” hypothesis formed on the basis that a damaged liver is unable to clear phosphatases made in other tissues, resulting in increased levels of alkaline phosphatase [170]. The “regurgitation” hypothesis is based on the assumption that elevated serum phosphatase originates in the liver [171] and is supported by several recent studies [172-174], despite little evidence that the human liver excretes serum ALK into bile. However, liver biopsies obtained from obstructive jaundice patients contain greatly increased levels of ALK compared with livers obtained from normal patients [168].

Increased serum γ -glutamyl transferase (GGT), involved in the transfer of amino acids across the cell membrane, has been previously associated with liver diseases, and predicts morbidity and mortality independently of both alcohol intake and liver disease [176, 177]. Moderate increases within a normal GGT range may be an early marker of oxidative stress and thus explain the strong association of GGT with many cardiovascular risk factors and disease [178]. Increases in GGT are predictors of hypertension, incident cases of type II diabetes, and coronary heart disease independent of alcohol intake or liver disease [176, 177, 179-182]. Additionally, several studies have linked increased levels of GGT with metabolic syndrome [177, 179, 183]. Waist circumference is a major determinant of circulating GGT levels [183] and may be a marker of hepatic steatosis with or without hepatic insulin resistance [184, 185].

The incidence of non-alcoholic fatty liver disease (NAFLD) is increasing in children and adults due to the increasing incidence of obesity and type II diabetes [186, 187]. Non-alcoholic fatty liver disease is often associated with metabolic syndrome and is considered to be the hepatic manifestation of the disease [188-190]. NAFLD is a result of ectopic, or abnormal, fat storage, an issue emerging as central to the pathogenesis of metabolic syndrome [191]. Molecular mechanisms leading to development of hepatic steatosis in the pathogenesis of NAFLD include enhanced lipolysis, increased lipogenesis, and decreased beta-oxidation [192]. Interestingly, it is commonly accepted that a second “hit” is required above baseline steatosis for NAFLD progression to occur [193, 194]. This second “hit” is described as steatosis in addition to oxidative stress in the liver [193, 194].

Summary

Childhood obesity represents a severe public health concern due to the potential chronic health effects for the individual as well as increasing costs of health care. Development of childhood obesity is not yet well understood but is likely due to excess energy intake, type of calorie consumed, or perhaps a combination. Excessive adipose tissue gain leads to severe chronic health issues, including diabetes mellitus and cardiovascular disease. Causative mechanisms underlying childhood obesity development and progression are not easily studied in humans due to ethical concerns, thus development of a translational animal model is warranted.

Childhood is a dynamic period of growth and compositional changes. Once maternal influence has been removed, children are most susceptible to external factors such as environment and nutritional manipulation. Increased reliance on highly processed, energy-dense, nutrient-poor foods consumed away from home has resulted in increased intake of fats and refined sugars as well as increased body fat. Effects of childhood obesity may be reversed with well-timed strategic interventions. As causative mechanisms of obesity are discovered, intervention strategies can be developed and implemented successfully.

Animal models have a distinct niche for studying causative mechanisms underlying childhood obesity. Pigs have been used as a biomedical model for decades and offer great potential for modeling childhood obesity. Simulating the development of diet-induced obesity using a high-fat, high-sugar diet in pigs will provide valuable clues to understanding the development of childhood obesity as well as providing additional clues to reverse and prevent manifestation of this disease.

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CHAPTER II

Dietary manipulation causes childhood obesity-like characteristics in pigs

Abstract

A translational animal model to study complications resulting from childhood obesity is lacking. Our objective was to develop a porcine model for studying mechanisms underlying diet-induced childhood obesity. Pre-pubertal female pigs, age 35 d, were fed a high-energy diet (HED; $n = 12$), containing tallow and refined sugars, or a control corn-based diet ($n = 11$) for 16 wk. Initially, HED pigs self-regulated energy intake similar to controls, but, by wk 5, consumed more ($P < 0.001$) energy per kg body weight. At wk 15 and 22, pigs were subjected to an oral glucose tolerance test (OGTT); blood glucose increased ($P < 0.05$) in control pigs and returned to baseline levels within 60 min. HED pigs were hyperglycemic at time 0, and blood glucose did not return to baseline ($P = 0.01$), even 3 h post-challenge. During OGTT, glucose area under the curve was higher and insulin area under the curve was lower in HED pigs compared to controls ($P = 0.001$). Pigs given 6 wk of dietary intervention marginally improved glucose area under the curve and blood metabolites although insulin area under the curve was unaffected. Chronic HED intake increased ($P < 0.05$) subcutaneous, intramuscular, and perirenal fat deposition, and induced hyperglycemia, hypoinsulinemia, and low-density lipoprotein hypercholesterolemia; however, a 6 wk dietary intervention partially recovered a healthy physiology. These data suggest pre-pubertal pigs fed HED are a viable translational large animal model for studying childhood obesity.

Keywords: adiposity, hyperglycemia, hypoinsulinemia, hypercholesterolemia, diabetes, metabolic syndrome

Introduction

Childhood obesity, defined simply as excess accumulation of body fat [1, 2] from infancy to puberty, represents a growing public health concern with increasing incidence. The American Heart Association currently estimates 1 in 3 children in the United States are overweight or obese [3, 4]. Childhood obesity increases risk for chronic health maladies including hypertension, increased blood pressure, sleep apnea, irregular heart rhythms, stroke, and diabetes [3-10]. Suspected leading causes of childhood obesity include excessive caloric intake and increased consumption of saturated and trans fats and cholesterol [3, 4, 9]; however, hypotheses relating to childhood obesity have not been tested mainly due to ethical concerns. As such, animal models are desperately needed to conduct studies on induction and development of obesity during pre-pubertal growth so that nutritional interventions may be developed and tested to reverse childhood obesity and the associated physiological aberrations. Thus, establishment of an animal model to further our understanding of biological mechanisms and consequences of childhood obesity is clearly warranted.

The pig is a well-regarded translational model for studying obesity and metabolic syndrome because of similarities to humans in regards to whole body metabolism, cardiovascular system, and digestive system [11-22]. Ossabaw pigs fed a high-fat and high-calorie diet develop characteristics of metabolic syndrome, including insulin resistance, glucose intolerance, dyslipidemia, and hypertension [12, 14, 18, 19, 23-25]. Miniature pigs have increased cholesterol and triglycerides as a result of consuming a high fat diet, with females exhibiting a greater response than males [13]. However, previous studies using these models were conducted with pigs at least 5 months of age

[18, 24], which is the end of the pre-pubertal or childhood period. Pig childhood, defined as the period of life between weaning and puberty, lasts approximately 22 wk and growth during pig childhood is comparable to childhood growth in humans. Therefore, our objective was to induce obesity and alter glucose tolerance in commercially bred pigs during the childhood period. We also tested the validity of nutritional intervention of childhood obesity.

Materials and Methods

Animals and housing

The Virginia Tech Institutional Animal Care and Use Committee approved all experimental procedures; 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. Female pigs (Premium Genetics 1020, Murphy-Brown, Waverly, VA) obtained from a commercial swine farm (Waverly, VA) were weaned at 21 d of age and transported to Virginia Tech. Pigs were individually housed in 0.6 x 0.9 m double-deck pens as previously described [26, 27] and offered a basal diet (Table 1) which met or exceeded nutrient recommendations [28]. Pigs were given ad libitum access to food and water, unless otherwise indicated. At 35 d of age, pigs were weighed and randomly assigned to either control (CON) or HED treatments (CON: 12.88 ± 0.57 ; HED: 13.45 ± 0.55 kg BW, $P = 0.45$); trial commencement was defined as wk 0. At wk 3, pigs were moved to 1.22 x 1.22 m pens for the remainder of the study.

Diets and experimental procedures

Control (n = 11) and HED (n = 12) diets were formulated and fed in phases based on nutritional requirements of pigs. HED was formulated with a reduction in standardized ileal digestible amino acid content, thus more energy was diverted to fat accumulation rather than lean deposition. Diet phase was determined according to mean body weight (BW) within treatment (Table 1). Food disappearance, BW, and ultrasound measures were collected weekly. CON and HED diets were fed for 16 consecutive wk. A group of HED pigs was selected for a 6 wk dietary intervention (INT, n = 7) to simulate recovery from chronic HED consumption. Dietary intervention was accomplished by replacing the HED with a control diet for 6 wk. At the end of respective treatment periods, pigs were euthanized by electrical stunning followed by exsanguination. *Longissimus* muscle samples were collected immediately following exsanguination; liver samples were collected following evisceration. Samples for proximate analysis were snap frozen in liquid nitrogen and stored at -80°C. Halved carcasses were chilled at 4°C for 24 h. The cross-sectional area of the *longissimus dorsi* muscle (LMA) and the subcutaneous fat thickness were determined by cutting perpendicular to the long axis of the carcass between the 10th and 11th ribs [29]. Perirenal fat was manually dissected and weighed.

Ultrasound

Depths of subcutaneous (USubQ) fat and *Longissimus* muscle at the last rib were collected weekly using a portable real-time ultrasound scanner (Aloka SSD-500v, Aloka Co., LTD., Wallingford, CT) with a 7.5MHz (wk 1 to 7) or a 5MHz (wk 8 onward) transducer (all from Aloka Co., LTD., Wallingford, CT). Vegetable oil was used as the

ultrasound medium. Ultrasonic measurements were used to estimate changes in body adiposity and lean content in individual pigs throughout treatment.

Oral glucose tolerance test (OGTT) and clinical characteristics

Pigs were subjected to OGTT at wk 16 (CON and HED) and 22 (INT) of study. Pigs were food-deprived 12 h and then offered an amount of CON diet equal to 1% BW mixed with a 40% D-glucose solution for an offering of 2 g glucose per kg BW. Animals were physically restrained and jugular venipuncture blood samples were collected in lithium-heparinized vacutainers (BD, Franklin Lakes, NJ) 0.5 h before and at 0.5, 1, 2, and 3 h post offering. Blood samples were immediately analyzed for glucose (YSI 2300 STAT Plus, YSI Inc., Yellow Springs, OH), then centrifuged ($3,000 \times g$, 15 min, 4°C). Resulting plasma was collected and frozen at -80°C until analyses. Plasma insulin (Porcine ELISA, ALPCO Diagnostics, Salem, NH), low density lipoprotein (LDL), high density lipoprotein (HDL), triglyceride (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, γ -glutamyl transferase (GGT), and alkaline phosphatase (ALK) were determined using commercially available kits (all from Teco Diagnostics, Anaheim, CA) according to manufacturer instructions.

The insulin ELISA, a sandwich type immunoassay, uses mouse antibodies specific for insulin which are immobilized to the microplate as the solid phase. Samples are sandwiched between the solid phase and the conjugate, a horseradish peroxidase enzyme labeled monoclonal antibody. The plate is then incubated, washed to remove unbound conjugate, substrate is added and the plate is incubated a second time during which a blue color develops as a result of substrate reacting with bound conjugate. Stop

solution is added which stops the reaction and changes the color from blue to yellow, allowing optical density to be measured at 450 nm. Color intensity is directly proportional to the amount of insulin in the sample. Insulin resistance was estimated using homeostatic model assessment (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI) as previously described [30, 31]. Pancreatic β -cell function was estimated using HOMA-B [31] with the formula $[\text{fasting insulin (mU/L)} \times 20] \div [\text{fasting glucose (mmol/L)} - 2.0]$, which accounts for lower fasting glucose levels in pigs compared to humans.

Low density lipoprotein cholesterol was determined using a two part, liquid stable reagent. A detergent reagent was added to sample to solubilize non-LDL lipoprotein particles. Cholesterol is released and consumed by cholesterol esterase and cholesterol oxidase in a non-color forming reaction. A second detergent then solubilizes the remaining LDL particles with a coupler to allow color formation. Color produced is proportional to amount of LDL cholesterol present in the sample. The intra-assay coefficient of variation for LDL was 4.75%. High density lipoprotein cholesterol was determined using a two-reagent format. The first reagent stabilizes LDL, very low density lipoprotein, and chylomicrons while the second reagent contains modified enzyme which selectively react the cholesterol in HDL particles. The intra-assay coefficient of variation was 11.87%. Triglyceride assay involves hydrolysis of triglycerides by lipase, yielding glycerol and fatty acids. Glycerol concentration can then be determined by enzymatic assay coupled with Trinder reaction, terminating in the formation of a quinoneimine dye. The amount of dye formed, determined by absorption

at 520 nm, is proportional to the concentration of TG in the sample. The intra-assay coefficient of variation for TG was 5.76%.

Aspartate aminotransferase catalyzes the reaction of aspartate and oxoglutarate to yield oxalacetate and glutamate. The assay uses a diazonium salt which selectively reacts with oxalacetate to produce a photometrically measurable color complex. The intra-assay coefficient of variation was 3.51% for AST. Alanine aminotransferase catalyzes the reaction of alanine and α -ketoglutarate to form pyruvate and glutamate. Pyruvate then reacts with 2, 4-dinitrophenyl-hydrazine (2, 4-DNPH-ine) to form 2, 4-DNPH-one. Addition of sodium hydroxide dissolves the complex and allows measurement of 2, 4-DNPH-one at 505 nm. The intra-assay coefficient of variation for ALT was 5.12%. The bilirubin assay measures the intensity of color produced by the reaction of bilirubin with diazotized sulfanilic acid producing azobilirubin. Azobilirubin has a maximum absorbance at 560 nm in dimethyl sulfoxide solvent. Color intensity is proportional to the amount of bilirubin in the sample. The bilirubin assay has an intra-assay coefficient of variation of 11.30%. Γ -glutamyl transferase catalyzes the transfer of a γ -glutamyl group from γ -glutamyl-p-nitroanilide. Rate of liberation of p-nitroaniline is related to the GGT activity in the sample and is quantitated by measuring the increase in absorbance at 405 nm. The intra-assay coefficient of variation was 6.30% for the assay. Alkaline phosphatase acts on AMP-buffered sodium thymolphthalein monophosphate. Addition of an alkaline reagent stops enzymatic activity and simultaneously develops a photometrically measurable blue chromogen. The intra-assay coefficient of variation for this assay was 16.02%.

Proximate analysis

Moisture and extractable lipid content of *Longissimus* muscle and liver samples were determined according to Novakofski et al. [32] with modifications. Briefly, 2 g of frozen muscle was ground, weighed, and enclosed in dried filter paper. After freeze-drying to determine moisture content, lipid was extracted in a Soxhlet apparatus using chloroform: methanol (87:13) for 12 h. Samples were dried and weighed to determine extractable lipid content.

Statistics

Pig was considered the experimental unit. Data normality was tested using the univariate procedure of SAS (Ver. 9.1.3, SAS Institute, Cary, NC); all data were normally distributed. The mixed procedure of SAS with repeated measures using time (week) and treatments as fixed effects and pig, pen type, and room as random effects for randomized complete-block design [33] was used to test the effect of diet on tissue adiposity and plasma metabolites. BW was used as a covariate for compositional parameters; glucose intake during OGTT was used as a covariate for plasma glucose and insulin analyses. Glucose and insulin total area under the curve (AUC) during OGTT were calculated using a SAS macro [34]. Least square means were compared using a *t*-test and Tukey adjustment in SAS. Data are presented as least squared means \pm pooled SE. Statistical significance was determined as $P < 0.05$; tendency for statistical significance was determined as $P < 0.10$.

Results

Growth and carcass characteristics

Ratio of metabolizable energy (ME): standardized ileal digestible amino acid (AA) was modified in HED to alter nutrient partitioning to divert more energy toward lipid accretion. Average growth rate of HED pigs was 31% lower ($P < 0.001$) than CON (Figure 1A), in agreement with our ME:AA reduction. Because of the increased energy density, HED pigs consumed less ($P < 0.0001$) food daily than controls. Initially, HED self-regulated energy intake (ME/kg BW) similarly to CON ($P = 0.83$), but, by wk 5, HED pigs consumed more ($P < 0.001$) metabolizable energy per kg body weight (Figure 1B). Over treatment duration, HED pigs consumed 16.2% more ($P < 0.001$) calories per kg BW than CON pigs (Figure 1B inset).

Ultrasonic subcutaneous (USubQ) fat depth and muscle depth were determined over treatment duration to provide an index of fat and muscle compositional changes. Proportional muscle growth, depth of *Longissimus* muscle per kg of BW, was not different between treatments (Figure 2A). However, proportional fat deposition, USubQ fat depth at the last rib per unit of BW, was increased ($P < 0.05$) for HED pigs (Figure 2B). Moreover, by wk 6 HED pigs deposited twice as much ($P < 0.001$) USubQ fat per unit of lean compared to CON pigs (Figure 2C).

Final body weight and empty BW of CON were greater ($P < 0.01$) than HED pigs (Table 2). Carcass SubQ fat at the 10th rib was 87% thicker ($P < 0.05$) in HED than controls. A 21% reduction ($P < 0.01$) in SubQ carcass fat was measured in INT compared to HED pigs. Visceral fat tended to be increased ($P < 0.10$) in INT compared to HED, and was higher ($P < 0.001$) in HED than CON pigs. In HED, LMA was reduced ($P < 0.001$) compared to controls.

Proximate moisture content was higher ($P < 0.001$) in *Longissimus* muscle of CON compared to HED pigs (Table 2). Fat content of *Longissimus* muscle was higher ($P < 0.05$) in HED compared to controls. With dietary intervention, fat content of *Longissimus* muscle was reduced by 40% in INT compared to HED pigs. Liver moisture and fat content were not different among treatments.

Carcass fat content was greater in HED pigs than controls ($P < 0.001$), while those HED pigs switched back to a control diet (INT) did not differ ($P < 0.001$) from controls but were indeed numerically higher.

Clinical characteristics

At wk 16, fasting glucose was 16.5% higher (Figure 3A) and fasting insulin was 47.4% lower (Figure 4A) in HED compared to CON pigs ($P < 0.001$). Consequently, HOMA-IR and HOMA-B were lower whereas QUICKI was higher ($P < 0.001$) in HED pigs compared to controls (Table 3). A 6 wk intervention period did not alter fasting glucose, insulin, HOMA-IR, HOMA-B, or QUICKI in intervention pigs compared to HED; hence these measurements remained different from CON pigs ($P < 0.001$).

Fasting LDL levels at wk 16 were 35.7% higher ($P < 0.003$) in HED compared to CON, and INT pigs had reduced ($P < 0.01$) fasting LDL concentrations by 32% following the 6 wk dietary intervention. Similarly, plasma TG levels tended to be increased ($P < 0.06$) in HED compared to CON pigs. Dietary intervention tended to reduce ($P < 0.07$) plasma TG levels in intervention pigs compared to HED; values were not different ($P = 0.87$) from controls. No differences in plasma LDL, HDL, or TG were discernable among CON and INT treatments following intervention.

Plasma ALT was elevated 43.6% ($P < 0.03$) in intervention pigs compared to CON and HED pigs at wk 16 (Table 3). Plasma GGT was higher ($P < 0.03$) in HED and intervention pigs compared to controls. Plasma ALK and bilirubin levels were higher ($P < 0.05$) in HED relative to CON pigs. Plasma ALK levels in intervention pigs returned to levels similar to CON following dietary intervention. AST levels were not different among any treatments.

Oral glucose tolerance test

Pigs were subjected to an OGTT at wk 16 (CON and HED) and 22 (INT). Baseline blood glucose was 20% higher ($P < 0.05$) in HED compared to CON pigs (Figure 3A). In response to offering a glucose bolus, a parallel increase ($P < 0.05$) in blood glucose was observed from baseline to 30 min in CON, HED, and INT pigs. CON blood glucose returned to baseline levels by 60 min ($P < 0.05$), whereas blood glucose from HED and INT pigs remained elevated ($P < 0.05$). Further, 3 h after a sugar bolus, blood glucose levels in HED pigs had not returned to baseline and remained elevated near peak levels. Conversely, INT pigs returned to baseline blood glucose by 3 h. Glucose AUC was higher ($P < 0.004$) for HED (44%) and INT (29%) pigs compared to controls (Figure 3B). More importantly, glucose total AUC during OGTT tended to be reduced ($P < 0.09$) in those pigs receiving dietary intervention (INT) compared to HED pigs.

CON pigs exhibited a 78% increase ($P < 0.001$) in plasma insulin within 30 min of receiving the glucose bolus (Figure 4A). In contrast, HED and INT pigs failed to mount an insulin response to the glucose bolus. The AUC for plasma insulin was lower ($P < 0.005$) for HED (-65%) and INT (-60%) pigs compared to controls (Figure 4B).

Consistently, dietary intervention did not improve ($P = 0.84$) insulin total AUC compared to HED pigs.

Discussion

The incidence of childhood obesity is of great public health concern, especially in developed countries. An estimated 25% of all children in the United States are overweight and 11% are obese [35]. Others estimate the incidence of overweight or risk of becoming overweight is closer to 30% for children aged 6-19 years [36]. Further, many adulthood diseases have preadolescent origins where excess weight gain is the precursor or “trigger point” of a variety of physiological issues which ultimately increase the risk of morbidity and mortality during adulthood [37]. The epidemic proportions of childhood obesity underscore the need for more information regarding the causative mechanisms, development, and consequences of this disease which robs the world’s citizenry of future health and prosperity. Information regarding this rapidly growing disease is relatively scant, partially due to the lack of an effective model. To that end, we describe herein the similarities between adolescent pigs fed an abnormally high energy diet and children becoming obese prior to sexual maturity.

Childhood obesity is quite simply defined as excess adiposity created in an individual prior to puberty [38, 39]. Given sexual maturity occurs in most modern-day pigs around 22 wks of age [40, 41], dietary manipulations to pigs as reported herein equate nicely with the pre-pubertal stage of humans. Obesity, on the other hand, is defined as those individuals with a body mass index (BMI) in the 95th percentile of their contemporaries. BMI is calculated as mass (kg) divided by height squared (m^2).

Equating human BMI values to pigs is rather difficult because such an equivalent calculation does not currently exist for pigs. BMI measurements arose within the health sciences discipline as a quick estimate of muscling yet has evolved to become a measure of body fatness [42, 43]. Given the huge amount of variation in body types among humans and the rather abstract nature of the variables used to calculate BMI, such an index has limited usefulness in pigs. In contrast, the highly integrated nature of the swine industry mandates regular, accurate methods of determining whole body composition so that lean deposition can be modeled and inputs matched accordingly. Using these approaches to ascertain the weight of various tissues adjusted for carcass weight, feeding of a high energy diet dramatically alters body composition.

Pigs have proven a valuable model for the study of childhood obesity [44-46]. Herein, we show HED increases indices of adiposity and results in an estimated 23% increase in body fat composition based on prediction equations for fat-free lean [47] and bone [48]. This is a conservative estimate, as the actual difference in body fat is likely underestimated because differences in perirenal, mesenteric, and intramuscular fat were not considered. Moreover, the aforementioned equations were designed for heavier body weights, which may have biased the HED pigs because they were smaller.

We readily acknowledge diets used in this study were intentionally balanced and fed deficient in protein and this results in a well-documented and studied reduction in lean body growth, yet repartitions dietary nutrients toward fat deposition [40, 49-51]. This concept was clearly illustrated by the fact that pigs fed the HED grew slower and weighed less at the end of the study. This obviously represents a limitation to using this approach to create obese pigs as this is quite different from what occurs with most obese

children. Not only do obese children grow faster on a weight basis, they tend to be taller than contemporaries, up to age fourteen [52]. Moreover, children tend to over-consume and thus, a diet deficient in protein rarely occurs in developed countries. Even though our treated pigs grew slower and were smaller, when tissue weights were adjusted for body weight, muscle growth of pigs fed the high energy diet was similar to controls, but fat was arguably greater. This disparity in a growth-related composition was further exacerbated when the major indicator of fatness, subcutaneous fat, was adjusted for the major indicator of muscularity, the depth of the *longissimus* muscle. Again, the relative contribution of each to total body weight was vastly different based solely on the fact that we fed a high energy diet to young, growing pigs. These data show that feeding a high energy diet to pre-pubertal pigs for 16 wk creates animals with greater adiposity.

Because this approach reduced whole body growth, we cannot rule out the possibility of an altered endocrine status or nutritional physiology, which drives adiposity; however, many obesigenic or atherogenic diets routinely fed to mini-pig and Ossabaw models are deficient in protein [6-8, 11-16, 18-22, 24]. These dietary shortcomings are created experimentally by simply adding fat, sugar, or other high energy ingredients to regular pig chow [6-8, 11-16, 18-22, 24]. This approach to diet formulation effectively results in a dilution of the protein and amino acid component below the effective lean growth needs of the animal. Again, changing the ratio of metabolizable energy to amino acid is known widely throughout the animal agriculture sector for dramatically altering the composition of weight gain in growing pigs [6-8, 11-16, 18-22, 24, 40, 53]. Even so, animals fed these diets grew and accreted lean tissue,

albeit in different proportions and rates compared to those pigs fed diets finely adjusted to support maximized lean muscle gain.

Increased adiposity alone is not sufficient to define obesity. Development of secondary health maladies associated with obesity, on the other hand, strongly argues the value of the HED pig as a model of childhood obesity. Indicators of childhood obesity include: dyslipidemia, an inability to regulate blood glucose, and insulin resistance, all of which were exhibited by the pigs fed a high energy diet in this study. Similarly, childhood obesity is also associated with metabolic syndrome, a cluster of factors which increase the risk of chronic health maladies. Metabolic syndrome is characterized by hypertension and coronary artery disease in addition to insulin resistance, impaired glucose tolerance, central obesity, and dyslipidemia [12, 18, 19, 54]. Presence of at least three of these criteria constitutes metabolic syndrome [18, 19]. Herein, we show that chronic feeding of a HED to pre-pubertal pigs results in central obesity, as shown by greater visceral adipose tissue, dyslipidemia, impaired glucose tolerance, and insulin resistance, or 4 of 6 criteria of metabolic syndrome, all created in a pre-pubertal animal. Thus, the process of feeding a diet high in fat and sugars to pre-pubertal pigs for 16 wk induces development of an animal with a physiology symptomatic of metabolic syndrome and childhood obesity.

The underlying cause of childhood obesity is thought to result from an imbalance between total calories consumed versus calories expended [55]; however, others suggest the type of calorie consumed is as important as the total calories [56, 57]. Western diets consist largely of highly processed foods which are energy-dense yet nutrient-deplete [58]. Additionally, dairy products, cereals, and refined sugars and oils constitute

approximately 72% of the total daily energy consumed by people in the United States [59]. Total per capita consumption of refined sugars has increased in the past 3 decades both domestically and abroad [60, 61] arguing the type of energy may be causing increased incidence of obesity. Furthermore, highly processed foods possess higher glycemic loads and contain altered fatty acid and macronutrient composition, as well as micronutrient density [58]. To that end, a typical Western diet derives 36.2% of energy from refined sugars and oils [58], similar to the inclusion of 35% refined sugar in the HED in this study. This is in stark contrast to the typical commercial pig diet that contains less than 5% fat. The reason for this particularly high carbohydrate diet is most likely a reflection of the availability of carbohydrate-rich feedstuffs, though nutritionally-based justifications cannot be ruled out. Even so, naturally-selected and feral domestic pigs consume a diet closer in composition to that consumed by humans simply because of the natural vegetation in their omnivorous diets. One of the more notable examples of the aforementioned is the Ossabaw pig which has evolved to accommodate seasonal changes and availability of food sources. The diet of these pigs on Ossabaw Island traditionally consists of acorns that are high in fat, and therefore these pigs deposit significant amounts of body and visceral fat when acorns are widely available in the fall. Curiously, when provided an ad libitum access under more intensive management conditions, like most commercial pigs, Ossabaw pigs deposit higher amounts of total body lipids and contain less muscle mass [18, 19, 21, 62], similar to the dietary-manipulated pigs reported herein. Adult Ossabaw pigs develop 5 of 6 criteria of the metabolic syndrome when fed a high fat diet [12, 18, 19]. As a result, these pigs have been used extensively for studying obesity in humans, particularly given their predisposition to developing insulin resistance

in response to high fat diets [18]. Commercial pigs fed a high energy diet may prove more accessible to many investigators interested in studying childhood obesity. Moreover, defining the dietary energy components most responsible for those criteria used to define childhood obesity and the metabolic syndrome will require more detailed and complex studies.

Liver panel results also strongly argue we created an obese state in pigs fed a high-energy diet in this study. Specifically, elevated levels of ALT, ALK, bilirubin, and GGT reflect liver steatosis in our pre-pubertal pigs. Similar panels have been used to assess liver steatosis in 4 d-old-piglets given parenteral nutrition [63], however, diet-induced liver steatosis in pre-pubertal pigs is, to our knowledge, a novel finding.

In an attempt to reverse the effects of this diet-induced metabolic syndrome, a 6 wk dietary reversion-treatment was added to a subset of HED pigs. Curiously, re-feeding a control diet for 6 wk failed to reduce fasting blood glucose levels to that of control pigs. Although levels of LDL-cholesterol, TG, ALK, and bilirubin levels returned to normal, glucose AUC was only marginally improved and insulin area under the curve was unaffected. Failure to recapitulate glucose homeostasis with this intervention further argues the authenticity of our proposed model and supports the notion that dietary intervention may be more useful when used in combination with weight loss. To that end, dietary management often consists of reducing dietary energy to create a negative energy balance. Despite the fact that a 6 wk intervention failed to re-establish glucose and insulin homeostases, improvement in key measures of metabolic stress was observed, suggesting a longer intervention may indeed lead to a full recovery of a healthy

physiological state. Defining such strategies using the pig has great promise for children and mankind.

An intriguing observation made during our attempts to feed young pigs a diet based on the Western lifestyle is that pigs simply consumed less of the high calorie feed. Though detailed data regarding eating behavior were not collected, observational data alone suggested this reduced consumption was not a result of issues with palatability. In fact, pigs enjoyed the diet, which is consistent with the fact that pigs have a palate for sweet foods [64]. Likewise, pigs have the ability to self-regulate energy intake based on metabolic needs [49-51], a phenomenon not overtly obvious in humans but anecdotally obvious to many. Regardless, this reduction in intake strongly argues the existence of an as yet unknown biological mechanism, especially in juvenile pigs, that is capable of gauging dietary energy intake. However, this point of nutrient intake regulation, after considerable dietary energy assault, fails and an increase in caloric consumption ensues, often exceeding requirements. Components of this hypothetical whole-body energy-sensing mechanism are not known but deserve some attention. Leptin and insulin, for example, act on the hypothalamus to regulate energy balance and hepatic gluconeogenesis, and therefore, appear to control fasting hyperglycemia in diabetes [65, 66]. As such, low levels of circulating insulin in our pigs potentially mean low levels of hypothalamic insulin signaling, which may contribute to documented fasting hyperglycemia and a dysregulated energy intake. Identifying and better defining the existence of such a mechanism in pigs may provide valuable clues to controlling food intake in children and encourage healthier eating habits, potentially preventing excess weight gain that leads to childhood obesity. Understanding the roles of leptin and insulin

in controlling satiety may provide useful clues in controlling food intake. Whatever the case, a model for studying the existence of an energy-sensing mechanism that controls food intake is of great value to society.

Taken together, data presented herein demonstrate for the first time that feeding a high-energy and low protein diet results in obesity, insulin resistance, metabolic syndrome, and hepatobiliary disorder in commercial pre-pubertal pigs. Feeding high fat diets is an area of intense interest to the nutritional sciences. This area of research offers tremendous possibilities for understanding mechanisms underlying childhood obesity as well as development of nutritional intervention strategies to treat this malady. The findings herein strongly suggest pigs are a viable model for studying childhood obesity.

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Table 1: Formulation and estimated composition of control (CON) and high energy (HED) diets for pigs.

Ingredient	Diet							
	Basal ¹	Phase 1		Phase 2		Phase 3		Phase 4 ²
	6-9 kg	10-29 kg		30-59 kg		60-89 kg		≥90 kg
	CON	HED	CON	HED	CON	HED	CON	
Corn, g/kg	694.4	698.5	243.1	782.0	307.1	830.2	350.2	884.0
SBM, g/kg	240.0	240.0	230.0	195.0	170.0	150.0	130.0	100.0
Soy Oil, g/kg	10.0	10.0	-	-	-	-	-	-
Fishmeal, g/kg	30.0	30.0	-	-	-	-	-	-
L-lysine•HCl, g/kg	1.0	1.0	-	1.2	-	0.8	-	0.8
Monocalcium phosphate, g/kg	6.5	6.5	9.7	7.5	8.5	5.7	7.0	0.7
Limestone, g/kg	8.8	8.5	11.7	10.0	10.0	9.5	9.0	10.7
Vitamin premix, g/kg ³	0.8	1.2	1.2	0.8	0.8	0.8	0.8	0.8
Mineral premix, g/kg ⁴	0.5	0.8	0.8	0.5	0.6	0.5	0.5	0.5
Salt, g/kg	3.0	3.5	3.5	3.0	3.0	2.5	2.5	2.5
Antibiotic, g/kg	5.0	-	-	-	-	-	-	-
Beef tallow, g/kg	-	-	150.0	-	150.0	-	150.0	-
Sucrose, g/kg	-	-	200.0	-	200.0	-	200.0	-
D-fructose, g/kg	-	-	150.0	-	150.0	-	150.0	-

Nutrient Composition

Crude protein, %	19.14	19.18	12.94	15.87	10.62	14.09	9.08	12.16
Fat, %	4.69	4.71	16.34	3.63	16.41	3.69	16.46	3.75
Ca, %	0.69	0.68	0.68	0.58	0.58	0.52	0.50	0.46
Available P, %	0.29	0.29	0.25	0.22	0.22	0.18	0.18	0.07
Lysine, %	1.01	1.01	0.67	0.78	0.52	0.64	0.42	0.51
Methionine, %	0.30	0.30	0.18	0.24	0.15	0.22	0.13	0.20
Threonine, %	0.62	0.62	0.43	0.50	0.35	0.44	0.29	0.37
Tryptophan, %	0.19	0.19	0.15	0.15	0.11	0.13	0.09	0.10
Arginine, %	1.12	1.12	0.83	0.90	0.66	0.76	0.54	0.62
Isoleucine, %	0.69	0.69	0.50	0.55	0.40	0.48	0.33	0.39
Valine, %	0.78	0.78	0.54	0.63	0.44	0.56	0.37	0.48

¹ Diet given during the acclimation period prior to the beginning of dietary treatments.

² Only CON Phase 4 diet was fed. Diet phase was based on average body weight within treatment. Intervention pigs consumed CON Phase 4 diet for 6 wk.

³ Provided an average of the following per kg of diet: vitamin A, 2,640 IU; vitamin D₃, 363 IU; vitamin E, DL- α , 11 IU; vitamin E, D- α , 4 IU.

⁴ Provided an average of the following per kg of diet: Mn, 2.20 mg; Zn, 7.22 mg; Fe, 7.22 mg; Se, 120 ppm; Cu, 4,409 ppm; I, 198 ppm.

Table 2: Effect of chronic dietary treatment on carcass characteristics¹.

Characteristic	Treatment			SE ⁴	P-value
	Control	HED ²	INT ³		
Body weight, kg	108.0 ^a	76.0 ^b	99.0 ^a	5.3	0.001
Subcutaneous fat depth at the 10 th rib (SubQ) ⁵ , cm	1.5 ^a	2.9 ^b	2.3 ^c	0.12	0.001
<i>Longissimus</i> muscle area (LMA) ⁵ , cm ²	43.2 ^a	33.2 ^b	31.8 ^b	1.3	0.001
Perirenal fat, kg	0.36 ^x	3.03 ^y	4.18 ^z	0.34	0.001
<i>Longissimus</i> muscle Proximate moisture, %	73.3 ^a	70.4 ^b	73.7 ^a	0.54	0.01
<i>Longissimus</i> muscle Proximate fat, %	2.91 ^a	8.91 ^b	5.06 ^c	1.43	0.001
Liver Proximate moisture, %	72.9 ^x	73.7 ^y	72.4 ^z	0.20	0.01
Liver Proximate fat, %	7.62	7.70	8.73	0.91	0.39

¹Data are least-square means per treatment, CON, n = 11; HED, n = 5; INT, n = 7.

²High-energy diet.

³Intervention pigs after a 6-wk period of control diet consumption.

⁴Pooled SE of treatment groups.

⁵Data are body weight-corrected.

^{a,b,c}Means in a row without a common superscript differ, $P < 0.05$.

^{x,y,z}Means in a row without a common superscript differ, $P < 0.10$.

Table 3: Effect of chronic dietary treatment on plasma metabolites¹.

Characteristic	Treatment			SE ⁴	P-value
	Control	HED ²	INT ³		
QUICKI	0.510 ^a	0.563 ^b	0.565 ^b	0.013	0.01
HOMA-IR	0.243 ^a	0.154 ^b	0.154 ^b	0.019	0.003
HOMA-B	47.4 ^a	17.2 ^b	18.9 ^b	4.52	0.001
LDL-cholesterol, mmol/L	75.0 ^r	101.8 ^s	76.6 ^r	3.43	0.001
HDL-cholesterol, mmol/L	48.2	54.2	50.5	2.26	0.15
Triglycerides, mmol/L	59.2 ^x	67.8 ^y	58.3 ^x	3.30	0.09
Alanine aminotransferase, U/L	24.2 ^r	21.7 ^r	34.8 ^s	1.93	0.005
Alkaline phosphatase, U/L	13.3 ^r	26.9 ^s	15.8 ^r	1.86	0.003
Aspartate aminotransferase, U/L	25.5	24.3	22.5	2.20	0.69
γ -Glutamyl transferase, U/L	16.0 ^r	22.1 ^s	25.0 ^s	1.54	0.02
Bilirubin, mg/L	17.9 ^x	32.4 ^y	15.0 ^x	4.30	0.06

¹Data are least-square means per treatment, CON, n = 11; HED, n = 12; INT, n = 7.

²High-energy diet.

³Intervention pigs after a 6-wk period of control diet consumption.

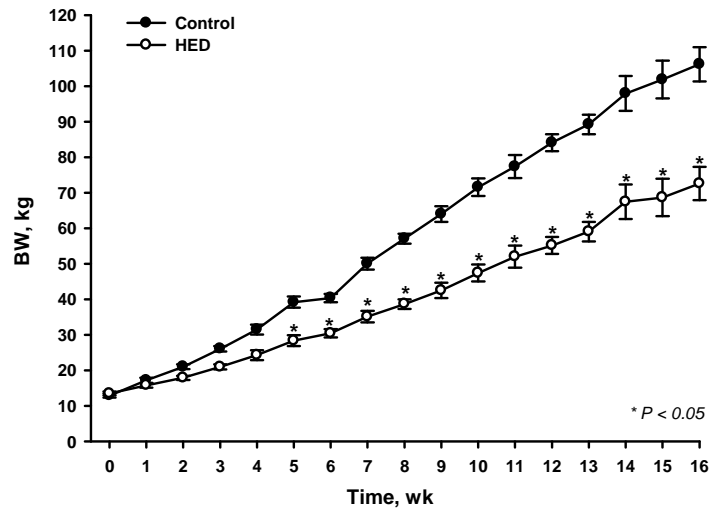
⁴Pooled SE of treatment groups.

^{a,b,c}Means in a row without a common superscript differ, $P < 0.01$.

^{r,s,t}Means in a row without a common superscript differ, $P < 0.05$.

^{x,y,z}Means in a row without a common superscript differ, $P < 0.10$.

A



B

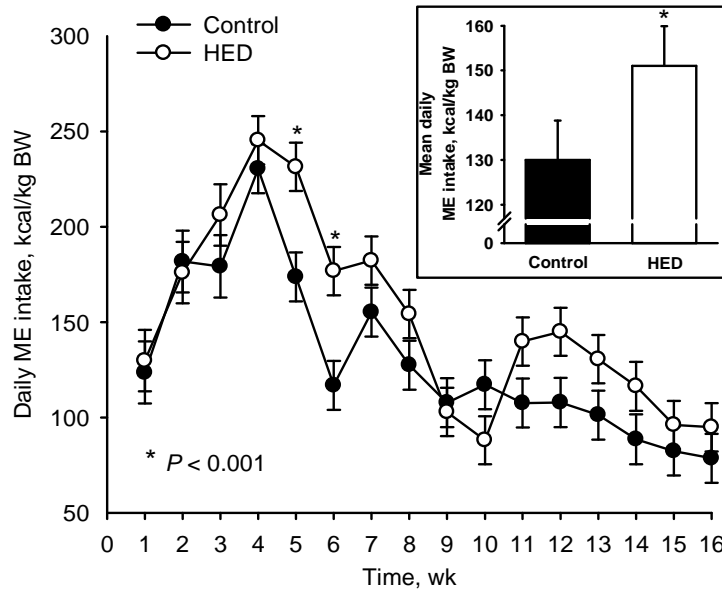
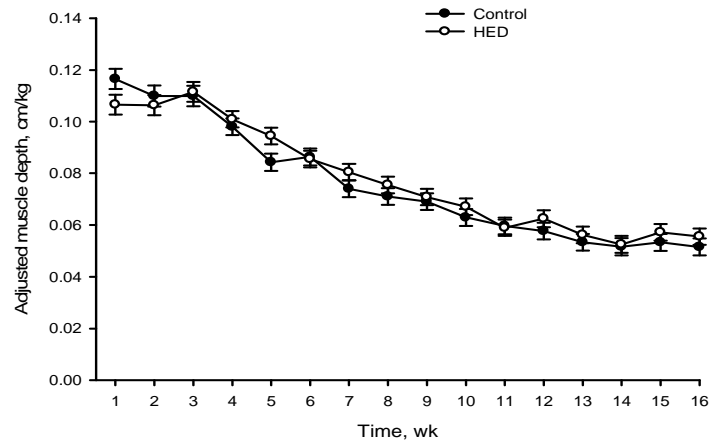
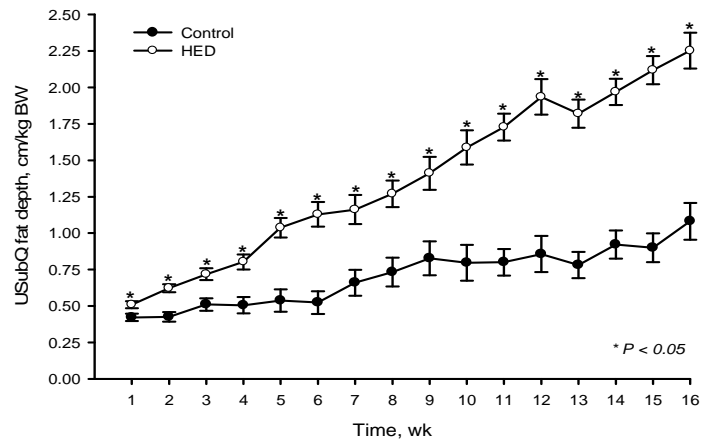


Figure 1: Changes in body weight (A) and metabolizable energy intake per kg body weight (B) of control (CON, n=11) and high energy diet (HED, n=12) pigs during a 16 wk dietary treatment. Data are mean \pm SEM. *Different from control at $P < 0.05$.

A



B



C

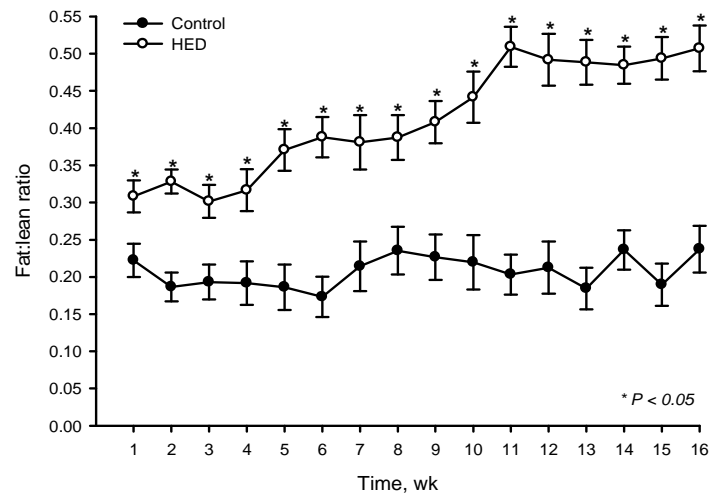


Figure 2: Growth traits of control (CON, n=11) and high energy diet (HED, n=12) pigs during a 16 wk dietary treatment. Adjusted ultrasonic *longissimus* muscle depth (A), adjusted ultrasonic subcutaneous (USubQ) fat depth (B), fat to lean ratio (C). USubQ fat and *longissimus* muscle depth are adjusted by body weight. Data are mean \pm SEM.
*Different from control at $P < 0.05$.

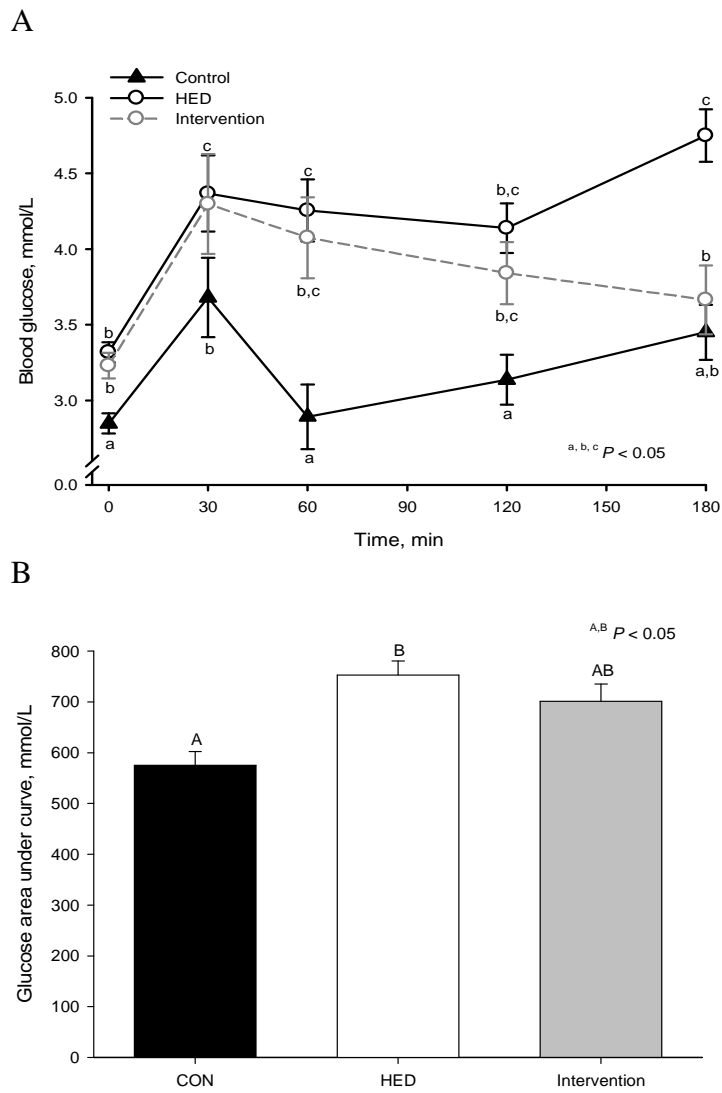


Figure 3: Blood glucose (A) and area under the curve (AUC, B) levels in control (CON, n=11) and high energy diet (HED, n=12) pigs during oral glucose tolerance test following 16 wk dietary treatment. Pigs were challenged with an oral bolus of 2 g glucose/kg body weight following a 12 h fast. Challenge was repeated following 6 wk of dietary intervention (INT, n=7). Data are mean \pm SEM. ^{a,b,c}Different from control at $P < 0.05$.

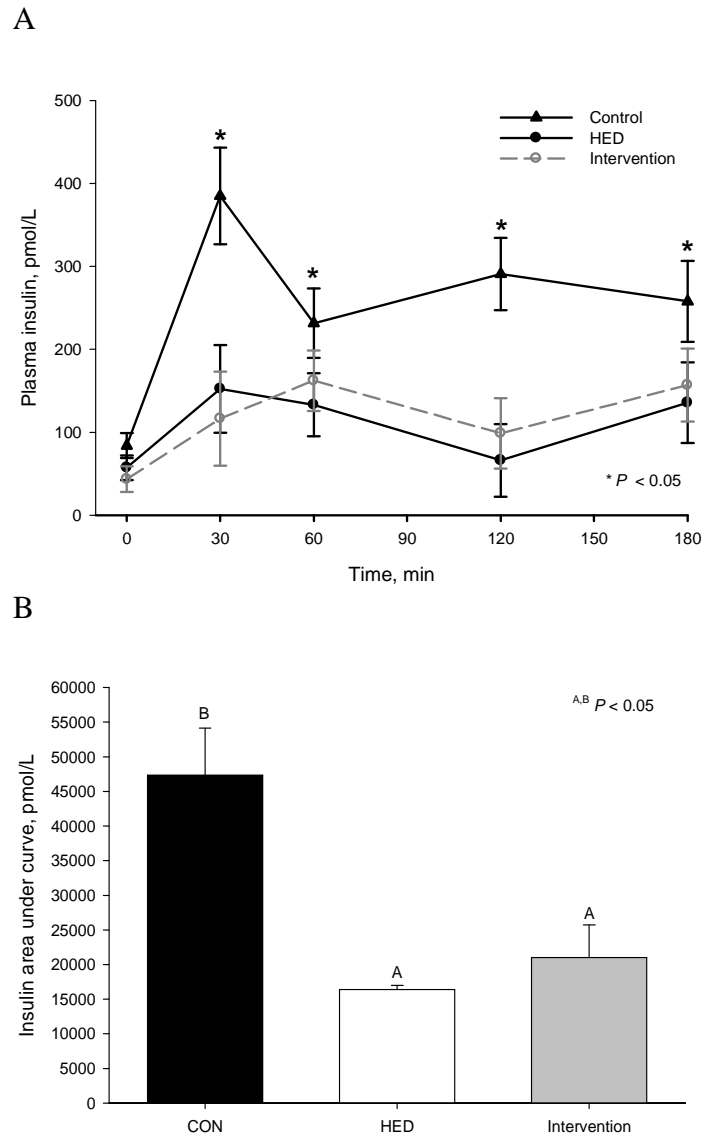


Figure 4: Plasma insulin (A) and area under the curve (AUC, B) levels in control (CON, n=11) and high energy diet (HED, n=12) pigs during oral glucose tolerance test following 16 wk dietary treatment. Pigs were challenged with an oral bolus of 2 g glucose/kg body weight following a 12 h fast. Challenge was repeated following 6 wk of dietary intervention (INT, n=7). Data are mean \pm SEM. ^{a,b,c}Different from control at $P < 0.05$.

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CHAPTER III
Summary and Future Research

Summary

The objective of this research was to develop a porcine model for studying mechanisms underlying diet-induced childhood obesity. The mechanisms contributing to obesity development are not well defined, although it is likely an imbalance of energy intake and expenditure accompanied by increased intake of fats and sugars [1-4]. Increased consumption of fat and refined sugars results in increased weight gain when energy intake exceeds expenditure. Previous studies of diet-induced obesity in pigs have resulted in insulin resistance, glucose intolerance, dyslipidemia, and hypertension [4-10] using Ossabaw and miniature pigs. We hypothesized that a 16 wk energy insult in pre-pubertal female pigs would result in obese pigs with metabolic and physiological aberrations including impaired glucose tolerance and insulin resistance.

Hyperglycemia and insulin resistance in HED pigs, combined with increased adiposity, suggest a state of obesity and induction of metabolic syndrome. Hepatobiliary injury, as evidenced by the liver panel, further underscores the effects of chronic high-energy consumption. Marginal glucose tolerance improvements, together with recovered LDL cholesterol, triglycerides, and alkaline phosphatase concentrations, suggest a 6 wk dietary intervention partially recovers a healthy physiology. Interestingly, some parameters, most notably insulin resistance, were unaffected by dietary intervention. Six weeks is a relatively short period of intervention and the body may require additional time in order to recover from a chronic energy insult. This also suggests that a longer intervention period may result in full recovery of a healthy physiology.

These findings provide insight regarding the development and progression of childhood obesity. First, consumption of a high-fat, high-sugar diet results in excess

weight gain due to fat accumulation, impaired glucose tolerance, insulin resistance, dyslipidemia, and hypercholesterolemia. The high-energy diet formulated herein is similar to Western diets in which 36.2% of energy comes from refined sugars and oils [11]. Weight loss and diet management are commonly prescribed to overweight and obese patients, unsurprisingly as diet is perhaps the single most important factor of excess weight gain. Second, a relatively short intervention period results in marginal improvements of physiology. This suggests the effects of childhood obesity may be reversed before foundations for adult health maladies fully manifest. With proper timing and intervention strategies, childhood obesity and the consequences associated with the disease may be reversed or prevented.

Future Research

Measurement of the abdominal circumference at the umbilical level has been shown to be a predictive biometric measure of amount of retroperitoneal, visceral, and subcutaneous fat in pigs of different strains and ages [7, 12], and has been shown to be more predictive of metabolic disorders than body mass index (BMI) in humans [13]. Creating a measure similar to BMI in pigs would allow for a more direct comparison of numerical estimates of obesity although a target BMI range would have to be developed for normal weight, overweight, and obese pigs. Waist circumference measurements are a quick, accurate predictor of obesity and associated metabolic disorders and can be performed in pigs with relative ease. This may form the basis for an objective quantitative determination of obesity in live animals and can be evaluated multiple times

as the pig grows, thus reflecting changes associated with childhood growth and obesity intervention.

Current definitions of metabolic syndrome are ambiguous and rationale behind sex-dependent thresholds is unclear. Including diabetes in the definition of metabolic syndrome does not offer additional information into the development and progression of the syndrome. Insulin resistance is a component of the metabolic syndrome although it is uncertain if it is the origin of the syndrome [14]. Additionally, there is no clear basis for inclusion or exclusion of other risk factors as cardiovascular disease is variable and dependent upon the specific risk factors present [14]. Interestingly, cardiovascular disease associated with metabolic syndrome does not seem to be more severe than the sum of its components and treatment of the syndrome is not different from treatments for individual components [14]. These observations make the value of clinical diagnosis unclear; however, clinicians should not rely on nor require a diagnosis of metabolic syndrome to encourage a healthy lifestyle plan including weight management, or loss, and exercise. Metabolic syndrome has been useful in drawing attention to the fact that some cardiovascular risk factors tend to cluster in patients and that presence of one risk factor should prompt the search for additional factors [14]. Patient cooperation with weight loss and lifestyle management may be improved if physicians could accurately predict that a certain amount of weight loss would improve the patient's health as evidenced by lower basal plasma glucose and improvements in insulin sensitivity without introducing insulin therapy.

In the current study, pigs aged 5 wk were selected, previous studies have selected pigs aged at least 5 months [7, 9]. Selecting pigs at a younger age, at 1-2 d post-

farrowing, is a potential area for future research. Testing fetal imprinting from sows fed a high fat diet is another area of interest gaining attention. These areas are of particular interest to determine if offspring from sows fed high energy diets have a higher propensity to consume high energy diets and become obese later in life. It has been demonstrated that animals exposed to mismatched nutritional environments in prenatal and postnatal environments develop obesity, adipocyte hypertrophy, insulin resistance, elevated blood pressure, endothelial dysfunction, and altered cardiovascular function [15-19]. Preliminary data from animal models of maternal obesity suggests that overfeeding during the pre-weaning period leads to impaired glucose tolerance, hypertension, hyperinsulinemia, and dyslipidemia [21-24]. Additionally, gestational diabetes and maternal obesity are becoming prevalent in Western societies and may perpetuate a transgenerational cyclic pattern of metabolic dysfunction [20]. Testing the next generation of piglets would determine if effects of chronic high energy diet consumption are transgenerational.

Clearly, diet composition has a key role in development of obesity. Pigs fed diets high in fat, sugar, or other high-energy, non-protein ingredients have consistently resulted in increased obesity, insulin resistance, and metabolic syndrome [5-9, 25-34]. Often, combinations of two or more dietary ingredients have been evaluated such as fat, sucrose, and fructose. Creating diets which will test effects of each dietary component or ingredient would provide insight as to whether the problem of diet-induced obesity is a single ingredient or interaction of multiple ingredients. Another area of future dietary research may be protein supplementation in combination with a high energy diet. Previous studies, ours included, have formulated diets with a combination of high

sucrose, fructose, and fat which has resulted in significant alterations to the ME:AA ratio of diets and therefore alters growth rate of pigs.

Thomas et al. concluded female miniature pigs given a high fat diet developed a greater magnitude of increased cholesterol and triglycerides than males [29]. Designing a study which incorporates both male and female domestic pigs to test effects of chronic high energy diet consumption may offer additional insight into development of obesity and potential differences in development and progression due to sex. Using intact males and females in biomedical models is a more perceptively appealing comparison to humans.

Catheters would allow for glucose challenge with minimal stress to the pig. Multiple catheters could be placed in a single animal to test glucose tolerance in various blood vessels in the body simultaneously. Additionally, intravenous (IV) glucose testing may be advantageous compared to oral glucose tolerance testing because IV glucose is administered quickly in a single bolus and by-passes the digestive system. This elicits a quicker insulin response in pigs.

Behavioral questions of obesity merit further investigation. Time spent consuming food could be evaluated and would offer insight into eating behaviors associated with obesity. Offering both a control and HED to piglets from sows given HED to determine if piglets develop taste preferences is another area for additional research. Both diets could be offered to weaned pigs as well to evaluate food preferences post-weaning. Finally, pigs fed a high-energy diet in our study developed a skin irritation which was not observed in control pigs. We attributed the irritation to increased fat intake, an equivalent to acne. This observation merits further consideration to determine

what the irritation is, how it manifests, if it would develop in another group of pigs, and if it has translational application to human medicine.

Finally, treatment period could be adjusted to allow pigs to reach puberty. This would allow for the study of effects of HED intake from birth to adulthood including evaluation of metabolic and physiological changes. Reports on how obesity affects onset of puberty have been conflicting [35, 36]. Allowing pigs fed a high energy diet to reach puberty may provide a more definitive explanation as to how obesity influences puberty and reproductive capabilities.

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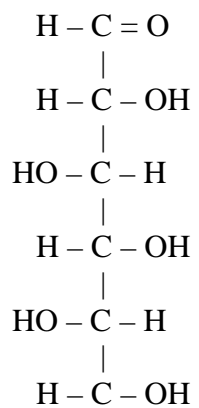
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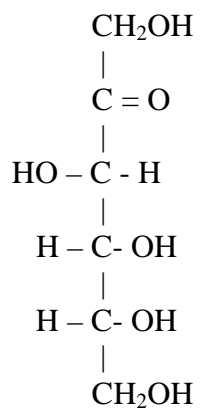
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APPENDICES

Appendix A. Structures of glucose and fructose



Glucose



Fructose

Structures are presented as Fischer projections.

Appendix B. Oral glucose tolerance test protocol

Procedure:

- Pigs were food-deprived overnight (12 h).
- A fasting blood sample (time 0) was collected. For all blood samples, pigs were physically restrained using a snare and jugular venipuncture blood samples were collected into lithium-heparinized vacutainers.
- An amount of control diet equal to 1% of body weight was mixed with a 40% D-glucose solution for an offering of 2 g glucose per kg body weight. Pigs were given a 30 min period to consume the glucose mixture.
- At 30 min post-offering, glucose mixture was removed and the refused portion was weighed. A second blood sample was collected (time 30 min).
- Additional blood samples were collected at 60, 120, 180, and 240 min post-offering of the glucose bolus.
- All samples were immediately analyzed for glucose (YSI 2300 STAT Plus, YSI Inc, Yellow Springs, OH) then centrifuged and stored at -80°C.

Appendix C. Proximate analysis protocol using Soxhlet apparatus

Sample collection and preparation:

- Collect *Longissimus dorsi* muscle samples immediately following exsanguination. Samples were cubed, frozen in liquid nitrogen, and stored at -80°C.
- Prepare filter paper packets using two filter papers folded into thirds, then folded into thirds again. This allows one end of the paper to fit into the other, creating a sealed packet.
- Secure packets with a paperclip and dry overnight (12 h) in a 105°C oven.
- Transfer packets to a desiccator, allow to cool, and weigh. Record weight as “empty weight”. Samples should be stored in a desiccator under vacuum for all subsequent steps.
- Remove samples from -80°C and keep on dry ice during the grinding process.
- Grind each sample to a powder form using a liquid nitrogen-cooled mortar and pestle. Weigh approximately 2 g of ground sample into prepared filter paper packets, label using a pencil. Record weight as “wet weight”. Place filter packets containing ground tissue in a desiccator and return to the -80°C.
- Freeze-dry samples and weigh (record as “freeze-dry weight”).
- Oven dry samples at 105°C overnight (12 h), weigh, record as “oven dry weight”.

Lipid extraction procedure:

- Vapors are harmful, extraction and subsequent steps should be performed under a ventilated hood.
- Place 500 mL flasks with boiling beads and approximately 300 mL of chloroform:methanol (87:13) in heating blocks. Place samples in Soxhlet and connect other pieces of the apparatus.
- Turn on heating unit and water cooling system. Allow the apparatus to cycle for 12 h. Turn off heating unit and allow system to cool.
- Turn off cooling system and remove samples. Allow samples to air dry at room temperature overnight.
- Dry samples in 105°C oven for 12 hours. Transfer samples to a desiccator under vacuum and allow to cool.
- Weigh samples, record weight as “extracted weight”.

$$\% \text{ lipid DM basis} = \frac{(\text{freeze-dried sample, g}) - (\text{extracted } 105^{\circ}\text{C dried sample, g})}{(\text{freeze-dried sample, g})} \times 100$$

$$\% \text{ moisture} = \frac{(\text{wet weight, g}) - (\text{dry weight, g})}{(\text{wet weight, g}) + (\text{paper} + \text{clip, g})} \times 100$$

Compound:	Formula:	Mol. Wt. (g):	Concentration:	Percentage:
Chloroform	CH ₃ Cl	119.38	99.9%	87.00
Methanol	CH ₃ OH	32.04	99.9%	13.00
Extraction solution				100.00

Notes:

- Simplification of how the Soxhlet works: Chloroform: methanol is heated to boiling in the solvent reservoir. Vapors rise and are cooled by the water in the condenser. Re-condensed chloroform: methanol drips into the sample reservoir, extracts lipid from the samples, and is automatically siphoned back into the solvent reservoir. The process then begins again, however, the lipid remains in the solvent reservoir and “clean” chloroform: methanol enters the sample reservoir.
- Use care when working with the Soxhlet apparatus as it is made completely of glass.
- Chloroform: methanol is extremely flammable and vapors are harmful, use caution and perform all steps under ventilated hood.
- Oven drying steps prior to lipid extraction in the Soxhlet apparatus are used to extract moisture, thus we assume weight differences pre- and post-extraction are due to lipid being removed.
- Do not allow flasks to boil down. Contents of the flask may ignite.

Appendix D. Freeze-dry procedure

Freeze drying of samples is often a preparatory step for specific analyses. There are certain analytes that can be damaged or lost with oven drying. When collecting samples, it is important to have prior knowledge concerning the eventual analysis to ensure proper sample handling.

Instructions for Labconco Freezone 12 with stoppering tray dryer:

1. Freeze samples for at least 24 h prior to placing in the freeze dryer. Grass, silage, or other feed samples should be frozen in a cloth or paper bag; liquid samples should be frozen in a plastic container covered with cheesecloth. Muscle samples should be frozen in filter paper or other appropriate storage mediums.
2. Capacity of the freeze dryer is limited, prepare samples accordingly. Greater surface area to volume ratio means faster drying time.
3. Place frozen samples in the freeze dryer and close the door. Check that the cover on the condensing chamber is in place.
4. Check the oil level of the vacuum pump and add oil if necessary. Periodically during each run cycle check the oil level of the vacuum pump; it may be necessary to add oil during a cycle.
5. Turn on the lower portion of the unit and set for automatic operation.
6. Close the vacuum release valve on the upper portion of the unit. Turn unit on, set to auto mode, select the appropriate program, and “run”. Appropriate program is dependent upon the type of sample.
7. Check that samples are completely dry once the cycle has completed.
8. Remove samples and turn off both the upper and lower portions of the unit. Open the cooling chamber and allow ice to melt. Pour warm water over the coils if rapid defrosting is required.
9. Open the drain plug and allow water to drain out of the condensing chamber.
10. Thoroughly dry the inside of the chamber prior to replacing the lid.

Pre-set programs for Labconco Freezone 12 with stoppering try dryer:

Program 1 – Forage and Silage

- Segment 1 – Ramp at 1.5°C/min to -34°C, hold 5 h
- Segment 2 – Ramp at 2.5°C/min to -10°C, hold 60 min
- Segment 3 – Ramp at 2.5°C/min to 10°C, hold 48 h

Program 2 – Ruminal Fluid

- Segment 1 – Ramp at 1°C/min to -25°C, hold 5 h
- Segment 2 – Ramp at 1°C/min to -15°C, hold 24 h
- Segment 3 – Ramp at 1°C/min to 0°C, hold 10 h

Program 3 – Adipose tissue, muscle tissue, and fecals

- Segment 1 – Ramp at 0.5°C/min to -34°C, hold 10 h

Segment 2 – Ramp at 1.5°C/min to -10C, hold 48 h
Segment 3 – Ramp at 1.5°C/min to 25°C, hold 10 h