VALIDATION OF THE OBESE OSSABAW MINIATURE PIG AS A MODEL OF THE OBESE POLYCYSTIC OVARY SYNDROME PHENOTYPE WITH CONCOMITANT METABOLIC SYNDROME: EXPLORATION OF POLYCYSTIC OVARY SYNDROME PATHOPHYSIOLOGY USING IN VIVO AND IN VITRO MODELS

BY

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DISSERTATION

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Polycystic ovary syndrome (PCOS) is the most common endocrine disorder of reproductive age women and has received substantial research time and financial support over the past few decades. Despite on-going research efforts, the etiology of this disease has not been elucidated. Many pre and post-natally androgenized animal models exist for this disorder but none completely recapitulate both the reproductive and metabolic features of the obese phenotype of PCOS with concomitant metabolic syndrome (MetS). Moreover, although PCOS is believed to arise from an interaction of heritable and non-heritable factors, there is no concrete evidence that in utero or environmental exposure to androgens forms the basis for the non-heritable aspect of its pathogenesis. This Ph.D. dissertation focused on the validation of the obese Ossabaw (OSS) pig as an animal model for the obese phenotype of PCOS with concomitant MetS. Unlike the currently available animal models for PCOS, this animal model incorporates both heritable (thrifty genotype breed) and non-heritable (obesogenic diet) components. The main findings of this work were that when OSS pigs were fed an excess calorie high fat/cholesterol/fructose diet: 1) both OSS sows and gilts developed android obesity and metabolic perturbations, including but not limited to dyslipidemia, hypertension and glucose intolerance, which met the Rotterdam Diagnostic Criteria for MetS; 2) both OSS sows and gilts developed persistent post-ovulatory size ovarian cysts; 3) both OSS sows (androstenedione only) and gilts (androstenedione and DHEAS) became hyperandrogenemic; 4) both OSS sows and gilts developed protracted estrous cycle length; 5) both OSS sows and gilts developed increased numbers of medium (3.5-6.5 mm) and large (6.5-12.5 mm) size follicles during the luteal phase of the estrous cycle; 6) OSS sows had increased LH and decreased progesterone serum concentrations; 6) OSS gilts had decreased LH and increased FSH serum concentrations throughout the
estrous cycle as well as increased progesterone serum concentrations during the luteal phase only; 7) OSS sows responded to a long-term GnRH agonist superovulation protocol similarly to OSS sows on a control diet; 8) the theca cells of OSS gilts did not demonstrate basal differences in the gene expression of steroidogenic enzymes but did respond to in vitro LH stimulation with increased production of androstenedione; 9) OSS gilts had an increased androgen:estrogen ratio in the follicular fluid and demonstrated down regulation of estrogen controlled genes on oocytes; 10) OSS gilts responded to an in vivo androgen challenge similarly to OSS gilts on a control diet. These findings demonstrate that both the obese OSS sow and gilt are excellent animal models for obesity and MetS in humans. There is also evidence that each of these animal model variations manifest some of the features of the obese phenotype of PCOS. However, based on the current evidence generated by this Ph.D. research neither of these model animals completely recapitulates all of the reproductive features of the obese phenotype of PCOS. It is possible that the obese OSS sow and/or gilt may be better suited as models of the effects of obesity on reproductive and endocrine physiology. Future research that examines the role of the adrenal gland and adipose tissue as well as expands on current knowledge of theca, granulosa, and oocyte physiology in this model animal will characterize further the molecular mechanisms underlying the biochemical and physical attributes of the obese OSS pig.
DEDICATION

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ABBREVIATIONS

ACTH=adrenocorticotropic hormone
AMH=anti-Müllerian hormone
AMPK=AMP-activated protein kinase
AR=androgen receptor
AR=androgen receptor gene
ART=assisted reproductive technologies
BMI=body mass index
BSA=bovine serum albumin
cAMP=cyclic adenosine monophosphate
CL=corpus luteum
COC=cumulus-oocyte-complex
CRI=continuous rate infusion
CT=computed tomography
CVD=cardiovascular disease
CVMVDL=College of veterinary medicine veterinary diagnostic lab
CYP11A=cytochrome P 450 11A (cholesterol side chain cleavage enzyme)
CYP17=cytochrome P 450 17
CYP19A=cytochrome P 450 19A (aromatase)
DHEA=dehydroepiandrosterone
DHEAS=dehydroepiandrosterone-sulfate
DNTTIP2=estrogen receptor binding protein gene
DVD=digital versatile disc
EGF=epithelial growth factor
ER=endoplasmic reticulum
ERα=estrogen receptor alpha
ERβ=estrogen receptor beta
ERBP=estrogen receptor binding protein
ERK ½=extracellular signal related kinase ½
ESR2=estrogen receptor β gene
E1=estrone
E2=estradiol
FAI=free androgen index
FBS=fetal bovine serum
FSH=follicle stimulating hormone
FSHR=follicle stimulating hormone receptor
GLUT 4=glucose transporter type 4
GnRH=gonadotropin releasing hormone
GnRHR=gonadotropin releasing hormone receptor
GPR30=G-protein coupled estrogen receptor 1
GV=germinal vesicle
GVBD=germinal vesicle break down
hCG=human chorionic gonadotropin
HDL=high density lipoprotein
HOMA-IR=homeostatic model assessment-insulin resistance
HPOA=hypothalamic-pituitary-ovarian axis
HSD3β1=3β-hydroxysteroid dehydrogenase 1 (gene)
HSD17β4=17β-hydroxysteroid dehydrogenase 4 (gene)
H&E=haematoxylin & eosin stain
IACUC=Institutional animal care and use committee
IGF-1=insulin like growth factor 1
IGF-1R=insulin like growth factor 1 receptor
IHC=immunohistochemistry
IM=intramuscular
IOF=incipient ovarian failure
IR=insulin receptor
IRS-1=insulin receptor substrate 1
IU=international unit
IUPUI=Indiana University-Purdue University Indianapolis
IV=intravenous
IVF=in vitro fertilization
IVGTT=intra-venous glucose tolerance test
IVM=in vitro maturation
Kcal=kilocalorie
KLF15=Krüppel-like factor 15
LDL=low density lipoprotein
LH=luteinizing hormone
LHCGR=luteinizing hormone receptor
MAPK=mitogen-activated protein kinase
MetS=metabolic syndrome
mRNA=messenger ribonucleic acid
MII=metaphase II
NAFLD=non-alcoholic fatty liver disease
NO=nitric oxide
OHSS=ovarian hyperstimulation syndrome
OGTT=oral glucose tolerance test
OSS=Ossabaw gilt
PBS-gel=phosphate buffered saline with gelatin
PCOS=polycystic ovary syndrome
PEG=polyethylene glycol
pFSH=porcine FSH
PGR=progesterone receptor
PGR=progesterone receptor gene
pLH=porcine LH
POF=premature ovarian failure
Pr=pregnenolone
PRKAG3=5'-AMP-activated protein kinase subunit gamma-3 gene
PSA=penacillamine streptomycin actinomycin
PVA=polyvinyl alcohol
PVC=polyvinyl chloride
P4=progesterone
qRT-PCR=quantitative real time polymerase chain reaction
RIA=radioimmunoassay
RN18S1=RNA ribosomal 18s 1 gene
SCC=side chain cleavage enzyme (CYP11A)
SEM=standard error of the mean
SHBG=sex hormone binding globulin
Sub-Q=subcutaneous
T=testosterone
TKX=telazol-ketamine-xylazine
TOF=transitional ovarian failure
TT=total testosterone
UIUC=University of Illinois-Urbana-Champaign
US=ultrasound
17α-OH=17-α-hydroxylase
17β-HSD=17-β-hydroxysteroid-dehydrogenase enzyme
17OHP=17-hydroxyprogesterone
3β-HSD=3-β-hydroxysteroid-dehydrogenase enzyme
5αA=5α-androstane-3,17-dione
Δ4=androstenedione
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CHAPTER 1

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most prevalent female reproductive endocrine disorder, affecting approximately 5-10% of women [1-4]. It is defined as otherwise unexplained hyperandrogenic anovulation [5], with a polycystic ovary being widely accepted as evidence of ovarian dysfunction [6, 7]. This disease is a complex endocrine and metabolic syndrome in which the characteristic hyperandrogenemia results from dysregulation of steroidogenesis due to theca cell dysfunction [8, 9]. Luteinizing hormone (LH) excess is also a common feature of the disease [10] and may contribute to abnormal folliculogenesis and the hallmark accumulation of small antral follicles (2-8 mm) [11]. Hormone imbalances in androgens, estrogens, progestagens and LH contribute to the aberrant folliculogenesis, oligo-/anovulation, and infertility found in PCOS patients [12]. This syndrome also is associated with metabolic syndrome (MetS), which results from the interaction of obesity and insulin resistance [6, 13, 14] and which aggravates the hyperandrogenism of PCOS [15-17]. In fact, PCOS patients have an 11-fold increase in the incidence of MetS compared to aged-matched controls [18]. Furthermore, compared with the lean PCOS cohort, obese PCOS patients with concomitant MetS are at increased risk for anovulation, infertility, and have lower conception rates [19, 20]. Currently, PCOS is classified as a heterogeneous disorder influenced by both heritable and non-heritable traits [21-23].

From 2005-2008, treatment of PCOS-related infertility accounted for approximately $500 million per annum in the United States [24]. Moreover, within a given population, higher obesity rates are associated with an increased prevalence of PCOS [25]. As of 2008, two thirds of the American population was overweight or obese [26], which may indicate an increased risk for PCOS among reproductive age women in this
country. Therefore, the public health implications and emerging economic burden of PCOS necessitate an appropriate, widely accessible animal model in which to examine heritable and non-heritable influences and molecular mechanisms of the disease, as well as to develop novel treatments. Although the pre-natally androgenized rhesus monkey model exhibits most PCOS symptoms [27], the high cost of primates and the long lag time to reach reproductive maturity render them impractical for large scale research [28].

Pigs have an estrous cycle that shares several common features with the menstrual cycle in women, such as the length of the overall cycle and the length of the luteal phase [29]. One particular breed of pig, the Ossabaw pig (OSS), has a mutation in the Val199\(\rightarrow\)Ile region of the PRKAG3 gene (the γ isoform of AMP-activated protein kinase (AMPK)), which causes increased accumulation of intramuscular fat and underlies their thrifty genotype [30, 31]. Obese OSS fed an excess calorie high fat/cholesterol/fructose diet have previously been used as appropriate animal models of coronary disease and MetS [30], obesity [32], and non-alcoholic fatty liver disease (NAFLD) [33] in humans.

Based on this information, we hypothesize that the obese OSS is an appropriate model of the endocrine and paracrine pathophysiology of the obese PCOS phenotype with concomitant MetS. We believe that the characteristic android obesity and insulin resistance of the obese OSS will drive a host of reproductive and metabolic perturbations in this animal model which will mimic the pathophysiology of the obese PCOS phenotype with concomitant. Furthermore, this animal model will permit the interaction of genetics (pig breed) and environment (obesogenic diet) on the development of the obese PCOS phenotype with concomitant MetS. Our working scientific model is outlined in Figure 1. Chapter 2 contains an overview of the follicular dynamics, aberrant steroidogenesis, metabolic disturbances, and intra-follicular environment characteristic of PCOS, particularly in the obese phenotype. Chapter 3 investigates the obese OSS sow as a model of the obese PCOS phenotype with
concomitant MetS and describes the response of this animal model to a long-term GnRH agonist ovarian stimulation protocol. Chapter 4 outlines our findings on metabolic perturbations in obese OSS gilts. Reproductive pathophysiology, including folliculogenesis, theca cell function, and systemic and intra-follicular endocrine environments, in the obese OSS gilt are discussed in Chapter 5. Lastly, Chapter 6 summarizes the research findings and outlines the direction of future research to assess the utility of the obese OSS pig as an animal model for reproductive and endocrine pathologies.
2.1 FOLLICULAR DYNAMICS

2.1.1 The Hypothalamic-Pituitary-Ovarian Axis (HPOA)

Under normal physiology, the sensitivity of the pituitary to gonadotropin-releasing hormone (GnRH) changes throughout the estrous or menstrual cycle in response to fluctuations in E2 [34]. A threshold level of estradiol (E2) activates the hypothalamic surge center, resulting in increased GnRH pulse frequency and amplitude, increased LH synthesis and release, and the LH surge followed by ovulation and luteinization [35, 36]. A hypothalamus primed by E2 will experience a physiological decrease in LH frequency post-ovulation through the interaction of progesterone (P4) with endogenous brain opioid peptides [37-40]. Thus, pituitary gland response to GnRH depends on cycle phase, steroid milieu, and GnRH pulse amplitude and frequency [41], in addition to neuropeptides. Slow frequency favors follicle-stimulating hormone (FSH) secretion, and fast frequency favors LH secretion [42, 43] (Figure 2 A).

Although not a diagnostic criterion, many PCOS patients, particularly lean PCOS patients, have an increased LH and LH:FSH ratio [44]. This defect in gonadotropin secretion may be caused by primary hypothalamic pathology or altered ovarian-pituitary-hypothalamus steroid hormone feedback. Some PCOS patients have a tonic high frequency output of GnRH with increased amplitude and frequency of LH pulses [45] which may implicate an intrinsic hypothalamic defect [46-48]. On the other hand, centrally-mediated hyper-secretion of LH in PCOS patients may occur due to decreased opioid and dopaminergic tone [49-51] and altered adrenergic tone [52, 53] within the hypothalamus. PCOS patients require larger doses of P4 than control patients to slow
the frequency of GnRH pulse secretion which supports a hypothesis of decreased opioid and dopaminergic tone [54]. Furthermore, constant exposure to tonic E2 in anovulatory PCOS patients also is hypothesized to interfere with LH and FSH secretion [55]. Therefore, the increased LH:FSH ratio in PCOS likely arises from abnormalities in the E2-P4/opioid/GnRH feedback mechanism which cause high GnRH frequency, high LH secretion, and low to normal FSH secretion [56, 57]. In support of this theory, selective estrogen receptor modulators, cause increased GnRH pulse frequency, increased secretion of FSH, and recruitment of follicle growth in PCOS patients [58]. Sensitivity of the GnRH pulse generator to E2 and P4 may be influenced by androgens as treatment with flutamide decreases LH pulse frequency [59] and hyperandrogenism interferes with P4 suppression of LH pulse frequency and secretion [60, 61]. Mounting evidence indicates that the pathophysiology of PCOS in the hypothalamus and pituitary is secondary to feedback from ovarian factors, including steroid hormones [40, 62-67] (Figure 2 B).

In contrast to the lean PCOS cohort, obese PCOS patients represent a functionally distinct population in which body mass index (BMI) may negatively correlate with serum LH concentrations [10, 68, 69]. The role of genetics and environmental influences on the obese PCOS phenotype remains to be elucidated [25]. However, studies in rats have shown that chronic exposure to a high fat diet results in elevated leptin concentrations and subsequent decreased serum E2 concentrations due to negative feedback of leptin on the ovary [70]. Furthermore, a reduction in LH secretion from the pituitary is also seen in rats fed a high fat diet that have low serum E2 concentrations [71]. Additionally, some animal models show that LH secretion is suppressed by elevated insulin [72]. A recent paper by Lawson et. al. (2008) demonstrates that hyperinsulinemia suppresses LH secretion in obese PCOS patients.
as well. The relationship between LH secretion and pulsatility, obesity, neuropeptides, and steroid hormone feedback on the HPOA remains to be fully elucidated.

2.1.2 Follicular development

Folliculogenesis is controlled by autocrine and paracrine intra-ovarian signals early in the process and by gonadotropins in the later stages. Anti-Müllerian hormone (AMH) is expressed in primary and growing follicles in humans [73] and appears to be essential for regulation of follicular recruitment [74, 75]. In vitro studies of human follicles found both inhibition [76] and initiation [77] effects of AMH on primordial follicle growth which indicates that AMH may have dual roles in folliculogenesis. Such results implicate AMH in the pathogenesis of diseases like premature ovarian failure (POF) and PCOS.

In the primary follicle, granulosa cells proliferate, change to a cuboidal formation, and develop FSH receptor (FSHR) [78]. Additionally, E2 and/or P4 may play a role in the transition from primordial to primary follicles [79]. Mid-gestation baboon fetuses express estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) in the germ cell cyst epithelium surrounding the oocytes, which suggests a direct role for E2 in primary follicle development in primates [80]. Development of the antral follicle, on the other hand, is dependent on gonadotropins. In conjunction with gonadotropins, progression from preantral to small antral to pre-ovulatory follicle corresponds with a shift in synthesis of inhibit B to inhibit A to activin A, respectively [81-83]. As the antrum forms and the follicle grows, ERα and aromatase function together, along with gonadotropins, to modulate androgen production and to effect negative feedback on the HPOA [84]. Furthermore, androgens may prime granulosa cells to respond to FSH through up regulation of FSHR [85, 86]. Selection of dominant antral follicle(s) is dependent on increasing serum FSH concentrations, up regulation of FSHR on the follicle, and
subsequent increased follicle responsiveness to FSH [87]. As the dominant follicle(s) develop they become less dependent on FSH and more dependent on LH [88]. In preparation for ovulation, the LH surge triggers expansion of cumulus cells surrounding the oocyte [89] as well as oocyte meiotic maturation via activation of mitogen-activated protein kinase (MAPK) signaling [90].

The classic ovarian morphology of PCOS is the accumulation of large numbers of small antral follicles of 2-9 mm diameter [11]. However, increased numbers of primary and secondary follicles also are found in the ovaries of PCOS patients [91-93], suggesting defects at multiple stages of follicle development. Decreased AMH expression in the granulosa cells of primordial and early growing follicles of PCOS patients could cause increased numbers of follicles to enter the growth phase [94]. Moreover, androgens inhibit AMH expression, which also may promote follicle recruitment [95, 96]. Paradoxically, systemic serum AMH concentrations are often high in PCOS patients [97]. Interestingly, obese PCOS patients with hyperinsulinemia respond more readily than lean PCOS patients to treatment with metformin via decreases in systemic AMH and ovarian volume [98]. This finding suggests that AMH affects folliculogenesis through an insulin-mediated pathway. Furthermore, small pre-antral follicles from PCOS patients remain viable longer in culture compared with control follicles which indicates there may be less follicular atresia in PCOS patients in general [99]. Also of significance, androgens promote expression of oocyte insulin-like growth factor 1 (IGF-1) and IGF-1 receptor (IGF-1R) in the primate ovary, which may initiate primordial follicle growth, and contribute to the large pool of antral cysts seen in PCOS patients [100, 101].

The PCOS ovary has a decreased ability to select a dominant follicle from the small antral follicle pool. This decreased ability may be due, in part, to the inability of
FSH concentrations to attain the threshold necessary for normal follicular maturation [65, 102]. Furthermore, tonic hyper-secretion of LH in some PCOS patients may promote premature arrest of follicular growth in response to increased intra-follicular cyclic adenosine monophosphate (cAMP) concentrations and terminal follicle differentiation [102, 103]. Mathematical modeling of follicular dynamics in PCOS suggests that follicles that mature early and are hypersensitive to gonadotropins will arrest and fail to ovulate [104]. Moreover, some PCOS patients have increased LH pulse frequency and amplitude, both of which have been hypothesized to contribute to oligo-ovulation and subsequent oligo-menorrhea [44, 105, 106]. While anovulatory PCOS patients undergo active follicle growth, dominant follicle selection does not occur in these patients, which results in the accumulation of many arrested small antral follicles [107].

2.2 HYPERANDROGENISM

2.2.1 Relationship between androgen synthesis and follicular development

Androgens (androstenedione, testosterone) produced by the theca interna are essential for normal follicular growth and for synthesis of E2 [108, 109]. The rate-limiting step in steroidogenesis is formation of pregnenolone (Pr) from cholesterol by side chain cleavage enzyme (SCC). Steroidogenesis consists of the Δ5 pathway, which converts Pr to dehydroepiandrosterone (DHEA) and the Δ4 pathway, which converts P4 to androstenedione (Δ4) [109-111] (Figure 3 A). The rate-limiting step in Δ4 formation is gene expression of cytochrome P450 17 (CYP17), which is dependent on LH stimulation in the ovary [109].

LH acts on the theca-interstitial-stromal cells to produce Δ4, which is used as a precursor for the production of E2 by aromatase in granulosa cells under the influence of FSH (Figure 3 A). Small antral follicles are dominated by androgen production. However,
once a dominant follicle is selected, there is a shift from an androgenic environment to one predominated by E2. In contrast, non-selected follicles contain a persistent androgenic environment, which results in follicular maturation arrest and eventual atresia [108, 112, 113]. Intra-follicular modulation of LH-induced androgen production is essential for proper development of the dominant follicle. Increasing LH stimulation results in follicular desensitization to LH via down-regulation of LH receptor (LHCGR) on theca cells [109, 114]. Desensitization of the theca cell to LH causes down-regulation of SCC, 17,20 lyase, and 17 alpha hydroxylase (17-αOH) enzymatic activity which ultimately results in an increased 17-hydroxyprogesterone:androgen ratio [115]. Androgens and estrogens inhibit LH action whereas insulin and IGF1 positively modulate LH activity in the follicle [109, 114] (Figure 3 A).

2.2.2 Hyperandrogenism, aberrant steroidogenesis, and metabolic dysfunction in PCOS

Hyperandrogenism is the essence of PCOS [116]. Hyperandrogenemia in PCOS patients results primarily from overproduction of androgen by theca cells [114], although a subset of PCOS patients also experience increased androgen production from the adrenal gland [116]. Excessive androgen production in the ovary arises from intrinsic dysfunction that makes theca cells hypersensitive to direct LH stimulation and indirect FSH stimulation via inhibin B [116-118] (Figure 3 B). Although 3-beta-hydroxysteroid-dehydrogenase enzyme (3βHSD) also has aberrant function, the defining feature of the steroidogenic abnormality in PCOS is dysregulation of CYP17, which results in excessive 17-hydroxyprogesterone (17OHP) synthesis in response to LH or human chorionic gonadotropin (hCG) [110, 116, 117]. Theca cells of PCOS patients produce increased amounts of testosterone (T), however, this abnormality results from increased
abundance of Δ4 precursors rather than aberrant 17-beta-hydroxysteroid-dehydrogenase enzyme (17βSHD) function [119].

PCOS patients may have low, normal, or elevated circulating serum E2 concentrations [56, 120, 121] but often have low intra-follicular E2 concentrations [122, 123]. However, in response to gonadotropin stimulation, granulosa cells of PCOS patients both in vivo [118, 124, 125] and in vitro [126] produce increased amounts of E2 compared with controls which demonstrates normal granulosa cell functionality (Figure 3 B). Abnormalities in aromatase function in PCOS may be caused by direct inhibition by intra-follicular 5α-androstane-3,17-dione (5αA) [123] or may be caused indirectly by relative FSH deficiency [114] or paracrine inhibition of FSH via increased local inhibin B concentrations ([117]; Figure 3 B).

Theca cells from PCOS patients show increased steroidogenic enzyme activity resulting in increased basal and LH/insulin stimulated androgen production [9, 109, 127, 128]. The hyperinsulinemia that occurs as a response to systemic insulin resistance in PCOS patients exacerbates hyperandrogenemia and associated reproductive pathologies [2, 12, 129-131]. Paradoxically, although tissues like muscle, adipose, and liver are insulin resistant in PCOS patients, the ovaries remain sensitive to insulin binding which allows stimulation of the androgen-producing steroidogenic pathways [132]. Moreover, phosphorylation of extracellular signal related kinase ½ (ERK1/2) is decreased by 50% in theca cells of PCOS patients and is directly associated with increased CYP17 expression and androgen production [133]. In fact, the severity of cycle irregularity [134] and hyperandrogenism [20, 135] positively correlates with metabolic parameters. As 50-75% of PCOS patients are insulin resistant [136], 40-50% are obese [137], and 33% have MetS [14], the impact of metabolic disturbances on the characteristic hyperandrogenemia of PCOS should not be underestimated. Furthermore,
hyperinsulinemia and obesity act synergistically to increase levels of androgens and androgens possibly increase insulin in a self-perpetuating cycle [15-17]. As current treatments for hyperandrogenemia in obese PCOS patients include insulin-sensitizing drugs and weight loss, it is evident that the interaction of metabolism and steroidogenesis may form the backbone of PCOS pathogenesis in obese patients [138].

To assess the effect of metabolic dysfunction on the clinical manifestations of PCOS, patients often are classified into lean and obese phenotypes [139-141]. Compared with the lean PCOS cohort, obese PCOS patients are at increased risk for anovulation, infertility, and lower conception rates [19, 142]. Obese PCOS patients, who have higher serum androgen and insulin levels than lean PCOS patients, may experience more severe infertility due, in part, to these exacerbated endocrine pathologies [19]. Although PCOS patients are hyperandrogenemic, irrespective of BMI, obese PCOS patients have higher androgen concentrations than obese women without PCOS or lean PCOS women [143]. Moreover, the location of fat deposition is also a key element of hyperandrogenemia, as obese PCOS women with android fat distribution experience androgen levels elevated above those experienced by either lean PCOS women or obese PCOS women with peripheral fat distribution [144]. The android fat deposition typical of obese PCOS patients is correlated with insulin resistance and androgen biomarkers such as sex hormone binding globulin (SHBG) and free T [144]. Central adiposity works in tandem with hyperinsulinemia to compound the reproductive effects of the disease [14, 145]. Additionally, hyperandrogenism and oligo-ovulation with E2 unopposed by P4, act in concert with obesity to result in P4 resistance in the endometrium [146-148] and decreased pregnancy rates in obese PCOS patients [19]. Thus, interaction of the metabolic and endocrine abnormalities that characterize PCOS is essential to the perpetuation of the disease.
2.3 INTRA-FOLLICULAR STEROID ENVIRONMENT AND THE OOCYTE

2.3.1 Follicular fluid steroid shifts during follicle development and oocyte maturation

Steroid hormones work in conjunction with gonadotropins and epithelial growth factor (EGF) to stimulate oocyte meiotic maturation [149-151]. In mammals steroid hormones are not sufficient, without gonadotropins, to cause oocyte meiotic maturation [152-154]. However, the appropriate ratio of [155, 156] and temporal changes in [157-159] follicular fluid steroids are essential to cellular processes involved in both cytoplasmic [160, 161] and meiotic maturation [162-164] as well as cumulus cell proliferation and differentiation [165] which are essential steps in oocyte maturation and directly related to oocyte developmental competence.

In swine, E2 and, to a lesser extent, Δ4 concentration remain relatively high throughout the growing, pre-ovulatory period until the LH surge and germinal vesicle break down (GVBD) [158] (Figure 4). A physiologic block, imposed by the high systemic E2 and inhibin B concentrations suppresses FSH concentrations and, coupled with high local Δ4 and AMH, prevents small growing follicles and atretic follicles from entering the follicular pool such that only ovulatory size follicles remain by Day 21 of the swine cycle [166]. As follicular growth progresses, meiotically arrested oocytes occur most frequently in follicles with high steroidogenesis [167]. After the LH surge and GVBD, intra-follicular P4 and prostaglandin concentrations rise to affect the arrest of meiotic maturation at metaphase II (MII) [158, 166] (Figure 4). While high E2 concentrations drive initial oocyte maturation, increased P4 is essential for final oocyte maturation and cumulus cell proliferation and expansion in swine [165, 168].

In humans, high E2 concentrations also are essential for initiation of oocyte maturation [169]. High peri-ovulatory, intra-follicular P4 concentrations are correlated with improved oocyte maturation [170] but a high androgen:estrogen ratio is associated
with degenerate oocytes [108, 158]. Similarly, healthy dominant follicles are
categorized by an estrogenic microenvironment with an Δ4:E2 ratio < 4 [113]. A high
androgen:E2 ratio is an indicator of follicular atresia [159, 169, 171] as androgens are
predominant in the follicular fluid of both atretic and small, selectable follicles in primates
[172]. The dominant follicle suppresses other follicular growth through increasing E2
concentrations, which subsequently decreases FSH concentrations [173, 174]. After the
LH surge, granulosa cells synthesize primarily P4 and its metabolites [175, 176], which
results in the arrest of mitosis in granulosa cells [172, 177]. Substantial P4
concentrations are found in follicular fluid only in follicles > 16 mm [176, 178].

E2 [156], P4 [156, 179], and the androgen:estrogen ratio [108, 155, 169, 180]
may be correlated with oocyte maturation and fertilization in primates. Follicular fluid
Δ4:E2 ratio is lower in follicles containing oocytes at germinal vesicle (GV) stage than in
follicles containing necrotic oocytes [108]. Carson et. al. (1982) found that follicular fluid
P4 was positively correlated with successful in vitro fertilization (IVF) whereas follicular
fluid E2 was associated with successful pregnancy [156]. Similarly, in both stimulated
[125] and spontaneous [108, 178] cycles follicular fluid E2 concentrations are higher in
follicles containing meiotically competent oocytes. Increased intra-follicular androgen
concentrations, on the other hand, may adversely affect oocyte quality [181]. Lastly, it
has been shown that pregnancy outcome after IVF is more closely associated with the
androgen:E2 ratio rather than the absolute concentration of E2 [155].

2.3.2 Potential effects of steroid environment on oocyte maturation

When immature oocytes are removed from the follicular environment they
undergo spontaneous meiotic maturation in vitro [182, 183] which suggests the
existence of maturation inhibiting factor(s) within the follicular fluid [184]. However, this
capacity to spontaneously mature is limited to oocytes entering the final growth phase [162]. Despite, the propensity for spontaneous oocyte meiotic maturation in vitro, gonadotropins [185, 186], EGF [153, 187-189], cAMP [149, 190], nitric oxide (NO) [191-194], and steroids [154, 195, 196] all participate in meiotic maturation (Figure 5). Recently, steroid hormone receptors have been found in both oocytes and cumulus cells, lending support for the roles of steroid hormones in oocyte maturation. In swine cumulus-oocyte-complexes (COCs), two isoforms of progesterone receptor (PGR) [197] as well as ERα and ERβ [164] have been identified in cumulus cells and ERβ [164] and androgen receptor (AR) [198] have been identified in oocytes (Figure 5). By comparison, human oocytes express ERα [199] and AR [200] in addition to cumulus cell expression of PGR [195].

The role of steroid hormones in oocyte maturation has been assumed to be related primarily related to cumulus cell function. However, given the existence of steroid hormone receptors on the oocyte, steroid hormone ratios and the sequential presence of steroid hormones may play a prominent role in the cytoplasmic maturation of the oocyte [151, 201]. Sequential hormone maturation protocols mimicking in vivo follicular fluid conditions with increased levels of E2 and FSH for the first 10-20 hours of maturation, followed by a maturation media supplemented with either no hormones [202] or P4, LH and EGF [185] for the remainder of maturation improve cytoplasmic maturation.

At the onset of oocyte maturation, the endoplasmic reticulum (ER) [203] and mitochondria [204] are located in the periphery of the cytoplasm but congregate in the interior regions of the cytoplasm as the oocyte matures to MII. A diffuse distribution of mitochondria may be important to calcium (Ca^{2+}) changes during the final stages of oocyte maturation and at egg activation in response to fertilization [205]. Recently, ERβ has been found in the outer membrane of mitochondria [206], and G-protein coupled estrogen receptor 1 (GPR30) has been localized to the ER membrane [207], which
demonstrates a potential link between E2 and intracellular Ca\(^{2+}\) release. Human oocytes exposed to an E2-enriched in vitro environment fertilize at a rate 2.8 x that of control oocytes. Addition of Δ4 at a concentration equal to E2 in the maturation media adversely affects the non-genomic intracellular increase in Ca\(^{2+}\) and the final stages of oocyte maturation, possibly due to Δ4 inhibition of estrogen receptors on the oocyte [160]. Therefore, maturation media or follicular fluid devoid of E2 and rich in androgens or cumulus cells unable to synthesize E2 may cause inhibition of intra-oocyte Ca\(^{2+}\) release, egg activation, and fertilization [160, 161].

2.3.3 Intra-follicular steroid pathophysiology of PCOS

PCOS patients commonly have a high intra-follicular androgen:estrogen ratio [125, 126, 208] which may contribute to poor oocyte quality [181]. Elevated intra-ovarian androgen concentrations significantly impair fertility in PCOS patients as evidenced by the restoration of ovulation in anovulatory PCOS patients upon administration of flutamide [209]. To date there has only been one comprehensive study of follicular fluid steroid profiles in un-stimulated PCOS patients [122]. In this study, PCOS patients (n=27) had decreased follicular fluid estrone (E1) and E2 and increased follicular fluid androgen (DHEA, Δ4, T, androstandione) concentrations compared with controls (n=21). Moreover, the ratio of androgens:estrogens was higher in PCOS patients [122]. Such findings support evidence of high CYP17 activity [8, 210] and low aromatase activity [211] previously described in PCOS patients. Low aromatase activity in PCOS patients may arise from inhibition by 5αA, which is found in high concentrations in the follicular fluid of PCOS patients [123]. One study found that a component of follicular fluid from PCOS patients blocks aromatase activity [212]. However, local inhibition of aromatase
could also occur due to increased paracrine inhibin B suppression of FSH simulation of granulosa cells [117] (Figure 3 B).

PCOS patients are routinely stimulated with exogenous hormones for oocyte collection and IVF [121, 213]. The follicular fluid from stimulated PCOS patients has lower P4 [125, 208, 214] and higher E2 [215] and T [125, 181] concentrations than follicular fluid from stimulated controls. An increased intra-follicular androgen:E2 ratio in stimulated PCOS patients may be associated with an increased miscarriage rate [181] and with decreased rates of fertilization and embryonic development [216, 217]. Furthermore, increased intra-follicular T concentrations may affect fertilization rate through decreased intracellular Ca\(^{2+}\) oscillations during oocyte maturation and activation [160, 161, 213].

In response to stimulation, PCOS patients produce more oocytes for retrieval [124, 218] but experience lower fertilization rates than controls [215, 219, 220]. However, embryonic development in fertilized oocytes has been found to proceed normally in PCOS patients [221]. Such findings indicate that cytoplasmic factors may negatively influence fertilization in PCOS patients [218]. A meta-analysis of IVF procedures in PCOS patients demonstrated that despite a lower fertilization rate, PCOS patients have similar pregnancy and live birth rates to controls [124]. The fertilization and subsequent blastocyst developmental of oocytes from PCOS patients may be compromised due to intra-follicular E2 deficiency and an increased androgen:estrogen ratio [217, 222, 223]. Furthermore, microarray analysis has demonstrated that androgens may participate in differential gene expression in PCOS oocytes and contribute to defects in developmental competence [200]. The fertilization rate and developmental competence of an oocyte from a PCOS patient can be rescued by removal from the follicle and in vitro maturation.
[224]. Such a finding underlines the impact of the abnormal follicular environment in PCOS patients on subsequent oocyte developmental competence.
2.4 FIGURES

Figure 1. Scientific model to outline my proposed central working hypothesis that the obese OSS miniature pig is an appropriate model of the endocrine and paracrine pathophysiology of the obese PCOS phenotype with concomitant MetS.
Figure 2. A) Normal physiology of the hypothalamic-pituitary-ovarian axis B) Pathophysiology of the hypothalamic-pituitary-ovarian axis in PCOS. For simplicity, protein hormones secreted by the ovaries (follistatin, inhibin, activin) are not shown.
Figure 3. A) Two gonadotropin-two cell theory of steroidogenesis. B) Perturbations in two-cell steroidogenesis in the PCOS ovary. Modified from Ehrmann et. al. 1995.
Figure 4. Changes in follicular fluid hormones in follicles during oocyte maturation in swine (Xie et. al. 1990).
Figure 5. Model of gonadotropin-induced oocyte meiotic maturation with demonstration of steroid hormone receptor distribution in the porcine oocyte and granulosa/cumulus cells. Modified from Zhang et. al. 2009.
CHAPTER 3
INVESTIGATION OF THE OBESE OSSABAW SOW AS A MODEL FOR THE OBESE
PHENOTYPE OF POLYCYSTIC OVARY SYNDROME WITH CONCOMITANT
METABOLIC SYNDROME

3.1 ABSTRACT

This study characterizes the obese OSS sow as a model of the obese PCOS phenotype in humans and evaluates the response of this animal model to a superovulation protocol. Mature, cyclic, multiparous OSS sows (6-8 years old) were fed an excess calorie high fat/cholesterol/fructose (obese, n=4) or a control diet (control, n=5) for nine months prior to the study. During the four-month study, pigs remained on their respective diets and their reproductive and metabolic states were assessed with biweekly ovarian ultrasound and blood collection and weekly assessment of weights and measures. After such baseline measurements, they were superovulated with a long-term GnRH agonist protocol. Cycle length, and circulating Δ4, total testosterone (TT), P4, E2, FSH, LH, insulin, fructosamine, lipids, and glucose were measured. Obese pigs had longer estrous cycles, increased serum Δ4 concentrations, and increased luteal phase serum LH concentrations compared to control pigs. In the luteal phase only, obese pigs had more follicles in the medium, large, and cystic categories, whereas, control pigs had more small follicles. There were no differences in control and obese pigs in response to superovulation. Obese pigs were hyperglycemic, hyperinsulinemic, had elevated total cholesterol, triglyceride, and leptin concentrations, had abdominal adiposity, and had evidence of NAFLD. The obese OSS sow mimics some of the reproductive and metabolic abnormalities of obese PCOS patients according to the Rotterdam Diagnostic Criteria, indicating the potential value of this animal model for the study of PCOS.
3.2 INTRODUCTION

PCOS is characterized by irregular (oligo) or absent (an) ovulation, hyperandrogenemia and/or polycystic ovaries [6]. Current statistics estimate that 5-12% of reproductive age women are afflicted with PCOS [1-3, 225, 226]. Originally defined as a reproductive disorder, PCOS is currently considered a complex endocrine and metabolic syndrome in which insulin resistance and compensatory hyperinsulinemia drive hyperandrogenemia and other reproductive pathologies [2, 130, 131, 227, 228].

PCOS patients with MetS are characterized by abdominal obesity, elevated triglycerides, dyslipidemia, hypertension and/or fasting hyperglycemia [6]. Approximately 40-50% of PCOS patients are categorized as obese [229, 230]. Compared with the lean PCOS cohort, obese PCOS patients have increased risk for anovulation, infertility, and have lower conception rates [19, 142] as well as increased risk for metabolic pathologies such as NAFLD, cardiovascular disease, and type 2 diabetes mellitus [136, 231, 232]. Moreover, obesity exacerbates the effect of hyperinsulinemia on hyperandrogenism in obese PCOS patients [135].

Abnormal steroidogenesis and follicle recruitment, coupled with obesity, contribute to oligo-ovulation and unopposed estrogen levels in PCOS patients [12, 147]. Aberrant steriodogenesis in ovarian cells is one of the primary causes of dysfunctional follicle maturation [109]. Ovarian theca cells from PCOS patients, basally and under LH, insulin and IGF-1 stimulation, have increased steroidogenic enzyme activity and increased androgen production [9, 109, 233, 234]. Although liver, skeletal muscle, and fat tissues are insulin resistant in PCOS, the ovaries of PCOS patients demonstrate increased insulin stimulation of androgenic pathways [132]. In addition to increased androgen production, the ovary may have increased synthesis of E2 due to the bioavailability of Δ4 as an E2 precursor, and the exaggerated response of granulosa
cells to FSH [126]. Granulosa cells of PCOS patients inefficiently produce P4, sometimes resulting in an abnormal P4 to estrogen ratio [235]. Hyperandrogenism coupled with normal to high estrogen levels unopposed by P4, and the subsequent lack of an LH surge, results in abnormal folliculogenesis and anovulation [109]. PCOS patients who undergo superovulation are at increased risk for ovarian hyperstimulation syndrome (OHSS) and multiple embryo implantation [236-238]. The propensity for OHSS following superovulation in PCOS patients stems from increased FSH sensitivity, the recruitment of large numbers of follicles, and hyperestrogenemia [239].

Multiple animal models have been used to study PCOS [240]. Although the prenatally androgenized rhesus monkey model does exhibit many of the symptoms of human PCOS, including increased abdominal adiposity, [27, 241], the cost of primates, the long period required for them to reach reproductive maturity, as well as the increased zoonotic disease risk in these species render them inaccessible to large scale research [28]. Therefore, the development of an animal model that encompasses the complex reproductive and metabolic characteristics of the obese PCOS phenotype and is widely accessible would have an immense impact on the PCOS research field. Pigs are a valuable tool for the study of human diseases due to their anatomic, physiologic and biochemical similarities to humans [242]. There are considerable similarities between hormonal responsiveness and cycle length in humans and pigs [29]. With these similarities in mind, the authors sought to validate the obese OSS sow as an animal model for the obese PCOS phenotype with concomitant MetS. OSS have a mutation in the Val199→Ile region of the PRKAG3 gene (the γ isoform of AMPK), which causes increased intramuscular fat and may underlie their thrifty genotype [30, 31]. OSS pigs, when fed an excess calorie high fat/cholesterol/fructose diet, naturally develop features
of MetS including visceral obesity, glucose intolerance, and dyslipidemia [30, 31, 243-245].

Our study objectives were to: 1) characterize the obese OSS sow as a model for the obese PCOS phenotype with concomitant MetS according to the Rotterdam Diagnostic Criteria and 2) evaluate the response of this animal to a typical human superovulation protocol.

3.3 MATERIALS AND METHODS

3.3.1 Experimental animal procedures

All experimental animal procedures were performed in compliance with The University of Illinois at Urbana-Champaign (UIUC) Institutional Animal Care and Use Committee (IACUC) regulations, and followed the guidelines outlined in the Guide for the Care and Use of Laboratory Animals [246]. Surgical ovariectomy was performed on all pigs under anesthesia with an IV combination of 1.1mg/kg Telazol (Fort Dodge Animal Health, Fort Dodge, IA), 0.27mg/kg Ketamine hydrochloride (Ketaset, Fort Dodge Animal Health) and 0.27mg/kg xylazine (Ben Venue Laboratories, Inc., Bedford, OH) known as TKX. After surgical removal of the ovaries, pigs were euthanized by an intravenous (IV) injection of 87 mg/kg body weight of sodium pentobarbital (390mg/ml; Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI, USA) and other tissues were collected post-mortem. The TKX combination was comprised of tiletamine (50mg/ml), zolazepam (50mg/ml), ketamine (25mg/ml) and xylazine (25mg/ml).
3.3.2 OSS husbandry and diet treatment

The nine multiparous OSS sows (*Sus scrofa*) used in this study were aged 6-8 years at the project onset and were obtained from the breeding colony in the Comparative Medicine Program of Indiana University-Purdue University Indianapolis (IUPUI) School of Medicine (West Lafayette, IN). All females were cyclic at the initiation of the study. The IUPUI breeding records show that OSS sows as old as 7 years of age successfully breed and wean an average of 4.2 pigs/litter. While housed at IUPUI for a period of 10 months prior to the start of this study, five of these pigs were fed approximately 2840 kilocalories (Kcal) per pig per day of a standard, control diet (LabDiet® Mini-Pig HF Grower 5L80, Purina Mills Inc., St. Louis, MO) and four pigs were fed approximately 6770 Kcal of a high fat/cholesterol/fructose diet formulated to induce MetS (obese diet; [245]). The obese diet was comprised of a base pelleted pig feed (LabDiet® Swine Diet 20% Fructose 5KA6, Purina Mills Inc.) supplemented (percentage by weight) with soybean oil (17.1%), cholesterol (2%), cholate (0.7%), corn oil (2.3%), granular fructose (8.9%) and vitamins and minerals. For four additional months encompassing the study period at UIUC, the control diet (Rund diet, UIUC, Urbana, IL) was comprised of corn (57.45%) and soy (40%) supplemented with the recommended daily allowances of vitamins and minerals; the obese diet was the same formulation which had been fed to the females at IUPUI. During the four-month study period, control pigs (n=5) were fed approximately 2200 Kcal of the pelleted control diet per pig per day and obese pigs (n=4) were fed approximately 6770 Kcal of obese diet per pig per day. To keep the control pigs lean, the amount of Kcal per pig per day was decreased by 22% compared with what had been fed at IUPUI. Water was available *ad libidum*. Pigs were housed by dietary treatment group in pens of 2-4 pigs, and were exposed to a 12
hour light:dark cycle. At the time of euthanasia and tissue collection, all pigs in this study had been on their respective diets for 14 consecutive months.

3.3.3 Sample collection

To determine basal reproductive status, blood collection and ovarian ultrasound were performed on each pig (control, n=5; obese n=4) twice a week on fixed days during the first six weeks of the study. Pigs were trained to the use of a Panepinto low stress sling restraint system for purposes of ovarian ultrasound [247, 248]. Pigs were restrained manually for collection of blood from the caudal portion of the external jugular vein. Blood was collected for serum to assess circulating reproductive hormones, fructosamine and insulin and for plasma to assess blood glucose and lipid profiles. Blood samples were centrifuged at 1500g for 25 min and serum or plasma was removed and stored at -20 °C until sample analysis. Ovarian ultrasound was performed trans-rectally with a 7.5 mHz linear transducer (38 millimeter Linear Reproductive Transducer UST 5541-7.5 and Aloka prosound SSD-3500SV, Aloka, Wallingford, CT) fitted with a fixed-angle PVC handle [249]. Real time ultrasound images were DVD recorded (Sony RDR GX360, Sony, New York, NY) for subsequent in-depth analysis of follicular structures. Pigs were weighed once per week throughout the four-month study period. For assessment of body size, crown to rump length (between the eyes to the tail head), thoracic girth (in the axillary region), height (at the point of the shoulder), and abdominal girth (the widest point of the abdomen) also were measured once per week throughout the study. Pigs were checked daily for estrus by application of back pressure and monitoring for estrus behavior in the absence of a boar.

3.3.4 Superovulation protocol
The superovulation protocol was modeled to mimic a typical long-term administration GnRH agonist protocol used for superovulation in human in vitro fertilization clinics [250]. Starting on day 10-15 of the estrous cycle (mid luteal phase), pigs (control, n=5; obese, n=3) received a subcutaneous (Sub-Q) 100 μg dose of the GnRH agonist, triptorelin acetate (Trp, ProSpec-Tany TechnoGene Ltd, Rehovot, Israel) twice daily until there were no ovulatory size follicles on the ovaries (range: 15-19 days [250]). Only three obese pigs were included in the superovulation protocol because, after baseline parameter assessment, one obese female had to be euthanized due to lameness. During the GnRH agonist down-regulation period, blood collection and ovarian ultrasound were conducted biweekly. Once ovarian down regulation was achieved as determined by no follicles visible on the ovary, two Sub-Q doses (10-20 mg each) of dinoprost tromethamine (PGF₂α; Lutalyse, Pharmacia and Upjohn Company, Pfizer Inc., New York, NY) were administered 12 hours apart to induce luteolysis [251]. Subsequently, pigs received a total daily dose of 100 mg porcine FSH (pFSH; Folltropin-V, Bioniche Animal Health Canada, Inc., Belleville, Canada) and 1.67 mg porcine LH (pLH; Lutropin-V, Bioniche Animal Health Canada, Inc.) divided equally over three Sub-Q injections administered every eight hours. pFSH and pLH were administered for a period of 2-7 days, until follicles of ovulatory size (> 6.5 mm) were seen on ovarian ultrasound [250]. During administration of pFSH and pLH, blood samples and ultrasound scans were collected daily to follow hormonal and ovarian changes. Upon development of ovulatory size follicles, a single dose of 2500 IU of hCG (Chorulon®, Intervet Inc., Millsboro, DE) was administered intramuscularly (IM). The first superovulated control pig received an initial hCG dose of 750 IU which was not sufficient to induce ovulation; two days after the initial hCG administration, this control pig received an additional 2500 IU of hCG. All subsequent pigs in the protocol received 2500 IU of hCG, which was sufficient to induce ovulation. Daily blood sampling and ovarian ultrasound were
continued for three days post-hCG administration, or until the point of ovulation as detected by ovarian ultrasound. Pigs were euthanized when they had ultrasonic evidence of development of new ovulatory size follicles in the estrous cycle following superovulation.

3.3.5 Serum hormone radioimmunoassay (RIA)

Coat-A-Count direct P4 and Δ4 kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA) were validated for use with pig serum. Standards were made in charcoal-stripped domestic pig serum by adding a known amount of P4 (32-1000 pg) or Δ4 (20-640 pg; Steraloids Inc, Newport, RI), and preparing further dilutions. For both the P4 and Δ4 assays, parallelism was validated for sample volumes of 50, 100, and 200 µl and recovery of unlabeled ligand was conducted. Once validation was completed, assays were run according to kit protocol, using pig serum standards. The sensitivity of the Δ4 assay was 60 pg and this assay had an inter-assay precision of 3.0% (n=5) and an intra-assay precision of 6.9% (n=5). The P4 assay had a sensitivity of 250 pg and an inter-assay precision of 4.0% (n=7) and an intra-assay precision of 2.4% (n=7). Although rarely encountered, samples that were non-detectable were assigned the lower limit of detection for each respective assay.

Serum samples for E2 and T assessment were extracted with ether and hexane-methanol [252] prior to RIA. The ether extraction method was modified to use six ml of ether per sample to accommodate the large amount of serum lipids [253]. To monitor extraction efficiency, samples were spiked with tritiated hormone of interest (~1000 cpm/100 µl spike/1 ml serum) prior to extraction. Hormone concentrations obtained from RIAs were corrected mathematically for extraction efficiency. Extraction efficiency was > 65% for all samples. Extracted samples were reconstituted in 1% phosphate buffered saline-gel (PBS-gel) at the same volume of the original sample (1 ml). Coat-A-Count
double antibody E2 kits and single antibody TT kits (Siemens Medical Solutions Diagnostics) were validated for use in a buffer system. Standards were made in 1% PBS-gel by adding a known amount of E2 (2.5-150 pg) or T (10-1600 pg; Steraloids Inc) to ethanol and then preparing further dilutions. Parallelism was validated for sample volumes of 100, 200, 300 and 400 µl for E2 and for sample volumes of 50, 100, 200, and 300 µl for TT. Recovery of unlabeled ligand was conducted for each assay. Once validation was completed, the assay was run according to kit protocol, but with 1% PBS-gel standards. The sensitivity of the E2 assay was 2.5 pg, and this assay had an inter-assay precision of 4.9% (n=6) and an intra-assay precision of 1.2% (n=6). The TT assay sensitivity was 400 pg with an inter-assay precision of 3.2% (n=3) and an intra-assay sensitivity of 1.9% (n=3). Samples that were non-detectable were assigned the lower limit of detection for each respective assay.

Serum LH was analyzed according to a previously described double antibody RIA validated for pLH [254] with modifications as described. Standards (0.4-6.25 ng) were made with addition of purified pLH (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) to 0.1 M PBS buffer (pH 7.5) with 1% bovine serum albumin (BSA; Sigma Aldrich Inc., St. Louis, MO). Aliquots of 5 µg pLH in 5 µl distilled water were iodinated over 1 minute with 2.5 µg of chloramine-T (Chloramine-T hydrate 98%, Sigma Aldrich) and 0.25mCi of \(^{125}\text{I}\) (Iodine-125 Radionuclide, 1mCi (37MBq), Specific Activity: \(\sim\)17Ci (629GBq)/mg, 0.1M NaOH (pH 12-14), (Reductant Free), Concentration >350mCi/ml; Perkin Elmer, Waltham, MA). The iodination reaction was stopped with 20 µg of sodium metabisulfite (Thermo Fisher Scientific, Waltham, MA) applied to the reaction vial for 5 seconds followed by 200 ul of 0.05 M PO\(_4\) (pH 7.5) to rinse the reaction vial. The contents of the reaction vial were layered on resin packed column to separate free \(^{125}\text{I}\) from \(^{125}\text{I}\)-pLH. The iodinated pLH
(\(^{125}\)I-pLH) solution was counted and then diluted with 1% BSA in 0.01 M PBS to create a stock solution of 60,000 cpm/100 µl. Prior to use, the \(^{125}\)I-pLH stock solution was diluted to a working solution of 10,000 cpm/100 µl. Each time \(^{125}\)I-pLH was made a binding check was performed to verify adequate iodination.

For the LH assay, primary antibody (Rabbit anti-Swine, National Hormone and Peptide Program) and secondary antibody (Ovine anti-Rabbit, Lampire Biological Laboratories, Pipersville, PA) were titered with 1% BSA in 0.1 M PBS to 1:1,200,000 and 1:20, respectively, prior to use. The primary antibody was used at a final dilution in the assay of 1:4,800,000. Prior to analysis, serum samples were diluted 1:2 with 0.1M PBS buffer with 1% BSA to decrease potential interference of serum lipids with antibody-antigen assay interactions. Parallelism was validated for 1:2 diluted pooled serum sample volumes of 100, 200, and 300 µl. Upon assay validation, all 1:2 diluted serum samples were run in triplicate according to the following protocol. Standards or samples (200 µl) and primary antibody (200 µl) were coincubated for 24 hours at 4 °C at which point 100 µl (~ 10,000 cpm) \(^{125}\)I-pLH was added to each tube, the tubes vortexed briefly, and the assay incubated for an additional 48 hours at 4 °C. After 72 total hours of incubation, 700 µl of secondary antibody mixed with 6% PEG (Sigma Aldrich) in 0.01 M PO4 (pH 7.5) was added to each tube, the assay was incubated for 30 minutes at 4 °C, the tubes were centrifuged for 20 minutes at 1800g (Eppendorf 1510 R, Hauppauge, NY), the supernatant was aspirated off, and the tubes were counted for 5 minutes each. This assay was run at an average percent binding of 29% (n=4). The sensitivity of the assay was 450 pg and this assay had an inter-assay precision of 4.9% (n=4) and an intra-assay precision of 3.6% (n=4). Samples that were non-detectable were assigned the lower limit of detection for this assay.
Serum FSH was analyzed according to a previously described double antibody validated for pFSH [255]. Standards (0.08-5.0 ng) were made with addition of purified pFSH (National Hormone and Peptide Program, Harbor-UCLA Medical Center) to 0.1 M PBS buffer (pH 7.5) with 1% BSA (Sigma Aldrich). Iodination of purified pFSH and further dilution to a working solution was conducted as described for pLH above. Primary antibody (Rabbit anti-Swine, National Hormone and Peptide Program) and secondary antibody (Ovine anti-Rabbit, Lampire Biological Laboratories) were titered with 1% BSA in 0.1 M PBS to 1:100,000 and 1:20, respectively, prior to use. The primary antibody was used at a final dilution in the assay of 1:400,000. As with the pLH, assay serum samples were diluted 1:2 with 0.1M PBS buffer with 1% BSA. Parallelism was validated for 1:2 diluted pooled serum sample volumes of 100, 200, 300, 400 and 500 µl. After validation, samples were run in triplicate as described for the pLH assay. The FSH assay was run at an average binding of 34% (n=3). The sensitivity of the FSH assay was 627 pg. The FSH inter-assay precision was 1.9% and the intra-assay precision was 2.3%.

3.3.6 Follicular dynamics

DVD images of ovarian ultrasound were assessed on a Vizio LCD television (Vizio VO22LF 22-Inch 1080 pixel LCD HDTV, Vizio, Irvine, CA) using a Toshiba DVD player with step-through capabilities (Toshiba SD 6100 with 1080 pixel upconversion, Toshiba, New York, NY). Follicles and corpus luteum (CL) on each ovary were measured and counted individually and a conversion factor was used to determine the actual follicle size from the ultrasound image [256]. Follicle size categories were assigned as follows: <3.5 mm, small; 3.5-6.5 mm, medium; 6.5-12.5 mm, large; >12.5 mm, cyst [257]. Although the largest follicle size was termed cyst, for a follicle to be classified as a cyst it must also have persisted on the ovary longer than one week.
3.3.7 Assessment of glucose homeostasis

The Precision Xtra glucometer (Abbott Laboratories, Bedford, MA) was validated for use in the female OSS pig by the simultaneous collection of nine serum samples from each treatment group that were run at the University of Illinois College of Veterinary Medicine Diagnostic Lab (CVMVDL) and on the glucometer. A significant correlation (R=0.92, p<0.001) between the serum and plasma samples was achieved (SAS 9.1.3, SAS Inc., Cary, NC). Fasting (12-16 hour fast) plasma blood glucose (mg/dL) was monitored twice weekly prior to superovulation and once daily during superovulation. To monitor fasting blood glucose over the course of several weeks, and to rule out the development of diabetes in the obese pigs, serum fructosamine was assessed in each study pig every 4 weeks (CVMVDL). Leptin and insulin serum concentrations were measured bi-weekly with an RIA (Linco/Millipore Corporation, Billerica, MA). Insulin resistance by modified homeostatic model assessment-insulin resistance (HOMA-IR) was calculated by multiplying glucose and insulin [30].

3.3.8 Plasma lipids

Monthly total plasma cholesterol (mg/dL) and triglyceride (mg/dL) concentrations were assayed with standard enzymatic kits (Cholesterol EZ, Triglyceride EZ, Sigma Aldrich). Low-density lipoprotein (LDL; mg/dL) and high-density lipoprotein (HDL; mg/dL) were assayed using the Cholesterol EZ kit. To determine HDL, 50 µl of a 1:1 mixture of MnCl₂ and heparin was added to 250µl of plasma sample, precipitating out the LDL, which permitted assay of the supernatant for HDL using the Cholesterol EZ kit. MnCl₂ solution was a 0.002 M solution (Manganese Chloride Tetrahydrate, MP Biomedicals, LLC, Solon, OH) and 50,000 USP units of heparin (H 3393, Sigma-Aldrich) were dissolved in MilliQ water to a concentration of 4504.5 USP/ml. LDL was determined by
subtracting HDL and 1/5 triglycerides from total cholesterol. All plasma lipid analysis was conducted as previously described [30].

3.3.9 Liver tissue histologic preparation

Liver tissue was fixed in 10% buffered formalin and processed according to Lee [245]. Fixed pieces were embedded in paraffin wax blocks, blocks were serially sectioned at 5 µm thickness, and sections were transferred onto microscope slides. Slides were dried for 24 hours, deparaffinized with xylene, rehydrated, and stained with Mayer’s Haematoxalin (Sigma Aldrich) and Eosin Y Solution (Sigma Aldrich; H&E). Slides were examined at 400X magnification for evidence of steatosis.

3.3.10 Statistical analysis

The reproductive data were divided into two data sets: basal and superovulation. Response variables included in the multivariate model for both data sets were: Δ4, TT, P4, E2, FSH, LH, small, medium and large follicles, and cysts. Baseline estrous cycle data were divided into follicular and luteal phases. The percentage of estrous cycle length dominated by follicular and luteal phases was determined for each pig and each estrous cycle. Follicular phase was defined as sample dates preceded by a decrease in P4 below 2 ng/ml and followed by an increase in P4 above 2 ng/ml, corresponding with the lack of a CL by ultrasound visualization. Luteal phase was defined as a period with P4 greater than 2 ng/ml with visualization of CL on ultrasound. Estrous cycle length was calculated from one point in time characterized by a nadir in P4 (ie, below 2 ng/ml) coupled with lack of CL on ultrasound and standing estrus behavior, to the next point in time demonstrating these three criteria. The superovulation data were subdivided by
percentage of stimulation completion and number of days post-hCG administration. Measurement parameters and metabolic data were assessed over the four-month study period on a weekly and biweekly schedule, respectively. For purposes of statistical analysis, each measurement and metabolic parameter for each pig was averaged monthly. The measurement and metabolic data were analyzed as one complete data set. Normality of data was assessed using a Levene’s test of homogeneity and Shapiro-Wilke. Transformation of non-normal data was done logarithmically for quantitative data and using square root for count and ratio data. ANOVA analysis was done with PROC MIXED using type 3 sums of squares in SAS 9.2 (SAS, Inc.). Results from PROC MIXED were back transformed and the back-transformed data is presented herein as least square mean ± SEM. In all statistical tests, \( p<0.05 \) was the criterion for statistical significance.

### 3.4 RESULTS

#### 3.4.1 Reproductive Parameters

Obese pigs had a longer average estrous cycle length than control pigs (obese, 32.2 ± 1.3 days; control, 25.2 ± 1.0 days; \( p=0.002 \)), but each treatment group spent a similar amount of time in follicular (obese, 14.7 ± 1.1 %; control, 19.6 ± 7.0 %) and luteal (obese, 85.3 ± 1.1 %; control, 81.7 ± 7.6 %) phases of the cycle. Obese serum \( \Delta 4 \) concentration was significantly higher than control serum \( \Delta 4 \) concentration in both follicular and luteal phases, but serum TT was not different between the two treatment groups (Figure 6).

While there were no significant differences in baseline numbers of follicles between the two treatment groups during the follicular phase, obese pigs had
significantly more medium (3.5-6.5 mm), large (6.5-12.5 mm) and cystic (> 12.5 mm) follicles than control pigs during the luteal phase of the estrous cycle (Figure 7). Cystic size follicles persisted on the ovaries of control sows for 2.0 ± 0.0 days and on the ovaries of obese sows for 7.6 ± 6.2 days. Control pigs, on the other hand, had significantly more small (<3.5 mm) follicles than obese pigs during the luteal phase (Figure 7). There was no significant difference in the number of CL during the luteal phase in control (1.4 ± 0.2 CL) and obese (1.1 ± 0.2 CL; p=0.28) pigs. There was also no significant difference in the amount of P4 per CL in control (14.18 ± 3.52 ng/CL) and obese (14.52 ± 4.55 ng/CL; p=0.73) pigs. Luteal progesterone peaked at 47.4 ± 14.3 ng/ml during the late luteal phase in control sows and at 28.9 ± 15.9 ng/ml during the mid luteal phase in obese sows (Figure 8). Figure 9 shows an ultrasonic image of two typical ovarian cysts, one on each ovary, in an obese pig. During the luteal phase, obese pigs also had significantly lower serum P4 concentrations but had significantly higher serum LH concentrations as compared with control pigs (Table 1). All other hormones were similar between the treatment groups during basal sampling (Table 1).

Overall, obese and control pigs responded similarly to superovulation. During the superovulation period, two of three obese pigs and one of five control pigs developed symptoms of OHSS, including diarrhea, anorexia, and ascites. P4 concentrations rose and the average number of ovulatory size follicles present on the ovary fell for both groups on the second day post-hCG administration, demonstrating an ovulatory response to hCG administration. In response to superovulation, there were no significant differences between the two treatment groups in serum hormone concentrations or numbers of large follicles or cysts (Figure 10).
3.4.2 Metabolic Parameters

Weight (control, 108.5 ± 3.8 kg; obese, 162.8 ± 4.9 kg; \( p=0.0001 \)), thoracic girth (control, 123.7 ± 2.0 cm; obese, 151.2 ± 2.3 cm; \( p<0.0001 \)), and abdominal girth (control, 127.5 ± 2.5 cm; obese, 154.9 ± 2.8 cm; \( p=0.002 \)) were all significantly increased in obese compared to control pigs (Figure 11). Height did not differ between treatment groups (control, 70.1 ± 1.8 cm; obese, 73.9 ± 2.3 cm; \( p>0.05 \)).

Obese pigs had fasting hyperglycemia compared with control pigs (control, 59.1 ± 2.8 mg/dL; obese, 78.7 ± 5.1 mg/dL; \( p=0.001 \); Table 2). Additionally obese pigs were hyperinsulinemic (control, 12.0 ± 2.3 µU/ml; obese, 23.5 ± 2.6 µU/ml; \( p=0.003 \); Table 2) and had significantly larger HOMA-IR scores than control pigs (control, 942.5 ± 322.9; obese, 1821.4 ± 365.6; \( p=0.024 \); Table 2). Despite having significant perturbations in both fasting glucose and insulin levels, obese pigs were not diabetic as demonstrated by fructosamine levels which were similar to control pigs (control, 282.73 ± 6.01 µmol/L; obese, 288.0 ± 6.8 µmol/L; \( p=0.57 \); Table 2). Leptin concentrations were significantly higher in obese pigs compared to control pigs (control, 6.6 ±1.1 ng/ml; obese, 19.8 ± 1.1 ng/ml; \( p<0.0001 \); Table 2).

Obese pigs had elevated total cholesterol as compared with control pigs (control, 102.7 ± 16.7 mg/dL; obese, 215.4 ± 19.5 mg/dL; \( p<0.0001 \); Table 3). Although HDL (control, 32.6 ± 2.3 mg/dL; obese, 55.7 ± 6.1 mg/dL; \( p=0.021 \)) and triglycerides (control, 41.6 ± 6.1 mg/dL; obese, 87.6 ± 14.2 mg/dL; \( p=0.048 \); Table 3) concentrations were significantly higher in obese compared to control pigs, there was no significant difference between the treatment groups with respect to LDL concentrations (control, 61.7 ± 7.8 mg/dL; obese, 87.3 ± 34.2 mg/dL; \( p=0.389 \)) or LDL:HDL (control, 3.6 ± 0.4; obese, 4.8 ± 1.2; \( p=0.429 \); Table 3).
Fixed H&E stained liver sections from three obese females and five control females were examined for evidence of steatosis. Multiple small areas of steatosis were observed in one out of the three obese females, whereas steatosis was not observed in liver sections from control females (Figure 12).

3.5 DISCUSSION

The major finding of this study is that, compared with age-matched lean controls, obese OSS sows were hyperandrogenemic with respect to serum Δ4 concentrations, had prolonged estrous cycle length, and formed persistent ovarian cysts, thereby meeting the Rotterdam Diagnostic Criteria for PCOS [258]. Obese OSS sows also developed three of the five criteria of MetS: abdominal obesity, elevated triglycerides, and fasting hyperglycemia [6, 231]. To our knowledge, this is the first report of PCOS with concomitant MetS in a large animal model. As PCOS patients afflicted with hyperandrogenism, oligo-menorrhea and ovarian cysts comprise 53-70% of the PCOS cohort [259-262] and MetS affects 33% of PCOS patients [14], our animal model will impact research of benefit to the majority of PCOS patients [239, 263]. In addition to baseline parameter similarities to human PCOS, our obese pigs had an increased propensity for OHSS development in response to superovulation, but had a very similar response to superovulation as compared to control pigs. Similarly, the response of obese IVF patients and lean IVF patients to superovulation are parallel [264]. These novel findings provide evidence that the obese OSS sow could be an appropriate animal model in which to study the underlying pathology and potential treatment options for the obese PCOS phenotype with concomitant MetS.
Although not significantly different due to large standard error of the mean (SEMs), obese OSS pigs respond to superovulation with larger Δ4 increases than control OSS pigs. Although obesity is linked to hyperandrogenism in both PCOS patients and patients with simple-obesity [265], large increases in Δ4 in superovulated obese pigs are suggestive of a primary ovarian abnormality in this model animal. In on-going studies with OSS gilts, H&E histologic sections of ovarian follicles have demonstrated hypertrophy of the theca layer in obese OSS and primary theca cell cultures demonstrate a significantly increased production of Δ4 in response to LH treatment as compared to control OSS, which also supports the contribution of a primary ovarian abnormality to increased Δ4 concentrations in this model animal.

The lack of elevated TT concentrations in obese pigs does not fit with the characteristics of PCOS. The concentration of serum TT found in control and obese pigs is similar to previous assessments in swine [266] and humans [267], indicating accurate measurement of this hormone. It is possible that functional polymorphisms of 17βHSD [111] or homologous transcription factors for 17βHSD like Krüppel-like factor 15 (KLF15) [16] found in PCOS patients do not exist in swine, thereby limiting upregulated production of T in the porcine ovary. Furthermore, adipose tissue can convert Δ4 to T and vice versa via 17βHSD (AKR1C3) [268], with omental adipose favoring Δ4 production and subcutaneous adipose favoring T production [265]. It is likely that the adipose tissue in our obese pigs is helping to drive the androgen milieu, perhaps favoring Δ4 over T production.

Although there was little difference between the basal E2 concentrations of obese and control pigs and this finding is somewhat in contrast to reports of normal to elevated basal E2 concentrations in PCOS patients [109, 126]. Cystic follicles in human PCOS patients often have low follicular fluid E2 levels [269], possibly due to inhibition of
E2 biosynthesis [212] or direct inhibition of aromatase [122]. The steroid hormone concentrations of obese pigs did not correspond with the number of follicles present suggesting that, similar to cystic follicles in human PCOS patients, the larger sized follicles in our obese pigs had low E2 steroidogenesis. Despite differences in cyst size between human PCOS patients (2-8 mm) and our obese females (>12.5 mm), cyst basal physiology appears to be similar in favoring androgen excess and low to low-normal E2 concentrations. The collection of small antral cysts in PCOS may be attributed to increased follicular fluid androgen concentrations [122] which inhibit AMH expression and promote follicle recruitment [95, 270] coupled with an inadequate FSH threshold necessary to facilitate dominant follicle selection and growth [65, 103] and tonic hypersecretion of LH which promotes follicle arrest and terminal differentiation [102]. Follicular selection and maturation in swine occurs similarly to humans, however, a cohort of dominant follicles, rather than a single follicle as in humans, with increased sensitivity to increasing FSH concentrations is selected [257]. Unlike humans, swine have a large population of small antral follicles present on the ovary throughout the estrous cycle [256]. In our obese pig model, the collection of post-ovulatory sized cysts may indicate the lack of an effective LH surge and/or an inappropriate response of the follicle to the LH surge. Further in vivo testing is necessary to assess the dynamics of the LH surge and follicular response in our model animal.

Obese pigs had lower basal P4 concentrations during luteal phase compared with control pigs, but formed CL at the same rate and had no difference in the average P4 produced per CL compared with control pigs. *In vitro* studies of granulosa cells from PCOS patients demonstrate a markedly reduced ability of these cells to secrete P4 in response to a high dose of FSH and/or IGF-1 [126]. Furthermore, luteal cells from PCOS patients incubated in PCOS follicular fluid *in vitro* were slow to produce P4 [271]. It is
possible that the increased numbers of small antral follicles present on the ovary throughout the estrous cycle in control pigs are responsible for the increased luteal P4 concentrations in our control pigs. It is important to note that although we would expect to find 4-5 CL on the ovaries of these sows (based on average litter size for the breed), we found only 1-2 CL on the ovaries of these sows. Such a finding may indicate the approach of reproductive senescence in our sows. Lastly, unlike findings in PCOS patients, it does not seem that the obese pigs experience luteal insufficiency.

Elongated estrous cycles in the obese OSS sow are analogous to oligo-menorrhea in PCOS patients. Although we could not determine the exact date of ovulation, we were able to confirm from ovarian ultrasounds that ovulation had occurred. Although our obese sows seem to be oligo-ovulatory based on their elongated estrous cycle length, we did not observe constantly elevated serum LH in the obese sows. Furthermore, we did not find a difference in the LH:FSH ratio between our treatment groups. This is perhaps due to the decreased, as opposed to absent, ovulatory cycle frequency in our obese pigs. Increased serum LH in obese pigs during the luteal phase mirrored increases in Δ4 concentrations during this time. This increase in LH and subsequent production of ovarian androgens corresponds with decreased P4 concentrations. However, as LH was only measured twice weekly and LH tonic secretion is episodic, the LH results should be interpreted judiciously. Furthermore, low or low-normal P4 concentrations in PCOS patients may accelerate the GnRH pulse generator, increase levels of LH during the luteal phase, and lead to hyperandrogenemia and anovulation [272]. Typical PCOS patients have increased serum LH concentrations, which may contribute to oligo-ovulation and subsequent oligo-menorrhea [106, 273, 274]. However, in PCOS, BMI is negatively correlated with serum LH concentration [10, 68, 69]. This inverse relationship between BMI and serum LH concentrations may be
caused by accelerated metabolism and clearance of serum LH by the liver in obese patients [275].

The hyperandrogenemia in this animal model could be due to the hyperinsulinemia or adiposity of our obese pigs [30, 245], similar to that found in obese PCOS patients [276, 277]. Hyperinsulinemia and insulin resistance contribute to elevated levels of androgens and development of large numbers of antral follicles during ovarian stimulation in human patients [278]. Additionally, excessive follicle production coupled with hyperinsulinemia may precipitate overactive aromatization in granulosa cells and an elevated E2 to Δ4 ratio, rather than a strict elevation in Δ4 [279]. However, obesity alone is associated with increased serum androgen concentrations [143, 280]. Abdominal adipose tissue is particularly active in peripheral conversion of androgens to estrogens [143, 281-283] and T to Δ4 [265]. Some studies have found that androgen levels are comparable in obese and control subjects, unless the obese subject specifically has central deposition of adipose tissue [284]. Furthermore, obese women with abdominal, rather than omental, fat deposition may have increased hydroxysteroid dehydrogenase levels [280]. Whereas, women with omental, as opposed to subcutaneous, fat deposition have increased aromatase levels [285]. Several studies also have shown that androgen clearance rates are slower in obese as compared with normal women [135, 285]. Obese women with PCOS typically have higher androgen levels than obese women without PCOS due to adipose tissue steroid metabolism coupled with PCOS-related aberrant ovarian steroidogenesis [143]. Furthermore, obese PCOS women with android fat distribution experience androgen levels elevated above those experienced by either lean PCOS women or obese PCOS women with peripheral fat distribution [144]. It is possible that the increased Δ4 production in our obese pigs is the result of the combination of a primary ovarian defect, hyperinsulinemia, and the
intraconversion of steroid precursors by abdominal adipocytes. Similarly in obese PCOS patients, hyperinsulinemia and abdominal obesity are certainly major factors involved in hyperandrogenism and infertility [286], as significant weight loss alone can improve fertility and alter biochemical parameters in such patients [19].

In addition to reproductive pathology, obese OSS sows had MetS characterized by abdominal obesity, fasting hyperglycemia, and elevated triglycerides. Obese pigs weighed more and had larger circumferences in both the thoracic and abdominal regions compared with control females. The height of obese and control pigs was similar; therefore, the increased weight of obese pigs was due to android fat deposition, rather than skeletal size differences. Increased abdominal girth in OSS correlates (R=0.91) significantly with visceral or android fat distribution [30], which is highly associated with type 2 diabetes mellitus and an increased risk for cardiovascular disease in humans [232], as well as Δ4 production [265]. Furthermore, obese PCOS patients with android obesity have more pronounced insulin resistance and hyperandrogenism than obese PCOS patients with peripheral obesity or lean PCOS patients [142, 144]. The hyperglycemic, hyperinsulinemic obese OSS sow appropriately models the metabolic abnormalities seen in the majority of PCOS patients, as 70% of such patients are afflicted by hyperinsulinemia, insulin resistance, and hyperglycemia [136, 287]. Moreover, 33% of our obese pigs showed evidence of NAFLD, which has been associated with 41-55% of PCOS patients [288-290] and is intimately associated with MetS [291]. Obese pigs had leptin concentrations three-fold higher than control pigs, which may indicate that obesity alone does not explain elevations in leptin elevations. Although obese OSS pigs did not manifest dyslipidemia as seen in 65-95% of PCOS patients [6, 14, 292], they did have elevated triglyceride levels and total cholesterol. PCOS patients with atherogenic lipid profiles tend to be obese and have more elevated
androgen concentrations than PCOS patients without atherogenic lipid profiles [17]. In a previous study in which OSS were fed an excess calorie high fat/cholesterol/fructose diet, young pigs (5-6 months of age at diet onset) developed dyslipidemia with high LDL and an increased LDL:HDL ration [30]. However, a more recent study with a slightly different diet formulation from Dyson et. al. (2006) also using young pigs showed an increase in all lipid profiles [33]. It is possible that use of younger animals or a slightly modified diet would permit development of dyslipidemia as seen in PCOS patients. Although blood pressure was not evaluated in this study, in an on-going study with a young group of OSS gilts (aged 1-1.25 years), hypertension has been confirmed in the obese pigs as compared with control pigs. Our findings of abdominal obesity, fasting hyperglycemia and elevated triglycerides in obese OSS sows corroborate our conclusion that obese OSS sows not only have symptoms of PCOS but also manifest MetS with evidence of insulin resistance and NAFLD.

In conclusion, OSS sows, when fed excess high fat/cholesterol/fructose diet, develop many of the Rotterdam Criteria reproductive pathologies seen in obese PCOS patients. Obese OSS sows represent a potential animal model, applicable to large-scale research, in which to study treatment options for the obese phenotype of PCOS with concomitant MetS.
3.6 FIGURES AND TABLES

Figure 6. Basal serum TT (ng/ml; A) and Δ4 (ng/ml; B) over one to three estrous cycles per pig in control (n=5) and obese (n=4) pigs. Within a given phase of the estrous cycle (fOLLICULAR and luteal), bars with different letters indicate significantly different (p<0.003) serum hormone concentrations between control and obese sows.
Figure 7. Average number of follicles per sow in follicular (A) and luteal (B) phases of the estrous cycle in control (n=5) and obese (n=4) sow. Within a given follicle category, different letters indicate significance between control and obese sow (p<0.02).
Figure 8. Average number of large follicles per sow as indicated by columns on the figure and serum progesterone (ng/ml) as shown by the lines in the figure for a representative estrous cycle in control and obese sows. Within a given phase of the estrous cycle, columns with different letters are significantly different (p<0.05).
Figure 9. Transrectal ultrasound image of ovarian cysts in an obese sow (A) and large and medium sized follicles in a control sow (B). The ovarian cysts in the obese pig were
36.3 mm and 27.6 mm respectively and persisted on the ovary for approximately two weeks.

Table 1. Basal estrous cycle hormones in control (n=5) and obese (n=4) sows.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Control</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Follicular</td>
<td>Luteal</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>51.8 ± 3.6</td>
<td>30.2 ± 1.9</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>1.2 ± 6.1</td>
<td>30.8 ± 3.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>2.6 ± 0.2</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>LH:FSH ratio</td>
<td>0.76 ± 0.03</td>
<td>0.64 ± 0.01</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> p=0.04  <sup>c,d</sup> p=0.01
Figure 10. Response of follicular hormones and ovarian follicle growth to superovulation. Average serum Δ4 or TT (ng/ml, right Y axis) and average number of follicles per pig (left Y axis) in response to superovulation in control (n=5) and obese (n=3) sows. The table shows average E2 (pg/ml) and P4 (ng/ml) in each stage of the superovulation period.

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>First Third</th>
<th>Second Third</th>
<th>Complete</th>
<th>1 day post</th>
<th>2 day post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control E2</td>
<td>10.6 ± 68.1</td>
<td>24.1 ± 56.3</td>
<td>68.6 ± 56.3</td>
<td>69.3 ± 56.3</td>
<td>67.0 ± 56.3</td>
<td>59.3 ± 60.0</td>
</tr>
<tr>
<td>Obese E2</td>
<td>13.3 ± 112.2</td>
<td>29.6 ± 72.7</td>
<td>151.4 ± 72.7</td>
<td>241.2 ± 72.7</td>
<td>66.6 ± 72.7</td>
<td>47.3 ± 72.7</td>
</tr>
<tr>
<td>Control P4</td>
<td>1.6 ± 0.5</td>
<td>1.4 ± 0.4</td>
<td>2.0 ± 0.6</td>
<td>4.1 ± 2.3</td>
<td>4.5 ± 3.0</td>
<td>10.8 ± 7.9</td>
</tr>
<tr>
<td>Obese P4</td>
<td>1.7 ± 0.5</td>
<td>1.3 ± 0.5</td>
<td>2.1 ± 0.7</td>
<td>4.1 ± 3.0</td>
<td>5.2 ± 3.8</td>
<td>21.6 ± 10.2</td>
</tr>
</tbody>
</table>
Figure 11. Average abdominal and thoracic girths (cm; left Y axis) and weight (kg; right Y axis) in control (n=5) and obese (n=4) sows. Obese sows had greater abdominal (a, b; p=0.002) and thoracic (c,d; p<0.0001) girths than control sows and weighed more than control sows (e,f; p=0.0001).
Table 2. Assessment of glucose homeostasis in control (n=5) and obese (n=4) sows.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Obese</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>59.1 ± 2.8</td>
<td>78.7 ± 5.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>12.0 ± 2.3</td>
<td>23.5 ± 2.6</td>
<td>0.003</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>942.5 ± 322.9</td>
<td>1821.4 ± 365.6</td>
<td>0.024</td>
</tr>
<tr>
<td>Fructosamine (µmol/L)</td>
<td>282.7 ± 6.0</td>
<td>288.0 ± 6.8</td>
<td>0.570</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>6.6 ± 1.1</td>
<td>19.8 ± 1.1</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Table 3. Plasma lipid profiles in control (n=5) and obese (n=4) sows.

<table>
<thead>
<tr>
<th>Parameter (mg/dL)</th>
<th>Control</th>
<th>Obese</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol</td>
<td>102.7 ± 16.7</td>
<td>215.4 ± 19.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL</td>
<td>61.7 ± 7.8</td>
<td>87.3 ± 34.2</td>
<td>0.389</td>
</tr>
<tr>
<td>HDL</td>
<td>32.6 ± 2.3</td>
<td>55.7 ± 6.1</td>
<td>0.021</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>41.6 ± 6.1</td>
<td>87.6 ± 14.2</td>
<td>0.048</td>
</tr>
<tr>
<td>LDL:HDL ratio</td>
<td>3.6 ± 0.4</td>
<td>4.8 ± 1.2</td>
<td>0.429</td>
</tr>
</tbody>
</table>
Figure 12. Representative H&E stained sections of liver tissue obtained from an obese (A) and a control (B) sow at 400x magnification. Black arrows indicate areas of hepatic steatosis in the liver of the obese sow.
4.1 ABSTRACT

PCOS affects approximately 5-10% of the reproductive age female population [1]. Its life-long disease sequelae, type 2 diabetes, cardiovascular disease (CVD), obesity, and MetS, create a large financial burden on the American health care system [2]. This study characterizes the obese OSS gilt as cost-effective large animal model of the metabolic pathologies associated with the obese PCOS phenotype with concomitant MetS. Cyclic, nulliparous OSS gilts (~6 months old) were fed an excess calorie high fat/cholesterol/fructose (obese, n=10) or a control diet (control, n=9) for three months prior to the study. During the five-month study, gilts remained on their respective diets and were sampled twice weekly for blood glucose, once weekly for morphometric measurements, and every other month for measures of glucose homeostasis and lipid profiles. At the conclusion of the study, gilts underwent an intravenous glucose tolerance test (IVGTT) to determine their level of insulin resistance and had a single blood pressure measurement taken. Obese OSS gilts weighed more, had android obesity, and a larger body size than control OSS gilts. Obese OSS gilts also were hyperglycemic, hypertensive, insulin resistant based on IVGTT only, had elevated serum leptin, total cholesterol, triglycerides, HDL, LDL, and had an increased LDL:HDL ratio as compared with control OSS gilts. The obese OSS gilt when fed an excess calorie high fat/cholesterol/fructose diet for a minimum induction period of three months manifests the characteristics of the obese PCOS phenotype with concomitant MetS and, therefore,
may represent a potential superior, cost-effective, large animal model as compared with the obese OSS sow for the obese phenotype of PCOS with concomitant MetS.

4.2 INTRODUCTION

PCOS is the most common endocrine disorder in women of reproductive age [2]. This heterogeneous disease is tightly linked to MetS, which is characterized by hypertension, dyslipidemia, impaired glucose tolerance, and android obesity [231]. Android obesity is found in approximately 60% of PCOS patients [6] and directly influences the severity of insulin resistance and the development of type 2 diabetes mellitus and CVD [293] as well as exacerbating hyperandrogenism [142]. As up to 70% of PCOS patients are insulin resistant or hyperinsulinemic [294], and PCOS patients have an 11-fold increase in the incidence of MetS compared to aged-matched controls [18], the study of these sequelae and their relationship to the obese PCOS phenotype has held increasing importance in recent years.

Irrespective of their BMI, PCOS patients are at risk for insulin resistance due to a defect in post-insulin receptor (IR) signaling in which increased serine phosphorylation yields decreased insulin receptor substrate 1 (IRS-1) activation and decreased glucose transporter type 4 (GLUT4) expression [295]. However, obesity exacerbates the underlying IR defects in PCOS to effect more severe defects in insulin sensitivity [20]. Increasingly it has become apparent that a myriad of metabolic variants exist in PCOS, with perturbations in the metabolism of PCOS patients strongly influenced by the interaction of android obesity and insulin [296]. Insulin resistance may be exacerbated in obese PCOS patients through increased lipolysis and the subsequent elevation in levels of circulating free fatty acids [297]. Recently, it also has been demonstrated that obese
PCOS patients have defects not only in lipid but also amino acid metabolism which may underlie the chronic low-grade inflammation and athrogenic tendency found in these women [298]. The central role of obesity in the reproductive dysfunction of PCOS patients cannot be overemphasized given that resumption of ovulation in anovulatory patients is highly correlated with early and consistent loss of intra-abdominal fat deposits [299]. Furthermore, the $1.77 billion per annum economic burden of treating the metabolic sequelae of PCOS [300] warrants research focused on the mitigation of the long-term health aspects of the disease.

Given the prevalence of PCOS in the reproductive age female population, the heavy financial burden of this disorder on our healthcare system, and the lack of understanding about its etiology, in recent years the research community has seen increased interest in appropriate animal models of this syndrome [240, 301]. Pre-natally and post-natally androgenized animals, including various rodent models, the rhesus monkey, and the ewe, remain the most widespread animal models currently available for the study of PCOS etiology [301]. However, none of these animal models recapitulates the reproductive and metabolic features of the obese phenotype of PCOS with concomitant MetS. Furthermore, as prenatal exposure to high levels of androgens has not been correlated with increased risk of developing PCOS later in life [302] these models do not mimic the natural disease progression in humans. Our research group has previously examined the use of the OSS sow as a model for the metabolic and reproductive features of the obese phenotype of PCOS with concomitant MetS. Pigs share common estrous cycle features [29] and a similar LDL-dominated lipid metabolism with humans [303], which makes this species an attractive option for a potential animal model of the obese PCOS phenotype with concomitant MetS. The OSS has a mutation in the Val199→Ile region of the PRKAG3 gene (the γ isoform of AMPK), which causes
increased intramuscular fat and may underlie their thrifty genotype [30, 31]. Therefore, when fed an excess calorie high fat/cholesterol/fructose diet, OSS naturally develop features of MetS including visceral obesity, glucose intolerance, and dyslipidemia [30, 31, 243-245]. While the OSS sow holds promise as a model of some of the features of the obese phenotype of PCOS with concomitant Mets, the age of the animals and the required length of time on the obesogenic diet to induce the phenotype render this model extremely expensive. Therefore, the aim of this research was to validate the obese OSS gilt as a more cost effective animal model for the metabolic features of the obese PCOS phenotype with concomitant MetS.

Our objective with this study was to demonstrate that the obese OSS gilt when fed an excess calorie high fat/cholesterol/fructose diet for a minimal induction period of three months develops android obesity and meets the Rotterdam Diagnostic Criteria for the development of MetS.

4.3 MATERIALS AND METHODS

4.3.1 Experimental animal procedures

All experimental animal procedures were performed in compliance with UIUC IACUC regulations, and followed the guidelines outlined in the Guide for the Care and Use of Laboratory Animals [246]. All anesthetic drugs and dosages used in this live animal study were the same as outlined under section 3.3.1.

4.3.2 OSS husbandry and diet treatment
The 19 nulliparous OSS gilts (*Sus scrofa*) used in this study were aged 6-7 months at the project onset and were obtained from the breeding colony in the Comparative Medicine Program of IUPUI School of Medicine (West Lafayette, IN). All females were cyclic at the initiation of the study and were sire-matched by treatment group. The obese diet utilized in this study was of the same composition as described under section 3.3.2. The control diet fed in this study was the Rund diet (UIUC). During the eight-month study period, control gilts (n=9) were fed approximately 2200 Kcal of the pelleted control diet per pig per day and obese gilts (n=10) were fed approximately 4570 Kcal of obese diet per pig per day. A previous pilot study performed at Purdue University in the laboratory of Dr. Rebecca Krisher determined that it takes a minimum of 12 weeks on the obese diet for obese gilts to demonstrate significant differences in metabolic hormones and measurement parameters from control gilts. Therefore, OSS gilts were fed their respective diets for three months prior to the initiation of the actual study period. After the three-month diet induction period, gilts remained on their respective diets and were sampled for a five-month study period. Throughout the study water was available *ad libidum*. Gilts were housed by dietary treatment group in pens of 2-4 pigs, and were exposed to a 12 hour light:dark cycle. Gilts were euthanized after a total of eight months on diet treatment and when they had ovulatory sized follicles present on their ovaries.

4.3.3 Sample Collection

Blood collection was performed on each gilt (control, n=9; obese n=10) twice per week on fixed days during the five-month study. Blood collection and processing were conducted as described under section 3.3.3. Blood was assessed for fructosamine, insulin, leptin, glucose, and lipid profiles. Pigs were weighed and had morphometric measurements taken once per week throughout the five-month study period. As in the
OSS sow study, weekly measurements consisted of crown to rump length, thoracic girth, abdominal girth, and height. To verify that gilts were cyclic throughout the eight-month diet induction and study period, gilts were checked daily for estrus by application of back pressure and monitoring for estrus behavior in the presence of a boar.

4.3.4 Assessment of glucose homeostasis

The Precision Xtra glucometer (Abbott Laboratories) was used to measure fasting (12-16 hour fast) plasma glucose twice weekly in all OSS gilts. To rule out the development of diabetes in obese pigs, fructosamine was measured every other month at CVMDL. Leptin and insulin serum concentrations were measured monthly with a multi-species and porcine-specific RIA, respectively (Linco/Millipore Corporation, Billerica, MA). The leptin RIA was run at an average binding of 38.1% (n=2) and had an assay sensitivity of 930 pg. The inter-assay precision was 2.9% and the intra-assay precision was 4.5% for the leptin RIA. The insulin RIA was run at an average binding of 43.3% (n=9) and had an assay sensitivity of 0.34 μU. The inter-assay precision was 0.1% and the intra-assay precision was 4.0%. Insulin resistance by modified HOMA-IR was calculated by multiplying glucose and insulin [30].

4.3.5 Plasma lipids

Every other month, total plasma cholesterol (mg/dL) and triglyceride (mg/dL) concentrations were assayed with standard enzymatic kits (Cholesterol EZ, Triglyceride EZ). Low-density lipoprotein (LDL; mg/dL) and high-density lipoprotein (HDL; mg/dL) also were assayed every other month using the Cholesterol EZ kit. Kits were utilized as
described under section 3.3.8. All plasma lipid analysis was conducted as previously described [30].

4.3.6 Intravenous Glucose Tolerance Test (IVGTT)

The week prior to euthanasia an IVGTT was performed on gilts in the study. Only nine obese gilts had the IVGTT procedure, as one obese gilt died due to a cardiac incident prior to the end of the study. Gilts were placed in a Panepinto low stress sling restraint system [304] and administered isoflurane gas (Butler Schein, Dublin, OH) by facemask. Once gilts were in a deep anesthetic plane, they were placed in dorsal recumbency and a 14 gauge, 15 cm J wire IV catheter was placed percutaneously in the right jugular vein (Cook Medical, Bloomington, IN). A single blood pressure measurement was taken during the course of the IVGTT using a tail cuff sphygmomanometer (Critikon Dinamap 8100 / 8100T NIBP Monitor) [305]. Gilts recovered from isoflurane anesthesia for a minimum of two hours prior to initiation of the IVGTT to avoid isoflurane-induced inhibition of insulin action [305].

To perform the IVGTT, gilts were placed in a Panepinto low stress sling and a single baseline blood sample was obtained from the IV catheter after which an IV bolus of 0.5 g glucose/kg was administered. Post-bolus blood samples were collected every 10 minutes for analysis of blood glucose and serum insulin concentrations. Blood glucose was assessed on a YSI 2300 Stat Plus analyzer (YSI, Yellowsprings, OH) and serum insulin was measured on a porcine-specific RIA (Linco/Millipore). HOMA-IR was calculated for each time point as described above.
4.3.7 Statistical analysis

Blood glucose and morphometric measurements were assessed over the five-month study period on a bi-weekly and weekly schedule, respectively. Insulin, fructosamine, leptin and lipid profiles were assessed every other month during the study period. Data were analyzed as one complete set. Normality of data was assessed using a Levene’s test of homogeneity and Shapiro-Wilke. Transformation of non-normal data was done logarithmically for quantitative data and using square root for ratio data. For baseline data, ANOVA analysis was done with PROC MIXED using type 3 sums of squares in SAS 9.2 (SAS, Inc.). Analysis of the IVGTT data was done using repeated measures in time in PROC MIXED with an auto-regressive heterogeneous (ARH) covariance matrix structure. ARH was chosen as this gave the best fit statistics of all covariance matrix choices. Results from PROC MIXED were back transformed and the back-transformed data is presented herein as least square mean ± SEM. In all statistical tests, \(p<0.05\) was the criterion for statistical significance.

4.4 RESULTS

4.4.1 Morphometric measurements

Abdominal girth (control, 97.8 ± 3.6 cm; obese, 122.4 ± 3.6 cm; \(p=0.0001\)), thoracic girth (control, 95.3 ± 3.3 cm; obese, 116.1 ± 3.0 cm; \(p=0.0003\)), and weight (control, 57.8 ± 5.4 kg; obese, 91.1± 5.1 kg; \(p=0.0002\)) were all greater in obese than control gilts (Figure 13). Crown rump length (control, 111.0 ± 2.3 cm; obese, 122.0 ± 2.0 cm; \(p=0.02\)) and height (control, 58.6 ± 1.1 cm; obese, 62.7 ± 1.1 cm; \(p=0.02\)) also were significantly greater in the obese as opposed to control gilts.
4.4.2 Glucose homeostasis

Obese gilts had fasting hyperglycemia compared with control gilts (control, 63.9 ± 1.5 mg/dL; obese, 80.9 ± 5.1 mg/dL; \( p<0.0001 \); Table 4). However, there was no difference in insulin concentrations between obese and control gilts (control, 14.5 ± 1.5 \( \mu U/ml \); obese, 15.0 ± 1.4 \( \mu U/ml \); \( p=0.8 \); Table 4). HOMA-IR scores also were similar between obese and control gilts (control, 879.0 ± 155.9; obese, 1157.4 ± 148.0; \( p=0.15 \); Table 4). Despite being hyperglycemic, obese gilts were not diabetic as demonstrated by fructosamine levels, which were similar to control (control, 254.2 ± 8.2 \( \mu mol/L \); obese, 254.8 ± 7.9 \( \mu mol/L \); \( p=0.95 \); Table 4). Leptin concentrations were significantly higher in obese gilts compared to control gilts (control, 6.0 ± 1.0 ng/ml; obese, 16.0 ± 1.0 ng/ml; \( p<0.0001 \); Table 4).

4.4.3 Lipid profiles and blood pressure

Obese gilts had elevated total cholesterol (control, 163.5 ± 44.2 mg/dL; obese, 985.6 ± 41.9 mg/dL; \( p<0.0001 \); Table 5), LDL (control, 28.7 ± 42.77 mg/dL; obese, 808.7 ± 40.5 mg/dL; \( p<0.0001 \)), HDL (control, 112.0 ± 4.3 mg/dL; obese, 148.8 ± 4.1 mg/dL; \( p<0.0001 \); Table 5), and triglycerides (control, 65.4 ± 11.1 mg/dL; obese, 99.6 ± 10.5 mg/dL; \( p=0.0007 \)) as compared with control gilts. Additionally, the LDL:HDL ratio was significantly increased in obese as compared with control gilts (control, 0.4 ± 0.3; obese, 5.8 ± 0.3; \( p<0.0001 \); Table 5). Obese gilts were hypertensive compared with control gilts (mean arterial pressure: control, 103.0 ± 1.5 mmHg; obese, 121.5 ± 1.1 mmHg; \( p<0.0001 \); Figure 14).
4.4.4 Intravenous Glucose Tolerance Test

Although obese gilts had significantly higher blood glucose concentrations compared to control gilts at all time points except for baseline and 10 minutes post glucose bolus, serum insulin was only significantly greater in obese gilts compared with control gilts at 30 minutes post glucose bolus (Figure 15). Despite these findings, HOMA-IR, a measure of insulin resistance, was significantly higher in obese gilts compared to control gilts at all time points post glucose bolus except time 0 (Figure 15). Therefore, we can conclude that obese gilts were glucose intolerant and insulin resistant based on the results of the glucose tolerance test.

4.5 DISCUSSION

The major finding of this study is that the OSS gilt when fed an excess calorie high fat/cholesterol/fructose diet for a minimum induction period of three months develops four of the five features of MetS as described by the Rotterdam Diagnostic Criteria [6]: android obesity, elevated triglycerides, hypertension, and glucose intolerance. Our research group has demonstrated previously that the older OSS sow (6-8 years old) develops MetS when fed an excess calorie high fat/cholesterol/fructose diet for a period of 14 months. However, the ability to induce MetS in a relatively short length of time in a young OSS gilt (6-14 months old), decreases the financial cost of this model while maintaining its useful metabolic features. As 50-60% of PCOS patients are obese [6], 65% are insulin resistant or hyperinsulinemic [230], and approximately a third are afflicted with MetS [14], this animal model holds wide-spread promise for the study of novel therapeutics targeted at the metabolic dysfunction seen in the majority of the PCOS patient population.
Obese OSS gilts weighed more and had a larger overall body-size than control OSS gilts. The larger body size of obese OSS gilts is in contrast to our previous results in OSS sows, where obese sows only had significantly greater thoracic and abdominal girths compared with control sows. Furthermore, in previous studies of OSS gilts fed an excess calorie high fat/cholesterol/fructose diet for only 9-12 weeks, android obesity as measured by ultrasound, computed tomography (CT) scan, and body measurements increased while overall body size remained the same between obese and control gilts [30, 244]. In the current study, there were two control gilts with markedly smaller body sizes than the remaining control gilt pool, which may have skewed the morphometric measurements for the control gilt group. Removal of these two gilts from the measurement calculations does result in similar height measurements between obese and control gilts (control, 59.6 ± 1.1 cm, obese 62.7 ± 1.1 cm, p>0.05) but does little to alter the crown rump length measurements (control, 112.8 ± 2.3 cm, obese, 121.7 ± 2.0 cm, p=0.02). As both excess and restricted calories during puberty may affect not only reproductive status but also size of the animal [306] and the obesogenic diet was begun around puberty in these gilts, the excess calories could have caused increased overall growth of the body frame in obese OSS gilts. Future studies in young animals should consider initiation of the diet at 7-8 months of age to eliminate the potential for increased overall body size in obese OSS gilts. Despite the overall increase in body size, obese OSS gilts had increased girth compared to control OSS gilts. Increased abdominal girth in OSS correlates (R=0.91) significantly with visceral fat distribution based on previous studies [30] and, therefore, indicates that the obese OSS gilt would be a good model of the effects of android obesity on the development of type 2 diabetes mellitus and CVD in humans [232] as well as the relationship between obesity, insulin resistance, and hyperandrogenemia [138].
Obese OSS gilts were hyperglycemic and had elevated serum leptin concentrations compared with control OSS gilts but did not demonstrate baseline elevations in serum insulin concentrations or HOMA-IR values. Despite lack of evidence for insulin resistance on baseline testing, obese gilts demonstrated glucose intolerance and insulin resistance during the IVGTT procedure. While HOMA-IR is the commonly utilized measurement for the assessment of insulin resistance in humans [307], definitive determination of insulin resistance, particularly in subclinical cases, may require the use of an IVGTT or an oral glucose tolerance test (OGTT) [308]. Moreover, baseline measurements of both glucose and insulin are not reliable indicators of insulin resistance in children or adolescents who often require an OGTT to definitively diagnose insulin resistance and/or type 2 diabetes [309]. Based on age at puberty (~6 months) and reproductive senescence (~8 years), the obese OSS gilt approximates a 15-year-old female adolescent. Therefore, it is possible that definitive determination of insulin resistance in an OSS gilt also requires the use of a glucose tolerance test. In previous studies from our lab, obese OSS sows had a nearly two-fold increase in baseline insulin concentrations compared to control OSS sows; this finding stands in stark contrast to the nearly identical average baseline insulin concentrations seen in obese and control OSS gilts. In humans, both age [310] and chronic exposure to a high fat/cholesterol/fructose diet [311] are directly related to the development of insulin resistance. Therefore, it is difficult to compare the OSS sow and gilt animal models, which varied in age and length on diet. Leptin concentrations, on the other hand, were similar between obese OSS sows and gilts. Given that obese OSS gilts and sows both developed android obesity and leptin is known to be produced by adipose tissue [312], it is not surprising that these two variations of the obese OSS model of PCOS show similar adipokine profiles. However, as chronic elevations in serum insulin concentrations result in increased leptin production [313], one might expect the hyperinsulinemic obese OSS sows to
demonstrate higher leptin concentrations the obese OSS gilts. Elevated leptin concentrations in the follicular fluid have been associated with failed IVF cycles in humans [314], therefore, increased serum leptin concentrations in obese OSS gilts may be related to the reproductive dysfunction seen in these model animals.

Obese OSS gilts were hypertensive and dyslipidemic, two sequelae of the obese PCOS phenotype with concomitant MetS in humans that put such PCOS patients at risk for CVD [17]. Android obesity is an important factor in CVD development, leading to hypertension, altered lipoprotein metabolism, and may account for greater than 50% of the variation in insulin sensitivity observed in obese patients [315]. Furthermore, reproductive classification of PCOS patients, in particular hyperandrogenism, as it relates to obesity, insulin resistance, and lipid profiles, is predictive of CVD risk [316]. Interestingly, PCOS patients with severely abnormal lipid profiles have been found to be profoundly hyperandrogenemic as well [17], implicating a connection between altered lipid metabolism and steroidogenesis. As cholesterol is the direct precursor for the production of all steroid molecules, it is not surprising that increases in total cholesterol and LDL in PCOS patients would predispose them to hyperandrogenemia. Although the same diet was fed to obese OSS sows in our previous study and gilts in our current study, the gilts developed dyslipidemia (increased LDL and LDL:HDL ratio), whereas the sows did not. This finding appears to be an aberration because increasing age, in human women, is associated with increasing LDL and non-HDL cholesterol levels [317]. Another published study that utilized similarly aged gilts and fed the same obesogenic diet also found increased LDL and LDL:HDL ratio in the obese OSS gilts [30]. Therefore, it is possible that genetic variation between the OSS sows in our previous study, which were from the founder population on Ossabaw Island, and the OSS gilts in the current
study, which have resulted from multigenerational inbreeding in captivity, could explain the differences in lipid metabolism and resulting plasma profiles.

In conclusion, our results demonstrate that the obese OSS gilt when fed an excess calorie high fat/cholesterol/fructose diet for a minimum induction period of three months manifests the characteristics of the obese PCOS phenotype with concomitant MetS. Therefore, the obese OSS gilt, when compared with the obese OSS sow, may represent a potential superior, cost-effective, large animal model for study of the metabolic perturbations associated with the obese phenotype of PCOS with concomitant MetS.
4.6 FIGURES AND TABLES

Figure 13. Average abdominal and thoracic girths (cm; left Y axis) and weight (kg; right Y axis) in control (n=9) and obese (n=10) gilts. Obese gilts had greater abdominal (a, b; \( p=0.0001 \)) and thoracic (c,d; \( p=0.0003 \)) girths than control gilts and weighed more than control gilts (e,f; \( p=0.0002 \)).
Table 4. Assessment of glucose homeostasis in control (n=9) and obese (n=10) gilts.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Obese</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>63.9 ± 1.5</td>
<td>80.9 ± 1.5</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>14.5 ± 1.5</td>
<td>15 ± 1.4</td>
<td>0.80</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>6.0 ± 1.0</td>
<td>16.0 ± 1.0</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>879.0 ± 155.9</td>
<td>1157.4 ± 148.0</td>
<td>0.15</td>
</tr>
<tr>
<td>Fructosamine (µmol/L)</td>
<td>254.2 ± 8.2</td>
<td>254.8 ± 7.9</td>
<td>0.95</td>
</tr>
</tbody>
</table>
Table 5. Plasma lipid profiles in control (n=9) and obese (n=10) gilts.

<table>
<thead>
<tr>
<th>Parameter (mg/dL)</th>
<th>Control</th>
<th>Obese</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol</td>
<td>163.5 ± 44.2</td>
<td>985.6 ± 41.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL</td>
<td>28.7 ± 42.77</td>
<td>808.7 ± 40.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL</td>
<td>112.0 ± 4.3</td>
<td>148.8 ± 4.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>65.4 ± 11.1</td>
<td>99.6 ± 10.5</td>
<td>0.0007</td>
</tr>
<tr>
<td>LDL:HDL ratio</td>
<td>0.4 ± 0.3</td>
<td>5.8 ± 0.3</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Figure 14. Systolic and diastolic blood pressure in control (n=9) and obese (n=10) gilts. Both blood pressure measurements were significantly different ($p<0.0001$) between the treatment groups.
Figure 15. Intravenous glucose tolerance test in control (n=9) and obese (n=9) gilts. Points on the obese gilt glucose curve marked with an * are significantly greater than corresponding points on the control gilt glucose curve ($p < 0.01$). The insulin point on the obese gilt curve marked with a # is significantly greater than the corresponding point on the control gilt curve ($p = 0.05$). As compared with control gilts, obese gilts had greater HOMA-IR values at all post glucose bolus points except for time 0 ($p \leq 0.05$).
CHAPTER 5
REPRODUCTIVE CHARACTERISTICS OF THE OBESE OSSABAW GILT MODEL OF THE OBESE PHENOTYPE OF POLYCYSTIC OVARY SYNDROME WITH CONCOMITANT METABOLIC SYNDROME

5.1 ABSTRACT

PCOS is the most prevalent endocrine disorder in reproductive age women, affecting approximately 5-10% of that population [1-4]. The United States health care system spends approximately $4.4 billion per annum on PCOS treatment, nearly half of which is for infertility treatment [300]. This study characterizes the obese OSS gilt as cost-effective large animal model of the reproductive pathologies associated with the obese PCOS phenotype with concomitant MetS. Cyclic, nulliparous OSS gilts (~6 months old) were fed an excess calorie high fat/cholesterol/fructose (obese, n=10) or a control diet (control, n=9) for three months prior to the study. During the five-month study, gilts remained on their respective diets and twice weekly had blood collection and ovarian ultrasound. Cycle length, and circulating Δ4, TT, dehydroepiandrosterone-sulfate (DHEAS), P4, E2, FSH, and LH were measured. At the conclusion of the study, gilts underwent an in vivo androgen challenge to determine whether they had aberrant androgen steroidogenesis in response to IV insulin and LH. Ovaries were harvested for histology, theca cell culture, follicular fluid steroid analysis, and oocyte and theca cell gene expression studies. Obese OSS gilts had elongated estrous cycles but spent a similar amount of time in follicular and luteal phases as compared with control OSS gilts. Obese OSS gilts were hyperandrogenemic with respect to Δ4 and DHEAS, had decreased LH and increased FSH concentrations, increased luteal P4, and similar E2 concentrations compared with control OSS gilts. In both the luteal and follicular phases
of the estrous cycle, obese OSS gilts had ovarian cysts. Obese gilts also had increased numbers of medium and large sized follicles during the luteal phase of the estrous cycle only. The theca cells of obese OSS gilts responded to in vitro LH stimulation with increased production of Δ4 but there was no difference between obese and control OSS gilts with respect to the gene expression of primary theca cell steroidogenic enzymes. The follicular fluid of obese OSS gilts had an increased androgen:estrogen ratio and the oocytes of obese OSS gilts had down regulation of estrogen regulated genes, *ESR2* and *DNTTIP2*. There was no difference between the responses of obese and control OSS gilts to the in vivo androgen challenge. The obese OSS gilt when fed an excess calorie high fat/cholesterol/fructose diet for a minimum induction period of three months manifests some of the reproductive characteristics of the obese PCOS phenotype with concomitant MetS. Further assessment of the steroidogenic roles of the adrenal and adipose tissue in this model animal will help to determine whether the obese OSS gilt is a better model of the obese PCOS phenotype with concomitant MetS or the effects of obesity on fertility and reproduction.

### 5.2 INTRODUCTION

PCOS is one of the most common female gynecologic disorders, affecting approximately one tenth of reproductive age women [1, 2] and costing the American health care system approximately $1 billion for infertility treatment per annum [300]. The etiology of this disease remains to be elucidated but the general consensus amongst the PCOS research community is that the disease arises from a complex interaction of inherited [318] and environmental factors [21, 319]. The Rotterdam Diagnostic Criteria characterize the disease by oligo/anovulation, polycystic ovaries, and/or hyperandrogenism [6]. However, many PCOS researchers consider hyperandrogenism
due to intrinsic theca cell dysfunction [8] and aberrant steroidogenesis as the basis of the disorder [7, 320]. PCOS also is associated with MetS that results from the interaction of obesity and insulin resistance [6, 13, 14] and aggravates the characteristic hyperandrogenism of the disorder [15-17]. Due to the heterogeneity of the disease, a myriad of phenotypes that include both the reproductive and metabolic pathologies have been described [141, 259]. Recently, many epidemiologic studies have focused on the biochemical and clinical differences between the lean and obese PCOS phenotypes [321].

Although oligo/anovulation and polycystic ovaries contribute to the triad of reproductive pathology associated with PCOS, hyperandrogenism due to theca cell and/or adrenal cortex dysfunction is often considered the primary defect [116]. Theca cells from PCOS patients, basally and in response to LH, insulin and IGF-1, have increased steroidogenic enzyme activity, which yields increases in androgen production [9, 109, 127, 128]. The ovarian defect is indexed by selective 17OHP hyperresponsiveness to LH and poor dexamethasone suppression of T production [117, 118, 322]. Although increased free androgen index (FAI) and free T are required for a patient to be clinically diagnosed as hyperandrogenemic [323], at the ovarian level it is dysregulation of 17αOH/17,20 lyase, and to a lesser extent 3βHSD, with excessive production of 17OHP and Δ4 that characterizes the hyperandrogenism of PCOS [116]. Additionally, increased systemic LH concentrations and/or hyperinsulinemia may exacerbate hyperandrogenemia through hyperstimulation of theca cells [109]. In a self-perpetuating cycle, hyperinsulinemia and obesity act synergistically in PCOS patients to increase levels of androgens and androgens possibly increase insulin production [15-17]. Although theca cells manifest the primary steroidogenic abnormality in PCOS, granulosa cells have been shown to produce decreased P4 basally and in response to
FSH and IGF-1 [126]. By contrast, it has also been shown that granulosa cells from PCOS patients have upregulated LHCGR and cytochrome P450 11A (CYP11A) expression, which may predispose them to early luteinization [324]. Although theca cell dysfunction is accepted as an underlying cause for PCOS pathology, clearly the role of granulosa cells in the disorder remains to be definitively defined.

Despite the dramatic biochemical abnormalities that underlie PCOS, many patients with normal menses remain undiagnosed until they encounter difficulty with conception [232]. While the use of assisted reproductive technologies (ART) to improve fertility in PCOS patients has been routinely used in the past, increasingly fertility researchers are investigating various follicular fluid components as predictors of IVF success in PCOS patients [224]. In response to ovarian stimulation, PCOS patients produce more oocytes for retrieval [124, 218], experience lower fertilization rates than controls [215, 219, 220], but boast normal embryonic development in fertilized oocytes [221]. Such findings indicate not only hypersensitivity to FSH stimulation but decreased oocyte quality possibly due to the follicular environment of PCOS. The fertilization rate and developmental competence of an oocyte from a PCOS patient can be rescued by removal from the follicle and in vitro maturation (IVM) [224]. Therefore, it is plausible that follicular fluid compound(s) in the PCOS patient impair oocyte maturation and fertilization. While follicular fluid AMH has been examined extensively as an indicator of oocyte quality in PCOS patients [325-327], the concentrations and relative ratios of follicular fluid steroid hormones also appear to play a role in oocyte quality [181]. In the most comprehensive evaluation of follicular fluid steroid hormone concentrations in PCOS patients, follicular fluid E1 and E2 were decreased and follicular fluid androgen (DHEA, Δ4, T, androstandione) concentrations were increased compared with controls (n=21) [122]. Such findings support evidence of high CYP17 activity [8, 210] and low
cytochrome P450 19A (CYP19A) activity [211] previously described in PCOS patients and underline the importance of the follicular fluid androgen:estrogen ratio. In fact, an increased intra-follicular androgen:estrogen ratio in stimulated PCOS patients may be associated with an increased miscarriage rate [181] and with decreased rates of fertilization and embryonic development [216, 217].

While descriptive epidemiologic and clinical studies of PCOS patients help to further knowledge about the disease, the development of an appropriate animal model in which to examine the possible reproductive pathogenesis and test fertility-enhancing therapeutics would be extremely valuable. As a large percentage of the PCOS patient population is affected by obesity [140], MetS [6], and hyperinsulinemia [287] and these sequelae exacerbate the reproductive aspects of the disease, an animal model with these metabolic perturbations as well as the hallmark reproductive pathologies of PCOS would be extremely useful. Our research group has previously examined the use of the obese OSS sow as a model for the metabolic and reproductive features of the obese phenotype of PCOS with concomitant MetS. The OSS, has a mutation in the Val199→Ile region of the PRKAG3 gene (the γ isoform of AMPK), which interferes with insulin signaling and results in increased intra-abdominal and subcutaneous adipose accumulation when fed an excess calorie high fat/cholesterol/fructose diet [30, 31]. The strengths of this particular animal as a model for the obese phenotype of PCOS with concomitant MetS lie in the interaction of genetics (thrifty phenotype breed) and environment (obesogenic diet). Such an animal model for PCOS would help elucidate the complex interaction between obesity, hyperinsulinemia, and hyperandrogenism in the context of heritable and non-heritable factors. While the obese OSS sow holds promise as a model of some of the features of the obese phenotype of PCOS with concomitant Mets, the age of the animals and the required length of time on the
obesogenic diet to induce the phenotype render this model extremely expensive. Therefore, the aim of this research was to validate the obese OSS gilt as a more cost effective animal model for the reproductive features of the obese PCOS phenotype with concomitant MetS.

Our objective with this study was to demonstrate that the obese OSS gilt, when fed an excess calorie high fat/cholesterol/fructose diet for a minimal induction period of three months, meets the Rotterdam Diagnostic Criteria for the development of PCOS.

5.3 MATERIALS AND METHODS

5.3.1 Experimental animal procedures, husbandry, and diet treatment

All experimental animal procedures were performed in compliance with UIUC IACUC regulations, and followed the guidelines outlined in the Guide for the Care and Use of Laboratory Animals [246]. All anesthetic drugs and dosages as well as tissue harvest techniques used in the research associated with this chapter were the same as those outlined under section 3.3.1. The animals utilized in this portion of the dissertation were the same animals utilized in Chapter 4. Please refer to section 4.3.2 for details on dietary induction of phenotype and husbandry of these animals.

5.3.2 Sample collection

To determine reproductive status, blood collection and ovarian ultrasound were performed on each gilt (control, n=9; obese n=10) twice per week on fixed days during the five-month study. Pigs were trained to the use of a Panepinto low stress sling restraint system for purposes of ovarian ultrasound [247, 248]. Blood collection and
processing and ovarian ultrasound were conducted as described under section 3.3.3. Blood was collected for serum to assess circulating reproductive hormones. Throughout the dietary induction phase and study period (eight months), pigs were checked daily for estrus by application of back pressure and monitoring for estrus behavior in the presence of a boar. Estrus was used in conjunction with serum hormone and ovarian ultrasound data to determine cycle length (see statistical analysis).

5.3.3 Serum hormone RIA

The following hormones were assessed bi-weekly in the serum throughout the five-month study: E2, P4, Δ4, TT, DHEAS, FSH, and LH. All assays were validated for use with swine serum or extracted samples using parallelism and recovery of cold ligand. All samples that were non-detectable were assigned the lower limit of detection for each assay. To remove lipids from serum samples prior to RIA, serum for the assessment of all steroid hormones except DHEAS was extracted with the ether and hexane-methanol procedure described in section 3.3.5 above. To monitor extraction efficiency, samples were spiked with tritiated hormone of interest (~1000 cpm/100 μl spike/1 ml serum) prior to extraction. Hormone concentrations obtained from RIAs were corrected mathematically for extraction efficiency. Extraction efficiency was > 65% for all samples. After extraction, all samples were reconstituted in 1% PBS-gel to the volume of the original serum sample (1 ml). All steroids with the exception of DHEAS were assessed using Coat-A-Count kits (Siemens Medical Solutions Diagnostics). For the assessment of E2 (2.5-150pg), P4 (32-1000 pg), Δ4 (20-640pg), and TT (10-1600 pg) standards were made by adding a known amount of the respective steroid hormone to 1% PBS-gel and then preparing further dilutions. The sensitivity of the E2 assay was 4.5 pg, and this assay had an inter-assay precision of 2.0% and an intra-assay precision of
1.0%. The E2 assay was run at an average binding of 47.9% (n=6). The TT assay sensitivity was 230 pg with an inter-assay precision of 5.0% and an intra-assay sensitivity of 2.1%. The TT assay was run at an average binding of 32.3% (n=4). The sensitivity of the Δ4 assay was 360 pg and this assay had an inter-assay precision of 3.2% and an intra-assay precision of 0.9%. The Δ4 assay was run at an average binding of 33.3% (n=7). The P4 assay had a sensitivity of 50 pg and an inter-assay precision of 1.8% and an intra-assay precision of 1.8%. The P4 assay was run at an average binding of 56.5% (n=5).

DHEAS was assessed using a Beckman Coulter double antibody kit (DHEA-S-7 RIA, DSL 2700, Beckman Coulter Inc, Brea, CA). Standards (25-2500 pg) were made by adding a known amount of DHEAS to PBS-gel and then making further dilutions in charcoal stripped pig serum. The DHEAS assay was run at an average binding of 26.8% (n=7) and had an assay sensitivity of 1055 pg. The inter-assay precision was 5.0% and the intra-assay precision was 1.5%.

Serum LH and FSH were analyzed according to previously described double antibody RIAs validated for pLH [254] and pFSH [255], respectively, with modifications as described in section 3.3.5 above. The LH assay was run at an average percent binding of 24.8% (n=7). The sensitivity of the assay was 240 pg and this assay had an inter-assay precision of 12.9% and an intra-assay precision of 5.3%. The FSH assay was run at an average binding of 31.9% (n=7). The sensitivity of the FSH assay was 393 pg. The FSH inter-assay precision was 1.7% and the intra-assay precision was 4.2%.

5.3.4 Follicular dynamics
DVD images of ovarian ultrasound were assessed as previously described in section 3.3.6. Follicle size categories were assigned as follows: <3.5 mm, small; 3.5-6.5 mm, medium; 6.5-12.5 mm, large; >12.5 mm, cyst [257]. Although the largest follicle size was termed cyst, for a follicle to be classified as a cyst it must also have persisted on the ovary longer than one week.

5.3.5 Ovarian histology

Gilts were euthanized and ovaries harvested when there were ovulatory sized follicles (6.5-12.5 mm) present. At tissue harvest, a subset of gilts (n=3 from each treatment group) had one of their ovaries placed in Dietrichs fixative for ovarian histology while the other ovary was reserved for theca cell culture. After 48-72 hours in Dietrichs fixative, ovaries were transferred to 70% ethanol until processing. Fixed ovaries were sectioned transversely into 1 mm thick slices and then were embedded in paraffin wax blocks. Paraffin blocks were serially sectioned at 7µm thickness, and sections were transferred onto microscope slides. Slides were dried for 24 hours, deparaffinized with xylene, rehydrated, and stained with Mayer’s Haematoxalin (Sigma Aldrich) and Eosin Y Solution (Sigma Aldrich; H&E). Ovulatory sized follicles were examined at 100-400x for evidence of normal architecture of the theca and granulosa cell layers.

5.3.6 Primary theca cell culture

Only gilts (control, n=4; obese, n=6) not included in the in vivo androgen challenge test had primary theca cell culture. After harvest from the abdominal cavity, ovaries not designated for histologic techniques were placed immediately in cold (~ 4 °C) HEPES (Sigma Aldrich) containing 0.1% BSA (w/v), 1.0 mM glutamine and 1x
essential and non-essential amino acids (MP Biomedicals, Costa Mesa, CA) until processing. To isolate theca cells for cell culture, ovulatory sized follicles were isolated from the ovary and bisected. Follicular fluid from bisected follicles was frozen at -20 °C for assessment of steroid hormone concentrations and oocytes were isolated from the follicular fluid and processed as described below. Theca interna and granulosa cell layers were stripped from the theca externa and connective tissue with forceps. Granulosa cell layers were mechanically stripped from the theca interna by repeatedly running the theca interna through a pair of closed forceps. Theca interna tissue pieces were allowed to settle by gravity in PBS on ice after which they were digested in a 15 ml conical with 0.1% collagenase XI (Sigma Aldrich) on a rotating rocker for 15-20 minutes. Digested theca cells were washed 3x in DMEM/F12 media with phenol red supplemented with 15% charcoal stripped fetal bovine serum (FBS; Charcoal/Dextran Treated Fetal Calf Serum, Thermo Scientific HyClone, Logan, UT), 1x vitamins (v/v; 100x Vitamins MEM modification, MP Biomedicals), and 1% penicillin-streptomycin-actinomycin (v/v; PSA, MP Biomedicals). The theca cell pellet was resuspended in approximately 1 ml of the above media and the cell suspension was counted with 0.2% (v/v) trypan blue solution (Trypan Blue Solution 0.4%, Gibco, Invitrogen™, Carlsbad, CA) and a hemocytometer. Large pieces of theca cells were run through a 22 gauge needle and 3 ml syringe multiple times to further pulverize prior to plating. Theca cells were plated at a density of 250,000 live cells per a well in 750 μl of the above-described cell culture media in 24 well tissue-culture treated Costar plates (Costar 3524, Costar®, Cambridge, MA). Cells were cultured in humidified 95% air and 5% CO₂ at 37 °C and were allowed to reach at least 60% confluence prior to treatment.

Once cells reached at least 60% confluence, three wells per treatment were washed with the treatment media two times followed by application of 750 μl of treatment.
media per well. Treatment media included: control, LH (10 ng/ml), insulin (100 ng/ml), and LH + insulin (10 ng/ml and 100 ng/ml). All treatment media consisted of DMEM/F12 with 1% PSA, 1x vitamins and 1% SSS (v/v; Synthetic Serum Substitute, Irvine Scientific, Santa Ana, CA). Insulin from porcine pancreas (Sigma Aldrich) and pLH (Sioux Biochemical, Inc, Sioux Center, IA) were used to make the treatment media. After 48 hours, treatment media was removed from each well and frozen at -20 °C in a 1.5 ml tube for future steroid hormone analysis. Theca cells were trypsinized with 200 µl of 0.25% trypsin with EDTA (v/v 0.5% Trypsin EDTA, Invitrogen, Carlsbad, CA), counted using a 0.2% trypan blue solution and a hemocytometer, and frozen at -80 °C in 300 µl β mercaptoethanol RLT lysis buffer (Qiagen Inc, Valencia, CA) for subsequent PCR analysis.

5.3.7 Follicular fluid hormone assessment

Follicular fluid from gilts (control, n=4; obese, n=6) not included in the in vivo androgen challenge test cohort was analyzed. Follicular fluid collected from bisected ovulatory sized follicles (8-13 follicles) was assessed for the following steroid hormones: E2, P4, Δ4, TT and 17OHP. To remove lipids and proteins, the follicular fluid (500 µl) was extracted with 3 ml of diethyl ether (Sigma Aldrich). Extraction efficiency was monitored as described under section 5.3.3 and was > 90% for all follicular fluid extractions. Extracted samples were reconstituted in 1% PBS-gel to the volume of the original follicular fluid sample (500 µl). All steroid hormone assays (Coat-A-Count, Siemens Medical Solutions Diagnostics) were validated for a buffer-based system by parallelism and recovery of cold ligand. Samples for assessment of E2 were diluted 1:800 to 1:600 prior to analysis. Samples for assessment of 17OHP were diluted 1:20 prior to analysis. Samples for assessment of Δ4 were run at 3% of the kit-required
sample volume per tube, whereas samples for the assessment of TT were run at 6% of the kit-required sample volume per tube. Samples for assessment of P4 were run at 2% of the kit-required sample volume per tube.

5.3.8 Theca cell and oocyte gene expression

Theca cells and oocytes from gilts (control, n=4; obese, n=6) not included in the in vivo androgen challenge cohort were assessed for gene expression. Total RNA was extracted from primary theca cell aliquots of 100,000 cells using the RNeasy Mini Kit (Qiagen Inc) and an on-column DNase treatment (RNase-free DNase Set, Qiagen Inc). Total RNA was quantified on the Nanodrop (Nanodrop 3300, Thermo Scientific, Wilmington, DE). RNA samples with an OD_{260}/OD_{280} ratio > 1.8 on the Nanodrop had adequate RNA extraction and were bioanalyzed for RNA quality (Agilent Bioanalyzer, Functional Genomics Unit, Keck Center, UIUC). Total RNA was reverse transcribed to cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems Inc, Carlsbad, CA), diluted 1:5 with RNAse–free water, followed by qRT-PCR (TaqMan Gene Expression Assay, Applied Biosystems Inc) on a 7900HT Fast Real-Time PCR System (Applied Biosystems Inc) to determine relative fold differences in the following mRNA expression: 3-beta-hydroxysteroid dehydrogenase 1 (HSD3β1), 17-beta-hydroxysteroid dehydrogenase 4 (HSD17β4), and 17-alpha-hydroxylase/17,20 lyase (CYP17A1). Our endogenous gene, 18s ribosomal RNA (RN18S1), was tested between control and obese tissues and demonstrated similar replication amongst the treatment groups. Applied Biosystems TaqMan Gene Expression Assay for HSD3β1: Ss03391752_m1; Applied Biosystems TaqMan Gene Expression Assay for HSD17β4: Ss03394675_m1; Applied Biosystems TaqMan Gene Expression Assay for CYP17A1:
Oocytes harvested from ovulatory sized follicles were washed 3x in cold 0.1% polyvinyl alcohol (PVA) in PBS (v/v) and then snap frozen (-80 °C) in 3 μl volume in groups of 8-9 oocytes per pig per tube for gene expression analysis. Total RNA was extracted (PicoPure® RNA Isolation Kit, Applied Biosystems Inc) with an on-column DNase treatment (RNase-free DNase Set, Qiagen Inc), reverse transcribed to cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems Inc), diluted 1:3 with RNAse–free water, followed by qRT-PCR (TaqMan Gene Expression Assay, Applied Biosystems Inc or Sybr Green Quanti-fast, Qiagen Inc) to determine relative fold differences in the following mRNA expression: estrogen receptor beta (ESR2), estrogen receptor binding protein (DNTTIP2), androgen receptor (AR), progesterone receptor (PGR). 18s ribosomal RNA (RN18S1) served as the endogenous control gene for the oocyte qRT-PCR.

Due to the small amount of starting material, we were unable to quantify the total RNA we put into the cDNA reaction, and, thus, how much we were loading into the qRT-PCR assay. Therefore, we created a bulk pig cDNA pool (liver, oocyte, theca), which we quantified on the Nanodrop (Nanodrop 3300, Thermo Scientific) and from which serial dilutions (1:10, 1:50, 1:100, 1:500, 1:1000) were made to create a relative standard curve for each gene. The efficiency of the standard curve for each gene was between 88 and 106%. qRT-PCR was performed on ESR2, DNTTIP2, PGR using Taqman Gene Expression Assays: Applied Biosystems TaqMan Gene Expression Assay for ESR2: Ss03394947_m1; Applied Biosystems Taqman Gene Expression Assay for DNTTIP2: Ss03391479_m1; Applied Biosystems Taqman Gene Expression Assay for PGR: Ss03374439_m1. qRT-PCR for AR was performed using Sybr Green (Sybr Green
Quanti-fast, Qiagen Inc) on a MasterCycler Realplex\textsuperscript{2} (Eppendorf North America, Inc, Westbury, NY). Primers were tested on pooled theca cells or oocytes and primer specificity was determined by melt curve analysis and gel electrophoresis. \textit{AR} primer sequences: forward 5’-AGACGCGGAGACAGGATAAA-3’, reverse 5’-CCTGGGTCTGGATCCTTT-3’. \textit{RN18S1} primer sequences: forward 5’-CGGCTACCACATCCAAGGAA-3’, reverse 5’-CTCAATCGGATCCTCGTTAAAGG-3’.

5.3.9 \textit{In vivo androgen challenge}

Eight gilts (control=4, obese=4) underwent the in vivo androgen challenge the day of euthanasia. Bilateral percutaneous IV catheters were placed as described in section 4.3.6. Prior to initiation of the challenge, a baseline blood sample was collected. For the first hour of the challenge, IV insulin (Humulin-R, Eli Lilly, Indianapolis, IN) was administered at a rate of 0.4 U/kg/hr as a continuous rate infusion (CRI). For the second hour of the challenge, IV insulin continued to be administered and was coupled with IV pLH (Sioux Biochemical Inc) administered at a rate of 1 μg/kg/hr as a CRI. To maintain blood glucose levels within normal physiologic levels (50-100 mg/dL), IV 20% dextrose was administered continuously throughout the challenge. During the challenge, blood was collected every 10 minutes for assessment of steroid hormones and blood glucose. After the two-hour challenge period, blood was collected every 30 minutes until euthanasia two hours post-challenge.

5.3.10 \textit{Statistical analysis}
Response variables included in the multivariate model were: Δ4, TT, DHEAS, P4, E2, FSH, LH, small, medium and large follicles, and cysts. Two consecutive complete estrous cycles were analyzed per gilt. Baseline estrous cycle data were divided into follicular and luteal phases. The percentage of estrous cycle length dominated by follicular and luteal phases was determined for each gilt and each estrous cycle. Follicular phase was defined as sample dates preceded by a decrease in P4 below 2 ng/ml and followed by an increase in P4 above 2 ng/ml, corresponding with the lack of a CL by ultrasound visualization. Luteal phase was defined as a period with P4 greater than 2 ng/ml with visualization of CL on ultrasound. Estrous cycle length was calculated from one point in time characterized by a nadir in P4 (ie, below 2 ng/ml) coupled with lack of CL on ultrasound and standing estrus behavior, to the next point in time demonstrating these three criteria. Normality of data was assessed using a Levene’s test of homogeneity and Shapiro-Wilke. Transformation of non-normal data was done logarithmically for quantitative data and using square root for count and ratio data.

ANOVA analysis was done for reproductive response variables, follicular fluid hormone, and cell culture media data with PROC MIXED using type 3 sums of squares in SAS 9.2 (SAS, Inc.). Repeated measures in time analysis with an auto-regressive (AR1) covariance structure in PROC MIXED using type 3 sums of squares was used to analyze the in vivo androgen challenge test data. Results from PROC MIXED were back transformed and the back-transformed data is presented herein as least square mean ± SEM.

For theca qRT-PCR results, the mRNA abundance of target genes was normalized to an endogenous control gene, RN18S1, for sample-to-sample comparisons. The relative fold induction of each gene was then compared between obese and control gilts using the ΔCT method. For oocyte qRT-PCR, the mRNA
abundance of target genes was normalized to an endogenous control gene, \textit{RN18S1}, using a bulk pig cDNA standard curve. Oocyte data was analyzed using the relative expression software tool, REST 2009 version 2.0.13 \cite{328}, with \textit{RN18S1} set as the reference gene. Expression ratios were generated using PCR efficiencies of the target and reference genes and the $\Delta$CT values of the control and obese samples \cite{329}. In all statistical tests, $p<0.05$ was the criterion for statistical significance.

5.4 RESULTS

5.4.1 Baseline reproductive status

Obese gilts had a longer average estrous cycle length than control gilts (obese, 22.2 ± 1.1 days; control, 19.6 ± 1.1 days; \(p=0.0001\)), but each treatment group spent a similar amount of time in follicular (obese, 20.6 ± 7.4 %; control, 20.8 ± 4.9 %; \(p=0.5\)) and luteal (obese, 79.9 ± 8.0 %; control, 79.2 ± 6.9 %; \(p=0.4\)) phases of the cycle. Obese serum $\Delta 4$ concentration (0.35 ± 0.3 ng/ml) was significantly higher than control serum $\Delta 4$ concentration (0.28 ± 0.03 ng/ml; \(p=0.005\)) in the luteal phase only (Figure 16). Serum TT was not different between the two treatment groups (Figure 16). Obese gilts had higher DHEAS concentrations during both the follicular (control, 0.17 ± 0.10 ng/ml; obese, 0.23 ± 0.01 ng/ml; \(p<0.0001\)) and luteal (control, 0.19 ± 0.01 ng/ml; obese, 0.25 ± 0.01 ng/ml; \(p<0.0001\)) phases of the estrous cycle (Figure 16).

Obese gilts had significantly more cystic follicles (>12.5 mm) than control gilts in both the follicular and luteal phases of the estrous cycle (Figure 17). Cystic size follicles persisted on the ovaries of control gilts for 3.0 ± 1.4 days and on the ovaries of obese gilts for 3.8 ± 6.2 days. Obese gilts had significantly more medium (3.5-6.5 mm) and large (6.5-12.5 mm) follicles than control pigs during the luteal phase of the estrous cycle only (Figure 17 B). There was no significant difference in the number of CL during the
luteal phase in control (4.7 ± 0.4 CL) and obese (4.6 ± 0.3 CL; \( p=0.9 \)) gilts. Control gilts had higher serum LH concentrations than obese gilts, while obese gilts had higher serum FSH concentrations than control gilts (Table 6). Therefore, control gilts also had a significantly higher LH:FSH ratio than obese gilts (Table 6). Obese gilts had significantly higher serum P4 concentrations during the luteal phase only. There was no difference in serum E2 concentrations the two treatment groups (Table 6).

5.4.2 Ovarian histology

All ovaries from control gilts demonstrated normal theca interna and granulosa cell layers in all large follicles visualized within the ovarian cortex. One of the three obese gilts had an ovary with hypertrophy of both the theca interna and granulosa cell layers in two out of four large follicles visualized within the ovarian cortex (Figure 18).

5.4.3 Follicular fluid steroid hormone concentrations, theca cell androgen production, and theca and oocyte gene expression

While there was no difference in the absolute concentrations of \( \Delta 4 \) (control, 50.0 ± 43.6 ng/ml; obese 97.9 ± 39.0 ng/ml; \( p=0.4 \)), TT (control, 18.5 ± 9.5 ng/ml; obese, 23.7 ± 8.5 ng/ml; \( p=0.7 \)), E2 (control, 72.8 ± 25.2 ng/ml; obese 31.3 ± 22.5 ng/ml; \( p=0.4 \)), P4 (control, 35.9 ± 17.8 ng/ml; obese, 60.7 ± 16.0; \( p=0.3 \)), and 17OHP (control, 15.3 ± 6.8 ng/ml; obese, 20.8 ± 6.1 ng/ml; \( p=0.6 \)) in the follicular fluid of control and obese gilts, obese gilts had significantly greater \( \Delta 4: E2 \) and TT:E2 ratios than control gilts (Figure 19).
Primary theca cells from obese gilts produced increased amounts of Δ4 in cell culture in response to treatment with LH (10 ng/ml) as compared with control gilts (control, 0.15 ± 0.03 ng/ml; obese, 0.25 ± 0.03 ng/ml, *p*=0.03; Figure 20). All other theca cell culture treatments yielded similar Δ4 production amounts between the control and obese gilts.

Primary theca cells did not demonstrate differential gene expression with respect to *HSD3B1, HSD17B4* or *CYP17A1* between control and obese gilts (Figure 21 A). *ESR2* and *DNTTIP2* were significantly down regulated in obese as opposed to control gilt oocytes, whereas *PGR* and *AR* were similarly expressed in oocytes from the two treatment groups (Figure 21 B).

### 5.4.4 In vivo androgen challenge

Control and obese gilts produced similar amounts of serum Δ4 in response to insulin infusion alone and insulin + LH infusion. However, two hours after the completion of the in vivo androgen challenge, the average Δ4 production by obese gilts became significantly greater than the production by control gilts (Figure 22).

### 5.5 DISCUSSION

The major finding of this study is that, compared with age-matched lean controls, obese OSS gilts fed an excess calorie high fat/cholesterol/fructose diet for a minimum induction period of three months were hyperandrogenemic with respect to serum Δ4 and DHEAS concentrations, had prolonged estrous cycle length, and formed persistent ovarian cysts, thereby meeting the Rotterdam Diagnostic Criteria for PCOS [258]. Our research group has demonstrated previously that the older OSS sow (6-8 years old) develops similar reproductive pathologies when fed an excess calorie high
fat/cholesterol/fructose diet for a period of 14 months. However, the ability to induce many of the reproductive pathologic features of PCOS in a relatively short length of time in a young OSS gilt (6-14 months old) decreases the financial cost of this model while maintaining its usefulness. The OSS gilt model also has been shown by our research group to develop android obesity and MetS. Therefore, the obese OSS gilt may be a cost efficient large animal model of the obese phenotype of PCOS with concomitant MetS in which to study some of the effects of obesity and metabolic disturbances on hyperandrogenism and reproductive dysfunction.

Although obese OSS gilts had elongated estrous cycles, which are analogous to oligo-menorrhea in human PCOS patients, they did not demonstrate elongation of either the follicular or luteal phases. This stands in stark contrast to PCOS patients whose oligo-menorrhea is due to persistent small antral cysts (2-9 mm) and elongation of the follicular phase of the menstrual cycle [6]. Interestingly, our obese OSS gilts did develop cysts, however, unlike human PCOS, these cysts were post-ovulatory size (>12.5 mm) and could be found in the presence of formed CL tissue. The fact that the cysts in obese OSS gilts were post-ovulatory size and coupled with large numbers of medium and large sized follicles during the luteal phase indicates a dysfunction in folliculogenesis during the later stages of recruitment and dominant follicle selection and persisting past the point of normal dominant follicle ovulation [257]. Furthermore, in contrast to what is seen in obese PCOS patients who have low to normal serum LH and normal serum FSH concentrations [69], the obese OSS gilts in this study had low serum LH and high serum FSH concentrations. The fact that obese OSS gilts had low LH indicates that obesity and insulin resistance may have caused suppression of LH secretion [72], as is seen in some obese PCOS patients. Moreover, such low LH concentrations in obese OSS gilts may cause the development of post-ovulatory size cysts due to an inability to mount an LH
surge necessary for ovulation [330]. The elevation in FSH concentrations, however, does not fit with any PCOS phenotype. Increases in FSH concentrations normally occur around the time of menopause in humans [331]. Furthermore, pathologic increases in FSH concentrations in humans may occur during the reproductive disease states of incipient ovarian failure (IOF; normal menses), transitional ovarian failure (TOF; oligo-menorrhea), premature ovarian failure (POF; oligo/amenorrhea) [332-334]. Given that obese OSS gilts had irregular estrous cycle lengths and elevated FSH concentrations and normal E2 concentrations, they could have a reproductive pathology similar to TOF. However, the production of copious numbers of selectable and dominant follicles and cysts and the remaining hormone milieu does not mimic TOF. Nonetheless, elevated FSH concentrations in obese OSS gilts may be related to the increased recruitment of medium sized follicles during the luteal phase of the cycle.

Interestingly, steroids produced in the adrenal cortex like cortisol have been linked to post-ovulatory size ovarian cyst production in pigs [335] and obesity is known to increase the production of cortisol via stimulatory effects of hyperinsulinemia [336]. Furthermore, menstrual cycle abnormalities are a common occurrence in hypercortisolemic conditions like Cushing’s disease [337]. In an on-going study with the OSS gilt, preliminary assessment indicates that the obese OSS gilt has elevated cortisol concentrations compared with the control OSS gilt (data not shown). Therefore, the role of the adrenal gland in the reproductive abnormalities of this model animal should not be underestimated. In fact, the two androgens found in excess in the obese OSS gilt, Δ4 and DHEAS, are produced by theca cells and the adrenal cortex in pigs [338] and humans [116]. One may argue that since TT was not increased in obese OSS gilts and this androgen is considered the hallmark of hyperandrogenism in PCOS patients [323], obese OSS gilts are not hyperandrogenemic. However, intrinsic theca cell dysfunction of
CYP17 [8, 9] and a parallel defect in the adrenal cortex [339] comprise the primary steroidogenesis defects in PCOS. Therefore, increased serum Δ4 and DHEAS concentrations are supportive of theca and/or adrenal cortex dysfunction in obese OSS gilts. TT concentrations were similar between the obese OSS gilts and sows, indicating accurate hormone measurement and similar physiology between our model animals. Given that visceral adipose tissue is known to interconvert steroids, favoring Δ4 production from T [265], and that our obese OSS gilts had android obesity, it is possible the adipose tissue in obese OSS gilts contributes to the hyperandrogenism, favoring Δ4 over T production. However, adipose tissue alone cannot be responsible for the steroid milieu in obese OSS gilts, because there is no evidence of increased aromatization [340] nor of general androgen sequestration due to fatty acid esters present in the tissue [341].

Ovarian theca and granulosa cell hypertrophy in a single obese OSS gilt is a somewhat similar histopathologic finding to what is seen in PCOS patients. Although the typical PCOS ovary has attenuated granulosa cell layers and/or luteinized theca within the follicles, a subset of follicles may maintain granulosa cell layers [342]. Evidence of architectural abnormalities within several follicles of an obese OSS gilt is suggestive of possible cellular dysfunction. Interestingly, we received conflicting results with respect to basal and stimulated theca cell function, possibly due to individual pig differences overshadowing overall treatment differences due to the small sample size. We did not find any differences between control and obese OSS gilts in gene expression of steroidogenic enzymes in primary theca cells. However, we did find increased production of Δ4 in primary theca cell cultures of obese OSS gilts in response to in vitro stimulation with LH. Such findings support a translational, rather than transcriptional, mechanism by which CYP17 causes increased steroid production. Methods to assess
protein concentrations of CYP17 and 3βHSD in theca cells (ie, IHC or western blot) would help elucidate the mechanism of Δ4 hypersecretion in our model animal. We hypothesize that the lack of difference in in vitro Δ4 production in obese versus control OSS gilts in response to insulin based treatments is due to down-regulation or lack of IR function on theca cells of obese OSS gilts. We know from our previous studies that obese OSS gilts are insulin resistant; therefore, supplementation of theca cells in culture with insulin-based treatments is unlikely to cause a response. Such a finding is in contrast to PCOS patients who are systemically insulin resistant but retain insulin sensitivity within the ovary [132]. We see a similar response with the in vivo androgen challenge, as there is no difference in serum Δ4 concentrations during IV supplementation with insulin or insulin+ LH. However, after removal of exogenous insulin and LH, we see a “Somogyi effect” or rebound of serum Δ4 concentrations in obese OSS gilts. It is likely that the dosing of the insulin, the LH, or both was too high for the short-term administration period of the test. Therefore, down-regulation of IR and LHCRGR on the theca cells may have occurred which would have muted subsequent steroid release. In our previous study with obese OSS sows, the results from our superovulation protocol suggested that the obese OSS ovary is hyperresponsive to LH in an FSH-primed environment. For future in vivo studies of theca cell function in obese versus control OSS gilts, it is recommended to perform an hCG or gonadotropin stimulation test on an unprimed ovary as is the gold standard for clinical diagnosis of subtle PCOS cases [118].

Despite indications that theca cells of obese OSS gilts aberrantly produce Δ4 both basally and in response to LH stimulation, the follicular fluid of obese OSS gilts does not demonstrate absolute increases in androgens or androgen precursors. However, relative to estrogens, androgens are increased in the follicular fluid of the
obese OSS gilt, which may indicate CYP19A dysfunction [343]. Interestingly, although absolute E2 is not decreased in the follicular fluid of obese OSS gilts, the gene expression of two estrogen-regulated genes, ERS2 and DNTTIP2, is down regulated on oocytes from obese OSS gilts. PCOS patients also have increased androgen:estrogen ratio in their follicular fluid [122] and may show decreased aromatase activity [123, 211, 212]. Although ERα and estrogen receptor binding protein (ERBP) are found in human oocytes [199], the exact mechanism of their action in the oocyte is unknown nor have these proteins been examined in the context of oocyte quality in PCOS. Future studies that examine oocyte quality and competence in obese versus control OSS gilts as they relate to the intra-follicular environment and oocyte gene expression may elucidate the roles of steroid hormones and their receptors.

In conclusion, our results demonstrate that the obese OSS gilt when fed an excess calorie high fat/cholesterol/fructose diet for a minimum induction period of three months manifests some of the characteristics of the obese PCOS phenotype with concomitant MetS. Therefore, the obese OSS gilt, when compared with the obese OSS sow, may represent a potential superior, cost-effective, large animal model for study of the obese phenotype of PCOS with concomitant MetS. Future studies that focus on both in vivo and in vitro stimulation of theca cells and the adrenal cortex will further characterize the sources of excessive androgen production in this model animal.
5.6 FIGURES AND TABLES

Figure 16. Serum androgens in control (n=9) and obese (n=10) gilts. Within a given phase of the estrous cycle (follicular and luteal) and androgen hormone, bars with
different letters indicate significantly different ($p<0.005$) serum hormone concentrations between control and obese gilts.
Figure 17. Average number of follicles per gilt in follicular (A) and luteal (B) phases of the estrous cycle in control (n=9) and obese (n=10) gilts. Within a given follicle category, different letters indicate significance between control and obese gilts (p<0.03).
Table 6. Basal estrous cycle hormones in control (n=9) and obese (n=10) gilts.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Control</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Follicular</td>
<td>Luteal</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>18.3 ± 1.8</td>
<td>7.8 ± 1.0</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>1.3 ± 2.0</td>
<td>11.0 ± 0.9</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>1.7 ± 0.1</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>5.2 ± 0.6</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>LH:FSH ratio</td>
<td>3.1 ± 0.4</td>
<td>1.5 ± 0.3</td>
</tr>
</tbody>
</table>

\[a,b,p=0.002; c,d,p=0.005; e,f,p<0.0001; g,h,p=0.02; i,j,p=0.01; k,l,p=0.02; m,n,p=0.02.\]
Figure 18. Representative H&E stained sections of ovarian tissue from a control (A) and an obese (B) gilt at 100x magnification (400x magnification in inset). The obese gilt has
hypertrophy of the granulosa and theca interna layer with vacuolation in the theca interna.
Figure 19. Follicular fluid steroid hormone concentrations and ratios in control (n=4) and obese (n=5) gilts. Within a given ratio, different letters indicate significance between control and obese gilts (p=0.03). Steroidogenic enzymes in parentheses after a given ratio indicate the enzyme function the ratio approximates.
Figure 20. Primary theca cell Δ4 production in response to control, LH (10 ng/ml), insulin (100 ng/ml) or LH + insulin (10 ng/ml + 100 ng/ml) in control (n=4) and obese (n=5) gilts. Bars with different letters are significantly different (p=0.03).
Figure 21. Theca (A) and oocyte (B) gene expression in control (n=4) and obese (n=5) gilts. For a given gene, bars with different letters show significantly different relative fold induction ($p<0.04$).
Figure 22. Production of serum Δ4 in response to an in vivo androgen challenge with IV insulin (0.4 U/kg/hr) and pLH (1 μg/kg/hr) in control (n=4) and obese (n=4) gilts. Two hours after the challenge was complete serum Δ4 concentrations rose dramatically in obese gilts to become significantly greater than serum Δ4 concentrations in control gilts (*p=0.05).
CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Polycystic ovary syndrome is the most common endocrine disorder of reproductive age women. As the etiology of this disorder is not fully understood and as animal models of human disease are useful to determine disease pathogenesis, recent research efforts have focused on the development of an appropriate animal model for PCOS. In this Ph.D. dissertation I sought to validate the obese OSS pig as an animal model for the obese phenotype of PCOS with concomitant MetS. The main findings of this work are that when OSS pigs were fed an excess calorie high fat/cholesterol/fructose diet: 1) both OSS sows and gilts developed android obesity and metabolic perturbations, including but not limited to dyslipidemia, hypertension and glucose intolerance, that met the Rotterdam Diagnostic Criteria for MetS; 2) both OSS sows and gilts developed persistent post-ovulatory size ovarian cysts; 3) both OSS sows (Δ4 only) and gilts (Δ4 and DHEAS) became hyperandrogenemic; 4) both OSS sows and gilts developed protracted estrous cycle length; 5) both OSS sows and gilts developed increased numbers of medium and large size follicles during the luteal phase of the estrous cycle; 6) OSS sows had increased LH and decreased P4 serum concentrations; 6) OSS gilts had decreased LH and increased FSH serum concentrations; 7) OSS sows responded to a long-term GnRH agonist superovulation protocol similarly to OSS sows on a control diet; 8) the theca cells of OSS gilts did not demonstrate basal differences in the gene expression of steroidogenic enzymes but did respond to in vitro LH stimulation with increased production of Δ4; 9) OSS gilts had an increased androgen:estrogen ratio in the follicular fluid and demonstrated down regulation of estrogen controlled genes on oocytes; 10) OSS gilts responded to an in
vivo androgen challenge similarly to OSS gilts on a control diet. Based on these findings, I have revised my working model of the whole-animal physiology of the obese OSS pig (Figure 22).

The research in this dissertation demonstrates that the obese OSS pig is an excellent model animal for obesity and MetS in humans. Furthermore, the obese OSS sow and gilt possess at least some of the reproductive features of the obese phenotype of PCOS. However, based on the findings of my Ph.D. research, neither of these animal models completely recapitulates all of the reproductive features, nor the exact pathophysiology, of the obese phenotype of PCOS with concomitant MetS. Moreover, both sows and gilts demonstrated some differences in the response of their reproductive physiology to the obesogenic diet. As there were several sources of variation between the two animal models, including age (6-14 months old versus 6-8 years old), genetics (generational captive inbred versus wild-caught), and length of time on diet (8 months versus 14 months), it is difficult to pinpoint an exact reason for the differential response in the reproductive parameters. It is possible that the obese OSS sow and/or gilt may be better suited as models of the effects of obesity on reproductive and endocrine physiology. The bulk of this Ph.D. research was descriptive in nature and generated data primarily related to the systemic environment of the obese OSS pig. Therefore, many of the black, solid arrows linking different research findings on the revised working model are hypotheses rather than concrete facts (Figure 22).

The obese OSS gilt model is more cost effective, requires a shorter time frame to generate data, shares many of the core reproductive features with the obese OSS sow model, and generated a small amount of data on the molecular mechanisms and intrafollicular environment. Therefore, future research designed to further define the utility of this animal model should focus on the obese OSS gilt. Besides the metabolic features,
the hyperandrogenemic environment remains a strong suit of this animal model. Although several findings in the obese OSS gilt model (ie, increased theca cell production of Δ4 in response to LH in vitro, increased androgen:estrogen ratio in the follicular fluid, abnormal granulosa and theca cell appearance on histology) indicate a role for the ovary in the generation of androgens, the contribution of other androgen-producing tissues cannot be excluded. Therefore, research in the obese OSS gilt that examines the role of the adrenal gland and adipose tissue as well as expands on knowledge of the theca, granulosa, and oocyte physiology will further characterize the molecular mechanisms underlying the hyperandrogenemia in this animal model. Additionally, incorporation of a lean genetic control into future research studies of the obese OSS gilt would be helpful in discerning the roles of genetics and diet in the model.

In on-going research as a part of my NIH K01 grant I plan to address the following questions and complete the following in vivo and in vitro experiments in Yorkshire-cross gilts (n=6), lean OSS gilts (n=6), and obese OSS gilts (n=6). Such experiments are designed primarily to discern the roles of the ovary, adrenal, and adipose tissue in the generation of increased concentrations of androgens (Δ4 and DHEAS) in the obese OSS gilt model. The addition of a lean control pig breed will help determine if the OSS gilt has a genetic predisposition to the increased production of androgens by one or all of the above-mentioned tissues. Animals will be 6 months old at the start of the study. Pigs will be allowed to undergo 2-3 cycles prior to initiation of the “diet induction” period of the study (4 months). Pigs will be assessed for baseline parameters over 1-2 estrous cycles prior to the following experiments being conducted.
1) Do obese OSS gilts have elevated serum 17OHP and Δ4 in response to hCG ovarian stimulation?

The goal of this experiment is to assess ovarian steroidogenic response to hCG administration in our animals. After 5-6 estrous cycles, pigs will be ultrasounded daily starting eight days prior to the next estimated estrus. Three days before estrus, when CL regression and small follicles (<3.5 mm) are present on ovarian ultrasound (US), animals will be fasted 12 hours, placed in a low stress restraint sling, anesthetized with isoflurane via face mask, and an IV catheter will be passed into the external jugular vein. We will allow 24-48 hours between catheter placement and stimulation procedures. Prior to stimulation testing, a morning baseline blood sample will be collected after which 7.5 µg/kg dexamethasone will be administered IM to suppress coincident adrenal secretion of 17OHP and Δ4. Six hours later a blood sample will be collected and 2000 IU of hCG (Pregnyl®, Schering-Plough) will be administered IV [344]. Blood will be sampled at the following intervals post-hCG administration: 12, 24, 36, 48, and 72 hours [118]. Dexamethasone will be administered every 12 hours throughout the stimulation. To track ovarian changes, pigs will be trans-rectally ultrasounded every 12 hours for 4 days post-hCG administration. Serum collected during hCG stimulation will be assessed for 17OHP, DHEA, DHEAS, Δ4, cortisol, E2, P4.

2) Do obese OSS gilts have elevated serum DHEA post-ACTH stimulation?

The goal of this experiment is to assess adrenal steroidogenic response to ACTH stimulation. We will conduct US and place IV catheters as outlined above. Based on US, when there are medium size follicles and no CL present on the ovary, we will begin ACTH stimulation. A morning baseline blood sample will be collected from the jugular catheter after which 5 µg/kg dexamethasone will be administered IM to uniformly provide
low baseline secretion of adrenocortical steroids, which will enhance ascertainment of the response to exogenous ACTH. Six hours after dexamethasone administration, a blood sample will be collected and 1 µg/kg of ACTH (human ACTH(1-24); Neosystem) dissolved in 5ml sterile 0.9% saline will be injected via the jugular catheter [345]. Blood will be sampled at the following intervals post-ACTH administration: 15, 30, 60, 90, 120, 180 minutes. Serum collected pre and post ACTH stimulation will be assessed for 17OHP, Δ4, cortisol, and DHEA.

3) *Do obese OSS gilt theca cells demonstrate underlying elevations of CYP17 and HSD3β1 in vitro in response to LH and/or insulin?*

The goal of this experiment is to assess theca cell functional response to LH ± insulin in vitro. Such results will build upon data generated from theca cell culture conducted as a part of my Ph.D. research. Follicular phase ovaries with medium size follicles (to prevent luteinization in culture) will be collected from the live pigs in our study. Aliquots of 500,000 primary theca cells per animal will be frozen at -80 °C for qRT-PCR analysis. Remaining theca cells will be plated at a density of 10^5 cells/well [346] in DMEM/F12 with 10% charcoal stripped FBS (CSFBS) and penicillin-streptomycin-actinomycin (PSA) in 12-well plates, allowed to reach 60% confluence, and then treated with either control (no treatment), LH (10 ng/ml), insulin (100ng/ml), LH (10 ng/ml) + insulin (100 ng/ml) for 48 hours [346, 347]. Cell culture medium will be assessed for 17OHP and Δ4. Cultured and freshly isolated theca cells will be analyzed for gene expression of HSD3β1 and CYP17. Follicular fluid will be assessed for TT, Δ4, and 17OHP.
4) Do adrenal explants of obese OSS gilts demonstrate underlying elevations of CYP17, HSD3B1 and SULT2A1 in vitro in response to ACTH?

The goal of this experiment is to assess baseline steroid production and steroidogenesis in adrenocortical explants in vitro in response to ACTH. One adrenal harvested at euthanasia from each pig will be finely minced immediately and kept on ice in serum free DMEM/F12 media until further prompt processing [348, 349]. The other adrenal gland collected at euthanasia will be bisected with half placed into 10% neutral buffered formalin for IHC and half frozen for qRT-PCR. Cultures of explanted adrenal minces supplemented with 10 µM Pr (Steraloids), will be cultured for 24 hours then will be treated for 24 hours with: control (no treatment); 10⁻⁶ M ACTH; 10⁻⁷ M ACTH; and 10⁻⁹ M ACTH [350-352]. Cell culture media will be assessed for 17OHP, cortisol, DHEA, and DHEAS [349, 352]. Adrenal cortex gene regulation will be assessed from primary, non-cultured adrenal tissue frozen at euthanasia in tissue molds containing Tissue Tek® O.C.T. (Sakura Fintek Inc, Torrence, CA) and then dissected into compartments by laser capture microscopy. We will assess gene regulation (qRT-PCR) and protein expression (IHC) for the following genes: CYP17, HSD3β1, and SULT2A1.

5) Does the adipose tissue of obese OSS gilts have increased gene expression of HSD3β1, CYP17 and HSD17β4?

The goal of this experiment is to assess steroidogenic enzyme gene expression in Sub-Q and abdominal adipose tissue. Adipose tissue harvested from pigs at euthanasia will be divided three ways: frozen in RNA-later (Quiagen Inc), snap-frozen, and placed in 10% neutral buffered formalin. Adipose tissue saved in formalin will be utilized for IHC for HSD3β1, CYP17 and HSD17β4 protein expression. Snap frozen adipose tissue will be used for western blots for HSD3β1, CYP17 and HSD17β4 protein
expression. Tissue saved in RNA-later will be utilized for qRT-PCR to assess the gene expression of HSD3β1, CYP17 and HSD17β4.

Figure 23. Revised model illustrating known pathologies in the obese OSS sow (S) and gilt (G) models of the obese phenotype of PCOS with concomitant MetS. Solid, thick arrows indicate hypothesized downstream effects of the strengths of the model, obesity and insulin resistance, on reproductive and endocrine physiology. Dashed arrows
indicate unknown links in the pathophysiology of the whole animal model, some of which will be explored as future research aims.
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