

## Original Research

# Effects of Diet-Induced Obesity on Metabolic Parameters and Reproductive Function in Female Ossabaw Minipigs

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This study characterizes the effect of an excess-calorie, high-fat, high-cholesterol, high-fructose diet on metabolic parameters and reproductive function in female Ossabaw minipigs. Cycling sows were fed a hypercaloric, high-fat, high-cholesterol, and high-fructose diet (obese,  $n = 4$ ) or a control diet (control,  $n = 5$ ) for 13 mo. During the final 4 mo, ovarian ultrasonography was done, blood was collected, and weights and measures were taken. Pigs then underwent ovarian stimulation. Cycle length and androstenedione, total testosterone, progesterone, estradiol, follicle-stimulating hormone, luteinizing hormone, insulin, fructosamine, lipid, and glucose levels were measured. In addition, adipose tissue aromatase gene expression was assessed. As compared with control pigs, obese pigs were hyperglycemic and hyperinsulinemic; had elevated total cholesterol, triglyceride, and leptin levels, and demonstrated abdominal adiposity. Visceral adipose tissue of obese pigs, as compared with control pigs, showed increased aromatase gene expression. Obese pigs had longer estrous cycles, higher serum androstenedione, and higher luteal phase serum luteinizing hormone, compared with control pigs. During the luteal phase, obese pigs had more medium, ovulatory, and cystic ovarian follicles, whereas control pigs had more small ovarian follicles. When fed an excess-calorie, high-fat, high-cholesterol, high-fructose diet, female Ossabaw minipigs develop obesity, metabolic syndrome, and abnormal reproductive function. This animal model may be applicable to studies of the effects of obesity on fertility in women.

**Abbreviations:** CV, coefficient of variation; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; MetS, metabolic syndrome; qPCR, real-time quantitative PCR.

Adiposity is related to fertility in women<sup>14</sup> and female animals.<sup>21</sup> Both malnourishment and overnutrition<sup>5,25</sup> impair female fertility. Obesity causes perturbations in the hypothalamic–pituitary–ovarian axis via feedback from leptin and insulin,<sup>29</sup> which may contribute to the decreased mean serum luteinizing hormone (LH) concentration and amplitude found in obese subjects.<sup>18</sup> Furthermore, human epidemiologic studies have demonstrated a relationship between obesity and ovulatory dysfunction,<sup>8</sup> miscarriage,<sup>6</sup> and gestational complications.<sup>19</sup> Although obesity is known to affect fertility outcomes in women and female animals, the underlying mechanisms responsible for obesity-induced infertility remain unknown. For this reason, a suitable animal model is needed in which to study the molecular basis of the relationship between obesity and female reproductive function.

Multiple animal models have been used to study obesity-associated reproductive dysfunction.<sup>38</sup> Many of these models involve genetically modified strains of rodents fed high-fat

diets and, therefore, may not mimic the disease pathogenesis in humans. Although nonhuman primate models of obesity closely model the disease manifestation in humans,<sup>40</sup> the cost of primates and their long lag time to reproductive maturity render them inaccessible to large-scale research. Pigs are a valuable tool for the study of human disease due to their anatomy, physiologic, and biochemical similarities to humans.<sup>41</sup> There are considerable similarities between cycle length in humans and pigs,<sup>15</sup> and pigs have the same lipoprotein profile as do humans.<sup>12</sup>

Our goal was to validate Ossabaw minipigs as an animal model for the effects of obesity on metabolic parameters and reproductive function in women. Ossabaw minipigs have a mutation in *PRKAG3* that causes increased intramuscular fat, resulting in a thrifty phenotype.<sup>11</sup> When fed an excess-calorie, high-fat, high-cholesterol, high-fructose diet, Ossabaw minipigs naturally develop features that mimic metabolic syndrome (MetS) in humans, including visceral obesity, glucose intolerance, and dyslipidemia.<sup>11,28</sup>

Our study objectives were to characterize the effects of a hypercaloric, high-fat diet on metabolic parameters and reproductive function in female Ossabaw minipigs and to perform ovarian stimulation in female Ossabaw minipigs to compare their in vivo ovarian response to that reported for obese women.

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## Materials and Methods

All experimental animal procedures were performed in compliance with University of Illinois Urbana-Champaign IACUC regulation, and followed the guidelines outlined in the *Guide for the Care and Use of Laboratory Animals*.<sup>17</sup>

**Ossabaw husbandry and diet treatment.** Nine multiparous Ossabaw female pigs (*Sus scrofa*; age, 6 to 8 y) were acquired from Indiana University Purdue University Indianapolis. On the basis of physical exams, animals were deemed healthy and allocated to diet treatment groups. Pigs received their respective diet treatments for 9 mo prior to the initiation of this 4-mo study and throughout the study period. Five pigs were fed 2200 Kcal daily of the control diet (Rund diet, UIUC, Urbana, IL), which was composed of corn (57.5%) and soy (40%) and supplemented with vitamins and minerals, and the remaining 4 pigs were fed 6770 Kcal daily of a high-fat, high-cholesterol, high-fructose diet formulated to induce MetS (obese diet).<sup>28</sup> The obese diet was composed of a base pelleted pig feed (LabDiet Swine Diet 20% Fructose 5KA6, Purina Mills, St Louis, MO) supplemented (percentage by weight) with soybean oil (17.1%), cholesterol (2%), cholate (0.7%), corn oil (2.3%), and granular fructose (8.9%).<sup>27</sup> During the study, pigs were housed in isolated 100 ft<sup>2</sup> group pens, with 2 to 4 sows per pen. Pigs were on a 12:12-h light:dark cycle and had ad libitum access to water. One of the obese pigs died of an unexpected cardiac incident during the night before the ovarian stimulation test. Due to the timing of her death, her tissues could not be preserved for histology. Therefore, 3 obese pigs comprise the cohort from which the obese pig data was generated in the ovarian stimulation test and the histologic assessment of the liver.

**Sample collection.** Jugular blood collection and ovarian ultrasonography were performed twice weekly. Transrectal ovarian ultrasonography with a 7.5-mHz linear transducer (model UST 5541-7.5, 38-mm Linear Reproductive Transducer and Prosound SSD-3500SV, Aloka, Wallingford, CT) was conducted in a Panepinto low-stress sling.<sup>31</sup> Pigs were weighed weekly. Crown-to-rump length (between the eyes to the tail head), heart girth (in the axilla), height (at the point of the shoulder), and abdominal girth (the widest point of the abdomen) were measured weekly.

**Assessment of glucose homeostasis.** The Precision Xtra glucometer (Abbott Laboratories, Bedford, MA) was validated for use in Ossabaw pigs by the University of Illinois-Urbana-Champaign Veterinary Diagnostic Lab. Fasting plasma blood glucose (mg/dL) was monitored twice weekly. Serum fructosamine was assessed every 4 wk (University of Illinois-Urbana-Champaign Veterinary Diagnostic Lab). Serum leptin and insulin concentrations were measured biweekly by using a radioimmunoassay (Linco-Millipore, Billerica, MA). Insulin resistance according to the modified homeostatic model assessment for insulin resistance was calculated.<sup>11</sup>

**Plasma lipids.** Plasma lipid analysis was conducted as previously described.<sup>11</sup> Monthly total plasma cholesterol (mg/dL) and triglyceride (mg/dL) concentrations were assayed by using enzymatic kits (Cholesterol EZ, Triglyceride EZ, Sigma-Aldrich, St Louis, MO). HDL (mg/dL) was assayed (Cholesterol EZ), and LDL (mg/dL) was determined by subtracting the HDL concentration and 20% of the triglycerides content from the total cholesterol level.<sup>9</sup>

**Histologic preparation of liver.** Liver tissue was fixed in 10% buffered formalin, embedded in paraffin wax blocks, and serially

sectioned (5 µm). Slides were stained with Mayer and eosin Y solution (Sigma-Aldrich).

**Ovarian stimulation protocol.** The ovarian stimulation protocol mimicked a human long gonadotropin-releasing hormone agonist protocol.<sup>26</sup> Starting in midluteal phase, pigs received a dose of the gonadotropin-releasing hormone agonist triptorelin acetate (100 µg SC; Trp, ProSpec-Tany TechnoGene, Rehovot, Israel) twice daily until no ovulatory size follicles were observed (15 to 19 d).<sup>26</sup> At that time, 2 doses (10 to 20 mg SC each) of dinoprost tromethamine (PGF<sub>2</sub>α; Lutalyse, Pharmacia and Upjohn, Pfizer, New York, NY) were administered 12 h apart to ensure luteolysis. Subsequently, sows received a total daily dose of 100 mg follicle-stimulating hormone (FSH; Folltropin V, Bioniche Animal Health Canada, Belleville, Canada) and 1.67 mg LH (Lutropin V, Bioniche Animal Health Canada) divided equally and given subcutaneously every 8 h for a period of 2 to 7 d, until follicles of ovulatory size (larger than 6.5 mm) were observed.<sup>26</sup> Then, a single dose of 2500 IU of human chorionic gonadotropin (hCG; Chorulon, Intervet, Millsboro, DE) was administered intramuscularly.

**Serum hormone radioimmunoassay.** Coat-A-Count progesterone, androstenedione, estradiol, and total testosterone kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA) were validated for use with pig serum. Standards were made in charcoal-stripped pig serum for progesterone and androstenedione and in 1% PBS-gel for estradiol and total testosterone by adding a known amount of progesterone (32 to 1000 pg), androstenedione (20 to 640 pg), estradiol (2.5 to 150 pg), or total testosterone (10 to 1600 pg; Steraloids, Newport, RI) and diluting further. To validate each radioimmunoassay described, parallelism and recovery of unlabeled ligand were conducted. Serum samples for estradiol and total testosterone were extracted first with diethyl ether followed by hexane-methanol<sup>1</sup> before radioimmunoassay. The progesterone assay sensitivity was 250 pg per tube, with an interassay coefficient of variation (CV) of 4.0% ( $n = 7$ ) and an intraassay CV of 2.4% ( $n = 7$ ). The androstenedione assay sensitivity was 60 pg per tube, with an interassay CV of 3.0% ( $n = 5$ ) and an intraassay CV of 6.9% ( $n = 5$ ). The estradiol assay sensitivity was 2.5 pg per tube, with an interassay CV of 4.9% ( $n = 6$ ) and an intraassay CV of 1.2% ( $n = 6$ ). The total testosterone assay sensitivity was 400 pg per tube, with an interassay CV of 3.2% ( $n = 3$ ) and an intraassay CV of 1.9% ( $n = 3$ ).

Serum LH and FSH were analyzed according to previously described double-antibody radioimmunoassays validated for porcine LH<sup>24</sup> and FSH,<sup>33</sup> with modifications. Standards were made with purified porcine LH (0.4 to 6.25 ng) and FSH ((0.08 to 5.0 ng; National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA). Aliquots of 5 µg LH or FSH in 5 µL distilled water were iodinated with 2.5 µg of chloramine-T (Chloramine-T hydrate 98%, Sigma-Aldrich) and 0.25 mCi of <sup>125</sup>I (Perkin Elmer, Waltham, MA). The contents of the reaction vial were layered on a resin-packed column to separate free <sup>125</sup>I from iodinated LH (<sup>125</sup>I-pLH) and FSH (<sup>125</sup>I-pFSH). The primary LH antibody was used at a final dilution of 1:4,800,000. The primary FSH antibody was used at a final dilution of 1:400,000. The LH assay was run at an average percentage binding of 29% ( $n = 4$ ), with an assay sensitivity of 450 pg per tube, an interassay CV of 4.9% ( $n = 4$ ), and an intraassay CV of 3.6% ( $n = 4$ ). The FSH assay was run at an average binding of 34% ( $n = 3$ ), with an assay sensitivity of 627 pg per tube, an interassay CV of 1.9% ( $n = 4$ ), and an intraassay CV

of 2.3% ( $n = 4$ ). Nondetectable samples were assigned the lower limit of detection.

**Follicular description.** Follicles were measured and counted according to a previously established method.<sup>23</sup> Follicle size categories were: small antral, less than 3.5 mm; medium antral, 3.5 to 6.5 mm; ovulatory size, 6.5 to 12.5 mm; and cystic, larger than 12.5 mm.<sup>23</sup>

**Real-time quantitative PCR (qPCR).** Transcript abundance of aromatase was analyzed by real-time qPCR. Total RNA was isolated by using the RNeasy Mini kit (Qiagen, Valencia, CA) followed by on-column DNase treatment (RNase-Free DNase Kit, Qiagen). RNA was quantified (Quant-iT RiboGreen, Invitrogen, Carlsbad, CA). Single-strand cDNA was synthesized (MMLV reverse transcriptase, Invitrogen). Primers were designed by using Primer3.<sup>36</sup> Melting curve analysis and gel electrophoresis were used to confirm primer specificity. PCR products were cloned into pCR 2.1 TOPO vectors and transformed into *E. coli* (One Shot TOP10, Invitrogen). Plasmids were digested with EcoRI, sequenced to confirm amplicon identity, quantified (Quant-iT PicoGreen dsDNA Assay, Invitrogen), and serial dilutions prepared for the standard curve. qPCR was run on a 10-fold diluted sample of cDNA in duplicate.

**Statistical analysis.** Reproductive data were divided into basal and ovarian stimulation datasets. The first complete estrous cycle for each sow was divided into follicular (approximately days 1 through 7) and luteal (approximately days 8 through 21) phases. Follicular phase was determined by a decrease in serum progesterone to below 2 ng/mL and lack of corpora lutea on ultrasonography. To standardize across pigs, the ovarian stimulation period was subdivided into thirds (first, second, and last third) by percentage of stimulation completion for each pig (initial, first day of stimulation; last third includes the day of hCG administration), and number of days posthCG. Data normality was assessed by using a Levene test of homogeneity and the Shapiro–Wilke test, and nonnormal data were  $\log_{10}$ -transformed for continuous variables and square-root-transformed for noncontinuous variables. Analysis was done by using the Proc Mixed protocol for repeated measures (version 9.2, SAS Institute, Cary, NC). Proc Mixed results were back-transformed and are presented as least-square mean  $\pm$  SEM. qPCR data were analyzed by nonparametric ANOVA and the Wilcoxon rank-sum test. In all statistical tests, a  $P$  value of less than 0.05 was the criterion for statistical significance.

## Results

**Metabolic parameters.** Weight (control, 108.5  $\pm$  3.8 kg; obese, 162.8  $\pm$  4.9 kg), thoracic girth (control, 123.7  $\pm$  2.0 cm; obese, 151.2  $\pm$  2.3 cm), and abdominal girth (control, 127.5  $\pm$  2.5 cm; obese, 154.9  $\pm$  2.8 cm) were all significantly ( $P < 0.05$ ) greater in obese compared with control pigs (Table 1). Height did not differ between treatment groups (control, 70.1  $\pm$  1.8 cm; obese, 73.9  $\pm$  2.3 cm;  $P > 0.05$ ).

Obese pigs were hyperglycemic compared with control pigs (control, 59.1  $\pm$  2.8 mg/dL; obese, 78.7  $\pm$  5.1 mg/dL; Table 1). Obese pigs were hyperinsulinemic (control, 12.0  $\pm$  2.3  $\mu$ U/mL; obese, 23.5  $\pm$  2.6  $\mu$ U/mL; Table 1) and had greater insulin resistance scores than did control pigs (control, 942.5  $\pm$  322.9; obese, 1821.4  $\pm$  365.6; Table 1). Obese pigs were not diabetic, as demonstrated by fructosamine levels, which were similar to those of control pigs (control, 282.73  $\pm$  6.01  $\mu$ mol/L; obese, 288.0  $\pm$  6.8  $\mu$ mol/L; Table 1). Obese pigs were hyperleptinemic compared

**Table 1.** Metabolic parameters in control ( $n = 5$ ) and obese ( $n = 4$ ) female Ossabaw pigs

	Control pigs	Obese pigs	$P$
Weight (kg)	108.5 $\pm$ 3.8	162.8 $\pm$ 4.9	0.0001
Thoracic girth (cm)	123.7 $\pm$ 2.0	151.2 $\pm$ 2.3	<0.0001
Abdominal girth (cm)	127.5 $\pm$ 2.5	154.9 $\pm$ 2.8	0.002
Height (cm)	70.1 $\pm$ 1.8	73.9 $\pm$ 2.3	0.8
Glucose (mg/dL)	59.1 $\pm$ 2.8	78.7 $\pm$ 5.1	0.001
Insulin ( $\mu$ U/mL)	12.0 $\pm$ 2.3	23.5 $\pm$ 2.6	0.003
Insulin resistance score	942.5 $\pm$ 322.9	1821.4 $\pm$ 365.6	0.024
Fructosamine ( $\mu$ mol/L)	282.7 $\pm$ 6.0	288.0 $\pm$ 6.8	0.570
Leptin (ng/mL)	6.6 $\pm$ 1.1	19.8 $\pm$ 1.1	<0.0001
Total cholesterol (mg/dL)	102.7 $\pm$ 16.7	215.4 $\pm$ 19.5	<0.0001
HDL (mg/dL)	32.6 $\pm$ 2.3	55.7 $\pm$ 6.1	0.021
Triglycerides (mg/dL)	41.6 $\pm$ 6.1	87.6 $\pm$ 14.2	0.048
LDL (mg/dL)	61.7 $\pm$ 7.8	87.3 $\pm$ 34.2	0.389
LDL:HDL ratio	3.6 $\pm$ 0.4	4.8 $\pm$ 1.2	0.429

with control pigs (control, 6.6  $\pm$  1.1 ng/mL; obese, 19.8  $\pm$  1.1 ng/mL; Table 1).

Obese pigs had elevated total serum cholesterol compared with control pigs (control, 102.7  $\pm$  16.7 mg/dL; obese, 215.4  $\pm$  19.5 mg/dL; Table 1). Although HDL (control, 32.6  $\pm$  2.3 mg/dL; obese, 55.7  $\pm$  6.1 mg/dL) and triglycerides (control, 41.6  $\pm$  6.1 mg/dL; obese, 87.6  $\pm$  14.2 mg/dL; Table 1) were higher in obese compared with control pigs, there was no difference in LDL concentrations (control, 61.7  $\pm$  7.8 mg/dL; obese, 87.3  $\pm$  34.2 mg/dL) or the LDL:HDL ratio (control, 3.6  $\pm$  0.4; obese, 4.8  $\pm$  1.2; Table 1) between treatment groups.

Fixed, stained liver sections from 3 obese and 5 control pigs were examined for steatosis. Multiple areas of steatosis were observed in sections from one obese pig, whereas steatosis was not noted in sections from any of the control pigs.

**Reproductive parameters.** Obese pigs had longer estrous cycles than did control pigs (obese, 32.2  $\pm$  1.3 d; control, 25.2  $\pm$  1.0 d;  $P = 0.002$ ). Obese serum androstenedione was higher than control serum androstenedione in both the follicular and luteal phases, but serum total testosterone was not different between the 2 treatment groups (Table 2).

Whereas there were no significant differences in the number of ovarian follicles between the 2 treatment groups during the follicular phase, obese pigs had more medium antral, ovulatory, and cystic follicles than did control pigs during the luteal phase (Figure 1). In contrast, control pigs had more small antral follicles than did obese pigs during the luteal phase (Figure 1). During the luteal phase, obese pigs had lower serum progesterone concentrations but marginally higher serum LH concentrations than did control pigs (Table 2). All other hormone levels were similar between the treatment groups during basal sampling (Table 2). Aromatase gene expression in visceral adipose tissue was greater ( $P = 0.005$ ) in obese pigs compared with control pigs (Figure 2).

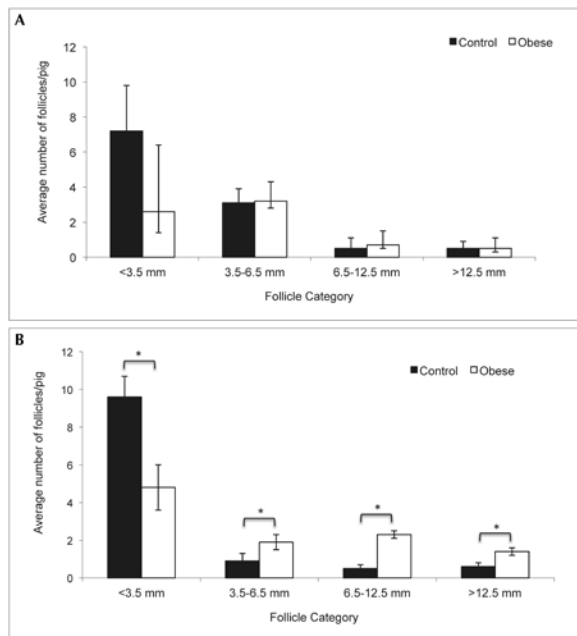
Overall, obese and control pigs responded similarly to ovarian stimulation (Table 3). During the stimulation period, 2 of 3 obese pigs and 1 of 5 control pigs developed symptoms of ovarian hyperstimulation syndrome. In response to ovarian stimulation, there were no differences between the 2 treatment groups in serum hormone concentrations or numbers of large follicles. At 2

**Table 2.** Basal reproductive hormone concentrations in control ( $n = 5$ ) and obese ( $n = 4$ ) Ossabaw pigs

	Control pigs		Obese pigs	
	Follicular	Luteal	Follicular	Luteal
Androstenedione (ng/mL)	$0.6 \pm 0.1^a$	$0.5 \pm 0.1^b$	$1.2 \pm 0.1^a$	$0.9 \pm 0.1^b$
Total Testosterone (ng/mL)	$0.3 \pm 0.1$	$0.3 \pm 0.1$	$0.3 \pm 0.1$	$0.2 \pm 0.1$
Estradiol (pg/mL)	$51.8 \pm 3.6$	$30.2 \pm 1.9$	$50.8 \pm 5.2$	$25.5 \pm 2.3$
Progesterone (ng/mL)	$1.2 \pm 6.1$	$30.8 \pm 3.9^b$	$1.7 \pm 9.0$	$16.8 \pm 4.5^b$
FSH (ng/mL)	$2.6 \pm 0.2$	$3.4 \pm 0.1$	$3.1 \pm 0.3$	$3.5 \pm 0.1$
LH (ng/mL)	$1.0 \pm 0.1$	$0.9 \pm 0.03^b$	$1.0 \pm 0.1$	$1.0 \pm 0.03^b$

<sup>a</sup>For a given hormone, significant ( $P < 0.05$ ) difference between the values for control and obese pigs during the follicular phase of the estrous cycle.

<sup>b</sup>For a given hormone, significant ( $P < 0.05$ ) difference between the values for control and obese pigs during the luteal phase of the estrous cycle.

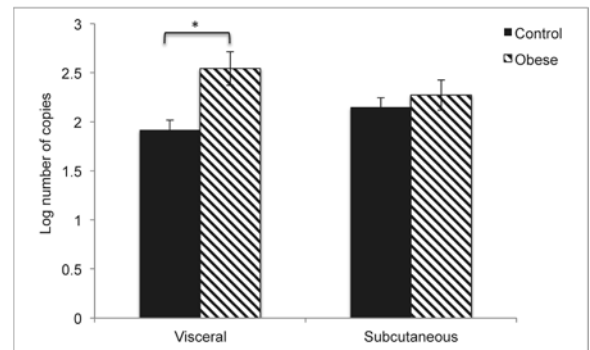


**Figure 1.** Number of follicles during the follicular and luteal phases in obese and control pigs. (A) Numbers of follicular-phase follicles. (B) Numbers of luteal-phase follicles. Results are expressed as least-square mean  $\pm$  SEM (control,  $n = 5$ ; obese,  $n = 4$ ). Within a given follicle category, \* indicates significant ( $P \leq 0.02$ ) difference from control value.

d post-hCG, obese pigs had significantly ( $P < 0.05$ ) more cystic follicles than did control pigs (Table 3).

## Discussion

The major findings of this study are: 1) mature female Ossabaw minipigs fed a hypercaloric, high-fat, high-cholesterol, high-fructose diet developed MetS (hyperinsulinemia, elevated fasting glucose, dyslipidemia) and hepatic steatosis; were hyperandrogenemic; had longer estrous cycles, and formed more persistent ovarian follicles than did age-matched lean control pigs; and 2) obese female Ossabaw minipigs developed ovarian hyperstimulation syndrome in response to ovarian stimulation but had a relatively similar hormonal and follicular response to that of control pigs. The responses of obese and lean in vitro fertilization patients to ovarian stimulation are similar to our findings in Ossabaw minipigs.<sup>37</sup> To our knowledge, this report is the first to describe the effects of obesity with concomitant MetS on female reproductive function in a large animal model. Given that



**Figure 2.** Aromatase gene expression in visceral and subcutaneous adipose tissue. Results are expressed as least-square mean  $\pm$  SEM (control,  $n = 5$ ; obese,  $n = 4$ ). \*,  $P = 0.005$ .

23% of American women have MetS<sup>13</sup> and that 64% are either overweight or obese,<sup>30</sup> the obese Ossabaw minipig model may be useful to characterize the mechanisms underlying reproductive dysfunction due to MetS and obesity and may help develop potential treatments for obesity, MetS, and the effects of these diseases on fertility and endocrine function in women.

Obese female Ossabaw minipigs 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 than did control sows. Given that the heights of obese and control pigs were similar, the increased weight of obese pigs can be attributed to android fat deposition. Previous studies have demonstrated that increased abdominal girth in Ossabaw minipigs correlates ( $R = 0.91$ ) significantly with visceral or android fat distribution.<sup>11</sup> In humans, android fat distribution is highly associated with type 2 diabetes, an increased risk for cardiovascular disease,<sup>20</sup> and increased androstenedione production,<sup>32</sup> which we observed in our obese pigs. Obese pigs had leptin concentrations that were 3-fold higher than those of control pigs. In humans, studies have demonstrated that increased serum insulin concentrations result in increased serum leptin.<sup>28</sup> Perhaps the hyperinsulinemia in the obese pigs also contributes to their severe hyperleptinemia. Although obese pigs did not manifest dyslipidemia, they did have elevated triglyceride and total cholesterol concentrations. Furthermore, atherogenic lipid profiles in obese patients are directly correlated with hyperandrogenemia due to accompanying hyperinsulinemia.<sup>7</sup> Hyperinsulinemia causes increased VLDL production from the liver, resulting in increased triglyceride secretion<sup>3</sup> as well as stimulation of androstenedione production by ovarian theca interna.<sup>2</sup>

**Table 3.** Response of follicular hormones and ovarian follicle growth to ovarian stimulation in control ( $n = 5$ ) and obese ( $n = 4$ ) Ossabaw pigs

		Stage of superovulation protocol					
		Initial	1st third	2nd third	Last third	1 d after hCG	2 d after hCG
Testosterone (ng/mL)	Control	0.6 ± 0.3	0.6 ± 0.3	1.0 ± 0.3	1.3 ± 0.3	1.2 ± 0.3	1.5 ± 0.3
	Obese	0.6 ± 0.4	0.7 ± 0.4	1.6 ± 0.4	1.7 ± 0.4	1.5 ± 0.4	1.5 ± 0.3
Androstenedione (ng/mL)	Control	0.8 ± 1.8	1.1 ± 1.5	2.4 ± 1.5	3.2 ± 1.5	2.7 ± 1.5	2.5 ± 1.5
	Obese	0.8 ± 2.0	1.3 ± 2.0	4.8 ± 2.0	8.5 ± 2.0	6.0 ± 2.0	3.9 ± 2.0
Estradiol (pg/mL)	Control	10.6 ± 68.1	24.1 ± 56.3	68.6 ± 56.3	69.3 ± 56.3	67.0 ± 56.3	59.3 ± 60.0
	Obese	13.3 ± 112.2	29.6 ± 72.7	151.4 ± 72.7	241.2 ± 72.7	66.6 ± 72.7	47.3 ± 72.7
Progesterone (ng/mL)	Control	1.6 ± 0.5	1.4 ± 0.4	2.0 ± 0.6	4.1 ± 2.3	4.5 ± 3.0	10.8 ± 7.9
	Obese	1.7 ± 0.5	1.3 ± 0.5	2.1 ± 0.7	4.1 ± 3.0	5.2 ± 3.8	21.6 ± 10.2
Ovulatory size follicles	Control	1.5 ± 0.7	0.7 ± 1.0	7.1 ± 3.0	17.7 ± 11.6	21.1 ± 10.2	17.0 ± 7.4
	Obese	0.5 ± 0.8	1.4 ± 1.2	15.4 ± 3.4	34.6 ± 13.4	39.8 ± 11.8	12.2 ± 8.5
Postovulatory size follicles	Control	0.0 ± 1.5	0.0 ± 1.5	0.0 ± 1.5	1.6 ± 1.5	2.5 ± 1.5	3.4 ± 1.5 <sup>a</sup>
	Obese	0.0 ± 1.7	0.0 ± 1.7	0.0 ± 1.7	0.0 ± 1.7	5.1 ± 1.7	8.8 ± 1.7 <sup>b</sup>

<sup>a,b</sup>Values are significantly ( $P = 0.04$ ) different.

Furthermore, given that 1 of the 3 obese pigs had hepatic steatosis, there is an indication that obese pigs are at risk for increased transport of fatty acids from adipose tissue to the liver.<sup>34</sup> Our findings of abdominal obesity, fasting hyperglycemia, and elevated triglycerides in obese female Ossabaw minipigs corroborate our conclusion that these animals manifest MetS with evidence of hyperleptinemia and insulin resistance.

Obesity in women is associated with variable oligomenorrhea with a lengthened follicular phase, decreased mean serum LH, decreased LH pulse amplitude,<sup>39</sup> decreased luteal progesterone,<sup>18</sup> and variable elevations in androgens.<sup>35</sup> Our obese female Ossabaw minipigs had elevated serum androstenedione but not total testosterone. In obese women, although the ovary, adrenal gland and adipose tissue may all contribute to androstenedione production, the ovary is the primary source of testosterone via 17 $\beta$ -hydroxysteroid dehydrogenase type 5 steroidogenic activity. There is very little information known about the existence of 17 $\beta$ -hydroxysteroid dehydrogenase type 5 in the pig ovary. A single study has demonstrated its presence in some sows during early estrus.<sup>22</sup> In our study, testosterone did increase marginally during the ovarian stimulation protocol, indicating the potential presence of 17 $\beta$ -hydroxysteroid dehydrogenase type 5 in the ovary of Ossabaw pigs. Serum androstenedione was elevated nonsignificantly in obese pigs during ovarian stimulation. This functional increase in ovarian androstenedione production suggests an active role of the ovary in the production of androstenedione in obese pigs. However, because adrenal androgens like dehydroepiandrosterone sulfate and androstenedione are known to increase in parallel with obesity,<sup>16</sup> it is possible that the basal elevation of androstenedione in obese Ossabaw minipigs is due to both adrenal and ovarian dysfunction. Furthermore, adipose tissue can convert androstenedione to testosterone and vice versa via 17 $\beta$ -hydroxysteroid dehydrogenase type 5,<sup>4</sup> with omental adipose favoring androstenedione production and subcutaneous adipose favoring testosterone production.<sup>32</sup>

Obese female Ossabaw minipigs demonstrated a protracted average estrous cycle length similar to the oligomenorrhea seen in obese women. Obese pigs had lower progesterone concentrations during the luteal phase, indicating possible luteal insufficiency similar to that found in obese women.<sup>18</sup> In future studies, numerical determination of corpora lutea would indicate whether obese pigs are oligoovulatory and thus corroborate potential luteal insufficiency. Obese pigs had a marginally significant increase in serum LH during the luteal phase compared with that in control pigs, which does not mimic LH pathology in obese women. However, given that LH was measured only twice weekly and that LH tonic secretion is episodic, this result should be interpreted judiciously. Moreover, the increased numbers of cystic follicles in obese pigs may indicate the lack of an effective LH surge or an inappropriate response of the follicle to the LH surge in obese animals. The presence of large numbers of medium antral, ovulatory, and cystic follicles during the luteal phase in obese pigs is suggestive of a hyper-responsiveness of growing follicles to FSH during this phase, coupled with a lack of appropriate responsiveness to LH during the follicular phase. Assessment of anti-Müllerian hormone in control and obese pigs would be prudent, given that anti-Müllerian hormone controls the responsiveness of antral follicles to FSH.<sup>10</sup>

In conclusion, when fed an excess-calorie, high-fat, high-cholesterol, high-fructose diet, female Ossabaw minipigs develop obesity, MetS, and ovarian and endocrine abnormalities. The inherent thrifty genotype of this animal model results in the development of diet-induced obesity and associated reproductive pathologies without a reliance on genetic manipulations that do not mimic the pathogenesis of obesity in humans. This pig model provides the opportunity to characterize the molecular mechanisms underlying obesity-induced metabolic and reproductive pathologies, which affect reproductive function and fertility in women.

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