

Fat and Fructose Consumption Affects Pre-pubertal Gilt Reproductive Tissues and Early Embryogenesis

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Abstract

Infertility among women has become a growing issue in the world requiring a significant number of women to seek treatment by means of assisted reproductive technologies (ART). One suggested reason for the fertility issue is modern diet, leading to diseases such as obesity and type II diabetes. In this study, twenty gilts three weeks in age, were placed on one of five dietary treatments (n=4 gilts per treatment) containing 15% fat (FAT), 35% fructose (FRU), both fat and fructose (HFHF), or two different controls: one standard industry (IND) diet meant to result in optimal lean growth and a second diet to account for the reduced lysine (LYS) intake in the treatment diets. Two experiments were performed to assess the reproductive outcomes of pre-pubertal gilts consuming a high fat and/or high fructose diet and to demonstrate interactions between diet and infertility using pigs as a model.

In the first experiment, follicular fluid was collected from these gilts and introduced into porcine *in vitro* maturation system to determine whether characteristics of the follicular fluid affect oocyte competence and embryo development. The follicular fluid of females consuming high fructose and fat diets did not alter nuclear maturation of oocytes ($p>0.10$). There were, however, detrimental effects on subsequent development of embryos, especially blastocyst formation, with the gilts having consumed the HFHF diet having reduced day 5 and 6 blastocysts formation when compared to the IVM follicular fluid free (FFF) group ($p=0.03$ and $p=0.01$, respectively). In regards to embryo quality, blastocysts from the FAT group had greater cell number when compared to all other groups.

In the second experiment, the reproductive tissues; ovary, oviduct, and uterus were analyzed for genes of interest: estrogen receptor alpha (*ESR1*), estrogen receptor beta (*ESR2*), insulin like growth factor I (*IGFI*), insulin like growth factor I receptor (*IGFIR*), and growth differentiation factor 9 (*GDF9*). Resulting data was analyzed in three ways: 1) across all 5 treatments, 2) with gilts grouped by whether or not they consumed fat, or 3) with gilts grouped by whether or not they consumed fructose. There were no differences detected between individual treatments for *ESR1* and *ESR2*. In the ovary samples, the fructose diets decreases *ESR2* ($p=0.05$). Also, *GDF9* ovarian expression tended to decrease with fructose consumption ($p=0.07$). Furthermore in the ovary, there was a positive correlation between *ESR2* and *GDF9* expression ($r=0.92$ and $p<0.01$). *GDF9* expression was lower in the oviducts of gilts consuming fat diets when compared to non-fat diets ($p=0.01$). Neither *IGFI* nor *IGFIR* were altered in the reproductive tissues analyzed.

Based on the results from both experiments, the consumption of fat and fructose alters both the developing embryo and gene expression in the reproductive tissues that support the growing embryo. Further investigation will provide more insight on the impact nutrition has on pre-pubertal reproductive development and subsequent fertility.

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List of Abbreviations

ART	Assisted reproductive technology
ATP	Adenosine triphosphate
BMI	Body mass index
BMP15	Bone morphogenetic protein 15
BSA	Bovine serum albumin
Ca ²⁺	Calcium ion
cAMP	Cyclic adenosine monophosphate
CH	Corpus hemorrhagica
CL	Corpus luteum
CO ₂	Carbon dioxide
COC	Cumulus oocyte complex
EGF	Epidermal growth factor
ESR1	Estrogen receptor alpha
ESR2	Estrogen receptor beta
FAT	Fat treatment
FFF	Follicular fluid free
FRU	Fructose treatment
FSH	Follicle stimulating hormone
GDF9	Growth differentiation factor 9
GnRH	Gonadotropin releasing hormone
GSH	Glutathione
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
HCO ₃ ⁻	Bicarbonate
HFHF	High-fat, high-fructose treatment
ICM	Inner cell mass
IGFI	Insulin-like growth factor I

IGFIR	Insulin-like growth factor I receptor
IND	Industry control
IP ₃	Inositol 1,4,5-triphosphate
IVC	<i>In vitro</i> culture
IVF	<i>In vitro</i> fertilization
IVM	<i>In vitro</i> maturation
LH	Lutenizing hormone
LYS	Lysine restricted control
MII	Metaphase II
MGC	Mural granulosa cells
mTBM	Modified tris buffered media
NADH	Nicotinamide adenine dinucleotide
NCSU	North Carolina State University
O ₂	Oxygen
PCOS	Polycystic ovarian syndrome
pFF	Porcine follicular fluid
PGE ₂	Prostaglandin E ₂
PGF _{2α}	Prostaglandin F _{2α}
PKA	Protein kinase A
PLCζ	Phospholipase C zeta
PPP	Pentose phosphate pathway
PVA	Polyvinyl alcohol
PZM	Porcine zygote media
ROS	Reactive oxygen species
STIM1	Stromal interaction molecule 1
TE	Trophectoderm
TCA	Tricarboxylic acid
TCM	Tissue culture medium

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Introduction

Infertility impacts numerous women in the United States, and they can seek assisted reproductive technology (ART) treatment to optimize their chance to conceive. Obesity is one factor that adversely impacts reproductive function, particularly oocyte quality, though the underlying mechanisms remain unknown. Obesity rates have increased since the 1970's, in adolescents from approximately 6% in 1976 to 17% in 2012 and in adults from approximately 15% in 1976 to 35% in 2012 (Flegal et al., 1998; May et al., 2013; Ogden et al., 2014a; Ogden et al., 2010). One suggested reason for the steady increase in obesity rates is the modern diet containing saturated fats and simple sugars, especially fructose. Consumption of fructose, specifically high fructose corn syrup, has increased by nearly 30% from 1970 and this trend is similar to the rise in obesity (Bray et al., 2004; Flegal et al., 2010).

The increase in childhood obesity rates has been a main concern, and likely affects subsequent adulthood fertility. Previous work from our laboratory demonstrated that inclusion of simple sugars in pre-pubescent gilt diets altered reproductive tract development and function. It has also been reported that high energy intakes during pre-pubertal growth, reduced the age of attainment of puberty however increased body fat content (Noblet et al., 1990). This signifies that nutrition will modify pre-pubescent growth and potentially alter reproductive tract development. Previously conducted research in rodents showed that consumption of high-fat or high-fructose diets affected reproductive parameters by reducing embryo development and the likelihood of pregnancy establishment (Gray et al., 2013; Luzzo et al., 2012). However, little work has been performed investigating diet-induced reproductive outcomes in the porcine model.

By using diet-induced obese pigs as a model for human infertility, it may reveal important insight to the physiological aberrations responsible for reproductive alterations and

infertility. For these reasons, two experiments were conducted to elucidate if nutritional supplementation of high fructose and/or fat affects 1) oocyte competence and embryo development in porcine *in vitro* system and 2) the gene expression of the developing reproductive tract.

Chapter 1: Review of the Literature

Female Swine Reproduction

Pre-pubertal Reproductive Growth

From birth until 100 days of age, primordial follicles account for the majority of follicles on the pig ovary (A.C.O. Evans, 2001). However, antral or tertiary follicles can be observed on the ovaries on 60 or more days of age (A.C.O. Evans, 2001; Oxender et al., 1979). In pre-pubertal females, the hypothalamic surge center is sensitive to positive feedback from estradiol. However, the ovaries are not producing sufficient levels of estradiol, therefore high pulses of gonadotropin releasing hormone (GnRH) cannot be released for ovulation to occur. Nevertheless, Oxender and coworkers (1979) demonstrated that follicles from 9 week old gilts (approximately 63 days of age) can ovulate by the induction of gonadotropins (discussed below) (Oxender et al., 1979). Observing ovary-independent (ovariectomized) gilt uterine growth, periods of growth were defined and characterized: infantile (before day 7), proliferative (days 7 to 14), and growth (after day 14) (Bartol et al., 1993; Spencer et al., 1993). At birth (infantile), the porcine uterus consists of simple columnar epithelium with the absence of endometrial glands. By day 7 (proliferative), simple, coiled tubular glands are present in the stroma and this is known as tubulogenesis. By day 14 (growth), many coiled tubular glands are apparent. By day 28, glandular epithelium is present throughout the endometrial folds and the coiled glands have branching toward the myometrium, this is known as adenogenesis. This continues and by day

120, the uterus is functionally mature and capable of supporting pregnancy (Bartol et al., 1993; Gray et al., 2001).

Puberty

As stated previously, follicular growth in gilts begins before puberty, or sexual maturation, and the estrous cycles following puberty are affected by numerous factors such as nutrition, environmental stress, and age. Gilts attain puberty at roughly 180 days of age (Whittemore, 1996). Development of the hypothalamic-pituitary axis is the first step toward attainment of puberty. Gonadotropin releasing hormone is released once the kisspeptin pathway is activated in the hypothalamus. Gonadotropin releasing hormone, released from the hypothalamus, will bind to its receptor on the gonadotrope cells of the anterior pituitary gland, causing the synthesis and secretion of the gonadotropins: follicle stimulating hormone (FSH) and luteinizing hormone (LH). Circulating FSH and LH will act on the ovary to stimulate follicular growth, thus increasing the production of estrogen and eventually initiating ovulation (Sanchez-Garrido and Tena-Sempere, 2013). However, puberty can be hastened with an injection of P.G. 600, containing pregnant mare's serum gonadotropin and human chorionic gonadotropin, which advances follicular growth and helps to induce the first estrus (Harper, 2009). From an animal production perspective, the purpose of P.G. 600 is to advance and synchronize pubertal estrus. Therefore, this management strategy increases the number of animals that ovulate and reduces nonproductive days for replacement gilts (Knox and Tudor, 1999; Knox et al., 2000).

Estrous Cycle

Once puberty is attained, estrus occurs approximately every 21 days throughout the animal's reproductive lifespan (unless the gilt/sow is pregnant). It is divided into two phases: the follicular phase and the luteal phase. The follicular phase is divided into proestrus and estrus,

whereas the luteal phase is divided into metestrus and diestrus. As progesterone decreases, estradiol will increase in response to hypothalamic GnRH stimulating the release of gonadotropins, which will act on the ovary. This characterizes the hormonal changes in proestrus. The follicles on the ovary will produce estradiol and will reach a threshold level during estrus, defined as the time during which the female is receptive to mating (A.C.O. Evans, 2001). Estrus can vary among gilts and sows, with gilts estrus length being roughly 40 hours and sows roughly 55 hours. The follicular phase as a whole lasts approximately 4-6 days and is characterized by final maturation of a dominant follicle and subsequent ovulation (Soede et al., 2011).

Post ovulation, the ruptured follicles will form structures called the corpora hemorrhagica (CH) in their transition to luteal tissue, otherwise called luteinization. Progesterone begins to increase during this period of metestrus. Mid-luteal phase (diestrus), the presence of the corpora lutea (CL) will dominate the ovary, and peak concentrations of progesterone will be attained 9-10 days post ovulation. Luteolysis (regression) of the CL occurs on roughly day 14 after ovulation and progesterone production decreases rapidly, signifying the end of diestrus and initiation of proestrus (Soede et al., 2011).

Folliculogenesis and Oogenesis

Follicular growth occurs during the luteal phase. However due to the high levels of circulating progesterone from the corpora lutea, follicles are unable to complete maturation and ovulate. As follicles grow, they undergo stages of recruitment, selection, and dominance. Follicular growth begins from primordial follicles (< 3mm) and culminates with ovulatory (Graafian) follicles (> 7mm). This process is known as folliculogenesis (Knox, 2005). Primordial follicles contain an immature primary oocyte arrested at meiosis I (prophase I), also

known as germinal vesicle (GV) stage. Primordial follicles are identified as an oocyte surrounded by a single layer of squamous cells (Knox, 2005; Sutton et al., 2003). The transition from primordial to primary follicles requires factors such as insulin, IGFI, and transforming growth factor β superfamily members (McGee and Hsueh, 2000). The resulting primary follicles are an oocyte surrounded by a layer of cuboidal cells. These cells will then undergo mitosis to form two or more layers of granulosa cells, thus forming secondary follicles (Knox, 2005). Finally, an antrum will form within the granulosa cells and create a tertiary (antral) follicle (Knox, 2005; Soede et al., 2011).

An important event during the recruitment process is the development of FSH receptors on small follicle granulosa cells, which will enable growth to over 1 mm in diameter (Guthrie et al., 1995). FSH is an important gonadotropin released from the anterior pituitary in response to GnRH released by the hypothalamus. Specifically, FSH will stimulate cumulus expansion and increase meiotic progression, as well as causing granulosa cells to convert androgens to estradiol via aromatase (Marchal et al., 2001). Granulosa cells undergo mitotic divisions to differentiate into cumulus cells surrounding the oocytes and mural granulosa cells (MGC), which are the innermost layer of the follicular wall *in vivo* (Zhang et al., 2010). During recruitment, FSH will increase thus promoting the growth of antral follicles from the ovarian follicular pool (McGee and Hsueh, 2000).

During follicular development, binding of LH and FSH to their receptors on the theca interna and granulosa cells aid in the production of estradiol. This is known as the “2-cell, 2-gonadotropin model” for the production of estradiol (Dorrington et al., 1975; Ryan et al., 1968; Short, 1961). Pre-selection, theca interna cells of the follicle have LH receptors, whereas granulosa cells have FSH receptors. At the time of selection, granulosa cells also gain LH

receptors (discussed below). Once LH from the anterior pituitary binds to its receptors, cAMP and protein kinase A (PKA) are activated which in turn will activate various enzymes to convert cholesterol to androstenedione in the theca interna cells. Androstenedione then moves across the basement membrane of the follicle and enters the granulosa cells. As gonadotropins bind to their receptors on the granulosa cells (FSH pre-selection and LH post-selection), cAMP and PKA are activated and PKA activates P450aromatase, the enzyme that converts androstenedione to estrone. Finally, the enzyme 17 β -hydroxysteroid dehydrogenase will convert estrone to estradiol. Concentrations of estradiol will increase as follicles grow larger during proestrus and reach a peak during estrus as dominant follicles (McGee and Hsueh, 2000).

As previously mentioned, recruited follicles undergo a selection process. The previously recruited follicles, which become selected will attain LH receptors on the granulosa cells, thus allowing them to develop further and producing moderate amounts of estradiol (Soede et al., 2011). Luteinizing hormone is the gonadotropic hormone crucial for continued development of follicles during and after the selection process (Guthrie et al., 1995). Non-selected follicles become atretic due to the fact that these follicles lack LH receptors on the granulosa cells. Because FSH is returning to basal levels at this time, follicles at this stage of development cannot survive without the ability to respond to the pulsatile secretion of LH from the anterior pituitary (Schwarz et al., 2008). Luteinizing hormone stimulation will support continued growth of follicles with LH receptors on granulosa (and theca interna) cells, causing follicles to reach 8-10 mm (Soede et al., 2011).

Follicular growth and development on the ovary has been linked to the transforming growth factor- β superfamily, specifically the protein growth differentiation factor-9 (GDF9), which was previously thought to only be synthesized by the oocyte (McGrath et al., 1995;

McPherron and Lee, 1993). Oocyte specific GDF9 plays an important role in the normal progression of early folliculogenesis by regulating the development of somatic cells in follicles (Dong et al., 1996; Hayashi et al., 1999). In pigs, GDF9 mRNA is found in oocytes, cumulus cells, and granulosa cells; with greatest GDF9 mRNA concentrations in oocytes (Prochazka et al., 2004). By injecting synthetic GDF9 in pre-pubertal gilts, the number of primary, secondary, and tertiary follicles increased while the number of primordial follicles was reduced (Shimizu et al., 2004).

These results suggest that GDF9 aids in the transition of primordial to primary follicles. Another transforming growth factor- β superfamily member similar to that of GDF9 is bone morphogenetic protein 15 (BMP15). Both GDF9 and BMP15 are oocyte-secreted factors with similar amino acid homology that play a role in folliculogenesis via paracrine signaling (Dube et al., 1998; Laitinen et al., 1998; Paulini and Melo, 2011). A strong positive correlation exists between the ratios of GDF9:BMP15 mRNA expression suggesting co-regulation of both factors in oocytes. However, the ratio of GDF9:BMP15 is low in pigs compared to other mammals with BMP15 mRNA expression being elevated in pig oocytes (Crawford and McNatty, 2012), suggesting species-specific regulation of GDF9 and BMP15.

Ovulation

Selected follicles will become dominant follicles and only dominant follicles will ovulate. A series of events must take place in order for ovulation to occur. As previously mentioned, the follicular phase is composed of proestrus and estrus. During proestrus, estradiol concentrations are increasing while progesterone concentrations are decreasing, which will eventually trigger the pre-ovulatory surge of GnRH. This will induce the pre-ovulatory LH surge, resulting in an immediate decrease in estradiol concentrations as granulosa and theca interna cells of the follicle

switch from producing testosterone to progesterone even before follicles rupture (Soede et al., 2011). Within the meiotically arrested oocyte, the LH surge removes meiotic inhibitors, causing germinal vesicle breakdown (GVBD) and resumption of meiosis. As a result, the first polar body is extruded forming a secondary oocyte. Secondary oocytes will arrest again in meiosis II (metaphase II) and will not complete meiosis until fertilization occurs (Brevini et al., 2007; Ferreira et al., 2009).

In pigs, ovulation takes place approximately 30 ± 3 hours after the peak of the LH surge (Soede et al., 2011). In polytocous species (litter bearing) such as pigs, there are multiple dominant follicles. The ovulation of the dominant follicles will occur over a 6 hour period near the end of estrus (Soede et al., 2011).

The number of follicles that ovulate or ovulation-rate determines the potential number of oocytes, embryos, or piglets that can be produced from one estrous cycle (Knox, 2005). This can be estimated by determining the number of dominant follicles at estrus and/or by the number of corpora lutea present on the ovary post-ovulation. However, this is not a clear indicator of the number of competent oocytes that ovulate and does not account for early embryonic loss (Knox, 2005). Thus, the number of piglets born at the end of gestation may or may not be representative of the number of follicles ovulated at the estrus that produced the pregnancy.

Luteinization and Luteolysis

Rupture of the dominant follicles at ovulation begins the luteal phase, which is significantly longer than the follicular phase. During the luteal phase, follicle development is less pronounced and the corpora lutea are the predominant structures present on the ovary. Luteinization is the term that describes the transformation of follicular cells into luteal tissue where granulosa cells become large luteal cells and theca cells become small luteal cells. The

luteal phase can be further divided into diestrus and metestrus. One week after ovulation, corpora lutea reach a maximal size of 6-10g (which is approximately 12mm maximum in size), and progesterone will reach peak concentrations 8-9 days after ovulation (McEntee, 1990; Soede et al., 2011). Progesterone is a steroid hormone that is produced by corpora lutea and is required for the maintenance of pregnancy. Post-ovulation, large luteal cells rarely multiply; however they increase in volume (hypertrophy) while small luteal cells increase in number (hyperplasia) (Senger, 2012). Both luteal cell types have LH receptors, but only the small luteal cells respond to LH by increasing progesterone production (Murphy et al., 2001). Even though binding of LH to receptors on large luteal cells does not increase progesterone production, these cells are responsible for synthesizing the majority of the progesterone. If the female does not become pregnant, the luteal phase ends with the regression of the corpora lutea, luteolysis. Luteolysis is caused by the hormone prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which is produced by the uterus.

Early Embryonic Development and Pregnancy

Post ovulation, if fertilization occurs, the sperm penetration of the oocyte causes the completion of the second meiotic division, which completes meiosis of the oocyte and extrudes the second polar body. The fertilized oocyte will then develop from zygote to blastocyst within the oviduct. The uterus will begin rapid elongating growth during the first few days of pregnancy, even before the blastocysts reach the uterus, in preparation for receiving and supporting the conceptuses (Rowlands, 1962). By day 8 and 9 post-fertilization, blastocysts have hatched from the zona pellucida and begin to elongate to roughly 4 mm in length (Rowlands, 1962). The rate at which morphological changes of the blastocyst occur in the pig is unsurpassed, because the conceptus will elongate from 4 mm to a long thin filament of roughly 150 mm within a four hour time span on gestational day 11 or 12 (Bazer, 2013; Blomberg et al., 2008;

Rowlands, 1962). In litter-bearing species, such as the pig, a large number of conceptus are lost in the early stages of development (gestational day 9 to 16) before placentation is fully established (Roberts et al., 1996; Rowlands, 1962).

In the pregnant sow, luteolysis must be prevented in order for the pregnancy to continue. Bazer and Thatcher (1977) proposed a widely accepted theory for maternal recognition of pregnancy in pigs. This theory states that the luteolytic hormone $\text{PGF}_{2\alpha}$ is secreted by the uterine endometrium in both pregnant and non-pregnant sows. In the pregnant sow, however, conceptus-derived estrogen causes $\text{PGF}_{2\alpha}$ from the uterus to shift from endocrine to exocrine secretion. Specifically, rather than entering the circulatory system through the uterine vein, $\text{PGF}_{2\alpha}$ is secreted directly into the uterine lumen so that it does not come into contact with the corpora lutea (Bazer and Thatcher, 1977). The rerouting mechanism of $\text{PGF}_{2\alpha}$ is not completely understood. However it is known that the critical window for estradiol secretion from the conceptus is gestational day 11 to 12. Furthermore, at least two conceptuses must be present in each uterine horn for the pregnancy to be maintained (Bazer, 2013).

With luteolysis prevented, the CL is able to produce progesterone during the entire length of gestation (Bazer, 2013; Ostrup et al., 2011). Placentation occurs in two phases: the apposition phase and the implantation phase. The apposition phase is the first epithelial contact between the embryo and the uterine wall and the implantation phase is when the trophoblast will adhere to the uterine wall, establishing the least invasive placental form (Ostrup et al., 2011). Pigs have an epitheliochorial placenta where both the uterine epithelium and outer placental layer of the chorion (trophectoderm) remain intact throughout pregnancy (Bazer, 2013; Roberts et al., 1996). There is no direct contact between maternal and fetal blood supplies throughout pregnancy. Instead, nutrients and growth factors necessary for fetal growth are exchanged via areolae that

are diffusely scattered across the placenta (Ostrup et al., 2011). If fertilization, maternal recognition of pregnancy, and placentation progress as normal, gestation will ensue and last approximately 114 days in the pig.

Porcine In Vitro Maturation

For the first time in the early to mid-1980's, live piglets were born as a result of *in vitro* fertilization (IVF) of oocytes that had matured *in vivo*. The first live piglets produced from oocytes matured *in vitro* (*in vitro* maturation [IVM]) were born in the late 1980's (Cheng, 1986; Mattioli et al., 1989). This provided a system to investigate factors such as nutrition, environmental stress, and age impacts on oocyte maturation and early embryonic development. Various maturation media were developed to mimic *in vivo* conditions for the normal progression of oocyte maturation in ovarian follicles. Common *in vitro* maturation media include tissue culture medium 199 (TCM 199), North Carolina State University medium (NCSU) 23, and NCSU 37 medium. Striking differences between the media would be varying ionic compounds such as sodium chloride (NaCl), sodium bicarbonate (NaHCO₃), monopotassium phosphate (KH₂PO₄), organic osmolytes, such as taurine, and amino acid concentrations (Wang et al., 1997). Typically, relatively low levels (compared to physiological) of sodium concentrations and/or supplementation of organic osmolytes in the maturation medium will aid in cytoplasmic maturation. This process includes the accumulation of mRNA and protein, reorganization of cell organelles, and changes in cellular metabolism (Day, 2000; Ferreira et al., 2009; Xie et al., 2016).

Regardless of the maturation media used, gonadotropins and growth factors are typically supplemented. The addition of gonadotropins, FSH and LH, as well as growth factors such as epidermal growth factor (EGF) aid in the progression of meiotic divisions, indicated by the

extrusion of the first polar body (Park et al., 2004; Schoevers et al., 2003). This process is otherwise known as nuclear maturation, and is necessary for oocytes to be competent for blastocyst formation (Sutton et al., 2003). This ability for oocytes to become fertilized, properly develop to blastocysts, initiate pregnancy, and have normal fetal development is known as oocyte competence (Watson, 2007).

Nuclear maturation of the oocyte is regulated by signals transmitted via gap junctions between cumulus cells and the oocyte (Rath et al., 1995; Sutton et al., 2003). With nuclear maturation mimicking oogenesis *in vivo*, resumption of meiosis and progression to metaphase II (MII) would mimic the effects of the pre-ovulatory LH surge *in vivo*. Gap junction communication in the follicle *in vivo* is vital in oocyte meiotic regulation by allowing passage of cyclic adenosine monophosphate (cAMP), metabolites, and amino acids (Sutton et al., 2003). *In vitro*, expansion of cumulus cells is associated with increased percentages of oocytes reaching MII and has beneficial effects on further development to morula and blastocyst stages. This process is enhanced by the presence of porcine follicular fluid (pFF) and FSH (Algriany et al., 2004; Marchal et al., 2001; Rath et al., 1995). By removing cumulus cells in porcine oocytes before IVM, this will decrease the quality and developmental competence of the oocytes. However, it has been demonstrated that co-culturing denuded oocytes with cumulus cells will improve developmental competence in oocytes (Zhang et al., 2010).

Porcine follicular fluid could enhance the response of granulosa cells to FSH and LH during oocyte maturation (Gilchrist and Thompson, 2007). Expression of the LH receptor is restricted to MGC, but factors released by MGCs could convey the LH response to cumulus cells and the oocyte (Park et al., 2004). EGF - like peptides are factors released by MGCs in response

to the LH surge, and induce resumption of meiosis and progression to MII (Gilchrist and Thompson, 2007).

Nevertheless, there has been conflicting data regarding the effect of pFF alone on nuclear maturation and blastocyst formation. Marchal and coworkers (2000) saw that oocytes matured in the presence of pFF (10% v:v) displayed reduced sperm penetration, cleavage rates, and development of embryos (Marchal et al., 2001). Potentially, the origin of pFF used could explain reduced developmental competence. In a few studies, supplementing maturation medium with pFF (10% v:v) from large follicles (5-8 mm) versus small follicles (2-4 mm) aids in cumulus expansion, nuclear maturation, and blastocyst formation (Algriany et al., 2004; Ito et al., 2008). Not only is developmental competence dependent on the size of the follicles from which the pFF is collected, but partially enhanced by the inclusion of FSH (Algriany et al., 2004; Ito et al., 2008; Rath et al., 1995).

Oocyte maturation *in vivo* is termed an “induced” process where gonadotropin cascades override the meiotic inhibiting factors in the follicle. *In vitro*, however, spontaneous maturation occurs when oocytes are removed from the follicles and placed into IVM systems (Gilchrist and Thompson, 2007; Van Blerkom, 1984). An induced oocyte maturation system has been developed in the murine *in vitro* system, where meiotic resumption is inhibited by cAMP and resumption is induced by either FSH or EGF (Eppig and Downs, 1987). The intracellular second messenger, cAMP, plays an important role in regulation of oocyte maturation and when added to IVM, activators of adenylate cyclase, such as FSH, will increase cAMP in oocytes (Gilchrist and Thompson, 2007).

Follicle stimulating hormone stimulates COC utilization of glucose. Glucose can be utilized in two ways, glycolysis or the pentose phosphate pathway (PPP) (Alvarez et al., 2013).

Glycolysis produces either lactic acid, ethanol, or pyruvate from glucose. Specifically, pyruvate could be further oxidized by the Krebs cycle within the oocyte mitochondria. Both glycolysis and the Krebs cycle generate two major energy currencies, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH). Glucose could also enter the pentose phosphate pathway (PPP) which is majorly involved in meiotic regulation (Sutton et al., 2003). The PPP oxidizes glucose to produce ribose, instead of ATP, for nucleotide and nucleic acid synthesis, and nicotinamide adenine dinucleotide phosphate (NADPH).

When both glucose and sodium pyruvate are added to the IVM medium, a greater number oocytes are accelerated in the progression of nuclear maturation (Sato et al., 2007). Finally, amino acids are utilized by the oocyte and cumulus cells as energy sources. The amino acid, cysteine, is involved with the production of glutathione (GSH), this is a thiol tripeptide which is important for the reduction and protection against reactive oxygen species (ROS), as well as, being essential for male pronucleus formation and subsequent early embryonic development (Sutton et al., 2003).

Porcine In Vitro Fertilization

Events for successful fertilization include sperm capacitation, acrosome reaction, zona pellucida penetration, and pronuclei formation. Capacitation is the process of mammalian sperm becoming capable of fertilizing oocytes by removing seminal plasma proteins coating the sperm surfaces (Ikawa et al., 2010). Post-capacitation, sperm will become hyperactively motile and bind to the zona pellucida of the oocyte. The binding will initiate a process known as the acrosomal reaction. This will allow the release of acrosomal enzymes to aid sperm-induced digestion through the zona pellucida (Shur et al., 2004). Once both capacitation, acrosome reaction, and penetration of the zona pellucida occurs, the sperm nucleus will de-condense and

form the male pronucleus, signifying successful fertilization (Shur et al., 2004; Wassarman et al., 2001).

To investigate fertilization location, unilateral resection of the isthmus of the oviduct were conducted in the porcine model finding that fertilization and early embryonic development occur in the ampulla rather than the isthmus (Hunter and Leglise, 1971). Hunter and Leglise (1971) saw that absence of the isthmus led to increased amounts of sperm entering the ampulla, thus increasing the incidence of polyspermy (Hunter and Leglise, 1971). Polyspermy is when multiple sperm fertilize an oocyte, resulting in embryo death. This suggests that the isthmus portion of the oviduct regulates the passage of fertilization-competent spermatozoa to the site of fertilization, though this step is bypassed by IVF (Hunter and Leglise, 1971; Ikawa et al., 2010).

In the mouse model, bicarbonate (HCO_3^-) is essential for fertilization because, in its absence the acrosome reaction cannot occur (Abeydeera and Day, 1997b; Lee and Storey, 1986). It has also been reported that HCO_3^- is necessary for penetration of pig oocytes during IVF (Suzuki et al., 1994). Sperm penetration can occur in HCO_3^- free media when supplemented with synthetic organic buffers such as Hepes, Tris, or Mops but not as efficiently as HCO_3^- buffered media (Abeydeera and Day, 1997b; Abeydeera et al., 1997). Tris is often used as a component of buffer solutions, such as in TAE and TBE buffer as alternative to sodium bicarbonate which aids in the release of CO_2 and neutralizes acidity. It has been demonstrated that *in vitro* pig oocytes can be penetrated by spermatozoa in a modified Tris-buffered medium (mTBM) without bicarbonate (Abeydeera and Day, 1997b) and that penetration is significantly decreased in the presence of Hepes (Suzuki et al., 1994). *In vitro* fertilization media supplemented with synthetic organic buffers should be at the ideal conditions with pH of 7.2, 5% CO_2 in air, and 38°C for sperm penetration of the porcine oocyte (Abeydeera and Day, 1997b).

Other factors aid in fertilization parameters *in vitro*, such as bovine serum albumin (BSA) and caffeine. By using a concentration of 0.4% BSA, the average number of sperm per oocyte was significantly increased, so this concentration could have stimulated the capacitation (Abeydeera and Day, 1997b). In this instance, the average number of sperm bound to the oocyte is a measurement of fertility, and is not necessarily indicative of polyspermy.

The presence of caffeine in IVF media can stimulate capacitation and penetration of porcine oocytes, as well as increase the motility of boar spermatozoa (Abeydeera and Day, 1997b; Wang et al., 1991). The optimum concentration of caffeine appears to be 1mM where the percent oocytes penetrated and percent polyspermic oocytes were optimized with a high percentage penetrated but lower incidences of polyspermy (Abeydeera and Day, 1997b; Wang et al., 1991).

Typically, supplementation of BSA would improve male pronucleus formation and prevention of polyspermic fertilization (Ito et al., 2008). However, these supplementary factors are “undefined” and lead to an inconsistency in development (Abeydeera et al., 1998). Thus, there are advantages to using a truly defined medium that does not contain any animal-derived components such as BSA. One commonly used non-biological alternative to BSA inclusion in the IVF medium is poly-vinyl alcohol (PVA). However, when included in IVF medium as a direct substitute for BSA, PVA compromises blastocyst development. Thus, it appears that a semi-defined fertilization media with BSA and caffeine and without bicarbonate can effectively aid in the penetration of pig oocytes (Abeydeera et al., 1998).

Immediately following *in vitro* maturation, porcine oocytes are freed from cumulus cells using hyaluronidase (enzymes that degrade hyaluronic acid). This allows identification of oocytes that have reached metaphase II (MII) by locating the extruded first polar body. This not

only aids in identifying oocytes that presumably completed nuclear maturation, but also assists sperm in penetrating the zona pellucida.

Extended fresh semen is predominantly used for porcine IVF due to the impracticality of using frozen boar semen, though some have attempted to use frozen-thawed spermatozoa. Preparation of sperm for IVF can be done by the “swim up” method and/or by Percoll density gradient centrifugation. These preparation methods ensure that sperm are free from seminal plasma, thus allowing them to undergo capacitation as previously stated (Ikawa et al., 2010). During centrifugation, sperm washing media can be used to prepare sperm for fertilization, and use of wash media is known to increase the percentage of cleaved oocytes, and blastocysts formed (Y.J. Yi, 2004). Common components in sperm washing media include, protein supplementation such as BSA or PVA and antibiotics such as penicillin or gentamycin.

Post-sperm washing, the sperm concentration is adjusted for optimal fertilization conditions (Abeydeera and Day, 1997a). Abeydeera and Day (1997) observed that sperm concentrations of 5×10^5 and 1×10^6 /mL did not differ statistically in penetration rates and polyspermy rates however, the male pronucleus per oocyte was significantly increased in sperm concentrations of 1×10^6 /mL (Abeydeera and Day, 1997a). This significant increase could be due to increased number of capacitated sperm during sperm-oocyte co-incubation and the degree of polyspermy is an indication of the number of capacitated sperm (Abeydeera and Day, 1997a; Abeydeera et al., 1997).

The timing of the sperm-oocyte co-incubation is critical because the acrosome membrane of boar spermatozoa, capacitated *in vitro*, will be unable to penetrate the zona pellucida by 6 hours post insemination (Yoshida et al., 1990). *In vivo*, within the oviduct, the ampulla is the site of fertilization and boar spermatozoa can reach and penetrate the zona pellucida within 2 hours

post-copulation and can enter the oocyte cytoplasm causing meiotic resumption within 3 hours (Abeydeera and Day, 1997a; Hunter and Dziuk, 1968; Hunter and Leglise, 1971). Abeydeera and Day (1997) investigated co-incubation lengths of 3, 6, 9, and 12 hours, finding the optimum co-incubation length to be 6 hours because there was maximum oocyte penetration (81%) with minimum incidence of polyspermy (39%) while other lengths either exhibited increased polyspermy or decreased penetration (Abeydeera and Day, 1997a). Furthermore, if the gametes are incubated for more than 18 hours, the boar spermatozoa will lose their ability to penetrate the zona pellucida of porcine oocytes (Yoshida et al., 1990).

During mammalian fertilization, the oocyte's intracellular free calcium ion (Ca^{2+}) oscillates at a low frequency to ensure stimulation of embryonic development (Lee et al., 2012; Machaty et al., 1999; Stricker, 1999). The Ca^{2+} oscillations are initiated by the sperm head diffusing into the oocyte cytoplasm and releasing phospholipase C zeta ($\text{PLC}\zeta$) (Saunders et al., 2002). Phospholipase C zeta is an enzyme that will stimulate inositol 1,4,5-triphosphate (IP_3), which will bind and activate the IP_3 receptor on the surface of the internal Ca^{2+} storage or endoplasmic reticulum (Lee et al., 2012; Saunders et al., 2002; Stricker, 1999). Stromal interaction molecule 1 (STIM1) also functions as a calcium sensor in the endoplasmic reticulum, and in the porcine oocyte model, aids in Ca^{2+} depletion of the endoplasmic reticulum and generating a Ca^{2+} influx to replenish (Koh et al., 2009; Lee et al., 2012). Due to the importance of Ca^{2+} for proper embryonic development, extracellular Ca^{2+} is required for successful fertilization (Abeydeera and Day, 1997b). Abeydeera and Day (1997) observed that Ca^{2+} plays a critical role due to the fact that no sperm penetration occurred in mTBM containing a low 0.5 mM of calcium and an optimal calcium concentration of 7.5 mM resulted in elevated sperm penetration (Abeydeera and Day, 1997a, b).

Porcine In Vitro Culture

The development of swine zygotes beyond two cleavage divisions *in vitro* had previously been limited (Davis, 1985). Pig embryos exhibit an especially long four-cell stage (20-24 hours) and, *in vitro*, resulted in the inability of defined media to overcome the four-cell block (Hunter, 1974). Methods have been developed to overcome the four-cell block. For example, zygotes can be produced *in vivo* and four-cell embryos can be recovered and cultured to blastocyst stage in a bicarbonate buffered salt solution supplemented with BSA (Davis, 1985; Davis and Day, 1978).

Inspired by work showing that mouse oviducts in organ culture will support the development of mouse zygotes to blastocyst stage (Biggers et al., 1962; Gwatkin and Biggers, 1963), Krisher and coworkers (1989) demonstrated that mouse oviduct in organ culture will also support development of pig zygotes to blastocyst stage (Krisher et al., 1989). Preimplantation mammalian embryos develop in the oviduct during the early luteal phase *in vivo*, suggesting that factors within oviductal fluid aid in the development from zygote to blastocyst stage. In contradiction to this hypothesis, culturing pig zygotes in pig oviductal fluid collected on day 3 of the luteal phase results in fewer zygotes developing to four-cell embryos (Archibong et al., 1989). However, culturing pig zygotes in 25% oviductal fluid in bicarbonate culture media after culturing in either oviductal fluid or culture media increased the percentage of blastocyst formation when compared to culturing in solely bicarbonate culture media, suggesting that dilution of oviductal fluid reduces the concentration of any embryotoxic factors (Archibong et al., 1989).

Developing a chemically defined media that can support porcine zygotes to blastocysts requires inorganic elements and energy substrates found in mammalian oviducts, however, these substances vary between species (Yoshioka et al., 2002). For example, potassium concentrations

in porcine oviducts are relatively high when compared to other domestic farm species (Iritani et al., 1974). Also, Nichol and coworkers (1992) found that the concentrations of lactate and glucose previously used in media for the development of pig embryos was elevated when compared to other species and the pyruvate concentrations were physiologically similar to those found in porcine oviductal fluid (Nichol et al., 1992).

One common media used for successful *in vitro* culture (IVC) of pig embryos is NCSU 23 medium containing substances such as BSA, calcium chloride dehydrate, and glucose while lacking substances such as sodium pyruvate and calcium lactate pentahydrate (Petters, 1993; Yoshioka et al., 2002). However, NCSU-23 is not a chemically defined media. Yoshioka and coworkers (2002) developed an IVC medium called porcine zygote medium (PZM) based on recorded inorganic and energy substrate compositions of pig oviductal fluid (Iritani et al., 1974; Nichol et al., 1992; Yoshioka et al., 2002). A defined PZM medium, known as PZM-4 with 3mg/mL of PVA added, as well as an undefined PZM medium, known as PZM-3 with 3mg/mL of BSA added, were developed to determine the effect of replacing BSA with PVA on zygote development (Yoshioka et al., 2002). There were no significant differences in blastocyst rates between PZM-3, PZM-4, and *in vivo* control. The percent that developed to the blastocyst stage in both PZM media were significantly higher than NCSU-23 (Yoshioka et al., 2002). This indicates that PZM provides a better IVC environment when compared to NCSU-23 and that porcine zygotes can successfully develop in the chemically defined medium, PZM-4.

Though many aspects of IVC conditions differ from *in vivo* conditions, oxygen tension under which embryos are cultured is one of the most critical (Karja et al., 2004). Porcine oocytes and embryos are exceptionally high in fat content. *In vitro*, however, the amount of triglyceride present decreases and there is a drop in oxygen consumption (Sturmey et al., 2009). Oxygen

consumption of early embryos reflect overall metabolic activity with a sharp increase during the morula stage, as a prelude to formation of the blastocoel cavity (Sturmey et al., 2009). Transfer of high oxygen consuming porcine embryos (more than $0.59 \times 10^{14}/\text{mol s}^{-1}$) led to 43% sows pregnant after an embryo transfer versus 0% sows pregnant from low oxygen consuming embryos. Thus, oxygen consumption rate can be indicative of embryo viability (Sakagami et al., 2015).

Oxygen concentrations for mammalian embryo culture have traditionally been maintained at the standard atmospheric oxygen tension, roughly 20%. But it has been demonstrated that the oxygen concentration in the oviduct is much lower, roughly 5% (Karja et al., 2004; Yoneda et al., 2004). Surprisingly, Yoneda and coworkers (2004) did not find significant difference in the developmental rates of porcine embryos between 5% and 20% oxygen concentrations using NCSU-23 media. Though reducing the oxygen concentration does not positively affect *in vitro* developed blastocyst rates compared to atmospheric oxygen levels (Yoneda et al., 2004), culturing embryos at atmospheric oxygen levels can lead to the formation of reactive oxygen species (ROS) (Johnson and Nasr-Esfahani, 1994; Karja et al., 2004; Yoneda et al., 2004). Interestingly, Yoshioka and coworkers (2002) found that porcine embryos cultured in PZM-3 were elevated under 5% than under 20% (Yoneda et al., 2004; Yoshioka et al., 2002). Suggesting that the PZM provides more suitable IVC environment for porcine embryos under a 5% oxygen concentration.

Reactive oxygen species, such as hydrogen peroxide and superoxide, are produced by mammalian mitochondria as oxygen is reduced along the electron transport chain and play an important role in cell signaling. By increasing oxygen concentration to 20% in IVC, there is an increase in hydrogen peroxide levels in porcine embryos (Yoneda et al., 2004). With the increase

in ROS, this potentially could lead to apoptosis or programmed cell death. Though some apoptosis is normal during embryonic development, increasing ROS in the embryo can increase apoptosis leading to the elimination of viable cells in the developing embryo (Hardy, 1997). Reactive oxygen species can lead to lipid peroxidation and since porcine embryos contain large amounts of cytoplasmic lipid, this can significantly lower cell division and cell number in the blastocyst (Yoneda et al., 2004).

With many varying IVC environments, the number of cells comprising the inner cell mass (ICM) and trophectoderm (TE) in the embryo is a good indicator of embryonic development and quality (Rath et al., 1995). Compared to porcine *in vivo* developed embryos, *in vitro* embryos have lower cell numbers (Yoshioka et al., 2002). Yoshioka and coworkers (2002) observed that compared to *in vivo* Day 6 embryos, porcine zygotes cultured to Day 6 in NCSU-23, PZM-3, and PZM-4 had significantly lower ICM and total cell numbers (Yoshioka et al., 2002). In the same experiment, embryos cultured in PZM-3 and PZM-4 had significantly greater ICM and total cell numbers when compared to embryos cultured in NCSU-23 (Yoshioka et al., 2002).

Not only did Yoshioka and coworkers (2002) observe difference in cell numbers between culture media but also between oxygen concentrations. Day 8 blastocysts cultured in PZM-3 under 20% oxygen had significantly less ICM and total cell numbers when compared to 5% oxygen (Yoshioka et al., 2002). A similar observation was made by Karja and coworkers (2004) where carrying out IVM, IVF, and IVC in atmospheric oxygen concentrations significantly lowered total cell numbers compared to carrying out IVM, IVF, and IVC in 5% oxygen concentrations (Karja et al., 2004).

Maternal Nutrition on Reproductive Performance

Nutritional factors before and during puberty play a crucial role in growth, successive estrous cycles, and reproductive productivity. Increasing dietary energy to 9,960 kcal metabolizable energy and giving 0.1 IU/kg body weight IV of insulin to cycling gilts, concentrations of LH and FSH increases ovulation rates (Cox et al., 1987; Flowers, 1988). Furthermore, high energy intake during pre-pubertal growth reduced the age at which puberty is attained and increased body fat content (Noblet et al., 1990). Rydhmer and coworkers (1992) proposed that selection against fat may delay the onset of puberty, especially if it reduces appetite and is not associated with improvements in growth rate (Rydhmer, 1992). Recognizing that nutrition will modify ovarian function in prepubescent gilts has led to the implementation of this practice on commercial pig farming. However, the overall objective is obtaining optimal gilt fatness without compromising age of puberty or subsequent reproductive performance.

Nutrition and metabolic function play an important role in follicular development and hormonal regulation. It is well known that feed restriction in female pigs leads to inhibition of GnRH and suppression of LH secretion. By reducing LH or follicular responsiveness to LH, fewer follicles pass the selection process (Hazeleger et al., 2005). Not only are LH pulses reduced during feed restriction, but insulin and FSH concentrations are reduced as well (Booth et al., 1996). Insulin treatment can reverse the reproductive effects of feed restriction by stimulating LH receptor formation and estrogen production in the granulosa cells of follicles, therefore, increasing ovulation rates (Cox et al., 1987; Hazeleger et al., 2005).

Dietary composition also affects reproductive processes. For example, sows fed a starch-rich diet rather than a fat-rich diet, had larger follicles two days post weaning (van den Brand et al., 2000). Zhou and coworkers (2010) also demonstrated the importance of diet composition,

showing that gilts fed high-starch diets had higher LH pulse frequency, circulating insulin, and number of follicles >4 mm, compared to gilts fed the high-fat diets (Zhou et al., 2010).

As previously discussed, GDF9 is an important growth factor in the process of mammalian folliculogenesis. In the pre-pubertal rat ovary, the monosaccharide or simple sugar, galactose based diet will induce ovarian toxicity by decreasing GDF9 protein expression and inhibiting follicular growth when compared another simple sugar, glucose (Liu et al., 2006). This suggests that there's a difference in specific monosaccharide metabolism acting on follicular growth and development.

Nutritional factors not only affect follicular development, but also alter luteal function. Suboptimal luteal function could be a major factor for infertility in pigs and luteal function can be affected by nutritional factors (Foxcroft, 1997). By increasing the amount of pre-mating diet (1.15 kg v. 3.5 kg) fed to gilts when compared to maintenance fed gilts, corpora lutea number on the ovary was greater (22.7 v. 19.0) and the corpora lutea were heavier. By continuing this diet post-mating, those gilts had heavier livers suggesting a greater capacity to metabolize progesterone (Ashworth et al., 1999a). A similar study where primiparous sows were subjected to a single week of reduced feed intake (from day 21 to 28 of lactation) resulted in reduced CL numbers when compared with that of ad libitum fed sows (15.4 v. 19.9 corpora lutea per sow) (Jindal et al., 1996; Zak et al., 2008).

During the beginning of pregnancy, progesterone concentrations supplied by the CL support embryo survival. Few studies observe the potential effects of poor nutrition on reproductive performance. Simulating a human modern diet with consumption of saturated fats and simple sugars, especially fructose, is associated with many metabolic diseases and infertility. It has been demonstrated that rats consuming a high fructose diet had difficulty becoming

pregnant when compared to the control diet (Gray et al., 2013). While excess sugar consumption can be detrimental to reproductive performance, consumption of non-esterified fatty acids or triglycerides and cholesterol should also be taken into consideration (Gray et al., 2013).

In the mouse, induction of maternal obesity by feeding a high fat diet results in oocyte meiotic aneuploidy, early embryonic loss, and fetal growth retardation occurred (Luzzo et al., 2012). In swine, consumption of a high intake pre-mating diet (1.15 kg v. 3.5 kg), results in day 12 blastocysts with more cells and greater production of CO₂ from glucose metabolism (Ashworth et al., 1999b). However, Ferguson and coworkers (2006) demonstrated that by increasing pre-mating feed intake in gilts with a starch-rich diet, fetuses were underdeveloped compared to the maintenance control diet (Ferguson et al., 2006). A similar observation was made by De and coworkers (2009) where embryonic survival in early pregnancy was significantly higher in the gilts fed a maintenance control diet compared to gilts fed for high consumption of their diet (De et al., 2009).

Hormone levels are also affected by feed consumption. Serum concentration of IGFI, leptin, and insulin tended to be higher in high fed gilts during early gestation (De et al., 2009). Not only do dietary-induced changes in concentrations of reproductive hormones impact embryo survival and uterine development in early pregnancy, but maternal diet can also affect fetal epigenetic reprogramming. High fat diets during gestation have been associated with offspring phenotypical changes such as obesity and diabetes. In a study where a high fat diet was consumed by Japanese macaques during gestation, there was an observed increase in hepatic triglycerides in fetal livers and increased expression of DNA methyltransferases 1 (*DNMT1*) which regulates methylated cytosine residues. This is significant because abnormal DNA methylation patterns are associated with developmental abnormalities (Seki et al., 2012). In

mice, consumption of a high-fat diet during gestation caused increased expression of insulin-like growth factor 2 (*Igf2*) which might cause epigenetic modifications that can be transmitted to the progeny (Chango and Pogribny, 2015; Masuyama and Hiramatsu, 2012). Taken together, these studies demonstrate that maternal diet affects reproductive hormone concentrations, ovarian environment, early embryonic development, and fetal epigenetic modifications. Unfortunately, the underlying mechanisms remain undetermined.

Swine Model for Human Infertility

Diet-induced obesity is one of many factors that adversely impact reproductive performance in women. Obesity is defined as having a body mass index (BMI) greater or equal to 30 kg/m² and has impacts on reproductive function by causing hormone imbalances and ovulatory dysfunction (Homan et al., 2007). Women who struggle with fertility issues can seek assisted reproductive technology (ART) treatment to optimize their chance to conceive. Unfortunately however, women with a high BMI respond poorly to ART treatment (Homan et al., 2007). Although obesity is known to affect fertility in females, the underlying mechanisms are not completely understood.

One condition associated with obesity and subfertility is an ovulatory dysfunction disorder known as polycystic ovary syndrome (PCOS). It is thought that pre-pubertal obesity promotes the development of PCOS in adolescents, which then continues into adulthood (Anderson et al., 2014). An early indicator of adolescent PCOS is insulin resistance. Although obesity and insulin resistance appear to be partly independent of each other, it is known that obesity intensifies insulin resistance in PCOS (Anderson et al., 2014). To determine if insulin signaling plays a role in infertility associated with obesity, Brothers and coworkers (2010) used a pituitary-specific insulin receptor knockout mouse model (PitRKO) in lean and diet-induced

obese models to assess reproductive parameters (Brothers et al., 2010). They found that when wildtype mice became obese, they resembled women with PCOS and reduced fertility.

However, obese PitRKO mice displayed improved reproductive function, suggesting a role of pituitary insulin signaling in obesity-associated infertility (Anderson et al., 2014; Brothers et al., 2010).

Typically, rodents are used as models to study metabolic parameters even though they do not always mimic disease pathogenesis in humans (Rosini et al., 2012). Pigs are becoming an ideal model to study human disease due to the anatomical, physiological, and biochemical similarities to humans (Newell-Fugate et al., 2014; Spurlock and Gabler, 2008). As such, pigs are particularly useful models for understanding obesity and insulin resistance in adolescents and adults. Newell-Fugate and coworkers (2014) used female Ossabaw minipigs to characterize the effects of diet-induced obesity on metabolic parameters and reproductive function which were then compared to that of obese women (Newell-Fugate et al., 2014). The Ossabaw pigs displayed many clinical signs of obesity including hypertension, insulin resistance, and increased LDL: HDL cholesterol (Spurlock and Gabler, 2008).

Metabolically and physiologically speaking, nutrient digestion and absorption in swine is remarkably similar to humans. One striking similarity is the structure and function of the pancreas. A key function of the pancreas in mammals is insulin secretion from the β -cells of the islets of Langerhans (pancreatic islets). Insulin's primary function is to promote the absorption of glucose from the blood. Glucose can stay in the circulation or be transported to the liver, or other tissues, for metabolism entering via GLUT4, which is insulin dependent (Bray et al., 2004).

The pig pancreatic islets cells function in a similar manner to that of humans, suggesting that pigs are an excellent model to study certain diseases such as type 2 diabetes (Swindle et al.,

2012). Obesity along with insulin resistance are characteristic risk factors for type 2 diabetes. Insulin resistance will eventually cause β -cell failure and apoptosis, which will lead to an increase in circulating glucose and type 2 diabetes (Ravnskjaer et al., 2016).

Glucose enter cells primarily via GLUT4 transporters (insulin dependent) while fructose enter cells using GLUT5 transporters (non-insulin dependent) (Bray et al., 2004; Joost and Thorens, 2001). Pancreatic beta cells lack GLUT5 transporters, for this reason, fructose does not stimulate insulin secretion like glucose (Bray et al., 2004). However, fructose is transported to the liver where it is converted to glucose, thus entering the circulation (Bray et al., 2004; Tappy and Le, 2010). In human livers, fructose can be phosphorylated by the enzyme fructokinase to form fructose-1-phosphate. This configuration is cleaved by the enzyme aldolase to ultimately form trioses, the backbone for phospholipid and triglyceride synthesis (Bray et al., 2004; Tappy and Le, 2010). In pigs, some of the fructose that enters the small intestines is converted to lactate, and both fructose and lactate are metabolized in the swine liver (Bjorkman et al., 1984). Though metabolically overconsumption of fructose does not directly lead to type 2 diabetes, it does lead to obesity and insulin resistance.

In a study done by Fisher and coworkers (2013), a high energy diet (HED) composed of 150 g/kg beef tallow, 200 g/kg sucrose, and 150 g/kg fructose was fed to 35 day old pigs for 16 weeks, after which, they were transitioned to a control diet for 6 weeks (Fisher et al., 2013). They observed metabolic dysregulation such as higher triglyceride levels, higher LDL-cholesterol levels, and impaired glucose tolerance and insulin response associated with reduced pancreatic β -cell function, demonstrating diet-induced obesity has an impact on metabolic function (Fisher et al., 2013). When HED pigs were exposed to a control diet for 6 weeks, in an attempt to reverse the diet-induced metabolic dysregulation, triglyceride and LDL-cholesterol

levels were lowered, but the glucose tolerance and insulin production did not improve. This suggests that consumption of the HED diet irreversibly damaged pancreatic β -cell function (Fisher et al., 2013). Similar results were observed when cycling female Ossabaw minipigs were fed an excess-calorie, high-fat, high-cholesterol, high-fructose diet (Newell-Fugate et al., 2014). Obese pigs (162.8 ± 4.9 kg) were hyperglycemic, hyperinsulinemic, had elevated total serum triglyceride concentrations, and had a greater insulin resistance score when compared to that of the control pigs (108.5 ± 3.8 kg) (Newell-Fugate et al., 2014). Together these experiments demonstrate the ability to induce metabolic changes in growing and adult pigs, which mimic that of childhood obesity and diabetic characteristics. Thus, under these conditions, the pig is a suitable metabolic model for humans.

Obesity and insulin resistance also affect female reproductive function and fertility. Though women have menstrual cycles whereas pigs have estrous cycles, obesity can alter both menstrual and estrous cycles. Oligomenorrhea or infrequency in menstrual cycles, which is typically the result of lengthened follicular phase, is seen in obese women and is similar to that of obese pigs that display longer estrous cycles (32.2 ± 1.3 days) (Newell-Fugate et al., 2014). These irregular reproductive cycles are associated with abnormal follicular activity on the ovary. As stated before, obese women are more likely to develop ovulatory dysfunction such as PCOS and this trend is also observed in the swine model.

During the luteal phase of the estrous cycle, obese pigs have more cystic follicles (>12.5 mm) and control pigs have more small antral follicles (<3.5 mm). In diabetic pigs, 61% of small antral follicles were atretic when compared to the 23% for control pigs (Meurer et al., 1991; Newell-Fugate et al., 2014). One hypothesis that supports the progression of PCOS is that prepubertal hyperandrogenemia enhances LH and interferes with normal follicular development

(Anderson et al., 2014; McCartney, 2010). Typically, obesity in women is associated with lower LH, variable elevations in androgens, and decreased luteal progesterone, which correlates with lengthened follicular phases (Newell-Fugate et al., 2014). In a similar manner, obese pigs had lower progesterone concentrations during the luteal phase, more cystic follicles (indicating either a lack of an effective LH surge or lack of LH response on the follicles), and elevated serum androstenedione (Newell-Fugate et al., 2014).

Androstenedione in females is released by theca cells, which is then converted to estrogen via aromatase in the granulosa cells. Androstenedione can also be converted to testosterone via 17β -hydroxysteroid dehydrogenase, but very few studies confirm the existence of 17β -hydroxysteroid dehydrogenase in the pig ovary (Newell-Fugate et al., 2014). However, diabetic gilts had lower intrafollicular concentrations estrogen and higher concentrations of testosterone when compared to control gilts potentially suggesting the presence of androstenedione and 17β -hydroxysteroid dehydrogenase in the pig ovary (Meurer et al., 1991). Taken together, the ovarian and hormonal characteristics of obesity-induced infertility in swine are similar to that of obese women.

One aspect that differs reproductively between swine and humans is that humans have one to two offspring per pregnancy while pigs are litter bearing. However, having litters rather than a single birth is advantageous for research, as it reduces genetic variation of subjects by having age-matched littermates (Swindle et al., 2012). Because pigs are litter bearing species, they have a bicornuate uterus (consisting of uterine horns). This differs from the human which have a simplex uterus consisting of a single uterine body (Gray et al., 2001). Unlike pigs, development of human uterine glands occurs slowly from the time of birth to puberty. For example, endometrial glands are absent in pigs at birth, however in humans endometrial gland

proliferation is initiated during fetal development. By day 14 of age in pigs the endometrial glands extend approximately a third of the distance from the myometrium and this process occurs in humans by 6 years of age. Although despite these differences, endometrial gland proliferation is completed postnatally in the human uterus and in a similar manner to that of pigs (Gray et al., 2001).

Fetal development and piglets are physiologically the most similar to infants when compared to other research animals. Nutrient requirements of humans and pigs are very similar between the two species in infancy, growth, reproduction, and lactation (Miller and Ullrey, 1987). However the biological age at which the two species reach these nutrient requirements varies. For example, adolescence is approximately 11 to 14 years of age in humans but 4 to 6 months of age in pigs (Miller and Ullrey, 1987). The nutrient requirements are similar because both have a monogastric stomach and the physiology of digestion is remarkably similar (Swindle et al., 2012). One additional noteworthy aspect of using a fetal pig model is to study various aspects of adipose tissue development and factors responsible for obesity (Miller and Ullrey, 1987). Therefore, the similarities between pigs and humans are notable and there is great potential to use the swine model for studying diet-induced obesity and diabetes along with its effects on reproductive performance.

Conclusions

In conclusion, multiple factors including nutrition contribute to reproductive performance, which can be measured as attainment of puberty, regular cyclity, and pregnancy in female swine and other mammals. Normal cyclity is important for proper ovulation and oocyte competency. When an ovulatory dysfunction (anovulation) occurs, women can seek out the assistance of IVF. *In vitro* fertilization is also an important tool to investigate factors such as

nutrition, environmental stress, and age impacting oocyte maturation and early embryonic development. By using swine as a model for human infertility, due to their similar metabolic parameters, we can better understand how diet-induced diseases such as obesity are influencing reproductive performance. Therefore, the purpose of the study is to investigate how a fructose and/or fat diet alters factors important in reproductive tract growth and function, such as embryo formation and support, in prepubertal gilts.

Chapter 2: Maturation of oocytes with follicular fluid from gilts consuming high fat and fructose diets affects subsequent embryo development

Abstract

Today's modern diet containing saturated fats and simple sugars has been suggested to play a role in the rising epidemic of obesity in humans. Consumption of a high fat and/or sugar diet not only correlates to obesity but also has an association with infertility. Diet-induced obesity could lead to hormonal imbalances that hinder oocyte competency and subsequent embryonic development. However, few studies have investigated the potential effects that consumption of a modern diet may have on oogenesis and embryogenesis. In this study, pre-pubescent gilts (n=20) were placed on one of five dietary treatments: 15% fat included (FAT), 35% fructose included (FRU), both fat and fructose included (HFHF), and two different control diets. The first control diet was a standard industry diet (IND) meant to result in optimal lean growth. The second control diet (LYS) was fed to match the lysine intake of the gilts receiving fat-containing diets. The treatment groups that received the fat (FAT) and fat + fructose (HFHF) diets were fed for ad libitum intake. The FRU and LYS groups were pair fed to match the intake of the FAT and HFHF groups. Porcine follicular fluid (pFF) collected from these gilts at 12 weeks of age, was introduced into *in vitro* maturation systems to determine whether characteristics of the follicular fluid affect oocyte competence and embryo development. There were no differences in the frequency of oocytes reaching metaphase II (MII) ($p=0.57$) between treatment groups, indicating the supplementation of pFF does not affect nuclear maturation. Frequency of day 5 blastocysts from IND group tended to be reduced compared to FFF, and that of FRU and HFHF group was reduced compared to FFF ($p=0.10$, $p=0.01$, and $p=0.03$ respectively). Frequency of day 6 blastocysts from FAT group tended to be reduced compared to

FFF, and the frequency from HFHF group was reduced compared to FFF ($p=0.08$ and $p=0.01$ respectively). Average total cell number of day 6 blastocysts from FAT group was increased compared to all other groups ($p<0.05$), except that of FRU group which only tended to be lower ($p=0.07$). Based on these results, we conclude that the inclusion of follicular fluid derived from HFHF, FAT and FRU fed pigs in the maturation medium does not impact nuclear maturation of oocytes but may affect oocyte competency, thus resulting in detrimental effects on subsequent development of embryos, especially blastocyst formation. Further studies will help us identify more specific effects of nutrition on oogenesis and subsequent embryo development.

Introduction

Modern diets including saturated fats and simple sugars, especially fructose, are associated with many metabolic diseases and infertility in humans (Sharma et al., 2013). With excessive caloric intake from consumption of high-fat foods and simple sugars, prevalence of obesity has increased to approximately 17% in adolescents and 35% in adults as of 2012 (Ogden et al., 2014a). One component of modern diets that has contributed to the rise in obesity is the development and consumption of high fructose corn syrup (Bray et al., 2004). High fructose corn syrup is found in almost all foods containing caloric sweeteners such as processed foods and soft drinks. Consumption of fructose has increased by nearly 30% from 1970 to 2000 and this trend is similar in the rise of obesity in the United States (Bray et al., 2004; Flegal et al., 2010). Not only is there an association between modern diet and obesity, but one of the devastating conditions associated with obesity is infertility.

Currently, the primary treatment for infertility is the use of assisted reproductive technologies (ART). One example can be the use of technologies such as *in vitro* fertilization (IVF) to aid in the treatment of infertility in humans. According to the CDC, the use of ART in

the United States has increased roughly 25% from 2004 to 2013 (CDC, 2015). Diet-induced obesity is related to hormonal imbalance and is further correlated with poor oocyte competence and embryonic development.

Few studies have considered the potential effects that consumption of a modern diet may have on female reproductive characteristics and function, especially on oogenesis and embryogenesis. It has been demonstrated in the rodent model that poor maternal diet negatively impacts oocyte and embryo quality. By inducing obesity with the consumption of a high-fat diet in the mouse model, this decreased the percent oocytes reaching metaphase II, cleavage rates, and blastocysts rates when compared to the control diet (Luzzo et al., 2012). In a similar model, rats consuming a high-fructose diet experienced difficulty becoming pregnant when compared to those consuming the control diet (Gray et al., 2013). In the swine model, there is evidence that both diets consumed before mating and during early pregnancy have a major impact on oocyte competency and embryo development (Ashworth et al., 1999a; Ashworth et al., 1999b; De et al., 2009; Ferguson et al., 2006). Altogether, these studies demonstrate that maternal diet has subsequent consequences for embryonic development and survival.

In vitro systems provide a way to investigate factors such as nutrition, environmental stress, and age impacting oocyte maturation and early embryonic development. Specifically aiding to observe components that aid in oocyte maturation *in vivo*, follicular fluid. On the ovary and within the antral follicles, fluid within the antrum cavity is called follicular fluid. The importance of follicular fluid in ovarian physiology include steroidogenesis, growth of the follicle to ovulation, maturation of the oocyte and its transport to the oviduct for fertilization (Edwards, 1974).

This current study was specifically designed to evaluate the direct effects of fat and/or fructose intake on oocyte competency and embryo development in the pre-pubescent porcine IVF model. Porcine follicular fluid (pFF) collected from these gilts was introduced into *in vitro* maturation systems to determine whether characteristics of the follicular fluid affect 1) oocyte maturation 2) subsequent blastocyst development and 3) embryo quality. Results of this study will provide justification for future experiments investigating the effects of fat and/or fructose consumption on fertility, specifically oogenesis and embryogenesis.

Materials and Methods

All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee (#13-103).

Animals and Diets

Twenty female cross-bred pigs from Virginia Tech Swine Center (Blacksburg, VA) were weaned at 21 days of age and offered a basal diet that met nutrient requirements as determined by the NRC (1998). The gilts were acclimated to the housing environment for three days and then began their treatment diets designating the start of the trial as week 0 (Wk0). At 24 days of age, all gilts were weighed and grouped into treatment pairs matched by weaning weights. The paired gilts were randomly assigned to one of five dietary treatment groups (n=4 pigs per treatment): 15% beef tallow included (FAT), 35% fructose included (FRU), both 15% beef tallow and 35% fructose included (HFHF), and two different control diets (Table 1). The first control diet (IND) was a standard industry diet designed to result in optimal lean growth. The second control diet (LYS) was designed to account for the reduced lysine intake by the gilts consuming the high fat diets. Daily feed intake was measured in the FAT and HFHF groups, the FRU and LYS groups were pair fed to match the intake of the pigs consuming fat-containing

diets to ensure balanced nutrient intake among treatment groups. The control IND group's intake was measured weekly. To ensure that weights were kept similar within each pen, gilts within each treatment were re-paired on Wk3 and remained in this new pairing for the duration of the trial.

Follicular Fluid Collection

At 12 weeks of age (Wk9), one gilt from each pen was transported to the animal facility at Litton-Reaves Hall, Virginia Tech for sacrifice. Pigs were sacrificed using Beuthanol and internal reproductive organs were collected and snap frozen in liquid nitrogen for further analysis (discussed in a later chapter). Visible antral follicles were counted and aspirated for each gilt. Follicular fluid was collected in labeled 1.5 mL tubes by pig ID number and stored at -20°C. Follicular fluid (pFF) was thawed and pooled by treatment groups for this study.

Preliminary study investigating supplementation percentage of pFF

A preliminary study was conducted on abattoir-collected follicular fluid supplemented at 0%, 10%, and 20% (v:v) in the maturation medium. This was done to investigate if there were any differences between 10% and 20% pFF supplementation. All procedures (IVM, IVF, IVC, and embryo quality analysis) were conducted in a similar manner described below.

This preliminary study was done to investigate if there were any differences between 10% and 20% pFF supplementation during the maturation (IVM) period. No differences were found in nuclear maturation, day 5 blastocyst frequencies, day 6 blastocyst frequencies, and total cell number ($p>0.10$) between supplementing 10% vs. 20% (Table 2). Therefore for the study 20% pFF was supplemented in order to achieve adequate results with the minimum amount of follicular fluid available.

In Vitro Maturation (IVM)

All chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. Ovaries were obtained from a local abattoir (Gunnose's Sausage Co., Inc.), washed in sterile saline (0.9% NaCl), and medium size (3-6 mm) follicles were aspirated using an 18 gage needle attached to a 10 mL sterile syringe. Cumulus oocyte complexes (COCs) were washed in 10 mM Hepes buffer wash medium three times. After the final wash, COCs were placed in a 60 mm x 15 mm culture dish to search for good quality oocytes using a micropipette (Drummond Science®, Captrol III®). Oocytes with circular, dark cytoplasm and intact cumulus cells were selected for maturation. Stock solutions of a TCM-199 (M 5017) maturation medium with 0.1 mg/mL cysteine, 0.55 mg/mL glucose, 0.13 mg/mL sodium pyruvate, 10 ng/mL epidermal growth factor (EGF), 0.5 µg/mL follicle stimulating hormone (FSH), 0.5 µg/mL luteinizing hormone (LH) and 20% (v:v) pFF from treatment groups, sterile filtered using 0.22µm SFCA Nalgene syringe filter (Thermo Scientific), and stored in 4°C refrigerator for subsequent replicates. Additionally, another group of COCs, labeled follicle fluid free (FFF), were matured in TCM-199 medium without pFF. Due to insufficient amounts of follicular fluid, the LYS group was omitted from this study. Roughly 10 selected cumulus oocyte complexes were matured *in vitro* in 50 µL TCM-199 drops for 40-44 h at 38.5°C, 5% CO₂ in air. Three to six replicate (n) experiments were conducted using a total of 951 oocytes, 246 FFF (n=6), 207 IND (n=6), 246 HFHF (n=6), 126 FAT (n=3), and 126 FRU (n=3).

In Vitro Fertilization and Culture (IVF and IVC)

At the end of the maturation, the surrounding cumulus cells were removed from the COCs by vortexing for 5 min in the presence of 0.1% hyaluronidase. Oocytes were selected based on the presence of the first polar body, indicating completion of metaphase II (MII). *In*

vitro matured oocytes were placed in 50 μ L droplets of IVF medium (modified Tris-buffered medium [mTBM] containing 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl₂, 11 mM glucose, 20 mM Tris, 2 mM caffeine, 5 mM sodium pyruvate, 1 mM caffeine, and 0.1% bovine serum albumin [BSA]) in groups of 30-35 oocytes.

Boar semen was collected weekly, diluted with AndroPRO® Plus (MOFA Global, Verona, Wisconsin) semen extender and kept in a 17-18°C refrigerator. For sperm wash, 9 mL of sperm washing medium (Dulbecco's PBS supplemented with 0.1% BSA, and 10 ng/mL gentamicin) was placed into a 15 mL tube, 1 mL of sperm suspension was added and washed three times by centrifugation at 1900 x g for 3 minutes at room temperature. Sperm pellet was re-suspended in 1 mL of mTBM and adjusted to a concentration of 0.25×10^6 cells/mL. Fifty μ L of the semen suspension was introduced into the droplets with oocytes. The gametes were co-incubated in the IVF medium for 5 h at 38.5°C in an atmosphere of 5% CO₂ in air. After co-incubation, the embryos were incubated in 20 μ L droplets of culture medium (porcine zygote medium 3 [PZM-3]) in groups of 15 embryos at 38.5°C, 5% O₂ and 5% CO₂ in air for 6 days.

Analysis of Embryo Quality

Embryos were assessed for the frequency of blastocysts on day 5 and day 6. Total cell count was performed on day 6 blastocysts. Briefly, the zona pellucida was removed by treatment with PBS/BSA 1% pH 1.9 at room temperature. The zona-free embryos were fixed using 4% paraformaldehyde for 30 minutes at room temperature. Fixed embryos were transferred to PBS/BSA 1% and then stained with 10 μ g/mL of Hoechst 33342 for 10 minutes at room temperature. The stained embryos were transferred to Permount™ Mounting Medium (Fisher Scientific) on a microscope slide and covered with a coverslip. Embryos were examined under UV light using a

Nikon Eclipse Ti-S Inverted Microscope. Nuclei labeled with Hoechst 33342 appeared blue and were counted for total cell number.

Statistical Analysis

Analyses were conducted by treatment (FFF, IND, FAT, FRU, HFHF). Nuclear maturation and embryo development (day 5 and day 6) were analyzed by the FREQ procedure of SAS (Cary, NC). Comparisons of frequencies were conducted using the chi-square method. Total cell numbers from day 6 embryos were analyzed using GLM procedure. Comparisons of means were conducted using the Tukey-Kramer method. Results are presented as least squared means $\pm$ SEM. Statistical significance was determined as $p \leq 0.05$; tendency for statistical significance was determined as $p \leq 0.10$.

Results

No effect of diet on porcine nuclear maturation

The effect of fat and/or fructose consumption on nuclear maturation was evaluated. The frequencies of oocytes reaching metaphase (MII) were observed after 40-44 hours of maturation. The frequencies of oocytes reaching MII were observed as 77.6% FFF, 73.4% IND, 74.6% FAT, 69.8% FRU, and 73.6% HFHF. There was no differences between nuclear maturation frequencies ($p=0.57$) between treatment groups (Figure 1A).

Fat and fructose consumption alter day 5 blastocyst development

The effect of fat and/or fructose consumption on day 5 blastocyst formation was evaluated. The frequencies of day 5 blastocyst formation were observed as 11% FFF, 5.9% IND, 6.4% FAT, 2.3% FRU, and 5% HFHF. Frequency of day 5 blastocysts from IND group tended to be reduced compared to FFF ($p=0.10$; Figure 1B). Frequencies of day 5 blastocysts from FRU

and HFHF group were reduced compared to FFF ($p=0.01$ and $p=0.03$; Figure 1B). However, there were no differences between FFF and FAT frequencies ($p>0.10$). A depiction of day 5 embryonic development can be found in Figure 2A-E (top).

Fat consumption effects day 6 blastocyst development and embryo quality

The effect of fat and/or fructose consumption on day 6 blastocyst formation and quality was evaluated. The frequencies of day 6 blastocyst formation were observed as 16.2% FFF, 10.5% IND, 8.5% FAT, 9.1% FRU, and 7.7% HFHF. Frequencies of day 6 blastocysts from IND and FRU groups did not differ from that of FFF ($p>0.10$). Frequencies of day 6 blastocysts from FAT group tended to be reduced compared to FFF ($p=0.08$; Figure 1C) and the frequency from HFHF group was reduced compared to FFF ($p=0.01$; Figure 1C). A depiction of day 6 embryonic development can be found in Figure 2A-E (bottom). Average total cell number of day 6 blastocysts were observed as 37.5 ± 2.1 FFF, 35.4 ± 2.9 IND, 51 ± 4.2 FAT, 35.9 ± 3.9 FRU, and 34.1 ± 2.9 HFHF. Average total cell number of day 6 blastocysts from FAT group was greater than all other groups ($p<0.05$), except that of FRU group which tended to be lower ($p=0.07$; Figure 1D). A depiction of stained nuclei of day 6 embryos can be found in Figure 3A-E.

Discussion

Typically, pre-pubertal porcine studies focus on the effects of altered plane of nutrition rather than the dietary composition (Ashworth et al., 1999b; Booth et al., 1996; Hazeleger et al., 2005). Studies have utilized high fat and high sugar diets to increase caloric intake, however these studies generally do not separate and investigate the effects of dietary fat versus dietary sugar on growth and reproductive performance (Fisher et al., 2013; Newell-Fugate et al., 2014; van den Brand et al., 2000). Therefore, this study focused on the effects of high fat and/or high sugar diets on pre-pubertal gilt reproductive performance. Additionally high caloric diet studies,

specifically high fat, typically do not account for the self-restricted feed intake, which limits the animal's protein intake. For this reason, the lysine control diet (LYS) was designed to account for the reduced lysine intake by the gilts consuming the high fat diets. However, due to previous work on these samples attempting to investigate triglyceride content of the follicular fluid (unpublished data), there was an insufficient amount of LYS follicular fluid for use in this study.

Based on the results, the inclusion of follicular fluid derived from HFHF, FAT and FRU-fed pigs in the maturation medium do not impact nuclear maturation of oocytes. This is contradictory to numerous studies suggesting a high fat diet would significantly decrease nuclear maturation and thus the progression to metaphase II (MII) (Luzzo et al., 2012; Sohrabi et al., 2015; Zhou et al., 2010). Though nuclear maturation was not impacted, oocyte competency may have been affected because oocyte competence also requires cytoplasmic maturation (Watson, 2007).

A reduction in oocyte competency may be responsible for the detrimental effects on subsequent development of embryos, especially blastocyst formation. We found that gilts consuming HFHF diets had reduced day 5 and 6 blastocysts frequencies when compared to FFF control group. Though this seems to signify negative outcomes in embryo development from consuming a high fructose and fat diet, unfortunately there were no differences when treatment groups (FRU, FAT, and HFHF) were compared to IND control. Although numerically, IND blastocyst frequencies were greater than FRU, FAT, and HFHF groups. This lack of difference could be due to pooling the follicular fluid samples from the four gilts in each treatment group or being unable to compare the differences in LYS treated embryos. Though it should be noted that previous findings from our lab (unpublished data), pigs fed HFHF were unable to become

pregnant with specifically fructose altering morphological characteristics of the reproductive tract. However, future studies would need to be conducted to confirm these findings.

Typically the number of cells in the inner cell mass (ICM) and trophectoderm (TE) is a good indicator of embryonic development and quality (Rath et al., 1995). Higher cell count signifies good quality embryos. Interestingly, the FAT group had greater total cell number when compared to all other treatments. However, a similar phenomenon has been seen in swine fed for high consumption pre-mating (1.15 kg v. 3.5 kg), where day 12 blastocysts had more cells compared to the maintenance pre-mating diet (Ashworth et al., 1999b). There is some evidence suggesting that preimplantation embryos release autocrine growth factors (Green and Day, 2013; O'Neill, 1998, 1997). Potentially, the high FAT dietary treatment altered the components of the follicular fluid leading to growth factors to be secreted in an autocrine manner and increasing the total cell number. This study presents a glimpse to the negative impact of high fat and/or sugar diets on oocyte competence and embryonic development. Altogether, further studies will help us identify more specific effects of nutrition on oogenesis, subsequent embryo development, and embryo quality.

Table 1. Wean diet formulation and composition for the five dietary treatments. Female pigs were fed control (IND; n=4), lysine restricted (LYS, n=4), fat (FAT, n=4), fructose (FRU, n=4), or fat and fructose (HFHF, n=4) diets for 8 weeks.

Ingredient, %	IND	LYS	FAT	FRU	HFHF
Corn	69.98	78.70	61.63	30.18	23.90
Soybean meal	24.00	19.00	20.50	20.50	23.20
Beef tallow	---	---	15.00	---	15.00
Fructose	---	---	---	35.00	35.00
Others	6.02	2.30	2.87	14.32	2.90
Calc. nutrients					
ME, Mcal/kg	3.39	3.33	3.95	3.33	3.93
Cal. from fat, %	11.0	9.1	34.5	7.3	32.2
Cal. from sugar, %	n/a	n/a	n/a	35.3	29.9
SID Lys, %	0.98	0.68	0.68	0.68	0.68
SID Lys/ME, g/Mcal	2.89	2.04	1.72	2.04	1.73

Table 2. Percentage of nuclear maturation, embryo development, and total cell number in each preliminary treatment groups.

	Metaphase II (total)	% MII	Day 5 blastocysts (total)	% blastocysts	Day 6 blastocysts (total)	% blastocysts	Total cell number (mean±SEM)
Treatment ¹							
0% (n=3) ²	101 (145)	69.66	10 (101)	9.90	16 (101)	15.84	36.00±4.36
10% (n=3) ²	106 (145)	73.10	10 (106)	9.43	22 (106)	20.75	41.11±5.01
20% (n=3) ²	96 (145)	66.21	9 (96)	9.38	18 (96)	18.75	36.58±4.13

¹ Three IVM preliminary treatment groups: 0% pFF added v:v (0%), 10% pFF added v:v (10%), and 20% pFF added v:v (20%) included.

² Number of replicates for each treatment group.

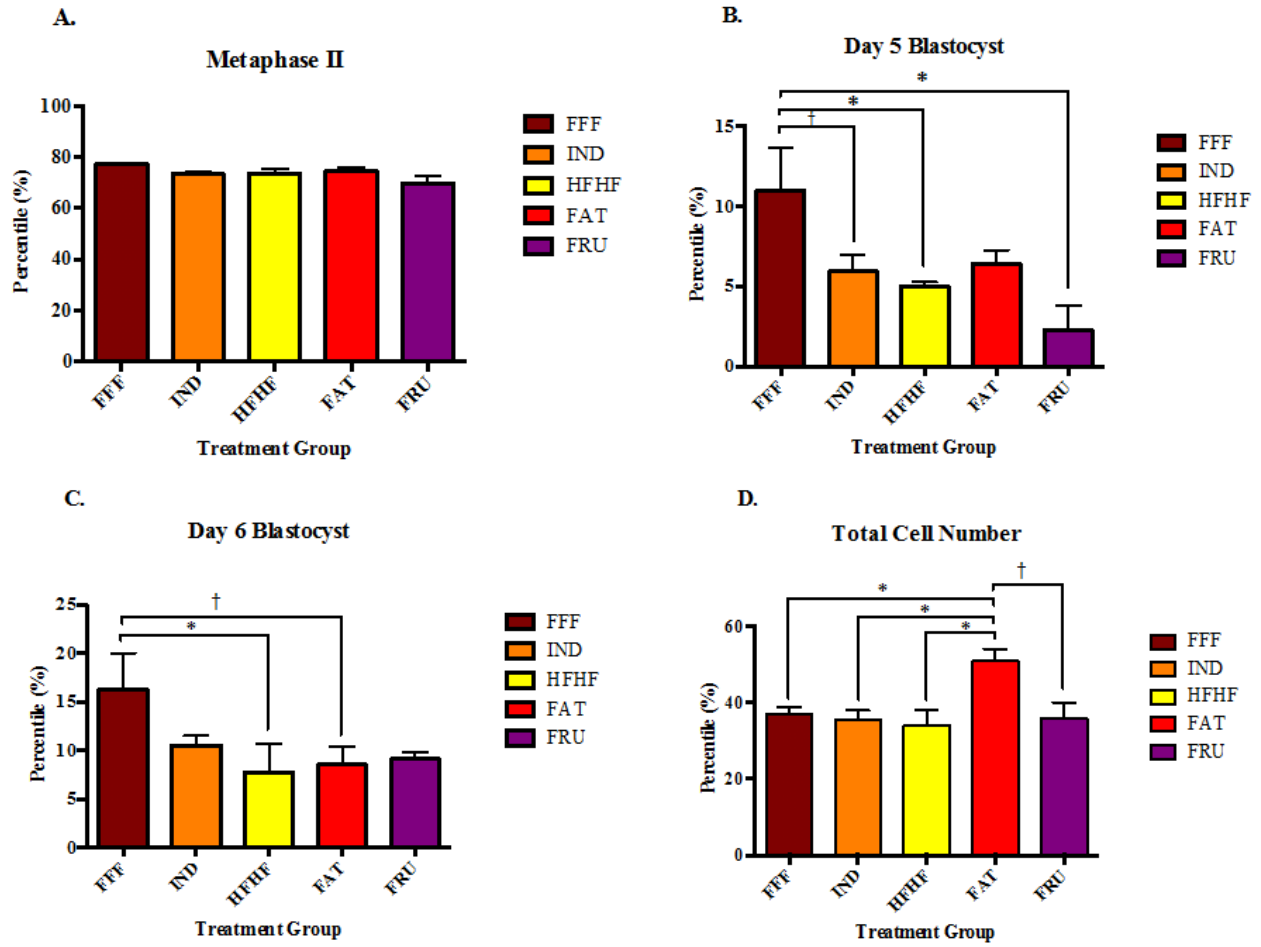


Figure 1. A. Nuclear maturation rates of oocytes developing in porcine follicular fluid from gilts fed different diets, B. Day 5 blastocyst rates, C. Day 6 blastocyst rates, D. Total cell number. $p^* < 0.05$, $p^\dagger \leq 0.10$.

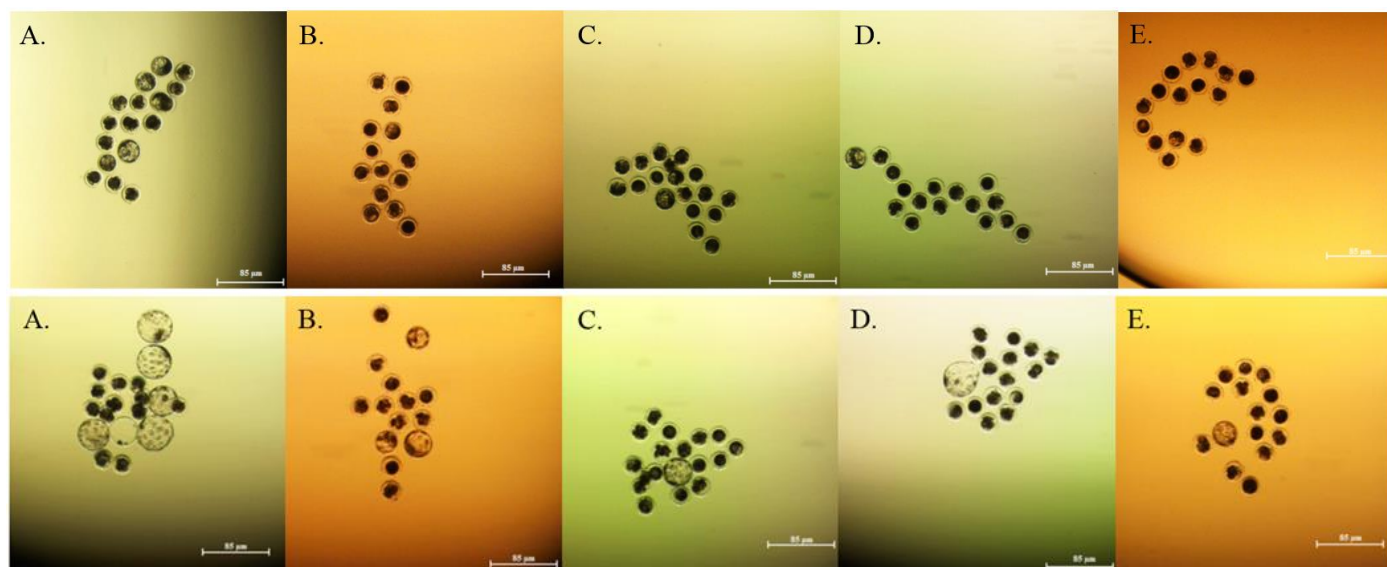


Figure 2. Porcine embryos 5 days (top) and 6 days (bottom) post fertilization.
A. Follicular fluid free (FFF) group, B. Industry control (IND) group, C. High fructose and fat (HFHF) group, D. High fat (FAT) group, E. High fructose (FRU) group. 10x objective lens.
Scale bar 85 μ m.

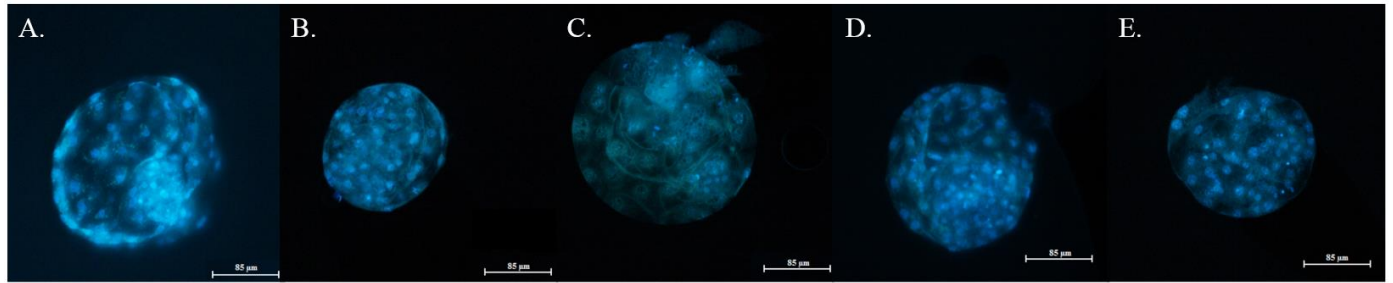


Figure 3. Hoechst stained day 6 blastocysts.

A. Follicular fluid free (FFF) group, B. Industry control (IND) group, C. High fructose and fat (HFHF) group, D. High fat (FAT) group, E. High fructose (FRU) group. 40x objective lens. Scale bar 85 μm .

Chapter 3: Gene expression in reproductive tract is altered in gilts consuming high fat and fructose diets

Abstract

Due to anatomical and physiological similarities to humans, pigs are a great model to investigate the potential relationship between diet-induced obesity and altered reproductive performance. In this study, twenty gilts three weeks in age, were assigned to one of five dietary treatments (n=4 pigs/treatment) containing 15% fat (FAT), 35% fructose (FRU), both fat and fructose (HFHF), or two different controls: one standard industry (IND) diet meant to result in optimal lean growth and a second diet to account for the reduced lysine (LYS) intake in the treatment diets. Tissues collected from the developing gilt (12 weeks of age) uterus, oviduct, and ovary were analyzed for transcript abundance of genes of interest: estrogen receptor alpha (*ESR1*), estrogen receptor beta (*ESR2*), insulin like growth factor-I (*IGFI*), insulin like growth factor-I receptor (*IGFIR*), and growth differentiation factor-9 (*GDF9*). There were no differences in expression for *ESR1* and *IGFIR* in all tissue samples. However, there was a tendency for *IGFI* expression to have a lower level of transcript in ovary samples from gilts consuming FAT diets when compared to IND diets. Also, *ESR2* was reduced in expression and *GDF9* tended to be reduced in the ovary with fructose consumption. Specifically in ovarian tissue, there is a positive correlation between *ESR2* and *GDF9* expression ($r=0.92$ and $p<0.01$). Also, *GDF9* oviduct expression was reduced in pigs consuming fat diets when compared to non-fat diets ($p=0.01$). However, *GDF9* expression tended to be more abundant in the oviduct with fructose consumption. Overall, the inclusion of fat and fructose seems to alter expression of genes responsible for proliferation and growth (*ESR2*, *IGFI*, and *GDF9*) at the transcript level in the

reproductive tissues. Further investigation will provide more insight on the impact nutrition has on pre-pubertal reproductive development.

Introduction

The prevalence of obesity is constantly increasing and becoming a worldwide epidemic. Specifically in the United States, approximately 17% of adolescents and 35% of adults are considered obese as of 2012 (Ogden et al., 2014b). Obesity is defined as having a body mass index (BMI) greater or equal to 30 kg/m^2 and has been associated with impacts on reproductive function, including hormone imbalances and ovulatory dysfunction (Homan et al., 2007). Childhood diet-induced obesity is particularly important to investigate because of the related tendencies for the development of various diseases and subsequent adulthood infertility. Childhood obesity has been associated with diseases such as type II diabetes, insulin resistance, and polycystic ovarian syndrome (PCOS) leading to difficulties in puberty attainment and maintenance of ovulatory cycles.

Typically, rodents are used as models to study metabolic parameters and reproductive physiology. Many studies have investigated diet-induced obesity on insulin resistance and infertility in rodents (Akamine et al., 2010; Gray et al., 2013; Tortoriello et al., 2004). However, pigs are becoming an ideal model to study human disease due to the anatomical, physiological, and biochemical (specifically dietary) similarities to humans (Fisher et al., 2013; Newell-Fugate et al., 2014; Spurlock and Gabler, 2008; Swindle et al., 2012). By using this model, we can characterize metabolic parameters and reproductive abnormalities in diet-induced obese pigs. Previous work from our laboratory (unpublished data) suggested pre-pubertal consumption of high fructose and fat diets altered reproductive tract development and led to difficulty establishing pregnancy later in life. This work suggested that nutrition will modify reproductive

function in prepubescent gilts and may lead to compromised age of puberty and/or subsequent reproductive performance.

Several hormones and their related components are important for proper function and reproductive development in pre-pubescent including estrogen receptors (ER), insulin-like growth factor I (IGFI), and growth differentiation factor 9 (GDF9). Estradiol and estrogen receptor (ER α and ER β) are important for the onset of puberty and reproductive behavior in females. Estradiol is a steroid sex hormone, predominantly produced by the granulosa cells of the follicles, and then acting on estrogen receptors on the entire reproductive tract (Matsuda et al., 2012). Estrogen receptors (ER α and ER β) are nuclear hormone receptors with ER α (*ESR1*) being the dominant receptor in the developed uterus and theca interna cells. Estrogen receptor β (*ESR2*) is lower in the developed uterus and expressed mainly in granulosa cells (Matsuda et al., 2012). However, *ESR1* and *ESR2* are expressed at comparable levels in the immature reproductive tissues, specifically the uterus (Koehler et al., 2005). Developmentally, function of the female reproductive tract is in response to the sensitivity of the steroid hormone receptors and has the potential to disrupt the growth of the pre-pubertal tract (Bartol et al., 1993).

Growth factors aid to stimulate cellular growth, proliferation, and differentiation. Insulin-like growth factor-I (IGFI) is a peptide hormone that is predominately produced by the liver and plays a reproductive role. Insulin-like growth factor -I specifically affects folliculogenesis (Soede et al., 2011; Wolfe et al., 2014). Both *IGFI* and *IGFIR* are expressed in porcine granulosa cells and increase responsiveness to gonadotropins, specifically FSH, thereby increasing estradiol secretion (Matsuda et al., 2012). A similar mechanism is seen in the porcine uterus with estradiol-induced uterine growth and differentiation associated with an increase in uterine IGFI secretion.

A growth factor in the transforming growth factor beta (TGF- β) superfamily, growth differentiation factor-9 (GDF9), is also thought to have a major impact on folliculogenesis (McGrath et al., 1995; McPherron and Lee, 1993). Porcine *GDF9* mRNA expression is seen in oocytes, cumulus cells, and granulosa cells; with greatest expression in oocytes (Prochazka et al., 2004). Growth differentiation factor-9 aids in the progression of folliculogenesis and development of pre-ovulatory follicles (Shimizu et al., 2004). Together these hormones function, either dependently or independently, to aid in pre-pubertal reproductive growth, folliculogenesis, and potentially attainment of puberty.

The current study was specifically designed to more clearly describe the direct effects of fat and/or fructose intake on pre-pubescent gilt reproductive tract gene expression. Tissue collection from the developing uterus endometrium, oviduct, and ovary aid to identify changes in abundance of 1) steroid hormone (*ESR1* and *ESR2*) receptor total RNA, 2) *IGFI* and *IGFIR* total RNA, 3) *GDF9* total RNA. Results of this study will provide justification for future experiments investigating the effects of fat and/or fructose on reproductive function and development pre-puberty and how this relates to human health.

Materials and Methods

All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee (#13-103).

Animals and Diets

Gilts were housed in pairs and fed as described in the previous chapter.

Tissue Collection

On week nine of the experiment (Wk9), one gilt from each pen was transported to the animal facility at Litton-Reaves Hall, Virginia Tech for sacrifice. Gilts were sacrificed as described in the previous chapter. The ovaries, oviducts, and uterus were weighed, the left uterine horn was removed from the tract and measured for total length; and the right ovary, right oviduct (isthmus and ampulla), endometrium of the uterus were snap frozen in liquid nitrogen. These tissues were selected because they support the growth and development of oocytes and embryos. All samples snap frozen in liquid nitrogen were stored at -80°C for analyses.

RNA Isolation

Tissue samples were weighed so that approximately 0.1g of each sample was placed in 12x75 tubes. For 0.1g of tissue, 1 mL of Isol-RNA Lysis Reagent (Fisher, Pittsburgh, PA) was added to the tube. Tissues were homogenized and then transferred to a 1.5 mL Eppendorf tube and incubated at room temperature for 10 minutes. Then, 200 µL of chloroform (Fisher Scientific) was added to each tube and inverted for 15 seconds. Samples were incubated for 2-3 minutes at room temperature and then underwent centrifugation at 12000 x g for 15 minutes at 4°C. The supernatant was transferred to a new 1.5 mL Eppendorf tube with 500 µL Isopropanol (Fisher Scientific) and inverted for 15 seconds. Samples were incubated for 10 minutes at room temperature and then underwent centrifugation at 12000 x g for 10 minutes at 4°C. The aqueous phase was decanted leaving a pellet at the bottom of each tube. Once all liquid was removed, 1 mL of 70% ethanol was added to the pellet and vortexed briefly until the pellet became white in color and popped off from the bottom of the tube. Samples were centrifuged at 12000 x g for 10 minutes at 4°C. The aqueous phase was decanted and the pellet was allowed to briefly dry, roughly 5 to 10 minutes. The pellet was re-suspend in DEPC water and stored at -80°C. All samples were analyzed on the NanoDrop (ND-1000) spectrophotometer (Thermo Fisher

Scientific) for RNA concentrations (ng/ μ L). On-column DNase digestion was performed, on all but oviduct samples, using the RNeasy® Mini Kit (Qiagen, Valencia, California) following the manufacturer's protocol. RNA cleanup (Qiagen) was performed on all samples following the manufacturer's protocol. All samples were stored at -80°C for RNA quality control.

RNA Quality Control

Although the NanoDrop (ND-1000) spectrophotometer (Thermo Fisher Scientific) can assess sample concentration and provide $A_{260/280}$ nm readings for purity, it does not provide data for RNA integrity. Quality and integrity of isolated total RNA was evaluated using the Experion™ automated electrophoresis system (Bio-Rad, Hercules, California). After extraction of total RNA, an aliquot (1 μ L) was taken to check RNA quality using the Standard Sense analysis chip (Bio-Rad) and followed the manufacturer's protocol. Parameters used to assess total RNA quality is 28S/18S rRNA subunit ratio (28S/18S), this is the ratio of dividing the area under the 28S peak by that of the 18S peak, and RNA Quality Indicator (RQI), and this is based on a Bio-Rad algorithm calculated by the Experion software (Bio-Rad).

Real- Time Quantitative Reverse Transcriptase-PCR (qRT-PCR)

Samples (2 μ g of total RNA) were used as a template to be converted to single-stranded cDNA using the random hexamer method. Conversion was done with a T100™ Thermal Cycler (Bio-Rad) in a 20 μ L reaction with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) for oviduct samples or iScript™ cDNA Synthesis Kit (Bio-Rad) for uterus and ovary samples and all samples diluted to 2 μ g/ μ L of cDNA. Transcript abundance was assessed by adding 10 μ L SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 1 μ L of each primer (10 μ M), 3 μ L nuclease-free water and 5 μ L cDNA template (total 20 μ L reaction

volume). The reactions were performed using the CRX96™ real-time PCR instrument (Bio-Rad). For absolute quantification, standard curves were generated using ten-fold dilutions of plasmid DNA carrying PCR amplicons (3×10^6 , 3×10^5 , 3×10^4 , 3×10^3 , 3×10^2 , and 3×10^1 copies). Standard curves as well as a negative control and no template control (NTC) were run on each real-time RT-PCR 96-well plate. Each sample was done in triplicates. Primer sequences for the genes analyzed are listed in Table 1.

Statistical Analysis

Analyses were conducted by treatment (IND, LYS, FAT, FRU, HFHF), by fructose content (FRU/HFHF vs IND/LYS/FAT), or by fat content (FAT/HFHF vs IND/LYS/FRU). Gene expression was analyzed by the GLM procedure of SAS (Cary, NC). Comparisons of means were conducted using the Tukey-Kramer method. Results are presented as least squared means $\pm$ SEM. Correlations in gene abundance within tissue samples and across tissue samples were analyzed by the CORR procedure of SAS. Comparisons of variables were conducted using the Pearson Correlation Coefficients. Statistical significance was determined as $p \leq 0.05$; tendency for statistical significance was determined as $p \leq 0.10$.

Results

Fat and fructose consumption alter estrogen receptor gene abundance

The transcript abundance of estrogen receptors alpha and beta (*ESR1* and *ESR2*) were analyzed in ovary, oviduct, and uterus. Across all five treatments, there were no differences in either ERs in ovary, oviduct, and uterus samples ($p > 0.10$). The data were also analyzed based on whether pigs received fructose (FRU and HFHF vs FAT, LYS and IND) or fat (FAT and HFHF vs FRU, LYS and IND) in their diets. In the ovary samples, the fructose diets decreases *ESR2*

expression ($p=0.05$; Figure 1B). Also in the uterus samples, the fat diets tended to decrease *ESR2* expression ($p=0.08$; Figure 3C). The oviduct samples displayed no differences in fructose or fat diets ($p>0.10$).

Fat consumption alter IGF1 but not IGFIR gene abundance

The transcript abundance of insulin-like growth factor I and its receptor (*IGF1* and *IGFIR*) were analyzed in ovary, oviduct, and uterus. Across all five treatments, there tended to be differences in *IGF1* expression in ovary samples ($p<0.10$), FAT tended to decrease expression when compared to IND ($p=0.09$; Figure 1A). However, there were no differences across all five treatments in *IGFIR* for all samples ($p>0.10$). There were no differences in *IGF1* and *IGFIR* based on fructose or fat content in any sample ($p>0.10$).

Fat and fructose consumption alter GDF9 gene abundance

The transcript abundance of *GDF9* was analyzed in ovary, oviduct, and uterus by treatment, fat, and fructose diets. Across all five treatments, *GDF9* expression tended to differ in oviduct tissue ($p<0.10$), FRU tended to be amplified when compared to FAT ($p=0.09$; Figure 2A) and amplified in FRU when compared to HFHF ($p=0.08$; Figure 2A). Looking at the data based on whether pigs received fructose (FRU and HFHF) or fat (FAT and HFHF) in their diets, *GDF9* oviduct expression was reduced in pigs consuming fat diets when compared to non-fat diets (IND, LYS, FRU) ($p=0.01$; Figure 2C). Again across all five treatments, *GDF9* expression tended to differ in the uterus ($p<0.10$), with a tendency to be increased in HFHF when compared to FRU ($p=0.06$; Figure 3A). Finally, fructose diets tended to reduce expression of *GDF9* in ovary tissue when compared to non-fructose diets (IND, LYS, FAT) ($p=0.07$; Figure 1B).

Pre-pubertal gilt gene abundance correlation in reproductive tissues

Transcript level of all genes in this study (*ESR1*, *ESR2*, *IGFI*, *IGFIR*, and *GDF9*) were analyzed for the sample coefficient of correlation within each tissue sample (ovary, oviduct, and uterus). In both ovary and uterus samples, a positive correlation (0.92 and 0.72 respectively) between *ESR2/GDF9* gene expression was found ($p < 0.01$). A summary of all the r and p values can be found in Table 2. Taking these results, expression of *ESR2* and *GDF9* were analyzed in ovary and uterus samples from pigs consuming fructose diets (FRU and HFHF) and non-fructose diets (IND, LYS, and FAT). Specifically in the ovarian samples (fructose diets and non-fructose diets), a positive correlation (0.82 and 0.93 respectively) between *ESR2/GDF9* gene expression was found ($p < 0.01$). A summary of the grouped r and p values can be found in Table 3.

Discussion

It is known that steroidogenesis and growth factors such as *IGFI* and *GDF9* work together in reproductive function. Estradiol is a steroid hormone produced by the granulosa cells of the ovary, stimulated by gonadotropins and locally produced *IGFI* (Cosgrove and Foxcroft, 1996; Meurer et al., 1991). Based on the results, fructose consumption reduces estrogen receptor beta (*ESR2*) expression in the ovary whereas fat consumption tended to decrease *ESR2* expression in the uterus. Studies suggest that *ESR2* plays a role in inhibiting cell proliferation (Lazennec et al., 2001; Martineti et al., 2005). Therefore, with a decrease in expression this could lead to cell proliferation. In regards to fructose consumption, this could be related to the increase in reproductive tract weight previously observed in these gilts consuming fructose diets (FRU and HFHF) when compared to non-fructose diets (IND, LYS, FAT). However, this phenomenon was not observed with fat consumption.

Though there were no differences observed in *IGFI* or *IGFIR* expression, there was a tendency for FAT to reduce expression in ovarian *IGFI* when compared to IND. This could be

associated with FAT having a numerically lower ovarian weight as a percentage of body weight due to the fact that IGF1 plays a role in growth (Wolfe et al., 2014). Also, Zhou and coworkers (2010) investigated follicular fluid composition in gilts consuming either high starch or high fat diets, similarly finding that gilts consuming a high fat diet tended to have lower IGF1 concentrations (ng/mL) when compared to gilts consuming a high starch diet (Zhou et al., 2010). This decrease in *IGF1* expression could also be related to the embryo development seen from the FAT group in the previous chapter. Potentially, with lower ovarian *IGF1* expression this would suggest lower IGF1 concentrations in the follicular fluid from FAT fed gilts. With lower IGF1 concentrations, oocytes and subsequent embryos could rely on autocrine secreted IGF1 for proper embryonic development (Green and Day, 2013; O'Neill, 1998, 1997).

In regards to *GDF9* expression, it seems that the oviduct was majorly impacted by either fructose or fat consumption. FRU tended to have an increase in expression when compared to FAT and HFHF which would suggest increased cell proliferation. The expression of *GDF9* induces cyclooxygenase 2 (COX-2), an enzyme that converts arachidonic acid to prostaglandin endoperoxide H₂ which is the precursor to prostaglandin E₂ (PGE₂) (Dunning et al., 2007; Elvin et al., 1999a; Elvin et al., 2000; Elvin et al., 1999b; Park et al., 2006; Russell and Robker, 2007). Prostaglandin E₂ in oviduct has been suggested to aid in muscle contraction regulation of gamete and embryo transport (Gabler et al., 2008; Weber et al., 1991). Interestingly, fat consumption decreases *GDF9* expression in the oviduct ($p=0.01$). This is similar to findings by Zhou and coworkers (2010), in which a fat-rich diet decreased mRNA expression of *GDF9* when compared to a starch-rich diet in gilts (Zhou et al., 2010). The decrease in *GDF9* expression would suggest a decrease in cell proliferation and this could hinder gamete transport by a subsequent decrease in PGE₂. Demonstrating the interaction between fructose and fat

consumption has an extreme impact on the oviduct and could be a reason why our lab previously found that gilts consuming a high fat and fructose diet had difficulty becoming pregnant (unpublished data).

Remarkably, fructose diets tended to decrease ovarian *GDF9* expression. However, a study by Elvin and coworkers (1999) demonstrated that *GDF9* deficient mice also expressed *ESR2* at reduced levels in the ovary (Elvin et al., 1999b). This phenomenon was also observed in this study with fructose consumption tending to decrease ovarian *GDF9* and significantly decreases ovarian *ESR2* expression. A positive correlation was found in both ovary and uterus samples between *GDF9* and *ESR2* expression. A positive correlation signifies that as one variable decreases, the other variable also decreases and vice versa. Potentially, *ESR2* expression is regulated by *GDF9* expression signaling pathway. The general mechanism is *GDF9* binds to its transmembrane receptor serine/threonine kinases (types I and II), and type II receptors phosphorylate type I receptors (Derynck and Zhang, 2003). Type I receptors then phosphorylate Smads (Smad2 or Smad3) and these receptor-activated Smads (R-Smads) dimerize with a common Smad4 (Co-Smad) in the cytosol (Derynck and Zhang, 2003). The R-Smad/Co-Smad complex will translocate into the nucleus where they regulate transcription of target genes (*ESR2*) through interaction with DNA-binding transcription factors and CREB-binding protein (CBP) or p300 coactivators (Derynck and Zhang, 2003; Hanstein et al., 1996; Imai et al., 2013). Furthermore under increased fructose consumption specifically in the ovarian tissue, potentially as oocyte specific *GDF9* expression decreases then granulosa cell *ESR2* expression will decrease as well. Previously stated, this suggests that *ESR2* plays a role in inhibiting cell proliferation (Lazennec et al., 2001; Martinetti et al., 2005). Therefore, with a decrease in expression this could

lead to cell proliferation and suggest why pigs consuming fructose had increased ovarian weights (unpublished data).

In conclusion, this study demonstrates that the inclusion of fat and/or fructose disrupts and alters pre-pubertal growth. Specifically, fructose consumption alters folliculogenesis and fat consumption alters oviductal gene expression, subsequently hindering gamete transport and early embryo development. Further investigation will provide insight to the detrimental effects poor pre-pubertal nutrition has on reproductive performance during adulthood.

Table 1. Oligonucleotide primers for targeted transcripts for which total RNA abundance was measured using real time quantitative reverse transcription PCR.

Gene ¹	GenBank number	Primer	Sequence (5' → 3')	Annealing temp. (°C)	Fragment size (bp)
<i>ESR1</i>	NM_214220.1	Forward Reverse	AGAATGTTGAAGCACAAGC GATCTCATGTCTCCAGGG	57.9	77
<i>ESR2</i>	NM_001001533.1	Forward Reverse	AGCCGTCACCTTCTGTATG TTTCAATGTCTCTCTGTTCAC	55.3	95
<i>IGF1</i>	NM_214256.1	Forward Reverse	ATGCCCAAGGCTCAGAAGGAAGTA AGGTAACTCGTGCAGAGCAAAGGA	64.6	146
<i>IGF1R</i>	NM_214172.1	Forward Reverse	GTGGAAGACGTACACATTATAACCAA AGAATGTTGAAGCACAAGC	59.2	106
<i>GDF9</i>	NM_001001909.1	Forward Reverse	TTCTAACAAGACTCTGCCTAA CCGTCACATCAATCTCAATC	59.2	98

¹ESR1, estrogen receptor alpha; ESR2, estrogen receptor beta; IGF1, insulin like growth factor 1; IGF1R, insulin like growth factor 1 receptor; GDF9, growth differentiation factor 9.

Table 2. Correlation in gene expression by tissue.

	Ovary r value ²	Ovary p value ³	Oviduct r value	Oviduct p value	Uterus r value	Uterus p value
Correlation¹						
<i>ESR1/ESR2</i>	0.1643	0.5585	-0.7344	0.8206	0.2400	0.4085
<i>ESR1/IGFI</i>	0.2966	0.3031	0.1804	0.5372	-0.1170	0.6662
<i>ESR1/IGFIR</i>	0.2299	0.3299	0.1533	0.6008	0.3521	0.1810
<i>ESR1/GDF9</i>	0.0667	0.8134	-0.1397	0.6340	-0.1219	0.6530
<i>ESR2/IGFI</i>	-0.0739	0.7935	-0.2080	0.5166	-0.0627	0.8313
<i>ESR2/IGFIR</i>	0.1339	0.6211	-0.0178	0.9563	0.3234	0.2594
<i>ESR2/GDF9</i>	0.9161	<.0001	0.2371	0.4827	0.7231	0.0035
<i>IGFI/IGFIR</i>	0.4023	0.1372	0.0053	0.9849	-0.1998	0.4583
<i>IGFI/GDF9</i>	-0.1395	0.6199	0.1850	0.5267	0.1259	0.6422
<i>IGFIR/GDF9</i>	-0.0405	0.8817	0.0896	0.7606	-0.1889	0.4835

¹ Correlation in gene expression. Estrogen receptor 1 (*ESR1*), Estrogen receptor 2 (*ESR2*), Insulin like growth factor I (*IGFI*), Insulin like growth factor I receptor (*IGFIR*), Growth differentiation factor 9 (*GDF9*).

² r value denotes linear association between variables.

³ p value of <0.05 indicates a statistically significant difference in gene expression.

Table 3. Correlation in gene expression by tissues from pigs consuming high fructose diets and non-fructose diets.

	Ovary r value ²	Ovary p value ³	Uterus r value	Uterus p value
Correlation¹				
<i>ESR2/GDF9</i> fructose	0.8241	0.0226	0.9382	0.0056
<i>ESR2/GDF9</i> non-fructose	0.9331	0.0002	0.1523	0.7445

¹ Correlation in gene expression. Estrogen receptor 2 (*ESR2*), Growth differentiation factor 9 (*GDF9*).

² r value denotes linear association between variables.

³ p value of <0.05 indicates a statistically significant difference in gene expression.

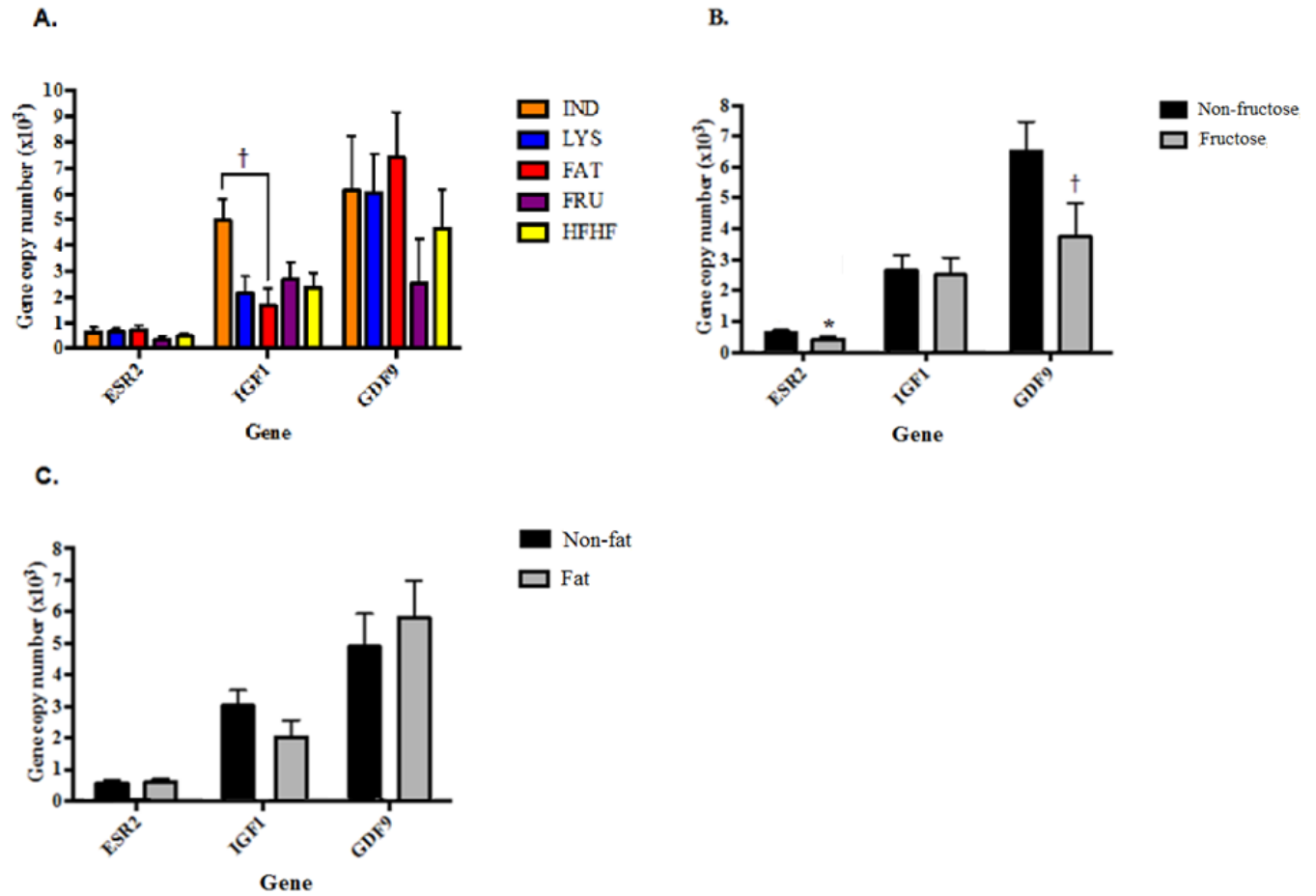


Figure 1. A. Ovary gene expression by absolute quantification by treatment. Gilts were fed one of five dietary treatments: Industry control (IND), lysine restricted (LYS), 15% fat (FAT), 35% fructose (FRU), or both fat and fructose (HFHF) included. B. Ovary gene expression by absolute quantification by fructose content. C. Ovary gene expression by absolute quantification by fat content. $p^* < 0.05$, $p^\dagger \leq 0.10$.

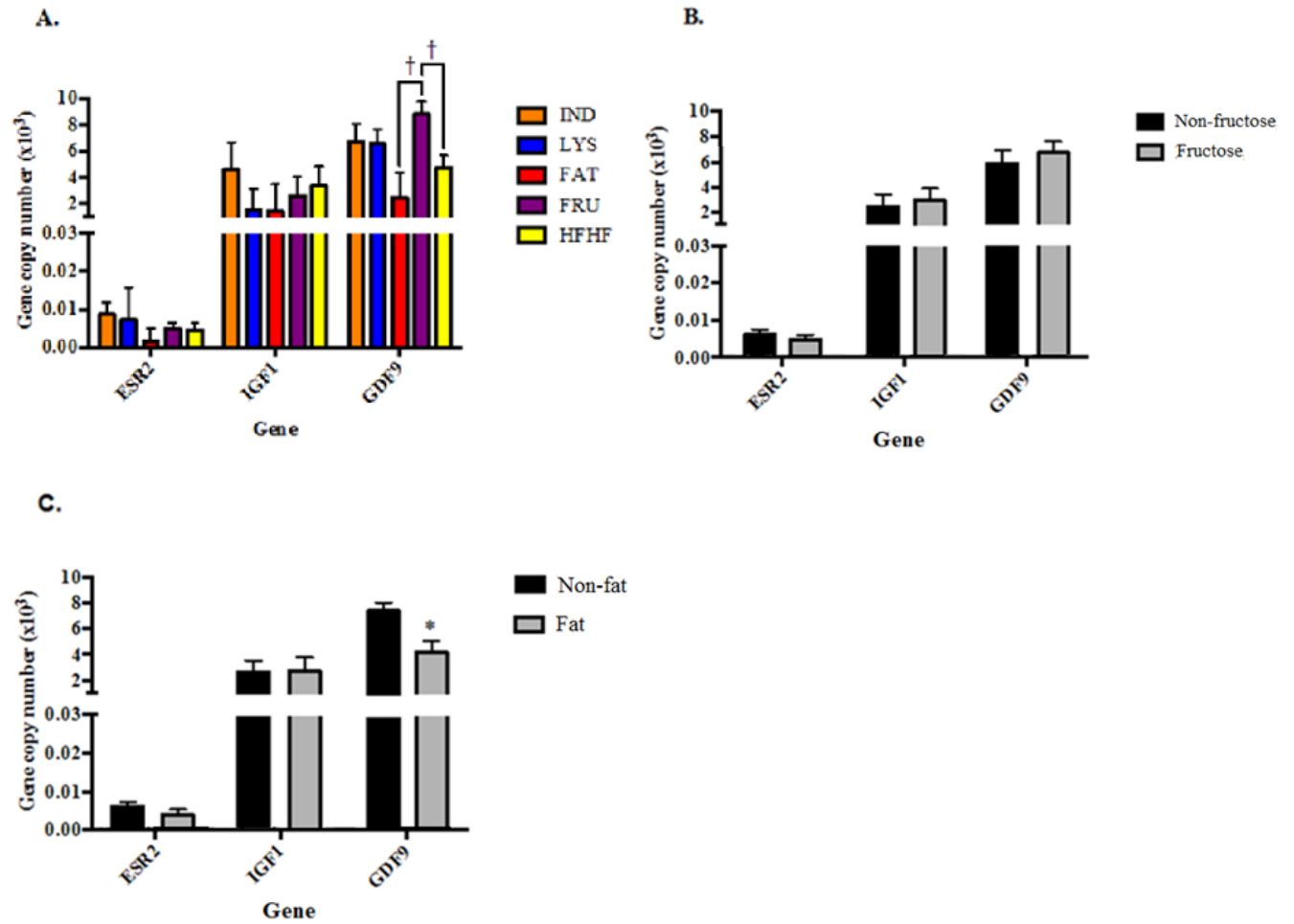


Figure 2. A. Oviduct gene expression by absolute quantification by treatment. Gilts were fed one of five dietary treatments: Industry control (IND), lysine restricted (LYS), 15% fat (FAT), 35% fructose (FRU), or both fat and fructose (HFHF) included. B. Oviduct gene expression by absolute quantification by fructose content. C. Oviduct gene expression by absolute quantification by fat content. $p^* < 0.05$, $p^\dagger \leq 0.10$.

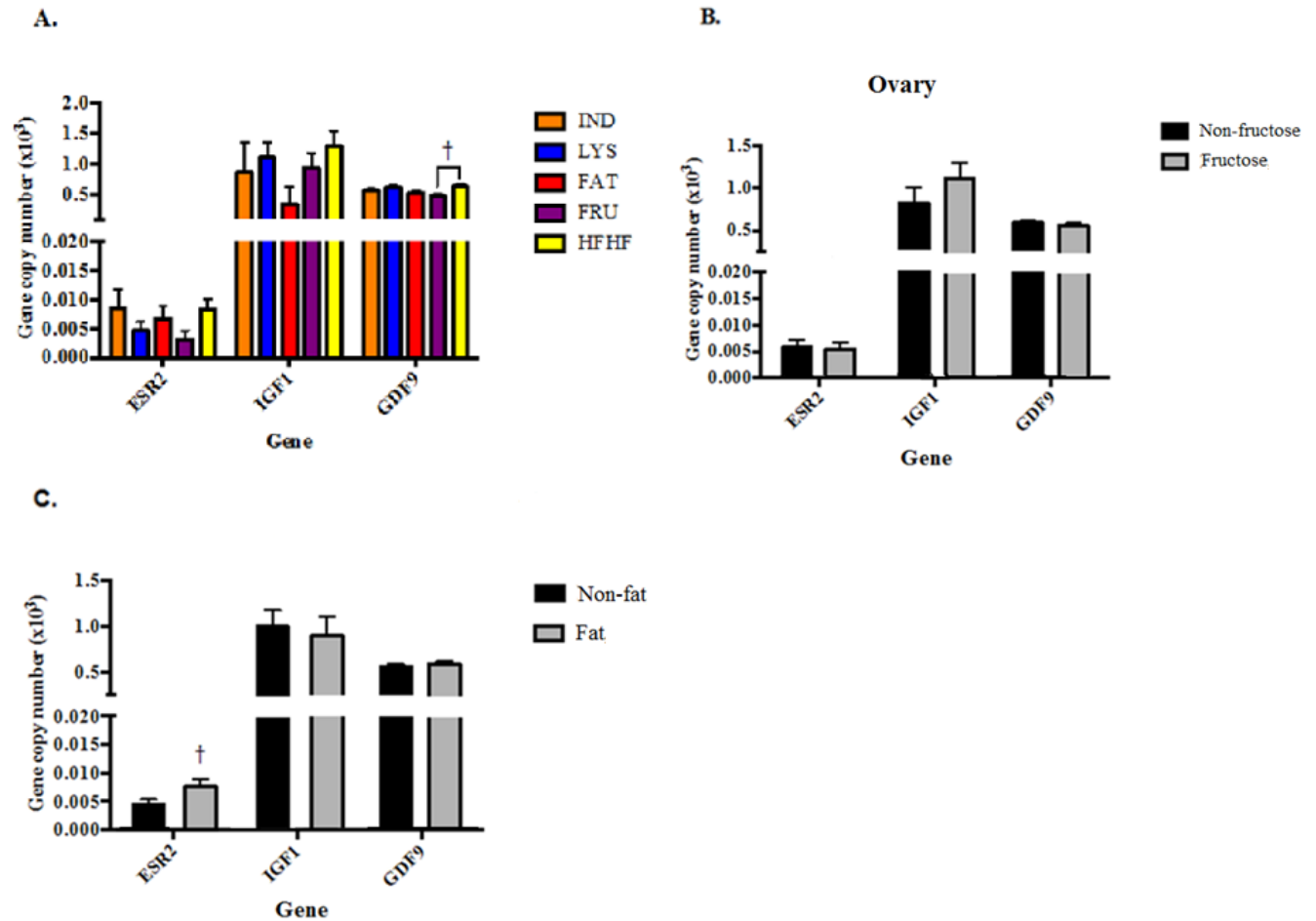


Figure 3. A. Uterus gene expression by absolute quantification by treatment. Gilts were fed one of five dietary treatments: Industry control (IND), lysine restricted (LYS), 15% fat (FAT), 35% fructose (FRU), or both fat and fructose (HFHF) included. B. Uterus gene expression by absolute quantification by fructose content. C. Uterus gene expression by absolute quantification by fat content. $p^* < 0.05$, $p^\dagger \leq 0.10$.

Chapter 4: Summary and future prospective

In conclusion, these studies have demonstrated that pre-pubertal diets containing high levels of fat and/or fructose may have detrimental effects on reproductive function and fertility in swine. During development, fructose consumption decreased *GDF9* and *ESR2* expression in the ovary and there was a significant positive correlation between these genes suggesting a similar pathway. To further investigate this, gene expression of follicle stimulating hormone receptor (*FSHR*) should be done on the ovarian tissues from these pigs. It has been demonstrated that as *GDF9* expression decreases, *FSHR* expression increases and vice versa (Spicer et al., 2006; Vitt et al., 2000). Therefore in pigs consuming fructose, ovarian *FSHR* expression should be increased when compared to pigs consuming non-fructose diets. Furthermore, an *in vitro* culture study with pre-pubertal porcine granulosa cells could also be done and a Smad2 and Smad3 activation inhibitor could be used to investigate *ESR2* and *FSHR* expression (Diaz et al., 2007). Smad (specifically Smad2 and Smad3) are intracellular proteins that induce extracellular signals from transforming growth factor beta family members (specifically *GDF9*) (Derynck and Zhang, 2003). Therefore, this system could aid in the understanding of the positive correlation in expression between *GDF9* and *ESR2* in the ovary.

Fat consumption significantly decreased *GDF9* expression in the oviduct. However, gilts consuming a FRU tended to have increased *GDF9* expression in the oviduct when compared to gilts consuming a FAT and HFHF diet. The expression of *GDF9* induces prostaglandin-endoperoxide synthase 2 (PTGS2), more commonly known as cyclooxygenase 2 (COX-2). This enzyme converts arachidonic acid to prostaglandin endoperoxide H₂ which is converted to prostaglandin E₂ (PGE₂) by the enzyme prostaglandin E synthase (PTEGS) (Elvin et al., 1999a; Elvin et al., 2000; Elvin et al., 1999b; Park et al., 2006; Russell and Robker, 2007). In the

oviduct, PGE₂ aids in gamete and embryo transport thus aiding in fertility (Gabler et al., 2008; Weber et al., 1991). Analysis of *PTGS2* and *PTEGS* gene expression in the oviduct could aid in the understanding the influence of fat and/or fructose consumption of oviductal *GDF9* total RNA expression. Also, it's important to note that protein expression should be analyzed as well in these tissue samples to account for post-transcriptional regulation.

In the ovary, gilts consuming a FAT diet had a tendency for lower *IGFI* expression when compared to gilts consuming an IND diet. Interestingly, the *in vitro* developed blastocysts from the FAT group had a significantly greater total cell count number. This suggests that with lower *IGFI* expression in the ovary tissue, oocytes and embryos from the FAT group could be secreting *IGFI* in an autocrine manner to aid in cell proliferation (Green and Day, 2013; O'Neill, 1998, 1997). To further investigate this, carry out the same dietary treatments on gilts and collect follicular fluid to add to an *in vitro* system. Also, oocytes from these gilts could be collected as well. Collected oocytes and developed *in vitro* blastocysts then could be analyzed for *IGFI* gene expression.

Liver samples were also collected from these gilts. The liver plays an important role for not only the metabolism of fructose (and other simple sugars like glucose) but in whole body function. Fructose enters the liver using GLUT2 transporter (non-insulin dependent), a low-affinity transporter that also has the ability to transport glucose or galactose (Douard and Ferraris, 2008). In other cells, fructose enters using GLUT5 transporters (non-insulin dependent) while glucose enter cells primarily via GLUT4 transporters (insulin dependent) (Bray et al., 2004; Joost and Thorens, 2001). Insulin's primary function is to promote the absorption of glucose from the blood and in mammals, insulin is secreted from the pancreatic beta-cells of the islets of Langerhans (pancreatic islets). Pancreatic beta cells lack GLUT5 transporters, for this

reason, fructose does not stimulate insulin secretion like glucose (Bray et al., 2004). However, fructose is transported to the liver where it is converted to glucose, thus entering the circulation (Bray et al., 2004; Tappy and Le, 2010). Therefore, analysis of GLUT2 transporter (*SLC2A2* gene) of the liver as well as Oil Red O staining of the liver to observe triglycerides and lipids (by products of fructose metabolism) can be done. Also, analysis of GLUT4 and GLUT5 transporters (*SLC2A4* and *SLC2A5* gene) can be done on the reproductive tract samples to account for fructose and glucose absorption by these tissues.

One aspect that would be interesting to investigate would be the influence of these diets on reproductive tract gene abundance in gilts post-puberty. This would be important to account for because in this study the age of the gilts were 3 weeks (21 days) to 12 weeks (84 days). This is the equivalent of a human child ranging from 2 to 6 years of age (Miller and Ullrey, 1987). In pigs primordial follicles account for the majority of follicles on the ovary from birth until 100 days of age, the uterus is functionally mature at 120 days of age, and puberty is approximately 180 days of age (A.C.O. Evans, 2001; Bartol et al., 1993; Gray et al., 2001; Whittemore, 1996). By carrying out this dietary study on pigs at puberty and after, both the ovary and uterus would be functionally mature and diet could have a more dramatic influence on gene abundance and reproductive function. Also, this would equivalent to a human adolescent ranging from 10 to 14 years of age (Miller and Ullrey, 1987).

Altogether, this work signifies that fat and/or fructose consumption hinders folliculogenesis and embryogenesis. Future work potentially could provide insight on the human health side demonstrating altered pre-pubertal development affects adulthood infertility.

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