Investigating impacts of altered central metabolism on ovarian function

Ahmad Abdulrahman Al-Shaibi

Iowa State University

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Investigating impacts of altered central metabolism on ovarian function

by

Ahmad A. Al-Shaibi

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in partial fulfillment of the requirements for the degree of

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Major Professor: Aileen F. Keating
Lance H. Baumgard
Jason W. Ross

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Ames, Iowa
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DEDICATION

"And of knowledge you were given not but a little." - Quran 17:85

"If I have seen further, it is by standing on the shoulders of Giants." Such were the words of Isaac Newton. This thesis is composed of the field notes taken during my clumsy attempt at the climb to be on those shoulders, and is an attempt at describing the view from where I stand. I dedicate this climb to my parents, my family, and my country, for I owe them everything. I also dedicate this thesis to the scientists whose footsteps I attempt to follow, so unworthy this thesis might be of them; the men and women who drove humanity's endless quest to elucidate our ignorance, and gave birth to the enterprise of science, one dedicated to the investigation of the laws of nature and enabling humanity to access the universe around us. Lastly, I dedicate this thesis as a stepping stone in my ongoing quest to reach a horizon I dreamt of as a boy many years ago, and continue to march towards.

Finally, please adhere to Alhazen's word as you read this thesis, for critique is the best that can happen to it: "The seeker after the truth is not one who studies the writings of the ancients and, following his natural disposition, puts his trust in them, but rather the one who suspects his faith in them and questions what he gathers from them and submits to argument and demonstration, not to the sayings of a human being whose nature is fraught with all kinds of imperfection and deficiency. Thus, the duty of the man who investigates the writings of scientists, if learning the truth is his goal, is to make himself an enemy of all that he reads, applying his mind to the core and margins of its content, and attack it from every side. He should also suspect himself as he performs his critical examination of it, so that he may avoid falling into either prejudice or leniency."
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ABBREVIATIONS

PGC: Primordial Germ Cells
PI3K: Phosphotidylinositol-3 Kinase
AKT: Protein Kinase B
GV: Germinal Vesicle
FSH: Follicle Stimulating Hormone
LH: Luteinizing Hormone
COC: Cumulus Oocyte Complex
StAR: Steroid Acute Regulatory Hormone
CYP: Cytochrome P-450
GnRH: Gonadotropin-Releasing Hormone
HS: Heat Stress
LPS: Lipopolysaccharide
ob: Obese
Db: Diabetic
LEPR: Leptin Receptor
INSR: Insulin Receptor
GVBD: Germinal Vesicle Breakdown
HIF: Hypoxia Inducible Factor
VEGF: Vascular Endothelial Growth Factor
IRS: Insulin Receptor Substrate
IGF: Insulin-Like Growth Factor
TLR: Toll-Like Receptor
PAMP: Pathogen-Associated Molecular Pattern
NF-κB: Nuclear Factor-Kappa B
TFN-α: Tumor Necrosis Factor Alpha
PGF2α: Prostaglandin F2 Alpha
Lpt: Leptin Pump
Sal: Saline Pump
WT: Wild Type
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Last, but not least, I would like to thank the staff at ISU, particularly those at the department of Animal Science, for their tireless work and for enabling everything to run as
smoothly as it does, and all the great people I've met during my stay in Ames since my admission to ISU as an undergrad.

While stumbles were aplenty, it is thanks to everyone that I'm here, that this is written, and that I'm still sane. Thank you.
Mammalian reproduction is highly dependent on the ovary. Ovarian health is crucial for the health of the female and the offspring. Observations of the timing of puberty and ovarian function reveal a major role for the metabolic status of the female on reproductive function. Many metabolic signals such as leptin, insulin, and LPS influence ovarian function as they participate in metabolic stress. All of these signals cross pathways at PI3K. Obesity, the accumulation of excessive fat, is an international health concern of wide prevalence. It causes an increase in circulating leptin, insulin, and LPS, and is associated with numerous reproductive disorders. Here, we investigated the effects of increasing systemic leptin during gestation on the offspring, and observed that gestational hyperleptinemia can alter the ovarian capacity to metabolize toxicants later in the offspring's life. Heat stress is a condition of increased core temperature, and it impedes the reproductive performance of production animals during summer, making it an increased threat to food security, climate change only worsening it. Heat stress causes increased circulating insulin and LPS. We heat stressed gilts cyclically after synchronizing their estrous cycles, and observed decreased phosphorylation of AKT and increased TLR4 abundance in their ovaries. This demonstrates that, during heat stress, ovarian TLR4 signaling is upregulated and that the ovary might be initiating a stress response as indicated by reduced AKT phosphorylation.
ORGANIZATION OF THE THESIS

This thesis is focused on ovarian function in the contexts of obesity and heat stress. Chapter 1 contains a general review of the relevant literature in which the major facets of ovarian function are introduced, as well as the pathways relevant to the stressors discussed herein. Chapter 2 contains the details of a project investigating the effects of leptin on ovarian function, where we observed the effects of systemic, maternal hyperleptinemia during gestation on the offspring, as well as the effects of exposing cultured ovaries to leptin ex vivo. Chapter 3 tells the details of our investigation into the effects of cyclical heat stress on the ovaries of synchronized, post-pubertal gilts. Chapter 4 is a general discussion of the reviewed literature and the findings presented herein, in addition to the author's thoughts on potential avenues of future research.
CHAPTER 1
A REVIEW OF THE LITERATURE

General background:

Reproduction is a cornerstone of evolution as it allows genes and traits to be inherited through the generations. It takes on a wide range of forms through the different domains of life, ranging from the simplicity and elegance of bacterial binary fission [1], all the way to the sophistication and intricacy of mammalian reproduction. Mammalian species are typically split into two sexes; male and female. Females, the subject of this thesis, specialize in the ovarian production of oocytes, the female gamete (sometimes called eggs), and their maintenance. On a cellular level, females contribute about 50% of the nuclear genome, all the cellular components, and all of the non-nuclear genomes including those in the mitochondria, while the male's contribution is limited to the other 50% of the nuclear genome [2–5]. This makes the health and quality of the oocyte of absolute importance, as the oocyte's metabolic machinery would constitute all machinery the offspring has as a single-cell embryo. Ovarian function is conducted through a finite reserve of functional units known as follicles. Follicles consist of up to three cell types; an oocyte, granulosa cells, and theca cells (Figure 1.1). The depletion of follicles discontinues ovarian function, leading to acyclicity and menopause [6,7].

Oogenesis:

As with all organs, the ovary is derived from specific cellular lineages during embryonic development [8,9]. As they develop, ovaries host the primordial germ cells (PGCs); a cell line which differentiates into oocytes [8,9]. Giving rise to oocytes is a process called oogenesis.
Basically, PGCs are directed by means of protein and small RNA signals which orchestrate the proliferation and differentiation of PGCs into prophase I of meiosis [8,9].

Oocytes arise from structures known as germ cell nests (also known as germline cysts). Germ cell nests are formed from a single cell which undergoes multiple rounds of mitosis with incomplete cytokinesis, resulting in an interconnected cluster of cells that divide in synchrony [10,11]. Cells within germ cell nests, called cystocytes, are connected through structures termed ring canals. In Drosophila, ring canals are threaded with microtubules that facilitate the transport of organelles and mRNAs [10,12]; processes that determine which of the cystocytes develop into an oocyte, while the rest become nurse cells that break down as the oocyte matures [12]. Cystocytes that are selected to become oocytes become arrested in prophase I of meiosis [9,10]. Germ cell nests break down after a period of time that varies between species [10]. For example, while nest breakdown begins right after birth in mice [13], it begins in the second trimester of gestation in humans [14]. As the nests break, oocytes separate into primordial follicles, which then develop through the process of folliculogenesis.

**Folliculogenesis:**

Development and growth of follicles, referred to as folliculogenesis, proceeds through four stages; primordial, primary, secondary, and tertiary. This classification was derived from a proposal by Pedersen and Peters [15]. After germ cell nest breakdown, oocytes become packaged into primordial follicles. Primordial follicles contain oocytes arrested in prophase I of meiosis [8–10]. Surrounding the oocyte at this stage is a layer of squamous granulosa cells, which are flat in morphology [8,16]. While considered metabolically inactive, primordial follicles are
responsive to circulating factors as shown by their activation in culture using serum-enriched media [17]. Primordial follicles are also capable of responding to metabolic signals like insulin [18] and insulin-like growth factor-1 (IGF-1) [19], which shows that, despite being metabolically dormant, primordial follicles are capable of responding to external stimuli.

Activation of the primordial follicles is one of two broad categories of folliculogenesis. The first is known as "initial recruitment," and refers to the process of initiating the growth of the follicle and transitioning it from the dormant, primordial stage. The second category is known as "cyclic recruitment," in which the signals associated with the ovarian/estrous cycle stimulate the development of follicles at the tertiary stage (see below) [20]. Initial recruitment initiates the growth of the oocyte and proliferation of the granulosa cells and transitions the oocyte into the primary stage [20]. Mechanistically speaking, initial recruitment has yet to be fully elucidated. It is thought that the phosphotidylinositol-3 kinase (PI3K) pathway plays a major role in this process, primarily through protein kinase B (PKB/AKT) [21]. Activation of PI3K promotes the translocation of forkhead transcription factor (FOXO3A) from the oocytic nucleus as signaled by its phosphorylation, in turn signaling the activation of the primordial follicle [22–24]. Other chemical factors, and even mechanical factors having to do with the vascular architecture surrounding the early ovary, are thought to participate in orchestrating this transition, both at the level of the individual follicle and at the level of the ovary as a whole [16,20]. A morphological characteristic of primary follicles is the cuboidal morphology that their granulosa cells acquire as they transition out of their primordial, flat morphology. It is also at the primary stage that the oocytic nucleus becomes the germinal vesicle (GV).
After the primary stage, the follicle transitions into the secondary stage. A follicle is morphologically characterized as secondary by having at least two layers of cuboidal granulosa cells surrounding its oocyte. When in the secondary stage, the follicle acquires follicle stimulating hormone (FSH) receptors. Studies have shown sensitivity of follicles to FSH during this stage of development, but not necessarily a dependency on it, especially in follicles isolated from neonatal animals [25]. Secondary follicles gain a third cell type, and those are the thecal cells [9], the development of which varies in timing between species [8].

As secondary follicles transition into the next stage of development, they gain a fluid-filled cavity, called an antrum, which is characteristic of tertiary follicles (also known as the antral or Graafian follicles). Antra are filled with follicular fluid that is derived from circulating blood. Follicular fluid is essentially filtered blood as it contains less of the larger molecular weight factors, about 50% of those smaller than 250kDa and almost no blood proteins above 850kDa [26]. From within emerging tertiary follicles, some are selected for further growth under the influence of luteinizing hormone (LH) and they become dominant follicles that mature towards ovulation [20]. Dominant follicles have a characteristic, morphological feature called the cumulus oocyte complex (COC), which is released from the follicle upon ovulation [8]. Folliculogenesis continues until the ovary is depleted of follicles, the point at which ovarian function ceases [6,7].

**Ovarian steroidogenesis and the two-cell model:**

In addition to housing the oocytes, the mammalian ovary produces sex hormones; mainly progesterone and estrogens. Follicles are responsible for the production of estrogens through a
synergistic process between the granulosa and thecal cells [27]. While Falck's 1959 publication demonstrated an interdependence between the granulosa cells and the thecal layer for estrogen production, it wasn't until 1962 that the "two cell" term was coined to describe a model of ovarian steroid production [28]. In the following years, experimental results were conflicted on which cell type produced which steroid(s) and whether or not both cell types were necessary for complete steroidogenesis [29–34]. In 1975 Makris and Ryan demonstrated a clear synergy between granulosa and thecal cells in steroidogenesis in hamsters [35]. Shortly afterwards, Erickson and Ryan demonstrated the specialized role of each cell type, and that gonadotropins played a crucial role in the stimulation of steroidogenesis in rabbits [36]. In the same year, in the same issue of Endocrinology, Dorrington et al. showed that granulosa cells produce estradiol specifically in response to FSH in rabbits [37]. The following year, Erickson and Ryan completed the picture by demonstrating that testosterone is produced by the thecal layer in rabbits when stimulated by LH [38]. Taken together, those studies created a model for ovarian steroidogenesis where thecal cells produce androgens under the influence of LH, and then the granulosa cells produce estrogens under the influence of FSH. This is known today as the two cell model of steroidogenesis. In the following year, experiments on rat [39] and ovine [40] cell cultures demonstrated similar results, lending more credence to the two cell model.

Steroidogenesis begins with cholesterol. Although not yet fully elucidated, cholesterol is imported into the mitochondria by a mechanism involving steroidogenic acute regulatory protein (StAR) [41,42], and is stimulated by LH in the thecal cells [43]. Once cholesterol is translocated into the inner membrane of the mitochondria, a 56kDa cholesterol side chain cleavage enzyme transforms it into pregnenolone. The enzyme is cytochrome P450, family 11, subfamily A,
polypeptide 1 (CYP11A1) [44,45]. Pregnenolone is then exported from the mitochondria into the cytoplasm where it is converted into progesterone by 3β-hydroxysteroid dehydrogenase [46–49]. Next, CYP17A1 converts progesterone to androstenedione [44,48,49], which is then converted to testosterone as catalyzed by hydroxysteroid 17-β dehydrogenase [44,49]. Testosterone then diffuses to the granulosa cells where it is acted upon by CYP19A1, or aromatase, and converted to estrogens, in a process known as aromatization [8,44,49]. Steroid production continues in a follicle even after ovulation, as the remnant of the ovulated follicle transforms into a structure known as the corpus luteum (CL) which specializes in the production of progesterone [8,35].

Estrogen production is necessary for the release of gonadotropins into circulation. Estrogen at the levels produced during the follicular phase of the estrous cycle is inhibitory to the production of gonadotropins, in that it prevents the release of large quantities of LH as seen in the LH surge [50]. This changes as more tertiary follicles are recruited for growth, increasing the ovarian capacity for estrogenesis, in turn leading to increased levels of circulating estrogen that it crosses a threshold of concentration, and gains a stimulatory effect that enables the LH surge [50–52]. Progesterone conveys a similar function with respect to inhibiting gonadotropin release as conferred by hypothalamic-pituitary signaling, particularly by influencing the release patterns of the gonadotropin-releasing hormone (GnRH) [50–52]. GnRH neurons were reported to express estrogen [53] and progesterone [54] receptors, indicating direct modes of action by which ovarian steroids can regulate GnRH release.
**Phosphatidylinositol-3 kinase (PI3K) signaling:**

PI3K is a membrane-bound enzyme which participates in regulating a wide variety of cellular processes. In the ovary, PI3K mediates its function primarily through the phosphorylation of protein kinase B (AKT; pAKT) [21]. Once phosphorylated, pAKT is imported into the nucleus, where it confers PI3K signaling by phosphorylating downstream, nuclear factors [55]. One such factor is the forkhead transcription factor 3a (FOXO3A). pAKT suppresses FOXO3A activity by phosphorylating it, which leads to its removal from the nucleus. Inhibition of FOXO3A through pAKT promotes cell survival and suppresses programmed cell death (apoptosis) [21,56,57]. While poorly understood, the initial recruitment of follicles is regulated, at least partially, by FOXO3A. Involvement of FOXO3A seems to be centered around activating the dormant oocyte, as the removal of FOXO3A from the oocytic nucleus as a consequence of pAKT signaling was reported to initiate follicular development [22–24].

The motif of promoting survival and suppressing stress responses continues with autophagy, as it is suppressed by pAKT activity [58]. Autophagy, the process of degrading damaged cellular components for recycling, is a stress response observed in conditions such as heat stress [59,60]. PI3K also participates in mediating the effects of gonadotropins. FSH was shown to induce the phosphorylation of PI3K and AKT, and upregulate estrogenesis in granulosa cells [61–64]. LH was also shown to act through PI3K to stimulate androgenesis by upregulating the expression of CYP17A1 [65]. With these studies, it becomes reasonable to consider PI3K and AKT as central regulators of general ovarian function.
Many stressors can alter these ovarian functions, and this thesis discusses alterations to ovarian function in the contexts of two; obesity and heat stress.

**Obesity:**

Obesity is the "abnormal or excessive fat accumulation that may impair health [66]." Clinically, the World Health Organization (WHO) uses BMI criteria to diagnose obesity, where a BMI of 25 or greater is considered overweight, while a BMI of 30 or greater is considered obese. WHO states that worldwide prevalence of obesity has doubled between 1980 and 2015. In 2014, 39% of adults were overweight, and 13% were obese, which equated to about 1.9 billion obese or overweight adults [66]. Global prevalence of obesity is comparable to its prevalence in the United States. Between 2011 and 2014, the percentage of obese adults exceeded 36% in the US [67]. Women have a higher prevalence of obesity (36.3%) than men (34.3%) in the US [67]. Obesity's prevalence and its association with a wide variety of disorders poses it as a public health concern worldwide [68,69].

Systemically, obesity is associated with a number of physiological changes such as increased circulating leptin (hyperleptinemia) [70,71], increased circulating insulin (hyperinsulinemia), and increased circulating endotoxins (metabolic endotoxemia). Reproductively, obesity is associated with a plethora of impairments to the female reproductive function, such as polycystic ovary syndrome (PCOS) [72], miscarriage [73], and anovulation [74]. Obesity is also associated with reduced chances of successful *in vitro* fertilization (IVF), as well as other assisted fertility treatments [75]. In adult humans, BMI is negatively correlated with circulating estrogen, LH, FSH, and inhibin levels [76]. Human childhood obesity, on the other
hand, was correlated with an earlier age of menarche [77]. In rats, obesity alone was shown to blunt the LH surge required for ovulation, but that effect was worsened by a high-fat diet [78]. In mice, obesity alters the estrous cycle, mainly by reducing the length of the estrus phase while increasing the length of the metestrus phase [79].

**Heat stress:**

Coinciding with the summer season, particularly the months of July, August, and September, American production pigs show a reduction in their overall reproductive performance known as seasonal infertility [80,81]. Seasonal infertility manifests as impaired overall reproductive performance due to reduced conception rates and increased embryonic deaths. Reduced farrowing can extend to the month of December, indicating a lasting effect of that period on the animals. During summer, animals are subjected to increased ambient temperatures, which leads to a sustained increase in core body temperatures. This is the condition known as heat stress (HS). US commercial swine production alone is estimated to suffer $299M in annual losses due to HS [82]. HS doesn't just reduce the production potential of livestock animals, but also compromises animal welfare.

HS challenges many facets of reproductive function. Investigations conducted mainly in ruminants showed HS to impact follicular development [5,6], steroidogenesis [5–8], embryonic [3,9,10] and fetal [10,11] development. HS also increases the onset of stress responses such as autophagy [59,60] and apoptosis [83,84]. Atresia, a partly apoptotic process [85] by which follicles die, was shown to be increased in ovaries of heat-stressed animals [86]. AKT phosphorylation was shown to suppress both apoptosis [57] and autophagy [58]. These effects of
HS pose challenges to animal agriculture and international food security. Such concerns are exacerbated by the grim outlook on climate change, as it is expected to increase the intensity of heat waves [87], which could subsequently intensify the impact of HS on animal agriculture.

HS is associated with many systemic changes. Circulating insulin was repeatedly shown to increase in heat-stressed production animals including pigs. This is paradoxical to the phenotype of reduced feed intake, also a hallmark of HS [88–93]. Another apparent contradiction is that, despite hyperinsulinemia, HS was shown to increase insulin sensitivity in pigs [94] and protected rats from high-fat diet-induced insulin resistance [95]. Understanding the role of insulin in HS is crucial as it influences ovarian function (explained below). Another systemic hallmark of HS is endotoxemia, a condition of increased circulating bacterial debris. This condition originates from the reduction in the integrity of the intestinal wall during HS as a consequence of blood flow being diverted to the skin in an attempt to cool it [96,97], and is characterized by the increased circulation of lipopolysaccharides (LPS) [92,98]. LPS signaling through toll-like receptor 4 is also capable of regulating ovarian function, which is explained in more detail later in this review.

**Fertility and Metabolic Energy Status:**

A link between the timing of puberty and body mass was described by Kennedy and Mitra in 1963 when they demonstrated a relationship between body mass in rats and the timing of the first onset of estrus, where rats of lower body mass showed a delayed time to first estrus [99]. Afterwards, in 1970, Firsch and Revelle published an observational study on human females in which they observed a similar correlation between body mass and the timing of the
onset of puberty, and hypothesized a correlation between the ratio of body mass and stature to the timing of the onset of puberty [100]. Their hypothesis later came to be known as the Firsch-Revelle model, sometimes called the critical body weight hypothesis or the critical fat mass hypothesis. Studies like this and others represented a growing body of evidence indicating a very strong link between energy status and the regulation of fertility in mammals. While there was no known mechanism linking energy status and the female reproductive system, scientists of the era suspected the hypothalamus to be the intersection connecting the two, and it wasn’t until the discovery of leptin that the mechanism linking energy status and fertility was recognized.

**Leptin:**

The characterization of leptin began with the 1950 discovery of the obese (ob) mutation in mice [101]. Mice homozygous for the mutation (ob/ob) displayed a behavioral phenotype of overeating, and thus were used as a model for studying obesity. ob/ob homozygous mice were also sterile [101]. In 1994, the ob gene was cloned and its homologue identified in humans [102], and a year later its protein product was purified and named leptin [103]. The protein is 16-kDa in size, produced by adipose tissue [103,104], and signals satiety, thereby suppressing appetite. Animals resistant to leptin, or incapable of producing it, develop a severe overeating phenotype, resulting in the development of obesity and subsequent complications including Metabolic Syndrome X and Type II diabetes. Administration of leptin to ob/ob mice reduced feed intake and body weight. It also suppressed feed intake in fasting, wild type mice [105], which was direct evidence of leptin's role in regulating food intake. Hypothalamic involvement in obesity was suspected before the discovery of leptin [106], and the hypothalamic involvement in leptin’s role in obesity was supported when hypothalamic expression of the leptin receptor (LEPR) was
shown in the human brain [107]. Microinjecting leptin into the ventromedial hypothalamus enhanced its effects compared to injecting it into other regions of rat brains [108], further supporting hypothalamic action of leptin. Together, the evidence suggested that leptin's control of food intake was mediated by hypothalamic signaling.

With the discovery of leptin, an indicator of the body’s adipose reserves, the missing link between energy storage and reproductive function, was identified.Injecting leptin into ob/ob female mice rescued the sterility phenotype [109], and injecting it into normal female mice accelerated the onset of puberty despite some retardation in growth [110,111]. Leptin was further determined to influence reproductive function through regulation of the levels of circulating gonadotropins [112]. Synthesis and release of gonadotropins is stimulated by GnRH which can be regulated by leptin [113–115]. Leptin's effects on GnRH neurons are best described as permissive as it functions following a pattern indicative of a threshold rather than a dose-dependent response [115,116]. Unlike estrogen, leptin lacks the ability to interact directly with GnRH neurons. Removal of LEPR from all neuronal populations prevented the onset of puberty and rendered mice infertile, but selective knockout of LEPR from GnRH neurons conferred no loss of fertility or blockage of puberty [117]. In addition, expression of STAT-3, a downstream signal of LEPR, was not detected in GnRH neurons after the direct intracerebral administration of leptin [117]. One indirect route by which leptin could influence GnRH neurons is by regulating Kiss1 neurons.

The kisspeptin-kisspeptin receptor (GPR54) system was identified and characterized in 2001 by three independent reports [118–120]. However, it wasn't until 2003 that its reproductive
role became apparent. Two independent reports investigating idiopathic hypogonadotropic hypogonadism – a disorder characterized by the blockage of the onset of puberty, reduced circulating gonadotropin levels, and reduced gonadal size – in patients who were born of multiple generations of first-cousin/tribal marriages discovered patients to be homozygous for loss of function mutations in the GPR54 locus [121,122]. Seminara et al. expanded their investigation using a model in which Gpr54 knockout (Gpr54^−/−) mice were evaluated and administered GnRH and gonadotropins. Gpr54^−/− were infertile, never reached sexual maturity, and had very low concentrations of circulating gonadotropins [121]. Administration of GnRH and gonadotropins rescued reproductive function, showing kisspeptin signaling to be upstream of both GnRH and gonadotropins [121]. A subset (10-40%) of kisspeptin neurons were shown to be targets of leptin [123,124]. Administration of leptin in a variety of models, including the ob mouse model, was shown to enhance kisspeptin production [124–127]. Leptin was also shown to act outside of the kisspeptin pathway [128]. This kisspeptin-independent effect was further explored by creating transgenic mice with Lepr knocked out in GABAergic neurons. Knockout mice showed delayed onset of puberty, blunted LH surge, and reduced overall fertility [129]. Together, these reports show leptin as a permissive regulator of cyclicity and the onset of puberty via indirect signaling to GnRH neurons through kisspeptin-dependent and -independent mechanisms.

Leptin's effects are mediated by the leptin receptor, encoded by the Lepr gene. Lepr was previously known as Db (abbreviation for diabetes) as mice homozygous for a loss of function mutation in this gene were used as a model for Type II diabetes. The mutation was first observed in an inbred strain of Jackson Laboratories mice (C57BL/Ks), and was first reported in a
publication by Hummel et al. in 1966 [130]. The authors showed this mutation to be recessive for obesity and other visible phenotypes [130]. With the discoveries in leptin and leptin function, it comes as no surprise that mice homozygous for the Db mutation had a similar behavioral phenotype of overeating to the ob mice. A homozygous genotype for the loss of function in the Db mutation also conferred infertility, which suggested a link between leptin signaling and fertility. Heterozygous individuals were originally considered phenotypically and morphologically indistinguishable from the wild type mice, however, this has since been shown to not be the case. Significant elevation in the levels of circulating leptin are characteristic of the heterozygous phenotype, potentially as a compensatory response to leptin resistance induced by the heterozygous loss of function in the leptin receptor [131]. In addition to its influence on circulating levels of gonadotropins, leptin also directly regulates ovarian function. LEPR was shown to be expressed in the ovaries of mice [132], cows [133], chickens [134,135], goats [136], humans [137], rabbits [138], and pigs [139], although ovarian LEPR localization varies between species.

Leptin signaling is primarily transduced through the PI3K pathway [140]. From a mechanistic standpoint, this provides a possible link between leptin and ovarian physiology through direct interactions with ovarian LEPR. As discussed above, many vital ovarian processes including folliculogenesis and steroidogenesis are regulated by the PI3K pathway through AKT, and leptin signaling has been shown to impact multiple stages of folliculogenesis both in vitro and in vivo. Leptin can regulate the later stages of folliculogenesis, specifically acting as a positive regulator of GV breakdown and ovulation. In in vitro culture of dominant follicles, leptin absence reduced the fraction of oocytes going through germinal vesicle breakdown
(GVBD) without the addition of any exogenous gonadotropins [132]. Leptin’s effects on early folliculogenesis on the other hand appear more contradictory. Administration of LEPR antagonists resulted in the depletion of the primordial pool without an observed increase in the accumulation of tertiary follicles in postnatal rats [141]. Conversely, reducing the effective levels of peripheral leptin through passive immunization was shown to enhance early folliculogenesis in prepubertal rats [142]. In addition, studies on caloric intake, which is directly related to circulating levels of leptin, show that a modest reduction in caloric content of the diet can extend fertility further into the lifespan, and improve the offspring’s health [143].

Leptin plays an additional role in influencing ovarian development via angiogenesis, and is an angiogenic factor [144] as well as an enhancer of fenestration [145]. The promoter of the leptin-coding gene, \( ob \), contains a hypoxia responsive element that enables it to respond to hypoxia-inducible factor (HIF) 1-alpha [146] and to work in concert with vascular endothelial growth factor (VEGF) [145]. Angiogenesis is crucial during folliculogenesis and is regulated on an individual follicle basis, potentially leading to the selective delivery of circulating factors such as gonadotropins [147]. Such a role of leptin provides a potential explanation for ovarian LEPR expression. Tertiary and larger secondary follicles are dependent on gonadotropins in advancing their development, making access to the circulatory system crucial for proper late folliculogenesis [147]. In addition, their large size makes vascularization necessary to facilitate the delivery of oxygen and nutrients. Because of this, appropriate follicular vascularization could be an important factor in selecting which follicles become dominant and which are lost to atresia, and ovarian expression of leptin could be one of its major regulators. Increased expression of
leptin was shown in breast [148], colon [115], and prostate [149] cancer cells, which fits with leptin's angiogenic properties.

**Insulin:**

The Islets of Langerhans were first described in the pancreas in 1869 [150]. However, it wasn’t until late 1921 that insulin was isolated and identified [150], followed by clinical use of insulin in 1922 to successfully treat a diabetic patient [151] due to its role in regulating glucose homeostasis. Insulin is a dipeptide; a polypeptide composed of two chains of amino acids linked with disulphide bonds [152]. It is encoded by the *Ins* gene first characterized in 1982 by Schröder and Zühlke, who also identified that mutations in this gene are among the causes of diabetes [153]. The receptor for insulin, insulin receptor (INSR), was discovered in 1971 in the liver [154]. Observing the rapid phosphorylation of tyrosine residues in proteins around 185kDa in size in response to insulin stimulation of insulin-sensitive cells paved the way for the discovery of the mechanism by which INSR transduces its signal [155].

Upon binding insulin, INSR phosphorylates a family of proteins called insulin receptor substrates (IRS). IRS-1 was cloned in 1991 and provided crucial insight into the molecular mechanism of insulin signaling [156]. IRS-1 knockout mice were capable of survival, and maintained some insulin and insulin-like growth factor-1 (IGF1) function, showing that IRS-1 mediated signaling was not the sole pathway of insulin signaling. However, it is important to note that IRS-1 knockout mice also displayed retarded growth beginning *in utero* [157,158], which indicated a major role of IRS-1 in insulin signaling, even if it was not its sole pathway. Redundancy in the mechanism of insulin signaling was explained by the discovery of IRS-2...
[159], IRS-3 [160], IRS-4 [161], and more recently, IRS-5 and 6 [162]. IRS proteins are typically activated through the phosphorylation of Threonine residues and are inhibited through the phosphorylation of Serine residues [163]. Action on IRS, mainly IRS-1 and 2, can confer insulin resistance, mainly via hyper-phosphorylation of Serine residues [163]. Reproductive tissues, including ovarian tissue, show reduced insulin sensitivity when compared to non-reproductive tissue [164]. The ovary and the hypothalamus also maintain insulin sensitivity in diet-induced hyperinsulinemia [164].

IRS-mediated signaling induces the phosphorylation of SH2 domains [165]. A major consequence of this is that PI3K becomes a target for insulin signaling [156,159–161] as it contains multiple SH2 domains [166–168], although it is worth noting that some insulin effects, such as the regulation of glucose transport, can function independently of PI3K signaling [18]. As explained above, PI3K is central to ovarian function at large, which enables insulin to exert a direct influence on ovarian phenotype. In pigs, administration of insulin was associated with improved rates of ovulation [169] and a decrease in the onset of atresia [170]. *Ex vivo* culture of human ovarian cortical tissue showed accelerated early folliculogenesis in response to insulin and IGF1 treatments, as treated tissues showed increased accumulation of primary follicles and decelerated the loss of larger follicles due to atresia [171]. Insulin was also shown to stimulate ovarian steroidogenesis [172]. As a general effect, the increase in steroidogenesis as a response to insulin is well conserved, such that porcine and bovine insulin can stimulate ovarian steroidogenesis in insects [173–175]. The sum of this evidence suggests that insulin has a direct mode of action in regulating ovarian function, making hyperinsulinemia a probable causative factor in the ovarian phenotypic perturbations displayed during obesity and HS.
LPS and TLR4 signaling:

Toll-like receptors (TLR) are cell-surface receptors that mediate the initiation of the innate immune response to pathogens [176]. Toll-like receptors are named as such due to the homology they share with Toll, a Drosophila receptor shown to participate in the innate, antifungal immune response [177]. Toll, and in turn TLRs, do this by recognizing antigens associated with pathogens. These antigens are named pathogen-associated molecular patterns (PAMPs) [176]. So far, 13 mammalian TLRs have been characterized [178,179]. Different TLRs specialize in recognizing different classes of PAMPs. For instance, TLRs 3, 7, 8, and 9 bind nucleic acids, while TLRs 2, 4, 5, 6 are capable of binding PAMPs found on bacterial surfaces [178]. Because the elevation of circulating lipopolysaccharides, or LPS, is a characteristic of the endotoxemia induced by obesity and HS, this thesis focuses on TLR4 as it is the primary receptor by which the innate immune response to LPS is initiated [180,181].

TLRs mediate their inflammatory signaling through nuclear factor-kappa B (NF-κB) and its family of transcription factors [182]; a response mediated by PI3K in the case of TLR4 [183]. Expression of TLR4 can be altered in response to circulating factors. For example, TLR4 expression can be altered during inflammation [182]. In renal epithelial cells, TLR4 expression was shown to increase in response to the inflammatory, tumor necrosis factor alpha (TNF-α) in vivo [184]. TLR4 is expressed in the reproductive tract and in the ovary [185], and its expression is differential between follicular cell types [186]. Granulosa cells were shown to have increased expression of TLR4 compared to the cumulus oocyte complex in mice [186]. Interestingly, stimulating follicles with gonadotropins increased the expression of TLR4 in both the COC and
in granulosa cells [186]. Expression of TLR4 in the reproductive tract in general explains, at least partially, the reproductive phenotypes displayed in response to an LPS challenge.

In mice, LPS administration induced early loss of pregnancy through both abortion and resorption [187–189]. These observations fit with the increased production of prostaglandin F2α (PGF2α), a factor which induces the degradation of the CL, in cultured bovine endometrial cells when challenged with LPS [190]. Administration of LPS to cows during the luteal phase of the estrous cycle caused shrinkage of the CL, and reduced circulating progesterone levels [191]. Additionally, LPS was shown to suppress the expression of progesterone receptors in mouse uteri, which could contribute to the increase in PGF2α production [192]. LPS also disrupted early folliculogenesis by depleting primordial follicles in mice and cows [193]. These data demonstrate LPS's wide capacity to disrupt reproductive function, making it a good candidate for participating in the reproductive impairment phenotypes observed during obesity and HS.

Summary:

The literature reviewed herein demonstrate the central role of AKT-mediated PI3K signaling in ovarian function both in normal and abnormal conditions. PI3K regulates folliculogenesis, steroidogenesis, and was shown to relay gonadotropic signaling. Stressors which compromise reproductive performance by upsetting the balance of ovarian functions often converge in their signaling, at least in part, at PI3K. Leptin exerts its effects through the PI3K pathway as mediated by LEPR. Similarly, insulin activates PI3K through INSR and IRS signaling. TLR4 also activates PI3K signaling once it binds LPS. Obesity induces many reproductive problems, which can partly be explained by hyperinsulinemia, hyperleptinemia, and
endotoxemia. HS compromises the reproductive capacity of livestock, and is associated with hyperinsulinemia, endotoxemia, increased apoptosis, autophagy, and atresia. All of these responses converge at PI3K, indicating that metabolic signals insulin and LPS could participate in said responses. We hypothesized a role for leptin in the reproductive phenotypes displayed during obesity (Chapter 2), and contribution of hyperinsulinemia and metabolic endotoxemia during HS-induced seasonal infertility (Chapter 3). Investigating these stressors is of great importance: Obesity is a worldwide, public health concern which has been on an upwards trend for decades. HS is also a significant issue as it reduces the capacity of animal production and compromises animal welfare, problems which are only projected to be worsened by the impending threat of climate change.
Figure 1.1. Histological Features of the Mammalian Ovary
This figure depicts the mammalian ovary and the follicles; the functional units which confer ovarian functions. Stages of follicular development are visible here, beginning with the primordial follicle shown as an oocyte (pink) surrounded by flattened granulosa cells (green). Once activated, the granulosa cells take on a cuboidal morphology and the follicle transitions into the primary stage. When gaining multiple layers of granulosa cells, the follicle becomes secondary, and at this stage it gains a thecal layer composed of theca cells (purple). At this stage, the follicle gains the capacity to catalyze the entire steroidogenic pathway to produce estrogen. With further development, the follicle acquires a fluid-filled cavity, the antrum (beige), which characterizes its entry into the tertiary, or antral, stage. Under the influence of LH, a tertiary follicle can grow into a pre-ovulatory, or dominant, follicle, characterized by the cumulus-oocyte-complex (COC) seen, in this figure, budding into the antrum. A surge in LH causes the oocyte to be ovulated as a part of the COC, while the rest of the follicle goes on to become the corpus luteum (CL; brown), specialized in producing progesterone. Unless a pregnancy takes hold, the CL is degraded and the cycle starts over. Activated follicles not selected to become dominant die through the process of atresia. With copyright permission. (Reproduced from Keating, A.F. and Hoyer, P.B. 2009. Mechanisms of reproductive toxicity. In: Drug metabolism in pharmaceuticals; Concepts and Applications. Chapter 24: 697-734.)
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CHAPTER 2
ALTERED OVARIAN EXPRESSION OF MICROSOMAL EPOXIDE HYDROLASE 1 DUE TO MATERNAL HYPERLEPTINEMIA DURING GESTATION

Al-Shaibi, A.A.¹, Schulz, L.C.², Pollock, K.E.², and Keating, A.F.¹*

¹Department of Animal Science, Iowa State University, Ames, IA, 50014
²Department of Obstetrics, Gynecology, and Women’s Health, University of Missouri, Columbia, MO 65212

Contribution Statement: Animal experiments were performed by LCS and KEP. AAA performed all of the molecular analysis in this chapter, and wrote the manuscript. AFK mentored AAA, planned the experiments and aided with data interpretation. AFK edited the chapter and will serve as corresponding author for the research paper submission.

*Corresponding author: Aileen F. Keating, Ph.D., Department of Animal Science, Iowa State University, Ames, IA 50014; Telephone 515-294-3849; Email akeating@iastate.edu
Abstract:

Obesity is a pathology of worldwide prevalence and is correlated with a plethora of reproductive problems such as infertility, polycystic ovary syndrome (PCOS), miscarriage, anovulation, and difficulties with assisted reproduction treatments. Obesity also likely affects oocyte quality. Systemic hyperleptinemia is caused by obesity as a consequence of increased adipose tissue accumulation. Leptin, a 16 kDa adipokine, regulates satiety and is also a permissive signal for the onset and continuation of ovarian cyclicity. Ovaries are sensitive to leptin as they express the leptin receptor (LEPR) with varying localizations between species. Leptin is a positive regulator of germinal vesicle breakdown (GVBD) and ovulation. Blocking leptin signaling via a LEPR antagonist depleted primordial follicles without increasing accumulated primary follicles while passive immunization against leptin increased the accumulation of primary follicles. Our previous investigation of obesity in both the high fat diet and the lethal yellow models revealed altered cyclicity, folliculogenesis, chemical metabolism, and steroidogenesis. We hypothesized that leptin is at least partly culpable for these effects due to its signaling being mediated by the phosphoinositide 3-kinase (PI3K) pathway, which participates in regulating all of these processes. Among others, epoxide hydrolase 1 (EPHX1) is a chemical metabolism enzyme regulated by PI3K. EPHX1 acts on epoxide groups to detoxify or bioactivate ovotoxicants. To investigate the effects of leptin, we used two gestational hyperleptinemia models: 1. Female mice who were heterozygous for a loss of function in the leptin receptor gene (Db/+), compared to wild type controls, and 2. Female mice administered leptin directly into circulation through a pump (Lpt) shortly before and during gestation compared to females fitted with a saline pump (Sal). Ovaries were retrieved from wild type offspring postmortem after 20 weeks of ad libitum feeding. We also used a rat ovarian culture
model where ovaries retrieved from postnatal day (PND) 4 ovaries were cultured in enriched DMEM and treated with 10 or 40 ng/mL of recombinant leptin for 7 d, and then fixed and sectioned for histological evaluation. Offspring of Db/+ females had reduced EPHX1 mRNA abundance, while offspring of Lpt showed reduced EPHX1 protein abundance. Ex vivo, leptin administration had no impact on the composition or health of the follicular pool in PND4 ovaries.

**Keywords:** Ovary, hyperleptinemia, gestation, microsomal epoxide hydrolase, folliculogenesis
Introduction:

The ovary is responsible for the maintenance of the female gamete, the oocyte, and production of hormones that regulate the female reproductive cycle. Functional units within the ovary, termed follicles, are composed of an oocyte, arrested in prophase I of meiosis, surrounded by granulosa cells. Another cellular layer, composed of theca cells, is recruited to the growing follicle during folliculogenesis [1]. Females are born with a finite number of primordial follicles which comprise the finite ovarian reserve [1]. Development of follicles, a process called folliculogenesis, begins with the recruitment of dormant primordial follicles coinciding with the proliferation of squamous granulosa and their acquisition of a cuboidal morphology [1]. Follicles mature through a number of stages in the order of primary, secondary, and tertiary, until the time of ovulation [1]. Under the influence of luteinizing hormone, the corpus luteum (CL) is formed from the remains of the ovulated follicle(s) [1,2]. Growing dominant follicle(s) and the CL produce the sex hormones 17β-estradiol and progesterone, respectively, by steroidogenesis. Steroidogenesis is coordinated between the theca and granulosa cells, where the theca cells produce androgens that are converted into estrogens by the granulosa cells in response to the gonadotropins, in what is known as the two cell model of steroidogenesis [1,3–9].

Recruitment and growth of primordial follicles are regulated by phosphoinositide 3-kinase (PI3K), primarily mediated by protein kinase B (AKT) [10]. Activation of the PI3K pathway results in the phosphorylation of forkhead transcription factor (FOXO3A), leading to its translocation from the nucleus to the cytoplasm, and causing a suppression in its activity. A number of reports support a role for FOXO3A in regulating the activation and growth of the
primordial follicle [11–13]. PI3K can be activated by external signals such as insulin [14], insulin-like growth factor-1 [15], and leptin [16].

Leptin, a 16kDa adipokine produced by the Ob gene [17–19], regulates satiety through hypothalamic signaling [20–22]. Leptin is also a permissive factor for the onset and sustainability of ovarian cyclicity [23–26]. The leptin receptor (LEPR) is expressed in the ovaries of a number of species with varying localization patterns [27–34]. In mice, LEPR is most abundant in the follicular thecal layer, indicating that secondary and tertiary follicles could be the most sensitive to systemic leptin [27]. Treatment of dominant follicles in culture with leptin was shown to promote ovulation, spontaneous germinal vesicle breakdown (GVBD), and enhanced gonadotropin-induced GVBD [27]. Blocking leptin signaling by administering leptin receptor antagonist to newborn rats induced a depletion of the primordial pool without a coinciding increase in the accumulation of primary follicles [35]. Passive immunization against leptin, however, increased the accumulation of primary follicles [36]. Leptin provides the link between energy stores and the reproductive system [37]. Changes in energy stores tend to manifest as changes in body mass as an individual gains or loses fat reserves, and reproductive function has been formally correlated to body mass for a number of decades [38].

Obesity is defined as the "abnormal or excessive fat accumulation that may impair health" according to the World Health Organization (WHO) [39]. A characteristic effect of obesity is an increase in circulating leptin [40,41]. Childhood obesity can reduce the age at which menarche (the first menstruation) occurs [42]. Obesity is also correlated with a host of reproductive problems such as infertility [43,44], polycystic ovary syndrome (PCOS) [45],
miscarriage [46], anovulation [43], and difficulties with assisted reproduction treatments like *in vitro* fertilization (IVF) [47]. Oocyte quality is also an area where obesity could have adverse effects. However, there is no clear consensus established in that regard [44,48,49].

As a human pathology, obesity is prevalent worldwide. The WHO cites that worldwide obesity has doubled in the period between 1980 and 2015. WHO diagnoses obesity based on body mass index (BMI), where a BMI of 25 or greater is considered overweight, and a BMI of 30 or greater is considered obese. Globally, 39% of adults aged 18 or older were overweight and 13% obese in 2014 [39]. This translates to 1.9 billion adults who are obese or overweight. In the United States, between 2011 and 2014, the prevalence of obesity among adults in the US was in excess of 36%, with a higher prevalence amongst women (36.3%) than men (34.3%) [50]. Due to both its prevalence and association with an increased risk of a wide range of disorders, obesity is an international public health concern [51,52].

We previously investigated the reproductive effects of obesity in the lethal yellow (LY) hyperphagia-induced progressive obesity and the high-fat diet-induced obese mouse models. In LY mice, changes in the abundance of ovarian steroidogenic proteins were observed [53]. Compared to wild type mice, progressive obesity increased abundance of CYP19A1 and CYP11A1, while the abundance of StAR was decreased. Additionally, LY mice had reduced primordial and primary follicle number at 12 weeks, and tended to have increased numbers of tertiary follicles starting at the same age [53].
Our investigation into obesity induced by high-fat feeding demonstrated alterations in ovarian PI3K signaling and the expression of xenobiotic metabolism enzymes [54]. Phosphorylation of ovarian AKT tended to be reduced in obese females. Ovarian mRNA abundance of Cyp2e1 was reduced, and ovarian EPHX1 tended to be reduced due to obesity [54]. Based on these data, we hypothesized that obesity-induced ovarian signaling alterations could be, at least partially, attributable to changes in circulating leptin concentrations. Utilizing two mouse models of gestational hyperleptinemia (leptin infusion and Db/+ mice) we assessed ovarian PI3K signaling, steroidogenesis, and xenobiotic metabolism in the offspring. In addition, we examined whether leptin exposure at normal or high levels would alter folliculogenesis in cultured neonatal rat ovaries.

Materials and Methods:

Reagents:

Primers were designed in-house as described in Nteeba et al. [53,54], and synthesized by the DNA facility at Iowa State University (Ames, IA). Superscript III one-step RT-PCR system, dNTP mix, Hanks’ balanced salt solution (without CaCl2, MgCl2, or MgSO4), and Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (Ham) 1x (DMEM/Ham's F12), Albumax, Penicillin-Streptomycin (5000U/ml), were purchased from Invitrogen, Co. (Carlsbad, CA). RNeasy mini-kit, QIAshredder kit, RNeasy MinElute kit, and Quantitect SYBR Green PCR kit were obtained from Qiagen, Inc. (Valencia, CA). N0, N0, N0, N0-tetramethylethlenediamine (TEMED), Tris base, Tris HCl, sodium chloride, Tween-20, ascorbic acid (vitamin C), sodium dodecyl sulfate (SDS), 30% Acrylamide/Bis-acrylamide, 2-b-mercaptopethanol, Glycine, Bovine Serum Albumin (BSA), paraformaldehyde (PFA), Ammonium persulphate, Recombinant Rat
Leptin, and transferrin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Ponceau S and hydrochloric acid was purchased from Fisher Scientific (Waltham, MA). Nonidet-P40 (N-P40) was purchased from US Biological (Salem, MA). SignalFire ECL reagent was acquired from Cell Signaling Technology (Danvers, MA).

Animal models:

Fisher 344 rat breeding scheme:

Fisher 344 female rats were housed individually in plastic cages in a climate-controlled room (22 ± 2°C) with a fixed light-dark cycle (12 h light/12 h dark), and provided a standard pelleted diet and water ad libitum. Rats were bred by placing two females in a cage with a male for one week before the females were separated into individual cages for parturition to occur. The Iowa State University Institutional Animal Care and Use Committee approved this breeding protocol.

Ex vivo ovarian culture:

Ovaries were excised from postnatal day (PND) 4 rats, excess tissue trimmed, and placed upon a Millipore cell culture insert floating on enriched DMEM/Ham’s F12 media (1 mg/mL BSA, 1 mg/mL Albumax, 50 μg/mL ascorbic acid, 5 U/mL penicillin, 5 μg/mL streptomycin, and 27.5 μg/ml transferrin). After the addition of treatment, each ovary was covered with a drop of media to prevent dehydration. Ovaries were cultured in media containing vehicle control or rat recombinant leptin at a final concentration of either 10 (n = 3-4) or 40 ng/mL (n = 4-8). Ovaries were maintained at 37°C and 5% CO₂ for 7 d with media and treatment being changed on alternate days. Following culture, ovaries were incubated in 4% paraformaldehyde for 2 h before
being transferred to 70% ethanol for storage. Fixed ovaries were sectioned and used for follicle counting.

*Gestational hyperleptinemia:*

Hyperleptinemia during gestation was induced using two animal models: 1) Control females (WT) or females heterozygous for the diabetic mutation (*Db/+*) were mated with wild type males. Wild type offspring from these dams were maintained and fed *ad libitum* for 20 weeks before euthanasia. 2) Females received direct infusion of saline (Sal) or recombinant leptin (Lpt; 350 ng/h) through a subcutaneous Alzet mini-osmotic pump. Offspring of this group were also maintained for 20 weeks and fed *ad libitum* before euthanasia. Ovaries were collected postmortem and were flash frozen for mRNA and protein analyses. Circulating leptin was measured using a mouse leptin ELISA kit (Intrassay variation, 15.5%; Millipore) as per the manufacturer’s recommendations, with the exception of extending the primary antibody incubation overnight. Animals procedures and serum assays were performed by the research group of Dr. Laura Schulz at the University of Missouri [55]. Feed intake corrected to body mass of *Db/+* female offspring was increased compared to WT offspring at 4 weeks, but was not statistically significant at 11 or 19 weeks. Female offspring of Lpt dams had increased feed intake compared to female offspring of Sal dams at 11 weeks but not at 4 weeks or 19 weeks [55].

*RNA isolation and qRT-PCR:*

RNA was isolated from whole ovaries using an RNeasy Mini Kit as per the manufacturer’s recommended protocol. RNA quantity and purity was measured using an ND-
1000 Spectrophotometer (k ¼ 260/280 nm; NanoDrop Technologies, Inc.). cDNA was synthesized from extracted RNA using a Superscript III system and an oligo-dT primer. Diluted cDNA (1:25) was amplified using a QuantiTect SYBR® Green PCR Kit on a MasterCycler RealPlex4. PCR was performed by heating the reaction vessels to 95°C for 15 min, then cycling through a 15 s melting at 95°C, a 15 s annealing at 58°C, and a 20 s extension at 72°C, 40 times. A melting gradient of 72°C to 99°C with a 1°C increase per step was used to assess product melt conditions. Gene-specific primers as listed in Table 1 were used. β-actin and Gapdh were used as endogenous controls. PCRs performed to test for genomic contamination were as follows: no template reaction for each primer, no primer reaction for each sample, and master mix without template or primer. The thermocycler was tested by including water-loaded wells on each plate.

Protein extraction and western blotting:

Whole ovaries were homogenized in tissue lysis buffer (1% NP40, 50mM Tris-HCl, 150mM NaCl, 1% SDS, pH 8) using a handheld homogenizer before being centrifuged through a Qiashredder column, and placed on ice for 30 min with occasional shaking. Lysate was then centrifuged at 9300 x g at 4°C for 15 min. The supernatant was transferred into a new tube and centrifuged at 9300 x g at 4°C for 15 min. Protein was separated by SDS-PAGE using a 10% Tris-Glycine gel, before being transferred to a nitrocellulose membrane via wet transfer. Transfer quality was assessed using Ponceau S staining. Membranes were washed of Ponceau S with Tris Buffered Saline (TBS; 5M NaCl, 20mM Tris-HCl, and pH 8) with 0.15% Tween-20 (TTBS) before blocking in 5% BSA in TTBS while rocking at 4°C overnight. Membranes were then immersed in primary antibodies targeting StAR (Cell Signaling; D10H12), mEH (Detroit R&D; MEH1), total AKT (Cell Signaling; 9272), or pAKT (Cell Signaling; C31E5E) at a dilution of
1:500 in 5% BSA in TTBS. Incubation in primary antibody dilutions were continued overnight with constant rocking at 4°C. Membranes were washed three times with TTBS for 10 min before being incubated in 1:5000 of goat anti-rabbit (Southern Biotech; 4030-05) or 1:10000 donkey anti-goat (Santa Cruz; sc-2304) in 5% milk in TTBS. Afterwards, membranes were washed three times with TTBS for 10 min before being immersed in SignalFire ECL reagent for 10 min, then X-Ray film was exposed to the membranes to visualize fluorescence. Densitometric analysis and quantification of the appropriately sized protein bands was performed using ImageJ (NCBI) and raw protein of interest values normalized to Ponceau S total protein measurements. Testing for random signal generated by the antibodies was performed by blotting pooled protein and testing fluorescence after incubating it with each primary antibody in the absence of secondary antibodies, normal goat serum in lieu of primary antibody with goat anti-rabbit as the secondary antibody, and with donkey anti-goat as a secondary with omission of primary antibody. Incubations and signal detection were performed as described above.

Slide preparation and follicle counts:

Ovaries were sectioned at a thickness of 5µM. Every sixth section was mounted and stained with Hematoxylin and Eosin (H&E). After blinding mounted sections, follicle classification and enumeration was performed using ImageJ (NCBI)’s Cell Counter plugin on images that had been captured using a QImaging QICAM Fast 1394 camera attached to a Leica DMI3000 B compound microscope. Healthy follicles were only counted if their nuclei were visible to avoid double counting of large primary and secondary follicles. Follicles were classified as primordial if the nucleated oocyte was surrounded by a partial or a complete layer of squamous granulosa cells, which have a characteristic flat morphology. When a nucleated oocyte
was surrounded by three to nine cuboidal granulosa cells, it was considered a small primary follicle. If it had 10 or more cuboidal granulosa cells, it was classified as a large primary. Follicles were classified as secondary if their nucleated oocyte was surrounded by at least two complete layers of cuboidal granulosa cells. Follicles showing pyknotic bodies, intense eosinophilic staining of oocytes, or any striking morphological abnormalities were deemed unhealthy.

*Statistical Analysis:*

Statistical analysis was performed on the ΔCt value for qRT-PCR data, on average densitometric values standardized to Ponceau S densitometric values for western blot data, and on average follicle counts of each category of follicles. Microsoft Excel was used to perform all calculations and statistics. *P*-values were calculated using Welch's t-test computed using the function `t.test(<array 1>;<array 2>;1;2)`. Significant difference between treatments was considered at *P* < 0.05. Graphs were created using GraphPad Prism 6 for Windows. Data bars represent mean values, and error bars denote the standard error of the mean as calculated by GraphPad.

**Results:**

**Gestational hyperleptinemia decreases circulating levels of leptin in offspring**

Offspring of *Db/+* females had reduced levels of circulating leptin (66.5%; *P* < 0.05) compared to offspring of wild type females (Appendix Figure 1A). Offspring of females infused with leptin via an osmotic pump also had reduced circulating levels of leptin (32.3%; *P* < 0.05)
when compared to offspring of females infused with saline (Appendix Figure 1B), courtesy of Dr. Laura Schulz [personal communication].

**Altered leptin concentration does not influence early folliculogenesis or morphological health of follicles in cultured PND4 rat ovaries**

PND4 rat ovaries exposed to a normal, physiological concentration of leptin *ex vivo* had no difference in the composition of their follicular pools compared to control treated ovaries. Primordial ($P = 0.32$; Figure 2.1A) and small primary ($P = 0.48$; Figure 2.1B) follicles were observed in equal abundance when comparing the control treated ovaries to those treated with leptin at a concentration of 10 ng/mL. Similarly, large primary ($P = 0.67$; Figure 2.1C) and secondary ($P = 0.74$; Figure 2.1D) follicles were equally abundant when comparing both treatments. The total number of healthy ($P = 0.13$; Figure 2.1E) and unhealthy ($P = 0.06$; Figure 2.1F) follicles did not differ due to leptin treatment.

PND4 rat ovaries were also exposed to a concentration of leptin measurable in obese mice (40 ng/mL [56]). Primordial ($P = 0.44$; Figure 2.2A), small primary ($P = 0.27$; Figure 2.2B), large primary ($P = 0.48$; Figure 2.2C), and secondary ($P = 0.14$; Figure 2.2D) follicles were not different in abundance compared to control treated ovaries. The overall abundance of morphologically healthy ($P = 0.39$; Figure 2.2E) follicles was not observed to differ between treatments, nor did the number of unhealthy follicles ($P = 0.08$; Figure 2.2F).
Gestational hyperleptinemia does not alter PI3K pathway member Akt1 mRNA in ovaries of female offspring

When normalizing to β-actin, offspring of Db/+ dams showed no difference (P = 0.08) in mRNA abundance of ovarian Akt1 compared to offspring of WT females (Figure 2.3A). A similar trend was observed in the offspring of Lpt females as their ovaries displayed no difference (P = 0.21) in the abundance of Akt1 mRNA (Figure 2.3D). Gapdh was not observed to vary significantly between Db/+ and WT offspring (P = 0.23) or between Lpt and Sal offspring (P = 0.39). Similarly, β-actin did not differ between the offspring of WT and Db/+ dams (P = 0.21) or Sal and Lpt dams (P = 0.30).

Steroidogenic gene expression is affected by gestational hyperleptinemia in ovaries of female offspring

StAR mRNA abundance was decreased (P < 0.05) in offspring of Db/+ dams compared to WT when β-actin was used as an endogenous control (Figure 2.3C). However, offspring of Lpt dams had increased (P < 0.05) abundance of ovarian StAR mRNA compared to offspring of Sal females when using β-actin as the endogenous control. There was no impact of gestational hyperleptinemia due to genotype (P = 0.07; Figure 2.3B) or Lpt infusion (P = 0.20; Figure 2.3E) on offspring Cyp11a1 mRNA abundance.

Gestational hyperleptinemia reduces ovarian Ephx1 mRNA abundance in offspring

Gestational hyperleptinemia in Db/+ or Lpt dams did not impact mRNA encoding Cyp2e1 (Figure 2.4A; (P = 0.25), E (P = 0.15)), Gstm (Figure 2.4B (P = 0.36); F (P = 0.40)), or Gstp (Figure 2.4C (P = 0.32); G (P = 0.10)) in offspring ovaries. Ovaries of Db/+ dams'
offspring had equally abundant \( P = 0.45 \) Ephx1 mRNA when compared to those of WT offspring (Figure 2.4D). Ovarian Ephx1 mRNA was less abundant (24.7\%; \( P < 0.05 \)) in offspring of Lpt compared to Sal females when standardized to \( \beta\)-actin (Figure 2.4H).

**Use of an alternative endogenous control gene impacts observed effect of maternal gestational hyperleptinemia on offspring mRNA abundance.**

When standardized to Gapdh, offspring of Db/+ females did not differ \( P = 0.15 \) in the abundance of ovarian Akt mRNA compared to wild type controls (Figure 2.5A). Consistent with this result, ovarian Akt mRNA abundance was not altered \( P = 0.16 \) in offspring of Lpt compared to Sal dams (Figure 2.5D). The abundance of mRNA encoding the downstream target of AKT, Foxo3a, was not different \( P = 0.18 \) in Db+ offspring compared to offspring of WT females (Figure 2.5B). Foxo3a mRNA abundance was not impacted \( P = 0.18 \) in offspring of Lpt dams relative to Sal dams (Figure 2.5E). The offspring of Db/+ dams had increased (14.6\%; \( P < 0.05 \)) Ephx1 mRNA abundance, relative to their respective WT controls. Offspring of Lpt dams tended to have increased \( P = 0.05 \) abundance of mRNA encoding Ephx1 when compared to the offspring of Sal dams (Figure 2.5F).

When using Gapdh as the endogenous control, mRNA abundance of the steroidogenic genes StAR (Figure 2.6A \( P = 0.28 \); D \( P = 0.26 \)), Cyp11a (Figure 2.6B \( P = 0.39 \)); E \( P = 0.27 \)) or Cyp19a (Figure 2.6C \( P = 0.27 \); F \( P = 0.43 \)) were not different between the offspring of dams experiencing genetically-induced (Db/+ or pump-induced (Lpt) hyperleptinemia during gestation compared to their respective control.
Offspring of hyperleptinemic dams have altered EPHX1 protein abundance

Compared to their respective controls, offspring of hyperleptinemic dams had no difference in phosphorylation of AKT ($P = 0.46$; Figure 2.7 A,E) or its total protein abundance ($P = 0.36$; Figure 2.7 B,F). In addition, there was no impact of gestational hyperleptinemia exposure on StAR protein level in the offspring of DB/+ dams compared to the offspring of WT dams ($P = 0.32$; Figure 2.7 C,G). In contrast, offspring of Lpt pump dams had increased ($11.2\%; P < 0.05$) EPHX1 protein abundance relative to the Sal offspring (Figure 2.7 D,H).

Discussion:

Body mass and the timing of puberty were formally correlated for the first time in rats in 1963 [38] and later extended to humans in 1970 [57]. Existence of a link between body mass and cyclicity as a mechanism to regulate fertility in response to the body's energy reserves made sense from an evolutionary standpoint, but such a signal was not characterized until the purification of leptin in 1995 [17]. This adipokine was soon shown to be capable of regulating the circulating concentrations of gonadotropins [37]. In addition, leptin can directly regulate ovarian function. LEPR is expressed in the ovaries of a variety of species, specifically in follicles, with varying distributions between species [27–34]. Mice in particular have an increased density of LEPR in the thecal layer compared to the oocyte and granulosa cells [27]. This potentially infers that follicles are most likely sensitive to leptin from the secondary stage onwards. Leptin's direct effects on ovarian function were demonstrated *ex vivo* by Ryan et al. when they demonstrated leptin as a positive regulator of GVBD and ovulation [27]. *In vivo*, administration of a LEPR antagonist to postnatal rats was correlated with the depletion of primordial and primary follicles without an observed increase in accumulated tertiary follicle
numbers [35]. In contrast, passive immunization against leptin was shown to enhance early folliculogenesis [36]. Both studies demonstrate a direct influence of leptin on ovarian function \textit{in vivo}. Such results support the view that leptin is capable of regulating reproductive function by directly interacting with the ovary.

Obese individuals tend to have increased serum leptin concentrations [40,41], attributable to the accumulation of adipose tissue in obese individuals, as adipocytes are major producers of leptin [17]. Due to elevated leptin concentrations during obesity and the expression of LEPR in the ovary, we investigated whether ovarian leptin signaling could be a participant in the reproductive phenotype observed during obesity.

Our interest in leptin arises from our findings of altered ovarian function in two models of obesity. Effects of hyperphagia-induced obesity on ovarian function were investigated using the LY mouse model [53]. LY mice have an abnormal agouti signaling pathway which leads to uncontrolled appetite and subsequent obesity when fed \textit{ad libitum}. We observed that ovarian steroidogenic enzymes had altered protein abundance: StAR was reduced, while the abundance of CYP19A1 and CYP11A1 were increased in LY mice. We also observed a depletion of primordial and primary follicles in LY mice beginning at 12 weeks of age, and a tendency for increased accumulation of tertiary follicles in the LY mice beginning at the same time point [53]. We also examined the effects of inducing obesity using a high-fat diet on ovarian function in mice [54]. In this study, obese females tended to have reduced phosphorylated AKT, reduced \textit{Cyp2e1} mRNA levels, and a non-significant trend for increased ovarian EPHX1 protein abundance.
We hypothesized that leptin exposure during gestation would cause offspring to display changes in ovarian physiology in similar areas as our previous studies in obese models, namely altered PI3K signaling and chemical metabolism. In addition, leptin's signaling through PI3K [58] combined with ovarian expression of LEPR could enable it to act as a modulator of growth and proliferation in the ovary. Our first hypothesis was that animals exposed to hyperleptinemia during gestation would have altered PI3K signaling, steroidogenic gene expression, and chemical metabolism. Our second hypothesis was that exposure to leptin would accelerate early folliculogenesis in the absence of circulating gonadotropins, which we examined using an ovarian *ex vivo* culture system.

In this project, two modes of inducing hyperleptinemia were examined. Hyperleptinemia was induced by direct infusion of saline or recombinant leptin into circulation using a subcutaneous osmotic pump prior to mating and during gestation [55]. The genetic *Db/+* mouse was also used since leptin resistance in this model is compensated for by increased production of leptin, causing increased circulating leptin without any other noticeable phenotypic changes. We then observed the effects of gestational hyperleptinemia on the WT offspring at 20 weeks of age to determine whether or not gestational leptin concentrations had lasting effects on their ovarian function. Recombinant leptin has been the standard practice when administration of exogenous leptin for many years and has been shown to be biologically active in many experiments prior to this [29,59–61].

Lpt mice had elevated fasting serum levels of leptin of around 50 ng/mL compared to around 30 ng/mL in their saline pump fitted counterparts. WT mice averaged about 50 ng/mL of
fasting serum leptin, while Db/+ animals had systemic leptin in the range of 125 ng/mL. Lpt females gave birth to offspring with leptin levels that were 32% lower than those born from Sal females. Parallel to this trend were the circulating levels of Db/+ females’ offspring, who had a reduction of 66% in their circulating leptin levels. The differences between the offspring exposed to pump-induced hyperleptinemia compared to offspring exposed to genetically-induced hyperleptinemia could be explained by the differences between the circulating leptin levels in the dams. Db/+ dams circulated about 2.5 times the leptin concentration compared to their leptin pump counterparts [55].

Leptin signaling is carried through the PI3K pathway [16]. PI3K controls the growth and development of follicles through phosphorylation of AKT [10]. Downstream of AKT is FOXO3A; a transcription factor that suppresses the activation of the primordial oocyte until it is removed from its nucleus [11–13]. We had previously observed reduced phosphorylation of ovarian AKT in mice who developed obesity induced by a high-fat diet, which indicated a decrease in PI3K signaling in these animals. Ovarian Akt1 mRNA content in offspring was not affected by hyperleptinemia in the dams, nor was the abundance or phosphorylation of ovarian AKT. These results indicate that exposure to increased leptin during gestation is insufficient to induce changes in offspring PI3K signaling at 20 weeks of age. However, this does not exclude any early effects that could imprint the ovaries.

Ovarian steroidogenesis is primarily inactive until puberty, and, similar to cyclic recruitment of follicles, is regulated by gonadotropins through PI3K signaling [62]. We previously observed a reduction in StAR protein during progressive obesity, beginning at 12
weeks onwards. In addition, CYP11A1 and CYP19A1 were decreased during progressive obesity [53]. Obesity shortened the time spent in the proestrus and estrus phases of the estrous cycle, and lengthened the time spent in diestrus [53]. Obesity was also demonstrated to be correlated with increased levels of circulating testosterone in human females [63], indicating altered steroidogenesis.

Our examination of steroidogenic genes in the mice exposed to hyperleptinemia in utero revealed no impact of leptin on mRNA abundance of Star, Cyp11a1, Cyp19a1, relative to their respective controls. Ovarian protein levels of StAR were also unchanged in offspring of Db/+ females. These results demonstrate a lack of effect of hyperleptinemia during gestation on the expression of steroidogenic genes at 20 weeks of age in this model, potentially indicating that hyperleptinemia might not be responsible for abnormalities in the concentration of circulating steroid concentrations during obesity, at least not through influencing the abundance of ovarian steroidogenic genes and proteins examined herein.

We have previously made the novel discovery that ovarian levels of proteins involved in metabolism of environmental toxicants are altered in obese females [64,65]. In addition, we have determined that the PI3K signaling pathway regulates ovarian expression of these genes [66,67]. In light of the crucial role of EPHX1 in detoxifying [67] or bioactivating [68] chemicals which can destroy ovarian follicles and induce DNA damage, we examined the impact of gestational hyperleptinemia on offspring ovarian EPHX1 abundance. Offspring of Lpt dams had increased total EPHX1 abundance at 20 weeks of age compared to the controls, with a concomitant trend for increased Ephx1 mRNA. Interestingly, the offspring of the Db/+ females had an opposing
trend as their Ephx1 mRNA levels was lower than their control females. Due to limited animal numbers, investigation of EPHX1 protein was not possible in the offspring of females with this genotype. Changes in EPHX1 abundance could reflect in the ovarian capacity to metabolize ovotoxicants, and could in turn augment the ovarian response to a variety of chemical stressors. This observation fits with our previous finding of increased ovarian EPHX1 during hyperphagia-induced obesity [64] and suggest that maternal hyperleptinemia during gestation could impact the ovarian capacity of the offspring to metabolize toxicants.

To test whether the observed depletion of small follicles in obese females could be attributed to differences in the concentrations of circulating leptin, we exposed PND4 rat ovaries to leptin in culture. We chose leptin concentrations of 10 ng/mL and 40 ng/mL as they are representative of circulating leptin concentrations in well-fed females and in obese females, respectively [56]. We anticipated that ovaries exposed to leptin to have enhanced early folliculogenesis. Leptin, however, had no impact on the composition or morphological health of the ovarian pool at either concentration tested.

These data suggest that circulating leptin does not directly regulate the recruitment of primordial follicles, or the development of follicles prior to the mid- to late-secondary stage. Observing no changes in early folliculogenesis in response to leptin is consistent with the distribution of leptin receptors in rodents. Postnatal ovaries used in the culture system used herein lack any tertiary follicles, and even when taking culture time into account, are not mature enough to develop large secondary follicles with a well-developed thecal layer, which could explain the lack of response to exogenous leptin. However, these results do not exclude an
indirect action of leptin on the ovaries. Leptin is capable of changing the localization of ATP-sensitive K⁺ ion channels in pancreatic beta cells, potentially leading to a change in glucose sensitivity [69], and in turn affecting the patterns of insulin secretion. If so, leptin could influence recruitment of primordial follicles through insulin, as insulin has been shown to positively regulate the recruitment of primordial follicles [70]. Being absent in culture, insulin's effect as regulated by leptin would not be apparent when treating ovaries with leptin *ex vivo*. Another indirect effect of leptin that could account for the disparity between *in vivo* and *ex vivo* observations is its role in augmenting vascularization. Leptin has been shown to promote angiogenesis and fenestration, processes thought to participate in folliculogenesis by inducing a selective delivery of circulating factors [60,71,72].

Taken as a whole, these results show that gestational hyperleptinemia did not impact PI3K signaling as mediated by AKT, nor did it change the expression of ovarian steroidogenic enzymes. Cultured ovaries lacking changes in folliculogenesis when exposed to leptin is consistent with these results. Interestingly, the expression of *Ephx1*, both at the mRNA and protein level, was impacted. This suggests that hyperleptinemia could impact the ovarian capacity to metabolize chemicals, as observed in obese females, and that the offspring of obese females could also have their ovarian chemical metabolism pathways augmented due to obesity-induced gestational hyperleptinemia.
**Table 2.1: Primer sequences used in this study**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5' to 3')</th>
<th>amplicon size</th>
</tr>
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<tbody>
<tr>
<td>β-actin</td>
<td>Forward: TCTATCCTGGCCTCACTGTC</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACGCAGCTCAGTAACAGTCC</td>
<td></td>
</tr>
<tr>
<td>Gapdh</td>
<td>Forward: GTGGAACCTCATGGCCTACAT</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGATGGAATTTGTGAGGGAGA</td>
<td></td>
</tr>
<tr>
<td>Akt1</td>
<td>Forward: ACTGTTCCCTCGCTACGGTCAAG</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>Reverse: TTGTAGCCAATAAAGGTGCCAT</td>
<td></td>
</tr>
<tr>
<td>Star</td>
<td>Forward: ATGTTCCTCGCTACGGTCAAG</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCCAGTGCTCTCCAGTTGAG</td>
<td></td>
</tr>
<tr>
<td>Cyp191</td>
<td>Forward: ATGTCTTGGAAATGCTGAAACC</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGGACCTGGATTTGAAGACGAC</td>
<td></td>
</tr>
<tr>
<td>Cyp11a1</td>
<td>Forward: ATGTTCCTCGCTACGGTCAAG</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCCCTGTAATGGGGCCCATAC</td>
<td></td>
</tr>
<tr>
<td>Ephx1</td>
<td>Forward: GTGCCTAAACACGACACTGCA</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCTCCAGAAGACACCACCTT</td>
<td></td>
</tr>
<tr>
<td>Cyp2e1</td>
<td>Forward: ATGTTCCTCGCTACGGTCAAG</td>
<td>210</td>
</tr>
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<td></td>
<td>Reverse: CCCCTGTAATGGGGCCCATAC</td>
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<tr>
<td>Gstm</td>
<td>Forward: GAGAGGATCCGTGCAGACAT</td>
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<td></td>
<td>Reverse: ACTTGGGGCTAAACCATACGG</td>
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<tr>
<td>Gstp</td>
<td>Forward: CCAAAGTTTGAGGATGGAAGA</td>
<td>212</td>
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<td></td>
<td>Reverse: CAGGGGCTTCACGTAGTCAT</td>
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<tr>
<td>Foxo3a</td>
<td>Forward: CTGGGGGAACCTGTCTATG</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCATTCTGAAACGCGCATGAAG</td>
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</table>
Figure 2.1. Impact of leptin exposure at basal physiological level on ovarian folliculogenesis. PND4 rat ovaries were exposed to 0 (n = 3) or 10 ng/mL (n = 4) of recombinant leptin for 7 d and different classes of ovarian follicles counted. Leptin treatment at 10 ng/mL induced no difference in the average abundance of follicles at the (A) primordial, (B) small primary, (C) large primary, or (D) secondary stages. Leptin exposure also did not affect the abundance of morphologically (E) healthy, or (F) unhealthy follicles.
Figure 2.2. Impact of leptin exposure at concentration observed during obesity on ovarian folliculogenesis.

PND4 rat ovaries in culture were exposed to 0 ng/mL (n = 4) or 40 ng/mL (n = 8) of recombinant leptin for 7 d. Leptin at a concentration observed during obesity, did not impact the number of (A) primordial, (B) small primary, (C) large primary, or (D) secondary follicles. The health of the follicular pool was not affected by leptin as (E) healthy and (F) unhealthy follicles were equally numerous between treatments.
Figure 2.3. Effect of maternal gestational hyperleptinemia on ovarian mRNA abundance in offspring.

Using whole mRNA extracted from whole ovarian homogenate, the expression of Akt1 and steroidogenic genes (Star and Cyp11a1) were evaluated in offspring of hyperleptinemic Db/+ (n = 3) and Lpt (n = 3) dams. Mean delta Ct values are depicted, which indicate higher mRNA expression when delta Ct values are lower. Standardized to β-actin, offspring of Db/+ had no changes in ovarian (A) Akt1 or (B) Cyp11a1 mRNA abundance compared to WT. (C) Db/+ offspring had decreased (41.8%; \( P < 0.05 \)) abundance of ovarian Star mRNA compared to WT. Compared to Sal offspring, Lpt offspring ovarian (D) Akt and (E) Cyp11a1 mRNA were equally abundant. (F) Star mRNA was increased (44.7%; \( P < 0.05 \)) in Lpt offspring compared to Sal offspring. * indicates different from control, \( P < 0.05 \).
Figure 2.4. Impact of maternal gestational hyperleptinemia on ovarian mRNA expression of genes encoding enzymes involved in xenobiotic biotransformation.

Whole ovarian homogenates from offspring of hyperleptinemic dams were utilized to compare the expression of chemical metabolism genes between treatments. Standardized to β-actin, Dbl+ offspring (n = 3) ovarian mRNA levels of (A) Cyp2e1 (P = 0.25), (B) Gst pi (P = 0.36), (C) Gst mu (P = 0.32), and (D) Ephx1 (P = 0.45) were not different from WT mice. Ovarian levels of Lpt offspring (n = 3) (E) Cyp2e1 (P = 0.15), (F) Gst pi (P = 0.10), and (G) Gst mu (P = 0.40) mRNA were not different compared to Sal offspring. (H) Ephx1 mRNA was decreased (24.66%; P < 0.05) in offspring of Lpt dams compared to Sal dams. * indicates different from control, P < 0.05.
Figure 2.5. Impact of maternal gestational hyperleptinemia on offspring mRNA.
The effect of maternal gestational hyperleptinemia on mRNA encoding ovarian genes of interest was also evaluated using Gapdh as the internal control. Offspring of Db/+ (n = 3) had no change in the abundance of ovarian (A) Akt ($P = 0.15$) or (B) Foxo3a ($P = 0.18$) mRNA compared to WT. Db/+ offspring had increased (14.65%; $P < 0.05$) abundance of ovarian (C) Ephx1 mRNA compared to WT. Lpt offspring (n = 3) had no difference in the abundance of ovarian (D) Akt ($P = 0.16$), (E) Foxo3a ($P = 0.18$), or (F) Ephx1 ($P = 0.05$) mRNA relative to Sal dam offspring.
Figure 2.6. Impact of maternal gestational hyperleptinemia on offspring steroidogenic mRNA.

The effect of maternal gestational hyperleptinemia on mRNA encoding ovarian steroidogenic genes was also evaluated using Gapdh as the internal control. Offspring of Db/+ (n = 3) had no change in the abundance of ovarian (A) Star ($P = 0.28$), (B) Cyp11a1 ($P > 0.39$), or (C) Cyp19a1 ($P = 0.27$) mRNA compared to WT. Lpt offspring (n = 3) did not differ in their abundance of ovarian (D) Star ($P = 0.26$), (E) Cyp11a1 ($P = 0.27$), or (F) Cyp19a1 ($P = 0.43$) mRNA when compared to the offspring of the Sal dams.
Figure 2.7. Maternal gestational hyperleptinemia impact on ovarian protein abundance. Whole ovarian protein homogenates from Lpt and Sal dam offspring (n = 3) were analyzed by western blotting to evaluate abundance of proteins involved in PI3K signaling (AKT and pAKT0, steroidogenesis (StAR) and chemical metabolism (EPHX1). Average densitometric intensities of appropriately sized protein bands were standardized to Ponceau S total protein staining. There was no difference in ovarian abundance of (A,E) AKT ($P = 0.36$) (B,F) pAKT ($P = 0.46$) or (C,G) StAR ($P = 0.32$) protein content. (D,H) EPHX1 levels were increased ($P < 0.05$) in ovaries of Lpt dam offspring compared to Sal. * indicates different from control, $P < 0.05$. 
Appendix Figure 1. Maternal hyperleptinemia during gestation reduced serum leptin in offspring. Serum leptin was quantified by ELISA in offspring who experienced exposure to maternal hyperleptinemia during gestation. Reduced average serum leptin concentration was demonstrated in offspring of dams who experienced hyperleptinemia due to (A) genotype or (B) Lpt infusion. * indicates different from control, $P < 0.05$. Performed by the research group of Dr. Laura Schulz, University of Missouri, and obtained through personal communication.
Bibliography:


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CHAPTER 3
IMPACT OF HEAT STRESS ON OVARIAN PATHWAYS REGULATING FOLLICULOGENESIS AND STEROIDOGENESIS IN SYNCHRONIZED POST-PUBERTAL GILTS

Ahmad Al-Shaibi, Ben J. Hale, Candice L. Hager, Jason W. Ross, Lance H. Baumgard, Aileen F. Keating*
Department of Animal Science, Iowa State University, Ames, IA, 50014

Contribution Statement: Animal experiments were led by CLH and the Baumgard and Ross research groups. BJH performed protein extractions and aided in ovary collection. AAA executed the molecular and statistical analyses, with the exception of estradiol and LBP measurements, and wrote this manuscript. AFK designed the experiments and aided with data interpretation. AAA was mentored by AFK, who edited this chapter and shall be the corresponding author when submitting the research article.

*Corresponding author: Aileen F. Keating, Ph.D., Department of Animal Science, Iowa State University, Ames, IA 50014; Telephone 515-294-3849; Email akeating@iastate.edu
Abstract:

Heat stress (HS) causes an estimated $299 million dollars in annual loss to the US swine industry. HS induces seasonal infertility; a period of reduced fertility in pigs observed during summer. The phenotypic response to HS manifests as altered folliculogenesis, steroidogenesis, embryonic, and fetal development. Systemic, physiological hallmarks of HS include hyperinsulinemia and endotoxemia. Insulin regulates ovarian function through the insulin receptor (INSR), and can influence ovarian function via phosphatidylinositol-3 kinase (PI3K) signaling, an event induced by phosphorylation of tyrosine residues of INSR substrates (IRS) by INSR. PI3K phosphorylates protein kinase B (AKT) which, in turn, regulates ovarian steroidogenesis and folliculogenesis. AKT regulation of folliculogenesis is mediated by forkhead transcription factor 3A (FOXO3A) as it is translocated from the nucleus of the primordial oocyte upon its phosphorylation, which begins the activation of the primordial follicle. Endotoxemia can also regulate ovarian activity through the PI3K pathway. Integrity of the intestinal barrier is reduced in heat-stressed animals which leads to debris from the intestinal flora to leaking into the bloodstream. This leads to endotoxemia, as endotoxins are part of the composition of this floral debris. Lipopolysaccharides (LPS) are endotoxins, and an increase in their circulating concentration is a classic consequence of endotoxemia. Toll-like receptor (TLR) 4 is the receptor that binds and initiates the cellular response to LPS, in part, through the PI3K pathway. We previously investigated the ovarian effects of HS in pre-pubertal gilts, and discovered increased abundance of INSR, IRS-1, AKT1, LDLR, and LHR mRNA during HS. We also observed increased phosphorylation of AKT and increased abundance of StAR and CYP19A (mRNA and protein). We hypothesized that HS would induce similar effects in post-pubertal gilts. Post-pubertal gilts (n = 6) were exposed to thermal neutral (TN) or cyclical HS (12 h per day) for 5 d
after 14 d of oral Matrix\textsuperscript{©} administration. Increased \((P < 0.05)\) abundance of ovarian INSR, reduced \((P < 0.05)\) phosphorylation of AKT, and increased \((P < 0.05)\) abundance of TLR4 were observed. Concentrations of 17\(\beta\)-estradiol and LPS binding protein (LBP) were unaltered in the follicular fluid of HS gilts compared to TN gilts. Protein abundance of AKT, CYP19A, and StAR were not changed in HS gilts compared to TN. Ovarian mRNA abundance of \textit{StAR}, \textit{IRS1}, \textit{LHR}, \textit{LDLR}, \textit{CYP17A1}, \textit{CYP19A1}, \textit{AKT1}, and \textit{INSR} did not differ between HS and TN gilts. These data indicate increased ovarian insulin sensitivity, an increased LPS response as well as reduced PI3K activation due to HS that could contribute to seasonal infertility in swine.

**Keywords:** Heat stress, Ovary, Steroidogenesis, Folliculogenesis
Introduction:

Heat stress (HS) is a condition of a sustained increase in core body temperature due to increased ambient temperature. HS jeopardizes livestock production and animal welfare. The American swine industry alone suffers an estimated $299M in annual losses due to HS [1]. Climate change is predicted to increase the intensity of heat waves [2], potentially leading to HS posing a sustained challenge to animal agriculture and food security.

The US swine industry experiences seasonal infertility as a consequence of reduced conception rates and increased embryonic deaths, particularly during July, August and September [3,4]. In the months of August, September, and October, day 28 pregnancy rates reach their lowest levels, with a subsequent reduction in farrowing rates occurring in November and December. In studies performed mainly in ruminants, HS impacted follicular development [5,6], steroidogenesis [5–8], embryonic [3,9,10] and fetal [10,11] development, indicating a major role of HS in seasonal impairment of reproductive performance.

Hyperinsulinemia was observed repeatedly in production animals, including pigs, in response to HS, which seems paradoxical to HS-induced reduction in feed intake [12–17]. Interestingly, HS was also demonstrated to increase insulin sensitivity in pigs [18]. Heat treatment also prevented high-fat diet-induced insulin resistance and improved glucose tolerance in rats [19]. Insulin functions by binding to the insulin receptor (INSR) which subsequently recruits and activates INSR substrates (IRS) [20–23]. IRS-mediated INSR signaling is largely conducted via phosphatidylinositol-3 kinase (PI3K) [24–26]. PI3K signaling is primarily mediated through the actions of protein kinase B (AKT) [27]. Ovarian AKT regulates the
activation and growth of primordial follicles through the forkhead transcription factor (FOXO3A) [28–30]. Primordial follicles are finite [31], meaning their depletion can be accelerated if they are overactivated. PI3K signaling also regulates ovarian steroidogenesis [26], which is responsible for the production of 17β-estradiol (E₂) and progesterone (P₄) from the tertiary follicles and corpus luteum, respectively [31,32].

We previously investigated the ovarian response to HS in pre-pubertal gilts [33], and demonstrated increased abundance of ovarian INSR and IRS-1 mRNA after 7 and 35 d of HS. Increased ovarian insulin signaling activation via IRS-1 phosphorylation at the Tyr⁶³² residue was also observed in heat-stressed animals. Additionally, AKT signaling was upregulated, evidenced by increased abundance of ovarian AKTI mRNA and phosphorylation of AKT in heat-stressed gilts compared to their thermal neutral controls. These data suggested that folliculogenesis could be altered due to HS. In addition, HS increased ovarian STAR and CYP19A (mRNA and protein), as well as LDLR and LHR mRNA abundance. Thus, despite being pre-pubertal, HS altered steroidogenic pathways in gilts.

Systemic HS reduces intestinal wall integrity resulting in subsequent endotoxemia; characterized by increased circulating lipopolysaccharide (LPS) [16,34]. Toll-like receptor (TLR) 4 is the primary receptor through which LPS signaling is mediated [35,36], and TLR4 can mediate its effects through the PI3K pathway [37]. In terms of ovarian function, LPS has been shown to deplete primordial follicles in mice and cows [38]. LPS is capable of inducing early loss of pregnancy through abortion or resorption in mice [39–41]. Administration of LPS to cattle increased prostaglandin F₂alpha production in cultured bovine endometrial cells [42], and
reduced the size of the corpus luteum and circulating P₄ concentrations in vivo [43]. In addition, LPS was demonstrated to reduce the expression of the P₄ receptor in the uteri of mice [44].

Based on our data in pre-pubertal gilts and the potential for LPS to be at least partially culpable in altering ovarian function, thereby contributing to seasonal infertility in swine, we hypothesized that HS during the follicular phase would alter ovarian function in post-pubertal estrous synchronized gilts in a manner similar to pre-pubertal gilts and that ovarian LPS signaling would be activated during HS.

**Materials and Methods:**

**Reagents:**

Primers were designed using Primer 3 output with the exception of GAPDH which was designed using Primer-BLAST. Primers were synthesized by the DNA facility at Iowa State University (Ames, IA). Ponceau S, hydrochloric acid, Sodium Phosphate Monobasic, and Sodium Phosphate Dibasic, were obtained from Fisher Scientific (Waltham, MA). SignalFire ECL reagent was acquired from Cell Signaling Technology (Danvers, MA). Ammonium persulfate, paraformaldehyde (PFA), Bovine Serum Albumin (BSA), Glycine, 30% Acrylamide/Bis-acrylamide, 2-b-mercaptoethanol, sodium dodecyl sulfate (SDS), Tween-20, Tris base, Tris HCl, sodium chloride, and N0, N0, N0, N0-tetramethylethylenediamine (TEMED), were acquired from Sigma-Aldrich, Inc. (St. Louis, MO). Superscript III one-step RT-PCR system, dNTP mix, iBlot 2 dry transfer system, and iBind western system, were purchased from Invitrogen, Co. (Carlsbad, CA).
Animals and sample collection

12 post pubertal gilts (126.02 ± 21.6 kg) were synchronized in their follicular phase through oral administration of Matrix® for 14 d. The gilts were then divided into two groups and exposed to thermal neutral conditions (TN; 20.3°C ± 0.1°C) or cyclical heat stress conditions (HS; 26°C during 12 dark hours and 32°C during 12 light hours) to simulate naturally-occurring HS patterns. Heat treatment was administered for 5 d following Matrix® withdrawal. During the treatment period, both TN and HS animals were limit-fed to 3 kg of feed per day. After 5 d, animals were euthanized using captive bolt penetration and the ovaries were excised. Dominant follicles were aspirated of their follicular fluid which was stored in pyrogen-free tubes on ice, followed by centrifugation at 9300 x g to produce a pellet. The supernatant was stored at -80°C in pyrogen-free tubes. One ovary from every animal was flash-frozen in liquid nitrogen and stored at -80°C while the contralateral ovary was fixed in 4% paraformaldehyde overnight before being stored in 70% ethanol. The Iowa State University Institutional Animal Care and Use Committee approved all experimental procedures involving animals.

RNA isolation and quantitative RT-PCR

Ovaries were powdered using a mortar and pestle and RNA was extracted using an RNeasy Mini Kit according to the manufacturer’s recommended protocol. Samples were treated with DNaseI to remove genomic DNA. RNA concentration and purity were measured using an ND-1000 Spectrophotometer (260/280 nm; NanoDrop Technologies, Inc.). mRNA was reverse-transcribed to cDNA using the Superscript III system and an oligo dT primer. Diluted (2 µl; 1:25) cDNA was amplified using the QuantiTect SYBR® Green PCR Kit on an Eppendorf MasterCycler RealPlex4 using gene-specific primers as detailed in Table 1. The thermocycler
program consisted of an initial hold at 95°C for 15 min, followed by 50 cycles of 95°C melting for 15 sec, annealing at 57°C for 30 sec, and extension at 68°C for 1 min, which was when amplification was measured. Melting curve analysis was conducted using a 60 to 99°C ramp of 1°C per step. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control gene to which all measurements were standardized as no variation between treatments was observed. Delta Ct values for the experimental genes of interest were calculated by normalization to the Ct values for GAPDH. To test for genomic contamination, a pool of purified RNA was created after extraction and was run through the cDNA synthesis protocol without including reverse transcriptase to the reaction. This reaction was then tested for GAPDH signal using RT-PCR protocol as explained above and no contamination was found. Additionally, samples primers were tested for template contamination by running them with no template, and cDNA was tested for primer contamination by being run with no primers.

**Protein extraction and western blotting**

Total ovarian protein was extracted from whole tissue homogenate. A sample of ovarian tissue was homogenized using a handheld homogenizer and lysed in phosphate lysis buffer (10mM; 0.69g monobasic sodium phosphate, 1.34g dibasic sodium phosphate, and 20g SDS in 1L of double-distilled water at pH 7.0). The protein homogenate was applied to a Qiashredder column and centrifuged at 9300 x g for 15 s. The lysate was centrifuged for 15 min at 9300 x g at 4°C and the supernatant placed on ice for 10 min before being recentrifuged for 15 min at 9300 x g at 4°C. Extracted protein was quantified using a Nanodrop® (A-280 nm; k = 260/280 nm; NanoDrop Technologies, Inc.) and was stored at -80°C. Protein was separated using Mini-PROTEAN® TGX™ 4-20% Precast Gels and then transferred onto a nitrocellulose membrane.
using Life Technology’s iBlot® 2 Dry Blotting System. Transfer quality was assessed by Ponceau S staining (0.5% Ponceau S in 1% Acetic Acid solution). Membranes were washed with phosphate-buffered saline (PBS) with Tween 20 (PBST) then blocked overnight in 5% BSA in PBST at 4°C with constant shaking. For TLR4 detection, the membrane was blocked in 2.5% BSA in PBST at 4°C with constant shaking. Primary antibodies were diluted as follows: anti-AKT (1:1000 in 5% BSA in PBST; Cell Signaling; 9272), anti-pAKT<sup>Thr308</sup> (1:1000 dilution in 5% BSA in PBST; Cell Signaling; C31E5E), anti-StAR (1:1000 dilution in 5% BSA in PBST; Cell Signaling; D10H12), CYP19A (1:500 dilution in 5% BSA in PBST; Cell Signaling; D5Q2Y), and anti-TLR4 (1:250 dilution in PBST; Santa Cruz; 8694). Membranes were incubated in their respective primary antibody dilutions overnight at 4°C with constant shaking then washed with PBST for three rounds of 10 minutes each at room temperature. Membranes were then incubated in a 1:5000 dilution of the species appropriate antibody (goat anti-rabbit in 5% BSA in PBST; donkey anti-goat in 1% BSA in PBST) for 1 h at room temperature with constant shaking. Membranes were washed with PBST for three rounds of 10 min each before being immersed in ECL Reagent for 10 min. X-ray film was then exposed to the membranes. Appropriate sized protein bands were quantified by densitometric analysis using ImageJ v1.49v software. Densitometric measurements of the protein band of interest was normalized by dividing by densitometric values obtained from Ponceau S images. Pooled protein samples were blotted as explained above and incubated in each primary antibody, each secondary antibody, and rabbit IgG or normal goat serum with rabbit anti-goat and goat anti-donkey secondary antibodies respectively. Signal was detected as explained above.
**Follicular fluid E\(_2\) quantification**

Aspirated follicular fluid was diluted in 1% BSA buffer (1 g BSA, 0.12 g sodium phosphate monobasic, anhydrous, 0.88 g sodium chloride, 0.01 g sodium azide, 100 mL ddH\(_2\)O, at 7.0-7.2 pH). Serial dilutions of 1:10, 1:100, 1:1000, and 1:10000 were prepared and measurement of E\(_2\) concentration in follicular fluid was via radioimmunoassay performed by Dr. George Perry at South Dakota State University.

**Follicular fluid LBP measurement**

Hycult Biotech's LBP ELISA kit (HK503) was used to quantify LPS binding protein (LBP) content of follicular fluid as per the manufacturer's recommendation.

**Statistical analysis:**

Statistical analysis was performed by comparing ΔCt values derived from qRT-PCR between TN and HS gilts. For protein abundance comparisons, densitometric measurements for the protein of interest were divided by densitometric measurements for total protein. Statistical comparison for both RNA and protein data between TN and HS treatments were performed using a 2-tailed Student's t-test calculated in Microsoft Excel using the function t.test(<array 1>;<array 2>;2;2). Graphs were generated using GraphPad Prism 6 software, where columns denote mean values while error bars represent the standard error of the mean. Statistical difference was considered if the \(P\)-value was less than 0.05.
Results:

Rectal temperature was increased in response to HS

Heat-stressed gilts averaged a rectal temperature of 39.48°C compared to the 38.68°C of TN controls ($P < 0.05$) during daytime.

HS alters ovarian insulin signaling

There was no effect of HS on mRNA encoding INSR ($P = 0.94$; Figure 3.1A) or IRS1 ($P = 0.29$; Figure 3.1B). Ovarian INSR1b abundance was increased (19%; $P < 0.05$) in HS compared to TN gilts (Figure 3.1C).

HS reduces AKT phosphorylation

The abundance of ovarian AKT1 mRNA was unaltered ($P = 0.46$; Figure 3.2A) in HS compared to TN gilts. Neither was there any effect of HS on FOXO3A mRNA level ($P = 0.71$; Figure 3.2B). Total ovarian AKT abundance did not differ ($P = 0.05$) between HS and TN gilts (Figure 3.2C). HS reduced ovarian pAKT (10%; $P < 0.05$) relative to TN control gilts (Figure 3.2D).

HS does not affect ovarian steroidogenic enzyme abundance

After 5 d of HS, no impact on ovarian LDLR ($P = 0.85$), StAR ($P = 0.53$), CYP17A1 ($P = 0.73$), CYP19A1 ($P = 0.85$), LHR ($P = 0.97$), or ESR1 ($P = 0.90$; Figure 3.3A-F) mRNA abundance was observed, nor was there any effect of HS on StAR ($P = 0.69$; Figure 3.4A) or CYP19A ($P = 0.58$; Figure 3.4B) protein abundance.
There is no impact of HS on follicular fluid E$_2$ levels

The concentration of E$_2$ in the follicular fluid aspirated from dominant follicles of TN and HS post-pubertal gilts did not differ ($P = 0.90$; Figure 3.5).

**TLR4 protein is increased in the ovary of HS gilts**

TLR4, the ligand for LPS, was increased in protein abundance in ovaries from HS gilts (4.9 fold; $P < 0.05$) relative to TN controls (Figure 3.6).

**HS does not alter follicular fluid concentrations of LBP**

Follicular fluid LBP concentrations were not different ($P = 0.93$) in HS gilts compared to TN gilts (Figure 3.7).

**Discussion:**

Coinciding with hot summer months, production animals undergo a period of reduced reproductive capacity known as seasonal infertility. Seasonal infertility is attributed to both HS and the longer photoperiod during the season [45,46]. The impact of HS on reproductive function manifests in its influence on folliculogenesis [5,6], steroidogenesis [5–8], embryonic [3,9,10], and fetal [10,11] development. We previously demonstrated altered ovarian signaling in response to HS in pre-pubertal gilts [33], in which increased activation of ovarian IRS-1 by phosphorylation of the Tyr$^{632}$ residue was observed. In addition, ovarian StAR and CYP19A (both mRNA and protein), and LHR mRNA were increased in abundance in heat-stressed gilts, despite those animals being pre-pubertal, indicating a potential impact on ovarian capacity for steroidogenesis. Additionally, our published data indicated increased PI3K signaling, as
evidenced by increased abundance of ovarian AKT1 mRNA and increased phosphorylation of AKT due to HS [33]. Based on this, we hypothesized that post-pubertal gilts would also experience perturbations to ovarian pathways involved in folliculogenesis and steroidogenesis. To test this hypothesis, post-pubertal estrus synchronized gilts were utilized and ovaries collected on the fifth day after Matrix® withdrawal, therefore our data is representative of the impacts of HS on the ovary during the follicular phase.

Insulin signaling can be directed through the PI3K pathway of signaling proteins. Upon binding insulin, INSR phosphorylates tyrosine residues in members of the IRS protein family [20–23], which go on to activate PI3K which, subsequently, activates AKT [24–27]. Cultured human ovarian tissue was shown to respond to insulin by increased accumulation of primary follicles, indicating a role of insulin in enhancing early folliculogenesis [47]. In addition, administration of insulin was shown to decrease atresia [48], and increase the rate of ovulation in pigs [49]. Together, these reports suggest that insulin can act as a growth factor in the ovary. Insulin signaling is a participant in the systemic response to HS, as insulin levels were shown to increase in heat-stressed production animals [12–16]. We observed increased abundance of ovarian INSR1b in HS gilts. HS can enhance insulin sensitivity, as evidenced by improved insulin sensitivity in pigs [18], and mitigation of high-fat diet-induced insulin resistance and improved glucose tolerance in rats [19]. Increased abundance of INSR1b is an indicator of increased insulin sensitization. Our previous findings of increased expression of INSR and IRS-1 are consistent with increased ovarian insulin sensitivity during HS [33]. If altered insulin sensitization is persistent, then ovarian function could be altered by HS even after the animal returns to thermoneutral conditions and normal circulating levels of insulin for as long as it takes
INSR abundance to return to normal. However, it is important to note that increased abundance of insulin receptors is not sufficient to confirm increased insulin sensitization. Insulin resistance can be induced through hyper-phosphorylation of serine residues on IRS-1, for instance, which could happen independently from any changes to INRS abundance [50]. Conversely, our previous investigation into the pre-pubertal ovarian response to HS revealed increased phosphorylation of IRS-1 at the Tyr\(^632\) residue [33], which indicates activation of insulin signaling [20–23].

PI3K regulates folliculogenesis by changing the localization of FOXO3A through AKT signaling [28–30]. Steroidogenesis is also regulated by PI3K as it regulates the production of E\(_2\) and P\(_4\) in tertiary follicles and CL, respectively [31,32]. No impact on total AKT was noted, however, AKT phosphorylation was decreased by HS. This is an interesting contrast to our previous findings of increased INSR signaling through IRS-1 and increased activation of ovarian AKT [33]. Also, these data are contrary to our previous findings of increased pAKT in the pre-pubertal gilt ovary in response to HS, potentially indicating an altered response due to the presence of circulating gonadotrophins and/or being at the end of the follicular phase of the estrous cycle. This could be interpreted as the ovary switching to a stress response state which requires a specific level of AKT phosphorylation lower than that required for normal ovarian function at the end of the follicular phase of the estrous cycle. AKT phosphorylation suppresses stress responses such as autophagy [51] and apoptosis [52]. HS was shown to enhance atresia [53], a process that is partly mediated through apoptosis [54]. In addition, HS induced apoptosis in human and bovine endothelial cells [55,56]. Autophagy is also upregulated under conditions of HS [57,58]. A reduction in ovarian AKT phosphorylation despite hyperinsulinemia and
circulating gonadotropins is potentially indicative of an ovarian stress response involving apoptosis and/or autophagy.

We previously identified increased StAR and CYP19A during HS in pre-pubertal gilts as well as altered mRNA abundance of genes involved in steroidogenesis [33]. Interestingly, in the ovaries from post-pubertal gilts, we did not observe any impact of HS on mRNA encoding LDLR, LHR, StAR, CYP17A1, CYP19A or ESR. LDLR was examined to elucidate any potential alterations to ovarian capacity to import cholesterol from the blood via LDL. LHR was examined to observe any responses which could change ovarian LH sensitivity as LH resistance is a potential mechanism of impairing ovarian function. StAR, CYP17A1, and CYP19A were investigated to evaluate any effect of HS on the ovarian steroidogenic capacity as they encode proteins responsible for importing cholesterol to the mitochondria, synthesizing pregnenolone, and synthesizing E2, respectively. ESR was examined to observe any potential changes in ovarian estrogen sensitivity. Additionally, HS did not affect ovarian StAR or CYP19A protein abundance. Since the gilts were heat-stressed during the follicular phase of the estrous cycle, these data are surprising. Additionally, we did not observe any effect of HS on 17β-estradiol concentration in the follicular fluid aspirated from the dominant follicles our observed values fell well within the range of concentrations previously observed in the dominant follicles of pigs [59]. It is possible for the ovaries of the HS gilts being close to their maximum steroidogenic capacity due to being in late proestrus, but this is an area for future investigation.

Endotoxemia results from compromised integrity of the intestinal wall observed in heat-stressed animals [16,34]. Elevated circulating LPS is characteristic of HS-induced endotoxemia.
TLR4 is the primary receptor responsible for detecting LPS and initiating a cellular response to its presence [35,36], and it does so by activating PI3K [37]. Administering LPS to gestating mice causes early loss of pregnancy [39–41]. It potentially causes that by suppressing the expression of uterine P₄ receptors [44], increasing circulating PGF2alpha [42], and reducing the size and P₄ production by the CL [43]. We observed a dramatic increase in the abundance of ovarian TLR4 in HS gilts. This implies increased ovarian TLR4 signaling in response to HS. However, the levels of LBP observed in the follicular fluid of HS gilts did not differ from their TN counterparts. While this could indicate that there is not a significant amount of LPS reaching the ovary, it is important to emphasize that our LBP measurements were taken from follicular fluid, which is derived, partly, by filtering blood [60], meaning that LPS could have been removed while the follicular fluid was passing through the thecal and granulosa layers.

In summary, cyclical HS in synchronized post-pubertal gilts increased the abundance of ovarian INSR1b but reduced ovarian AKT phosphorylation. HS did not alter E₂ concentration in follicular fluid nor the expression of steroidogenic genes. HS, however, did increase abundance of ovarian TLR4 indicating an ovarian response to HS-induced endotoxemia. Taken together, these data shed light on biological alterations to ovarian function that could contribute to seasonal infertility in swine.

**Acknowledgements:**

Our thanks are extended to Samantha Lei for her measurements of LPS binding protein in follicular fluid, and Dr. George Perry, South Dakota State University for measuring follicular fluid E₂ level.
**Funding:**

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**Table 3.1: Primer sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5' to 3')</th>
<th>Amplicon size</th>
</tr>
</thead>
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<td>GAPDH</td>
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</tr>
<tr>
<td></td>
<td>Right: GACCACTTCGTCACAAGCTCAT</td>
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</tr>
<tr>
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<td></td>
<td>Right: CTGGCCGAGTACAGGAGAAGCTG</td>
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</tr>
<tr>
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<td>160</td>
</tr>
<tr>
<td></td>
<td>Right: TGTGCCTGAAGTGAAGACAGG</td>
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<tr>
<td></td>
<td>Right: GGGTTTTTGGTGGAAGATCGG</td>
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</tr>
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<td>Left: CATGGCACCAGATCTCTTTCT</td>
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</tr>
<tr>
<td></td>
<td>Right: CGGAATGCCTTTTGGAATGAAT</td>
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</tr>
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<td>151</td>
</tr>
<tr>
<td></td>
<td>Right: AGGACCTGGTTATTGAAGACGAC</td>
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</tr>
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Figure 3.1. HS alters ovarian insulin signaling.

Total RNA or protein was isolated from ovaries of gilts who experienced thermal neutral (TN; 20.3°C ± 0.1°C) or heat stress (HS; 26°C) conditions during the follicular phase of the estrous cycle. The ovarian mRNA abundance of (A) INSR ($P = 0.94$) and (B) IRS1 ($P = 0.29$) were measured using qRT-PCR. Barcharts indicate delta Ct, normalized to GAPDH where an increased Delta Ct value reflects reduced mRNA abundance of the gene of interest. (C) Total ovarian INSR protein level was found to be increased (19%; $P < 0.05$) in HS animals compared to TN as quantified using (D) western blot. The data is expressed as average intensity of the INSR protein band, normalized to total protein loaded as determined by Ponceau S staining (PS). * indicates difference from TN controls at $P < 0.05$. 
Figure 3.2. HS reduces AKT phosphorylation.
Total RNA or protein was isolated from ovaries of gilts who experienced thermal neutral (TN; 20.3°C ± 0.1°C) or heat stress (HS; 26°C) conditions during the follicular phase of the estrous cycle. The ovarian mRNA abundance of (A) AKT1 (P = 0.46) and (B) FOXO3A (P = 0.71) were measured using qRT-PCR. Barcharts indicate delta Ct, normalized to GAPDH where an increased Delta Ct value reflects reduced mRNA abundance of the gene of interest. (C) Total ovarian AKT (tAKT) was not changed (P = 0.5) in abundance in HS animals, while (D) phosphorylated AKT (pAKT) protein level was decreased (10%; P < 0.05). Abundance of both proteins was quantified using western blot, and the data was expressed as average intensity of the protein band of interest, normalized to total protein loaded as determined by Ponceau S staining (PS). * indicates difference from TN controls at P < 0.05.
Figure 3.3. HS does not impact steroidogenic gene expression in post-pubertal gilt ovaries. Total RNA was isolated from ovaries of gilts who experienced thermal neutral (TN; 20.3°C ± 0.1°C) or heat stress (HS; 26°C) conditions during the follicular phase of the estrous cycle. The ovarian mRNA abundance of (A) LDLR \( (P = 0.85) \), (B) StAR \( (P = 0.53) \), (C) CYP17A1 \( (P = 0.73) \), (D) CYP19A1 \( (P = 0.85) \), (E) LHR \( (P = 0.97) \), and (F) ESR-1 \( (P = 0.90) \) were measured using qRT-PCR. Barcharts indicate delta Ct, normalized to GAPDH where an increased Delta Ct value reflects reduced mRNA abundance of the gene of interest.
Figure 3.4. HS does not affect StAR or CYP19A1 protein abundance.
Total protein was isolated from ovaries of gilts who experienced thermal neutral (TN; 20.3°C ± 0.1°C) or heat stress (HS; 26°C) conditions during the follicular phase of the estrous cycle. Ovarian protein abundance of (A) StAR ($P = 0.69$) and (B) CYP19A1 ($P = 0.58$) were quantified by western blotting. Data is expressed as average intensity of the protein band of interest, normalized to total protein loaded as determined by Ponceau S staining (PS).
Figure 3.5. Follicular fluid 17β-estradiol concentration is not impacted by HS. Follicular fluid was aspirated from dominant follicles on ovaries from gilts who experienced thermal neutral (TN; 20.3°C ± 0.1°C) or heat stress (HS; 26°C) conditions during the follicular phase of the estrous cycle. 17β-estradiol concentration was measured by radioimmunoassay. Data represents total 17β-estradiol concentration (ng/mL). Concentrations of E$_2$ was not changed ($P = 0.90$) in the follicular fluid aspirated from HS animals compared to TN.
Figure 3.6. HS increases abundance of TLR4 indicating LPS signaling.
Total protein was isolated from ovaries of gilts who experienced thermal neutral (TN; 20.3°C ± 0.1°C) or heat stress (HS; 26°C) conditions during the follicular phase of the estrous cycle. Ovarian protein abundance of TLR4 was quantified by western blotting. Data is expressed as average intensity of the protein band of interest, normalized to total protein loaded as determined by Ponceau S staining (PS). TLR4 abundance in the ovaries of HS gilts was observed to be elevated (4.9 fold; P < 0.05) compared to TN controls.
Figure 3.7. Follicular fluid LPS binding protein concentration is not impacted by HS.
Follicular fluid was aspirated from dominant follicles on ovaries from gilts who experienced thermal neutral (TN; 20.3°C ± 0.1°C) or heat stress (HS; 26°C) conditions during the follicular phase of the estrous cycle. LPS binding protein (LBP) concentration was measured by ELISA. Data represents total LBP concentration (ng/mL). No difference ($P = 0.93$) was observed in the concentration of LBP in the follicular fluid of HS gilts compared to TN.
Bibliography:


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CHAPTER 4
general discussion and conclusions

The reviewed literature and the data presented in this thesis underscore the important role(s) of metabolic status and ensuing signaling events on mammalian body on ovarian function. The rationale for these investigations are the systemic effects on the female body exerted by the ovary through the production of steroids, as well as the effects on future generations due to the ovarian role in production and maintenance of the maternal gamete. The capacity to sense and respond to global energy status demonstrate evolutionary importance to the reproductive system. This discussion contains the author’s overall interpretation of the reviewed literature, the conducted studies, and thoughts on the direction that future research could take.

General background:

Reproduction is a nutritionally expensive process on the female body, both in terms of caloric expenditure and in micronutrients. These expenses continue after parturition in mammals, and many other vertebrates, due to the offspring requiring nursing and care for a developmental period before they are capable of functioning independently. The reproductive system has evolved to recognize when the energy status of the female is insufficient to carry out these processes. Reproductive impairment is demonstrated in individuals who lead lifestyles of high caloric expenditure and low body fat. Female gymnasts possess extremely low body fat content, and this reflects on their reproductive performance as delayed onset of puberty [1]. Studies of the onset of puberty in the context of body mass support a correlation between energy status, measured mainly by body mass, and reproductive performance [2,3].
While plentiful nourishment is positively associated with ovarian function, excessive caloric intake leads to compromised reproductive performance. Polycystic ovary syndrome (PCOS) [4], miscarriage [5], anovulation [6], and difficulties with assisted fertility treatments [7], are all correlated to the condition yielded by excessive caloric intake; obesity. Metabolic factors such as leptin and insulin, as well as byproducts of extreme metabolic states like lipopolysaccharides (LPS), have been linked to altered reproductive performance. This impairment of reproductive performance in what is, essentially, a lifestyle of *ad libitum* feeding leads to the posit that a restriction in the quantity of available calories might be necessary for the reproductive system to function properly. Reduction in caloric intake has already been shown to increase lifespan [8] and prolong reproductive function [9]. Current animal research tends to include *ad libitum* feeding in its definition of "normal." A consequence of this might be that our understanding and expectations of what is biologically "normal" are skewed by convenience or by a biased human angle on the availability of food sources. Whether or not this bias is significant when it comes to overall research, I believe, is something worth investigating.

**Leptin:**

In this thesis, leptin signaling was discussed as a major regulator of reproductive function. Leptin is crucial in maintaining cyclicity due to its permissive effect over hypothalamic release of gonadotropin-releasing hormone (GnRH), mainly through the indirect routes of GABAergic neurons [10,11] and kisspeptin neurons [12–16]. Leptin is also capable of directly influencing ovarian function through its positive regulation of germinal vesicle breakdown (GVBD) and ovulation [17]. Initially, these observations suggest that systemic leptin could act as a general promoter of ovarian function. However, directly blocking leptin receptor (LEPR)
signaling via LEPR antagonists impedes early folliculogenesis [18], while reducing the efficacy of circulating leptin through passive immunization enhances it [19]. To add to the conflict, our experiments with cultured post-natal day (PND) 4 rat ovaries show no effects of leptin on early folliculogenesis (Chapter 2). We can consider two effects of leptin in an attempt to illicit an explanation for these conflicting reports.

Leptin is a factor which promotes both angiogenesis and fenestration [20,21], meaning that it has the capacity to recruit and make blood vessels more permeable. Ovarian vascular architecture is thought to be a limiting factor in early folliculogenesis due to its restricting of primordial follicles' access to circulating factors, which is supported by accelerated early follicular growth \textit{ex vivo} compared to \textit{in vivo} [22,23]. As follicles grow larger, their requirements of oxygen and nutrients increase, which leads to increased vascularization on individual follicles [24]. Leptin potentially participates in this process as ovaries of a variety of species were shown to produce leptin in all follicular cell types with variations in expression levels [17,25–31]. Recruitment of cells is a process typically carried out by chemical signal gradients, meaning more of a signal has to be at the destination relative to the rest of the environment. If one were to visualize a signal gradient by means of a drop of ink in a glass of clear water, the concept becomes clear as the gradient of ink would direct towards the drop. If the ink was leptin, then blood vessels would follow that gradient to where the droplet, in this case representing the ovary, is. Mixing some ink into the water beforehand would not negate the fact that ink is still most abundant at the source, but it would make finding the direction towards it more difficult due to the diminished relative strength of the signal. Passive immunization against leptin would, in effect, clarify the water as it reduces the effective concentration of circulating leptin, leading to a
stronger relative signal. Blocking leptin signaling via LEPR antagonists would block the sensors detecting the gradient, effectively "blinding" blood vessels to the existence of the signal gradient. The conflicting results could be interpreted as a result of ovarian vascularization being influenced by the blood vessels' ability to sense a leptin signal gradient produced by the ovary, which in turn could affect their recruitment and capacity to deliver circulating factors, thus influencing folliculogenesis. Our own results showing a lack of an effect of leptin on cultured ovaries also fall in line with this hypothesis as vascularization is of no concern to cultured ovaries (Chapter 2). An effect on vascularization could also explain the increased incident of anovulation during obesity. Reducing the efficacy of a leptin gradient in recruiting blood vessels to a growing follicle could restrict its access to luteinizing hormone (LH), which is already produced in lowered amounts during the LH surge necessary for ovulation in obese individuals [32]. Using models such as ob/ob, Db/Db, and high-fat diet mouse models, and examining their ovarian vascular architecture would help in tackling this hypothesis.

The second route by which leptin might influence early folliculogenesis is its influence on pancreatic beta cells. Leptin was shown to increase the localization of ATP-sensitive K+ channels in insulin-producing pancreatic cells [33]. If this was effective in influencing insulin response to glucose, leptin concentrations could influence early folliculogenesis by changing insulin secretion patterns and overall glucose tolerance. Monitoring systemic insulin levels in response to a glucose challenge after the administration of leptin into circulation could help in clarifying the picture and in providing more information to simulate such effects in culture.
Leptin is also a potential participant in heat stress (HS). During HS, animals display a consistent phenotype of reduced feed intake and seemingly paradoxical hyperinsulinemia [34–39]. Some hypothesize that as a result of inflammation due to endotoxemia, while others speculate it to be a mechanism of reducing thermogenesis. However, reduced feed intake could be mechanistically explained through leptin signaling. Chronic HS was shown to increase leptin production and leptin sensitivity in mice [40]. As a satiety-inducing factor, leptin could be a participant in reducing feed intake by directly suppressing feeding behavior. Elucidating this potential effect could be done by manipulating central leptin signaling to observe whether suppressing it would be effective in increasing feed intake during HS.

Another facet of appetite regulation is ghrelin; a promoter of appetite produced by the stomach [41]. Ghrelin is also a suppressor of insulin production [41,42]. Its production location, the stomach, is part of the gastrointestinal (GI) tract, which experiences hypoxia and reduced integrity during HS in the intestines due to circulation diverting blood to the extremities in an attempt to cool it [43,44]. If this hypoxia extends to compromising the stomach's capacity to produce ghrelin, then it could inhibit its stimulation of appetite and its suppression of insulin, contributing to reduced feed intake and hyperinsulinemia during HS.

Circulating leptin concentrations could be of transgenerational consequence. Our results show that gestational hyperleptinemia can have the ability to change the ovarian capacity to metabolize ovotoxicants through EPHX1 later in life (Chapter 2). These results suggest a possible capacity of leptin to imprint offspring in some way depending on its concentration in maternal circulation. If that is the case, investigating caloric intake during gestation might
elucidate an effect on offspring health, which would parallel the findings of general improvements in lifespan and reproductive longevity in response to reduced caloric intake.

**TLR4:**

Toll-like receptor (TLR) 4 is a specialized member of a larger family of receptors [45,46] concerned with initiating the innate immune response to invading pathogens by recognizing pathogen-associated molecular patterns (PAMPS) [47], specifically lipopolysaccharides (LPS) in the case of TLR4 [48,49]. LPS is increased in a condition known as endotoxemia. Both HS [38,50] and obesity have systemic endotoxemia as a phenotypic perturbation. Our observations in the ovaries of HS gilts showed a dramatic increase in the ovarian content of TLR4, indicating an increase in TLR4 signaling. Being associated with a plethora of impairments to the mechanisms of the reproductive system, LPS signaling is a potential target for rescuing reduced fertility in production animals when challenged by HS. The benefit of initiating an immune response outside the immune system is that it aids in the recruitment of immune cells and allows local tissues to resist active pathogens. However, in HS, this might not be entirely necessary. While endotoxemia is present, bacteremia might not be. The absence of active pathogens in circulation might justify a blockage of TLR4 signaling in the ovary as a method of protecting the corpus luteum (CL) from regression, and early follicles from depletion. An ovarian TLR4 knockout model would be ideal in elucidating the role TLR4 plays during early folliculogenesis, and whether innate immunity is necessary during HS-induced endotoxemia.
Insulin:

Insulin is a direct regulator of ovarian function. It functions as a stimulator of growth and a survival factor by enhancing the folliculogenic process and suppressing atresia [51–53]. Insulin also stimulates ovarian steroidogenesis [54] in such a conserved manner that exposing insect ovaries to mammalian insulin was shown to stimulate their steroid production [53–55]. Due to these effects, hyperinsulinemia during obesity and HS could stress the ovary by overactivating its metabolic machinery by overstimulating follicular growth and steroidogenic pathways. Manipulating systemic insulin through passive immunization and/or through INSR antagonists during HS and obesity might provide some insight into the role and the consequences of insulin under these conditions.

Phosphotidylinositol-3 kinase (PI3K):

When considering general ovarian function and all the factors discussed herein, it is important to remember that they all converge, at least partially, at a major signaling node. That node is PI3K and its downstream signal protein kinase B (AKT). PI3K not only mediates leptin, TLR4, and insulin signaling, but also controls folliculogenesis, steroidogenesis, apoptosis, and autophagy (Chapter 1). The reduction of AKT phosphorylation observed in HS gilts is indicative of a trend towards a stress response. PI3K regulates apoptosis and folliculogenesis through forkhead transcription factor (FOXO3A). pAKT-mediated inhibition of FOXO3A initiates folliculogenesis [56–58], increases cell survival, and suppresses apoptosis [59–61]. pAKT also suppresses autophagy [62]. HS is a condition which upregulates both autophagy [63,64] and apoptosis [65,66]. Upregulation of apoptosis could be attributed to the increase in atresia during HS [67] as it is a partly apoptotic process [68]. Our observed reduction in the phosphorylation of
AKT in response to HS despite hyperinsulinemia, endotoxemia, and gonadotropic signaling being positive regulators of PI3K signaling, is indicative of ovarian stress where apoptotic and autophagic pathways are being activated. Heat stressing post-pubertal gilts during the luteal phase or before the development of dominant follicles could help in elucidating that particular response of PI3K and AKT.

Apoptosis is another facet of ovarian function worth investigating. Apoptosis is the process of programmed cell death, where a cell could be eliminated either due to displaying signs of dysfunction, or having completed its role during a specific developmental stage. Apoptosis is activated in one of three ways: intrinsic, extrinsic, or by the granzyme pathway [69]. The characteristic markers of these pathways are activated caspase 9, caspase 8, and perforin/granzyme, respectively. Atresia's apoptotic portion is mediated by the extrinsic pathway through the Fas, cell-surface death receptor signaling system [70]. Histological investigation of sectioned ovaries from HS animals could show which, if any, apoptotic pathway is being activated as the activating signals are varied and could be independent of the common markers used to detect apoptosis (such as P53 and caspase 9).

Taken together, the studies described in this thesis provide starting points from which biological understanding of compromised reproductive function during obesity and HS can develop.
Bibliography:


