

**IMPACT OF ENVIRONMENTAL FACTORS ON OVARIAN FUNCTION AND ENDOCRINE ACTIVITY IN
LIVESTOCK**

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ABSTRACT

Ovarian function is important in reproductive efficiency since the ovary is the site of oocyte development, as well as production and secretion of hormones necessary for proper follicle development, ovulation, and maintenance of pregnancy. However, many environmental factors can affect female reproductive function. For example, physical exercise during gestation has previously demonstrated to increase availability of nutrients and oxygen to the developing offspring, decrease mortality rates, and increase birth weights. Unfortunately there is limited information regarding ovarian development and fertility of offspring born to exercised mothers. Nutrition has also been demonstrated to affect reproductive function by altering steroid and metabolic hormone concentrations which can overall affect ovulation rates, oocyte quality, pregnancy rates, and fetal development and survival. Therefore, the objective was to investigate how manipulation of certain environmental factors, such as exercise, diet, or arginine supplementation, can influence ovarian function and endocrine activity of female livestock species.

Keywords: Ovarian Function, Nutrition, Exercise, Endocrine Activity

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LIST OF ABBREVIATIONS

AA.....	Atretic Antral Follicle
Arg.....	Arginine
BCS.....	Body Condition Score
BW.....	Body Weight
C/CON.....	Control
cGMP.....	Cyclic Guanosine Monophosphate
CIDR.....	Controlled Internal Drug Release device
CL.....	Corpus Luteum
COR.....	Cortex
E2.....	Estradiol
eNOS.....	Endothelial Nitric Oxide Synthase
EX.....	Exercise
FSH.....	Follicle Stimulating Hormone
GnRH.....	Gonadotropin Releasing Hormone
GTP.....	Guanosine Triphosphate
H.....	Heavyweight
HA.....	Healthy Antral Follicle
IGF1.....	Insulin-like Growth Factor 1
IUGR.....	Intra Uterine Growth Retardation
IVF.....	In Vitro Fertilization
L.....	Lightweight
LH.....	Luteinizing Hormone
LI.....	Labeling Index
MED.....	Medulla
NO.....	Nitric Oxide
O.....	Overfed
P4.....	Progesterone

PGC.....	Primordial Germ Cells
Sal	Saline
SF.....	Secondary Follicle
SRY	Sex-Determining Region on Y Chromosome
TBS	Tris-buffered Saline
TDF	Testis Determining Factor
U.....	Underfed

CHAPTER 1: LITERATURE REVIEW

The Ovary

The Role of the Ovary

The female mammalian reproductive system consists of the ovaries, oviduct, uterus, cervix, vagina, and external genitalia. The functions of the reproductive system are to produce oocytes (eggs or ova), transfer those oocytes to the site of fertilization, and house the fertilized oocyte so growth of embryo and then fetus can occur inside the mother's womb. The reproductive system also produces hormones to help maintain a favorable environment for fertilization, the developing embryo/fetus, and reproductive cyclicity. Of these essential reproductive organs, the ovaries play gametogenic and endocrine roles. The ovary is the origin of the female germ cells, or oocytes, which further develop into a fetus upon fertilization. Without functional ovaries, there would be no progeny to continue on with the population, and that species would eventually become extinct. Thus, by lacking ovaries, the other reproductive organs would not be useful, and designating the ovaries to be one of the most indispensable organs for reproductive purposes.

Ovaries are paired, multi-compartmental organs, ovoid in shape, and relatively dense in structure (Bloom and Fawcett, 1975; Senger, 2003; Oketem and Oktay, 2008). Surrounding an individual mature ovary is an outer protective layer of tissue, referred to as the tunica albuginea, which functions in maintaining the ovarian shape. Beneath the tunica albuginea is a region of connective tissue, termed the cortex, which houses the population of oocytes, follicles, and other functional structures such as the corpus hemorrhagicum, corpus luteum, and corpus albicans. The central region of the ovary is the medulla, located caudally to the cortex, which encompasses the vascular, nervous, and lymphatic systems (Bloom and Fawcett, 1975; Senger, 2003). Blood vessels originating from the medulla traverse to the cortex delivering nutrients, hormones, and other factors and as a result the oocytes, follicles, and other structures can properly develop. When considering all the structures and regions, the ovary as a whole has two primary functions, 1) generation of a fertilizable oocyte that will grow and mature during follicle development until ovulation when an ovulatory follicle differentiates into a corpus luteum (CL), and 2) secretion of steroid and other hormones that are required for preparing the reproductive tract for fertilization, establishment of pregnancy, and maintaining the pregnancy until parturition (Rodgers et al.,

1994; Senger, 2003; Oktem et al., 2008). No matter what developmental stage the ovary is in, the foremost purpose is to produce oocytes. In fact, the ovary provides a lifetime supply of oocytes for reproductive lifetime of the female. Shortly after birth the newborn female's ovaries contain all of the oocytes she will have in her lifetime (Zuckerman, 1951) and when the ovary has depleted the oocyte supply, she will no longer reproduce. However, it has been demonstrated in the mouse that female germ-cell proliferation continues after birth (Johnson et al., 2004). Even though there may be a limit to the number of oocytes produced throughout the female's lifetime, the ever-changing ovary will produce a substantial amount of follicles ranging from primary to preovulatory stage. No other organ in the female body undergoes such predictable and dramatic series of changes in such a short period of time (Senger, 2003). From the time a female is born until she reaches menopause, her ovaries are constantly transforming in structure due to primordial follicles developing into antral and then dominant preovulatory follicles or follicles becoming atretic and degenerating, or differentiating to the CL which has limited lifetime. Because the ovary contains thousands of oocytes, within any region of the ovary there are several different types of follicles at different stages of development and maturity, and depending on the stage of development, follicles can either be found in the cortex or the medulla regions (Bloom and Fawcett, 1975; Senger, 2003).

Fetal Ovarian Development

When a male and a female participate in sexual union (coitus), where successful fertilization occurs, a fetus will begin to develop, starting with the undifferentiated (bipotential) gonads. Gonadal development involves several simultaneous events including 1) the migration of primordial germ cells (PGCs) to the gonadal ridge, 2) migration of the somatic cells to the gonadal ridge, and 3) differentiation and organization of the testis or ovary. Primordial germ cells, ectodermal in origin, originate from the outer covering of the blastocyst where they migrate to the embryonic mesoderm of the primitive streak, then further to the endoderm of the yolk sac (Nussbaum, 1880). Primordial germ cells are germline stem cells that give rise to the gametes (Soto-Suazo and Zorn, 2005), so the PGC are carriers of the sex chromosomes, XX or XY. As the PGCs migrate to the gonadal ridge, they proliferate, or increase in number. However, when the PGCs reach their destination, they are immature and do not differ

morphologically from migrating PGCs. Once gonadal development and differentiation has occurred, PGCs become recognizable as spermatogonia or oogonia depending on the sex of the fetus.

After the PGCs reach the gonadal ridge, they start to divide by mitosis which increases the number of cells. Division of PGCs stimulates other tissues on the gonadal ridge to proliferate. This stimulation induces the coelomic cavity or body cavity to produce three distinct renal systems, the pronephros, mesonephros, and metanephros originating from the nephrogenic cord. The pronephros forms first at the most caudal portion of the nephrogenic cord, but it is never functional and eventually regresses when the mesonephros starts to develop. The mesonephros forms in the middle of the cord and, unlike the pronephros, is functional and resembles a simple version of an adult kidney. However, the mesonephros is only functional to a certain point. The ventral cranial part of the mesonephros is termed the gonadal ridge; this is where the PGCs and somatic cells migrate to causing development to occur. Another function of the mesonephros is to act like a temporary kidney by producing excretory waste that is eliminated from the body by the mesonephric ducts. The mesonephric ducts act as ureters, but become even more important when the mesonephros regresses where they serve in differentiating the bipotential gonad. As the third renal system, the metanephros, starts to develop, the mesonephros begins to regress slowly. If the fetus is established male, the mesonephros will give rise to the future efferent ducts; however if established female the mesonephros will regress completely. While the mesonephros degenerates, the mesonephric duct will develop into the Wolffian duct, a structure that produces organs essential for male reproduction (Behringer, 1994; Sarraj and Drummond, 2012). The mature metanephros will develop into the fully functional kidney. While the mesonephros regresses and the metanephros matures, the paramesonephric duct forms. As fetal development continues, the paramesonephric duct eventually regresses and forms the Müllerian duct, a structure that will eventually develop into some of the reproductive organs of the female (Senger, 2003). The Müllerian and Wolffian ducts play an important role in sex-determination in governing what reproductive organs should develop.

Along with the PGCs migrating to the gonadal ridge, they also carry the sex chromosomes needed to determine the sex of the fetus. In mammals, a female offspring needs to have two X chromosomes, one X from the mother and one X from the father. A male offspring needs to acquire XY chromosomes, an X from the mother and a Y from the father. Whether the offspring is male or female

depends on the sperm of the father and what chromosome it is carrying. Located on the Y chromosome is a gene called the testis determining factor (TDF) or more commonly known as the sex-determining region on the Y chromosome (SRY). This SRY gene controls what sex the offspring will be. If the SRY gene is present, a male will develop with sex chromosomes XY; if the gene is not present a female will be born with the sex chromosomes XX (Sinclair et al., 1990, Koopman, 1992; Sarraj and Drummond, 2012). In this project we are interested in the reproductive capacity of the ovaries; therefore we will only be interested in female reproductive development.

Somatic cells originate from the mesoderm of the mesonephros and migrate to the gonadal ridge. The somatic cells and the PGCs form in close association with each other and form somatic cell-germ cell bridges. These bridges are important for the exchange of gases, nutrients, and molecular information by which the PGCs recognize their migratory pathway (Fujimoto et al., 1977; Pereda et al., 1988; Fujimoto et al., 1989; Soto-Suazo and Zorn, 2005). Once these interactions develop, the germ cells divide repeatedly by mitosis and become oogonia (Qi et al., 2007). As gestation continues to term, the fetal ovaries will begin to grow in size. When this occurs, the germ cells, originally PGCs, located in the medulla get pushed toward the cortex where a high density of germ cells will accumulate (Byskov and Westergaard, 2004). By the end of pregnancy, fetal ovaries approach a very fast growth stage, as ovarian weight increases more than ten-fold (Grazul-Bilska et al., 2009). Somatic cells in the medulla of the embryonic gonad give rise to the various cell types in the adult ovary, such as the granulosa cells (Matzuk and Lamb, 2002; Sarraj and Drummond, 2012), that support the differentiation of the germ cells that mature into gametes (Lindsley and Tokuyasu, 1980; Mahowald and Kambysellis, 1980; De Felici and Dolci, 1987; Boyle and DiNardo, 1995). Once the germ cells reach the cortex of the ovary, meiosis is initiated and the germ cells become oocytes. When the diplotene stage of meiosis is reached, the oocytes must become enclosed by granulosa cells forming a follicle or they will degenerate (Byskov and Hoyer, 1994; Oktem and Oktay, 2008). The oocyte surrounded by a single layer of granulosa cells is called a primordial follicle, located in the medulla (Moniruzzaman and Miyano, 2010). Clusters of primordial follicles will now develop in the medulla; these clusters are more commonly referred to as egg nests. When the majority of germ cells form into oocytes and are housed in egg nests, follicular development is initiated.

Folliculogenesis

Folliculogenesis, defined by Oktem et al. (2008), is the formation of the primordial follicle to its recruitment into the growing pool as a primary follicle and its further progression into the successive stages of preantral, antral, and finally preovulatory status. However, not all follicles will reach the preovulatory stage. Many will succumb to cell death or atresia. Atresia of follicles can occur at any developmental stage of folliculogenesis, only the follicles that can survive hormonal changes will further develop. Folliculogenesis has three stages of development. The first stage occurs when preantral and early antral follicles develop but are poorly vascularized and depend on locally produced growth factors. The second stage includes the small to medium-sized antral follicles which are now vascularized and sensitive to follicle stimulating hormone (FSH) levels. The third stage consists of the large antral and preovulatory follicles that are highly vascularized and their development is sustained by luteinizing hormone (LH; Mariana et al., 1991).

Initiation of folliculogenesis proceeds after the oocytes in the medulla reach the diplotene stage of meiosis during fetal ovarian development. In the pig, this occurs approximately 60 days postcoitus, when all of the gametes appear to be contained in egg nests (Oxender et al., 1979), as early as the 15th week of gestation in humans (Oktem and Urman, 2010), or day 90 of gestation in the cow (Yang and Fortune, 2008). Egg nests are located in the medulla of the fetal ovary, but once follicular growth ensues the follicles will begin to form in the inner part of the ovarian cortex (Byskov and Hoyer, 1994; Sawyer et al., 2002). At puberty and adult life, the preovulatory follicle, corpus hemorrhagicum, CL, and corpus albicans are forming close to the surface of the ovary. Follicle development up to the ovulatory stage is a highly complex array of events and has multiple features, such as primordial follicle recruitment, proliferation and atresia of granulosa and theca cells, steroidogenesis, oocyte maturation, ovulation, and luteinization, followed by CL formation (Mariana et al., 1991; Oktem et al., 2008).

Primordial follicles, also classified as egg nests, are the most immature and smallest follicles present on the ovary (Senger, 2003) at around day 62-64 postcoitum in the pig, making it the first recognizable ovarian follicle (Mauleon, 1964; Oxender et al., 1979). Primordial follicles consist of the oocyte surrounded by a single layer of flattened granulosa cells. After the formation of the primordial follicles, some of the oocytes begin to grow (Moniruzzaman and Miyano, 2010). During this growth the

primordial follicle will gradually and continually leave the resting pool to begin further development (Fortune, 1994). The primordial follicles will slowly start migrating from the medulla to the inner cortex region and develop into a primary follicle. Now the primordial follicle has acquired a cuboidal layer of granulosa cells rather than the flattened layer and is now termed a primary follicle. This recruitment of quiescent primordial follicles to a growing pool of primary follicles starts in fetal life and will continue postnatally until the ovarian reserve is depleted (Oktem et al., 2008). If the primordial follicles do not develop into primary follicles, the primordial follicles will undergo atresia and no longer develop. As follicles move into large developmental stages, both growth rate and frequency of atresia increases (Pedersen, 1970; Quirk et al., 2004).

After the recruited primary follicles are selected from the primordial pool they will either continue to grow and develop or become atretic. Primary follicles that continue to grow will develop into secondary follicles. As the follicle grows, it acquires multiple layers of cuboidal granulosa cells; this is what helps differentiate between primary and secondary follicles. Secondary follicles may also acquire a thin layer of theca cells surrounding the granulosa cell layers. However, the follicle does not have a fluid filled cavity, or antrum (Fortune, 2003; Senger, 2003). According to a study performed by Oxender et al. (1979) in understanding the ovarian development in fetal and prepubertal pigs, they found that secondary follicles were first observable around the time of birth and the percentage of secondary follicles present increased by 30% at 90 days of age. By the end of pregnancy in sheep, fetal ovaries may contain all types of follicles including primordial, primary, secondary follicle, and antral follicles (Grazul-Bilska et al., 2009). This helps understanding that follicles are constantly growing and developing toward becoming mature preovulatory follicles during the stages of fetal and postnatal development.

As the secondary follicles continue to grow, some of them will become atretic and other will develop further into antral follicles and enter the antral follicle pool. The secondary follicle will acquire an inner cavity called the antrum (Reynolds et al., 2005). Also during this growing stage, the antral follicles will gain successive layers of granulosa cells, grow in size, and the growing oocyte will become capable of resuming meiosis (Mariana et al., 1991; Moniruzzaman and Miyano, 2010). This resumption of meiotic activity is important because it will provide the oocytes that are available for fertilization. By continuing meiosis, the process will produce one female oocyte and polar bodies, these polar bodies will eventually

degenerate. The oocyte that is produced will ovulate from the preovulatory follicle and if fertilized will become an embryo and then a fetus. This process, once completed, will begin all over again. Because the antral follicle is continuing to grow, some of the follicles are able to be seen by the naked eye on the surface of the ovary (Senger, 2003). In pigs the first observable follicles were seen around 62-90 days after birth by Oxender et al. (1979). Again, as with all previous follicles during folliculogenesis, antral follicles will either experience atresia or continue to grow. If growth does continue, follicular waves will occur resulting in a follicle ready to ovulate, the destination of all follicles.

From the pool of antral follicles, follicles will be recruited, selected to grow, become dominant, and ovulate. The formation of the secondary follicle to the antral follicle has a major impact on follicular dynamics in terms of the number and quality of follicles (Soede et al., 2011). The initiation of follicular waves occurs around the time of puberty with the goal of producing a dominant preovulatory follicle that will release an ovum to be fertilized. During the time of recruitment, some antral follicles will be chosen, those recruited follicles will either continue to grow or become atretic. Those that continue to grow reach the selection phase of the follicular wave cycle. The selected follicles will again reach two fates- growth or atresia. Not many follicles are left at this stage of the wave. During the dominant stage, the follicles that survived selection will continue to grow towards being a preovulatory follicle. Preovulatory follicles, like antral follicles, in addition to the cumulus oocyte complex, have three very distinct cell layers, the theca externa, theca interna, and the granulosa cell layer. The difference between the two stages (i.e. antral vs. preovulatory) is that the preovulatory follicle is larger and closer to the cortex. Only one follicle will reach the preovulatory stage to ovulate in mono-ovulatory species. All other follicles become atretic and degenerate. However, in most species, there will be a total of two to four follicular waves that occur before successful ovulation occurs. While most livestock species (e.g., cows, sheep or goats) have wave-like patterns and typically ovulate one to three follicles, pigs are an exception. Pigs do not show the wave-like pattern of follicle development. Instead, there is continuous growth and atresia without evidence of dominant follicles or follicle waves (Dalin, 1987; Dalin et al., 1995, Guthrie and Cooper, 1996; Evans, 2003). Pigs may ovulate anywhere from 15 to 30 follicles at one time (Soede et al., 2011) because they are a polytocous species, giving birth to multiple offspring.

Endocrine Function of the Ovary

Endocrinology is the study of the biosynthesis, storage, chemistry, and physiological function of hormones, as well as the cells of the endocrine glands and tissue that secrete them. Three main endocrine organs in the reproductive system are 1) pituitary (anterior and posterior) gland associated with the hypothalamus, 2) gonads, and 3) uterus. The pituitary gland is located at the base of the brain and is composed of two lobes: the anterior and posterior. The anterior pituitary synthesizes and releases follicle-stimulating hormone (FSH) and luteinizing hormone (LH) along with other hormones not fully specific to the gonads. The posterior pituitary is responsible for the storage and release of hormones that aid in smooth muscle stimulation not fully specific to the gonads. The hypothalamus rests just above the pituitary gland and is responsible for secretion of factors regulating hormonal release by the pituitary. The hypothalamus releases gonadotropin-releasing hormone (GnRH) which controls FSH and LH secretion by the pituitary, and GnRH is essential for fetal gonadal development (Jost, 1956; Collenbrander et al., 1983). Gonads produce 1) estrogens which stimulate cell growth and division along with inducing heat/estrus, 2) progesterone (P4), which governs the estrus cycle, promotes gestation, and suppresses GnRH release, and 3) testosterone, which, in the male, promotes spermatogenesis and development of the male secondary sex characteristics, and in female is a precursor for estrogens. All of these steroids, or sex hormones, originate from cholesterol before they are converted to their respectable hormones.

Once the fetal gonads develop, they secrete a sex hormone (male=testosterone, female=estradiol) into the blood stream to travel to the developing brain. If male, testosterone will pass through the blood-brain barrier where it will be converted to estrogens via aromatase. Estrogens will “defeminize” the region of the hypothalamus termed the surge center. The surge center of the hypothalamus is responsible for the preovulatory release of GnRH that stimulates a surge of LH (Senger, 2003). The surge center releases hormones in high amplitude and high frequency pulses. The “defeminizing” of the surge center in the male will cause this region to not develop. However, the tonic center of the hypothalamus will still develop functions in which it regulates the tonic frequency of GnRH pulses. This will cause the GnRH to always be released in a tonic, or pulsatile, pattern much like a dripping faucet (Senger, 2003). In the female, estrogens are released into the blood stream and will travel to the brain. However, while in the circulatory system, estrogens will bind to alpha feto-protein causing the

estrogens to not pass through the blood-brain barrier. As a result, the surge center develops and GnRH is released in both tonic and surge patterns.

During early folliculogenesis in fetal ovaries, the granulosa cells of preantral follicles are producing and releasing estrogens into circulation. However, steroid production is low or undetectable during this stage (Byskov and Westergaard, 2004). During preantral follicular growth, GnRH is being secreted by the hypothalamus. Gonadotropins, such as GnRH, are required for antral follicle development, but not for the development of preantral follicles (Fortune, 2003). Once the follicles develop into the antral follicle pool, estrogens act on the tonic center of the hypothalamus causing the release of GnRH to the anterior pituitary. The anterior pituitary then releases FSH that, after reaching the ovary, initiates follicular growth and development of antral follicles into the antral follicle pool. Once the follicles reach a certain size, they release inhibin, which brings on a negative feedback to the anterior pituitary prohibiting the release of FSH. When the FSH levels fall, the antral follicles begin the selection process of the follicular wave (van der Hurk and Zhao, 2005). The reduction in FSH concentration induces atresia in most of the non-dominant follicles because most of them cannot survive under low levels of FSH (Adams et al., 1992b; Ginther et al., 2001; Ginther et al., 2002; Binelli, and Murphy, 2010). Estrogens are still continuing to be produced and secreted by the follicle causing a positive feedback to the surge center of the hypothalamus. This feedback to the surge center causes the release of GnRH to the anterior pituitary causing now an LH secretion that acts on the ovary. Those follicles that are able to survive under the low levels of FSH are now becoming dependent on LH. This dependency on LH causes the follicles to reach the dominant stage. Continued growth of the dominant follicles depends on the frequency of the LH pulses (Adams et al., 1992a). However, a loss of dominance is accompanied by a decreased capacity to produce estradiol-17 β (E2) and inhibin. Consequently, there is the release of a new surge of FSH and recruitment of a new pool of follicles begin accelerated growth (Adams et al., 1992a,b). This concludes the first follicular wave and the start of the second. As discussed above, most livestock species undergo multiple follicular waves before ovulation is successful.

If dominance is maintained, the frequency of LH pulses is high and the dominant follicle will continue to grow and develop (Binelli and Murphy, 2010). At this time, in mono-ovulatory species, only one follicle will reach the preovulatory stage. This is due to the ability of the dominant follicle to absorb

more LH forcing the other follicles to become atretic. The dominant follicle will secrete E2 and cause a positive feedback to the hypothalamus to induce the release of GnRH and the consequent pre-ovulatory peak of LH (Moenter et al., 1990), which in turn causes the dominant follicle to ovulate. Once ovulated, a CL will develop in the place of the ovulated follicle. After ovulation, fertilization may occur. The CL produces progesterone which creates a negative feedback on the anterior pituitary that regulates the LH surge. While the CL is present, which produces P4, follicular growth and development is postponed because of the suppression of FSH and E2 secretion (Zelevnik, 2001). The progesterone secreted by the CL will act on the tonic center to produce GnRH. This initiation of GnRH released to the anterior pituitary will stimulate LH secretion to support the developing CL. When the CL is established on the ovary pregnancy could occur resulting in the embryonic and then fetal development. However, if there is no fertilization the CL will regress through luteolysis process caused by prostaglandin F2 α , secreted by the non-pregnant uterus. During luteolysis, the CL regresses to a corpus albicans and the production of P4 declines. This decrease in P4 will cause the tonic center to release GnRH so the anterior pituitary can release FSH to the ovary and start the follicular development process over again.

Factors that Affect Fertility and Productivity in Livestock

Introduction

The continued genetic development of several livestock species, including cows, pigs, and sheep, are carried out to meet the needs for human necessities such as food and clothing (Dobson and Smith, 2000). In the United States alone, animal production for meat is a multibillion dollar industry; however, reproductive efficiency can have a major impact on the profitability of animal agriculture (Trenkle and Willham, 1977). Reproductive efficiency is the ability to maximize the output while minimizing the labor and costs put into each female. In the animal industry, reproductive efficiency is important to the farmer and/or producer by increasing pregnancy rates, decreasing services per conception, decreasing embryonic/fetal loss, and producing normal weight offspring that can all decrease the costs of production, as well as even creating a profit on carcasses at market. Unfortunately, if any of the previously mentioned situations are compromised, economic loss can occur. While there are many ways to enhance reproductive efficiency, there are also many environmental factors, both internal and external, that can affect the reproductive efficiency of the herd that may not be easily controlled.

Internal factors that can affect female reproductive function are hormone and growth and other factor production and secretion, as well as genetics of the parents, and aging of the female. In cows, it has been demonstrated that a decrease in concentrations of P4 in circulation can reduce the growth of the ovulatory follicle (Pursley and Martins, 2011), and if P4 concentrations are not elevated enough, the female will not be able to maintain her pregnancy and therefore will abort the developing fetus (Bridges et al., 2013). It has also been demonstrated that low levels of E2 can exert negative effects on ovarian function by causing a reduction in follicular development so that a dominant follicle does not form, and therefore cannot be ovulated. If not treated, low hormone production and secretion could potentially increase the number of services per conception or cause the female to abort; which would therefore increase production costs for the farmer and/or producer. Internal factors can also cause positive effects on reproductive function, one such internal factor is genetics. Selection for fertility and/or milking output are important characteristics that farmers/producers look for in their females. Increasing the desired characteristics in a female will increase the chances those characteristics will be passed down to her offspring and create profit for the farmer/producer. Not only can internal factors affect reproductive function, but external factors such as social position in the herd, temperature and transportation stress, disease, and nutrition can also affect function as well. Dobson and Smith (2000) reported that cows that were higher in the social hierarchy of the herd were more fertile and had better milk production than those with a lower social status. Those cows in the lower end of the hierarchy had decreased calving to conception days and increased inseminations per conception, which hinder not only the genetic process of those cows, but potentially their offspring as well. Nutrition can also have a major impact on reproductive efficiency, for example, ewes fed low or high planes of nutrition had decreased in vitro fertilization rates and low quality oocytes. These characteristics resulted in lower rates of cleavage and caused negative effects on early embryonic development (Grazul-Bilska et al., 2012). Effects of heat stress in cattle have demonstrated to impair prostaglandin secretion and reduced oviductal smooth muscle motility, which in turn could decrease gamete/embryo transport through the oviduct (Kobayashi et al., 2013). While there are many factors that can cause negative effects on reproductive performance, there are also ways to enhance not only reproductive efficiency, but fertility of that female and potentially her offspring as well.

Effects of Pregnancy on the Developing Offspring

Prenatal development is a critical phase of the fetal life cycle because events that occur during this period influence the subsequent growth, development, and productive life of the offspring (McGlone and Pond, 2003). In pigs, a polytocous species, multiple oocytes are ovulated at one time, and once fertilized, develop into blastocysts and implant into the uterine wall for further growth. Morgan (1987) found that the smaller blastocysts in a given litter that implant close to a larger blastocyst, may indirectly be destroyed by the advanced littermate because of the ability of the larger embryo to alter the uterine environment to their favor in pigs. It is also during this time that each developing embryo produces its own protective membrane and is dependent on maternal blood that flows across the placental attachments for nutrient and oxygen exchange (McGlone and Pond, 2003). Since the developing embryo that is now attached to the uterine wall is extremely dependent on the uptake of nutrients and oxygen from maternal circulation, the embryo is extremely sensitive to uterine conditions (Hughes and Varley, 1980). Therefore, if the mother is under any type of stress, disease, or receiving inadequate nutrition, it could potentially cause a negative impact on embryonic development.

Uterine and umbilical blood flow maintains placental transport and exchange of respiratory gases, nutrients and wastes, and thereby supports fetal growth and metabolism (Reynolds and Redmer, 1995). The pig has multiple fetuses developing in her uterus at one time that are competing for the same nutrients and oxygen from maternal circulation. This competition within the litter could potentially be why piglets can undergo in utero growth retardation (IUGR), or also be considered runts. In utero growth retardation, besides other factors such as uterine capacity and the number and position of the fetuses in the uterus, is mainly caused by limited oxygen and nutrient supply via the placenta (Ashworth et al., 2001). Even et al. (1994) discovered that the more offspring that are being fed by the uterine artery causes blood pressure in that particular artery of the placenta to be lower than normal. This decrease in pressure would also be associated with a decrease in the amount of oxygen and nutrients supplied to the more distal placentae and, ultimately, to the fetuses nourished by those placentae. Thus, insufficient blood flow could account for the local effect on organogenesis (Da Silva-Buttkus et al., 2003), fetal mass at birth (McLaren and Michie, 1960), as well as influencing litter size and postnatal survival (Vallet and Freking, 2007). Since the sow gives birth to multiple offspring, there is a greater chance for IUGR to

occur. In these IUGR offspring, there is more potential for reproductive disorders later in life, including altered time of onset of puberty (Ibáñez et al., 2000a) and reduced size of the uterus and ovaries (Ibáñez et al., 2000b). These postnatal disorders may be derived from altered fetal reproductive organogenesis (Da Silva-Buttkus et al., 2003).

As previously mentioned, the prenatal period is recognized as a unique physiological window in which maternal and fetal adaptations can have major consequences for the long-term health and well-being of the offspring (Hopkins and Cutfield, 2011). Specifically, what occurs during early embryonic development can highly impact the reproductive performance of the female offspring. Female reproductive performance and efficiency depend on what occurs during ovarian development. The ovary begins to develop in utero and is fully differentiated or developed after 23% of elapsed gestation has occurred (Senger, 2003), which is approximately 31 or 32 days postcoitus in the pig (Oxender et al., 1979). Primordial follicle assembly during embryonic development has direct implications for improving reproductive productivity and predicting the reproductive lifespan of the female (Geber et al., 2012). Since the number of oocytes are largely determined during gestation, maternal environment at this critical time may impact oogenesis proliferation and thus follicle numbers postnatally (Evans et al., 2012). The number of follicles present on the ovary is directly proportional to her fertility. For example, cows with a high number of antral follicles had higher pregnancy rates, had shorter calving to conception intervals, and received fewer services during the breeding season compared to cows with a low antral follicle count (Mossa et al., 2012). Therefore, what events occur during gestation, specifically early embryonic and fetal development, can greatly impact the reproductive abilities of the female offspring. A study by Da Silva-Buttkus et al. (2003) have shown that body weight of piglets originating from the same litter was significantly lower in the IUGR piglets, as expected, as well as absolute ovarian mass (IUGR: 51 ± 3.0 mg; normal: 108 ± 9.6 mg). Interestingly, IUGR piglets had higher numbers of primordial follicles compared to their normally sized littermates; however, there were significantly fewer primary follicles and no observed secondary follicles present on the IUGR piglet ovaries. The results of this study confirmed that compromised prenatal growth may have delayed the normal pattern of follicular development in the pig ovary (Da Silva-Buttkus et al., 2003). Many researchers are currently working to improve different methods of managing livestock species during pregnancy to not only impact the dam's reproductive

success but her offspring's growth potential and her reproductive performance later in life (Vonnahme and Lemley, 2012).

Exercise during Gestation

In many litter-bearing species, such as the pig, there is a negative correlation between litter size and fetal mass (Even et al., 1994). The litter size in a contemporary commercial sow unit can range between 10-15 piglets per sow (Foxcroft et al., 2009). With these large litters, the uterine environment can get crowded as litter size increases, which, as previously described, can affect the distribution of oxygen and nutrients to the fetus in order to support normal growth and development. Reynolds et al. (1985) demonstrated that as litter size increases so does uterine blood flow; however, even though blood flow is increasing, blood flow per fetus decreases with the increasing size of the litter. This could potentially be detrimental to the growing fetuses, especially those that are of small body weight compared to the rest of the litter. Those lightweight piglets have extremely low birth weights, a lower chance of survival, and difficulty generating viable economic returns (Foxcroft et al., 2009). Such economic loss can be attributed to those piglets having a harder time gaining weight and reaching puberty, which is not genetically beneficial to a producer; therefore, those lightweight piglets are more likely to be sent to market, and the producer would not receive much profit for that carcass. Thus, in order to produce lightweight piglets that are similar to the remainder of the litter, some external factor has to occur during gestation in order to alter the outcome of those compromised offspring.

One possible way to compensate for those lightweight offspring would be to increase blood flow which would increase the amount of nutrients and oxygen that is essential for normal growth and development. Exercise, in general, has been known to cause an increase in blood flow to major organs and tissues in the body. However, exercising during gestation has shown to cause a decline in total uterine blood flow (Metcalf and Stock, 1993). Unfortunately, there are many variations among studies due to the species of interest, duration and type of exercise being performed, stage of gestation the mother was in when exercise transpired, and when blood collection occurred (during or after exercise). Jones et al. (1990) performed a study looking at the effects of exercise training on blood flow during gestation in the rat. In this study, one group of rats was trained prior to the onset of the experiment and the other group did not receive any prior exercise training. Pre-exercise blood flow measurements were

taken and showed that the trained rats, while not significant, had on average 19% greater blood flow ($\text{mL} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$) at rest to the uterus (66 ± 9 vs. 54 ± 9), placenta (61 ± 8 vs. 46 ± 4), and ovary (3059 ± 467 vs. 2582 ± 279) compared to the untrained rats. After one minute of exercise and again after ten minutes of exercise, blood measurements were taken and results showed that blood flow to the reproductive tissues decreased significantly as a result of the exercise bout. However, rats in the trained group had on average 39% greater blood flow occurring than the untrained rats undergoing the same exercise regime although it was not significant. Therefore, Jones et al. (1990) concluded that training before and during gestation did not significantly reduce the blood flow to reproductive tissues observed in the untrained animals during an acute bout of exercise, although the absolute blood flows were generally higher in the trained rats during exercise. A similar study looking at the distribution of uterine blood flow occurred in trained and untrained pregnant sheep (Curet et al., 1976). It was demonstrated that prior to exercise, 79% of the uterine blood flow went to the cotyledons on the placenta while only 21% went to the myometrium and endometrium of the maternal uterus. Following 45-60 minutes of exercise on a treadmill, the percentage of uterine blood flow going to the cotyledons increased to 88% while that going to the maternal uterus was reduced to 12%. It is interesting to note that the redistribution of noncotyledonary flow to the cotyledons following exercise in the healthy mother tends to favor, and is not hazardous to, the developing offspring (Curet et al., 1976; Orr et al., 1972).

A study performed by Harris et al. (2013) looked at the effects of maternal activity during mid to late gestation on fetal growth, umbilical blood flow, and farrowing characteristics in sows. Sows in this study were either exercised three times per week for 30 minutes each day from day 40 to 104 of gestation or they remained individually penned throughout the study. Results demonstrated that fetal girth and biparietal distance, measured via Doppler ultrasonography, was not affected by maternal exercise. However, there was an effect of day of gestation, demonstrating that fetal growth increased as days of gestation increased. Umbilical blood flow was also measured using Doppler ultrasonography on the same days fetal measurements were taken. Exercised females had an overall increase in umbilical blood flow when compared to the females that remained in their pens. This suggests that since umbilical blood flow increases there is a possibility that there is also an increase in the nutrients and oxygen that are going toward the developing fetuses, and possibly those compromised fetuses will be similar in size to the

remainder of the litter. Unfortunately, that was not the case in this study, farrowing results showed that non-exercised females had more fully formed piglets born than exercised mothers (14.0 ± 0.8 , 11.0 ± 0.8 , respectively; $P = 0.03$). However, there were no differences in total litter birth, individual birth weight, or the size of the litter. A similar study by Hale et al. (1981) also looked at farrowing characteristics of maternal physical exercise during gestation; however, this study was performed starting in early gestation rather than mid gestation as Harris et al. (2013). Again there was an exercise and a non-exercise group, where the exercise group walked on a treadmill five days a week for 15 minutes daily, or a distance of 0.48 km starting seven days post-breeding and lasting for 100 days. Results from this study found that exercised females weaned 3.4% more piglets and piglets weighted 4.7% more than piglets born to non-exercised females. The studies by Harris et al. (2013) and Hale et al. (1981) demonstrate that exercise during gestation in the sow increases overall umbilical blood flow to the fetus, but does not cause any negative effects on farrowing characteristics or birth weights of the offspring. However, it seems that exercising sows in early gestation rather than mid gestation has a more positive effect on the number of piglets born and increases the birth weight of those exercised offspring, again without causing any negative effects. Both the studies by Harris et al. (2013) and Hale et al. (1981) performed a low-intensity exercise regime during gestation, it is currently unknown if a high-intensity regime cause greater amount of blood flow to the developing offspring and would it cause more observable effects on farrowing and offspring characteristics?

Schenck et al. (2008) performed two different exercise regimes, a low- and high-intensity. The low-intensity group walked/ran 122 meters per day five times per week from day 35 to 110 of gestation. The high-intensity group was also encouraged to walk/run but on an increasing schedule from day 35 to 110 of gestation so they reached a final distance of 427 meter per day for three days per week with the remaining two days being 122 meters per day. While umbilical blood flow was not measured, farrowing data showed that the females in the high-intensity group did have a greater live litter birth weight than both the low-intensity and control groups (16.08 ± 1.4 , 12.18 ± 1.4 , 11.04 ± 1.4 kg, respectively), and similar results were observed for number of piglets weaned (9.53 ± 0.4 , 8.12 ± 0.4 , 7.90 ± 0.4 , respectively). Interestingly, both the low- and high-intensity groups had significantly less piglet preweaning mortality when compared to the control group (1.55 ± 3.4 , 4.11 ± 3.4 , 12.26 ± 3.4 ,

respectively). These results suggest that a high-intensity exercise regime may cause a significant increase in the nutrients and oxygen going towards fetal development in utero as observed by the live litter birth weight being significantly greater than the low-intensity and control groups. Also, exercise in general caused a decrease in the piglet preweaning mortality indicating that exercise, whether low- or high-intensity, causes more piglets to make it to weaning age and potentially to reproductive age.

In conclusion, exercise during gestation in the sow seems to have positive effects on farrowing and weaning performance. Exercise, even at low-intensity, was shown to increase the overall umbilical blood flow towards the fetus (Harris et al., 2013), which in turn increases the nutrients and oxygen going towards fetal growth and development. When exercise was initiated in early gestation (Hale et al., 1981), there was an increase in the litter size and neonatal birth weight compared to females that initiated exercise during mid-gestation (Harris et al., 2013). So it seems that the uterine environment in early gestation creates a favorable environment for piglets born to mothers that exercised early in gestation. Also, a high-intensity exercise creates a larger litter bodyweight and increases the number of piglets that survive until weaning age. Overall, exercise did not create a negative impact on the developing offspring; instead it seems to create a litter similar to or better than that of a control environment. However, the effects of maternal exercise on reproductive characteristics of the offspring have yet to be elucidated (see Chapter 2).

Effects of Plane of Nutrition on Reproductive Function

Reproductive function can be affected by numerous environmental factors, including nutrition. Because the ovary is the source of developing oocytes, as well as the site of production and secretion of circulating steroid and other hormones, the ovary plays a major role in the reproductive process of the female. Many animal models have been extensively studied to determine how plane of nutrition affects ovulation rates, oocyte quality, fertilization, hormone production, embryonic development and survival, and pregnancy outcome (Robinson, 1990; Webb et al., 1999; Boland and Lonergan, 2005; Forcada and Abecia, 2006; Ashworth et al., 2009; Scaramuzzi et al., 2006, 2011; Wu et al., 2011; Grazul-Bilska et al., 2012; Naqvi et al., 2012). Therefore, determining how plane of nutrition affects ovarian function will help provide an improved understanding of methods to enhance reproductive performance of females that may have compromised function due to inadequate nutritional status.

During folliculogenesis, the oocyte is developing and maturing within the follicle. Also during this time, quality, or developmental competence, of the oocyte is becoming established (Krisher, 2004). Oocyte quality is important in ovarian and reproductive function because it not only impacts the ability of the oocyte to become fertilized, but also effects early embryonic survival, establishment and maintenance of pregnancy, fetal development, and offspring outcome (Krisher, 2004; Grazul-Bilska et al., 2012). Therefore, any negative influences on the ovary during the time of oocyte growth and maturation can impact oocyte quality. It has previously been demonstrated that inadequate nutrition can cause detrimental effects on oocyte quality (O'Callaghan et al., 2000; Adamiak et al., 2005; Borowczyk et al., 2006; Grazul-Bilska et al., 2012; Abecia et al., 2013). A study performed by Borowczyk et al. (2006) demonstrated that underfeeding ewes for four weeks with a diet that provided 60% of their daily intake requirements (2.6 Mcal Metabolizable Energy (ME) and 13% crude protein) yielded fewer blastocysts and lower rates of cleavage compared to oocytes derived from control ewes. A similar study performed by the same laboratory included the effects of oocyte quality on both overfed and underfed ewes (Grazul-Bilska et al., 2012). Ewes were fed control (100% NRC requirements), overfed (ad libitum), or underfed (60% of control) diets 60 days prior to oocyte collection. Results from this study indicate that the proportion of cleaved oocytes and both morula and blastocyst stage formation were reduced in overfed and underfed ewes compared to the control. Interestingly, there were no differences observed between overfed and underfed treatments on cleavage rate and morula and blastocyst formation. It was also shown that providing the diet with enhanced energy level to the ewe's diet prior to breeding (known as flushing) not only caused an increase in body weight but also resulted in an increase in ovulation rates of those supplemented ewes (Scaramuzzi et al., 2006; Naqvi et al., 2012). Thus, providing an excess diet during the breeding season can impact ovulation rates of those females; conversely, altering the diet by either restricted or excess feeding are associated with decreased oocyte quality measured by the rate of in vitro fertilization and early embryonic development (Borowczyk et al., 2006; Grazul-Bilska et al., 2012). However, inconsistent results concerning the diet effects on oocyte quality were obtained from several studies, which were possibly due to differences in the experimental design, such as length of nutritional treatment, level of excess or restricted diets, composition of the diet, and breed/species of animal (McEvoy et al., 1995; Lozano et al., 2003; Kakar et al., 2005).

Effects of Nutrition on Hormone Production and Secretion

Numerous studies have shown that nutrition has direct effects on reproductive functions by affecting hormone production and secretion (O'Callaghan and Boland, 1999; Lucy, 2003; Hunter et al., 2004; Grazul-Bilska et al., 2012). For example, Lozano et al. (2003) demonstrated that overfeeding and underfeeding ewes either decreased or enhanced peripheral concentrations of P4, respectively; indicating that plane of nutrition may be linked through the regulation of hormone secretion. As previously mentioned, nutrition can influence the peripheral concentrations of reproductive hormones, metabolic hormones, and metabolites.

In pigs and cows, it has been previously demonstrated that overfeeding of non-pregnant females resulted in decreased peripheral concentrations of P4 compared to females that received a maintenance diet (Dunn et al., 1974; Ferguson et al., 2003). Similarly, when pregnant ewes were subjected to overfeeding and underfeeding throughout gestation, peripheral P4 concentrations were reduced in overfed compared to control or underfed ewes (Cumming et al., 1971; Parr et al., 1982, 1987; O'Callaghan et al., 2000; Boland et al., 2001). In contrast, ewes that received either a maintenance or overfed diet resulted in no differences between P4 concentrations (Viñoles et al., 2005). These conflicting results could be due to duration of dietary treatment, and the content and/or level of energy in the diet. In conclusion, P4 plays a major role in the successful establishment and maintenance of pregnancy in domestic livestock species. Therefore, adequate concentrations of circulating P4 are vital to the successful establishment of pregnancy (Ashworth et al., 1989; Stronge et al., 2005; McNeill et al., 2006).

Metabolic hormones such as insulin-like growth factor 1 (IGF1), insulin, and leptin have specific roles in the control of ovarian follicular development, and are likely to be important mediators of the effects of dietary intake and/or energy balance on livestock reproductive function (Diskin et al., 2003). In a study performed by Grazul-Bilska et al. (2012), serum concentrations of insulin were greater in overfed than in underfed ewes, with control ewes being intermediate. These results are consistent with those by Caldeira et al. (2007) and Viñoles et al. (2005) who performed long- and short-term nutritional treatments, respectively. Similarly to insulin, leptin was also directly associated with nutritional intake; however, IGF1 concentrations remained unaffected by dietary intake (Adamiak et al., 2005; Viñoles et al., 2005; Scaramuzzi et al., 2006). In contrast, Caldeira et al. (2007) compared serum concentrations of IGF1 in

ewes with differing body weights and body condition and observed that IGF1 concentrations have a direct relationship with nutritional status of the female, meaning that as dietary intake increases IGF1 concentrations increase as well. Feed flushing of ewes prior to breeding also had an effect on metabolic hormones. It has been demonstrated that ewes receiving an additional energy supplement had increased ovulation rates compared to control ewes. These supplemented ewes had an increase in IGF1, insulin, and leptin concentrations in circulation compared to ewes that had only grazed on pasture (Naqvi et al., 2012). Therefore, dietary intake and body condition seem to have an effect on peripheral concentrations of metabolic hormones.

Glucose and cholesterol are important metabolites that are involved in the regulation of reproductive functions such as quality of oocytes and production of sex steroid hormones (Rabiee and Lean, 2000; Sutton-McDowall et al., 2010; Rato et al., 2012). Ying et al. (2011) demonstrated that overfeeding and underfeeding of non-pregnant ewes altered peripheral glucose concentrations. Results from their study showed that glucose concentrations in underfed ewes were significantly lower than those of the control and overfed ewes. This suggests that glucose concentration and dietary intake are directly related. Similar results were observed in non-pregnant cattle (Adamiak et al., 2005; Chelikani et al., 2009) who demonstrated that high levels of feeding caused glucose concentrations to increase compared to the control. Peripheral concentrations of cholesterol, on the other hand, have been demonstrated to be inversely related to dietary intake. Ying et al. (2013) observed that in the ewe, cholesterol concentrations increased with decreasing dietary intake, therefore underfed ewes had greater cholesterol concentrations than control and overfed ewes. In contrast, Hashem and El-Zarkouny (2013) observed that supplementing fat into the diet of ewes increased the concentrations of cholesterol compared to the control fed group. Therefore, these results indicate that peripheral cholesterol concentrations may depend on the duration, level of energy, and composition of diet.

Nutrition Impacts Fetal Development and Offspring Outcome

Maternal nutrition plays a critical role in fetal growth and development (Wu et al., 2004). The nutrients that the mother receives throughout gestation get broken down and released into circulation. These nutrients then enter fetal circulation through the placenta where they provide the requirements for proper fetal growth and development. Previous studies have indicated that both under and overnutrition

can negatively impact fetal development and can affect the reproductive performance of the offspring. Bell and Ehrhardt (2002) demonstrated that undernutrition during late-gestation reduced both placental and fetal growth in domestic animals. Specifically in the ewe, undernutrition during the first nine weeks of gestation significantly retarded aspects of fetal ovarian development (Borwick et al., 1997) and follicular development (Rae et al., 2001). Similarly, overfeeding during gestation retarded placental and fetal growth, and increased fetal and neonatal mortality in rats, pigs, and sheep (Wallace et al., 2003). Therefore, plane of nutrition during any stage of gestation, can cause significant effects to fetal growth and development. Not only can effects be observed during fetal development, but effects can also be observed well after birth. Studies performed by Rhind et al. (1989) and Gunn et al. (1995) showed that undernutrition during gestation resulted in a reduced lifetime reproductive capacity of the offspring, measured by the incidence of barrenness. Therefore, this evidence suggests that fetal growth and development, and the subsequent reproductive function of the offspring are vulnerable to maternal plane of nutrition.

Arginine Supplementation

One way to manipulate the maternal diet to compensate for compromised reproductive function, fetal growth, and offspring outcome is to provide a dietary supplement to the mother. One supplement that is currently of particular interest is arginine. Arginine is an essential amino acid that is a building block for proteins as well as a precursor for the synthesis of nitric oxide (NO) and polyamines (Wu and Morris, 1998). Specifically, NO has been demonstrated to stimulate cellular proliferation and migration, cellular remodeling, angiogenesis, and the dilation of blood vessels (Wu et al., 2009, 2013). Dilation of blood vessels is important in reproductive function since it increases blood flow to many different types of tissues, organs, and systems, such as the reproductive tract. Arginine enters the endothelial cell where it is converted to L-citrulline and NO via endothelial nitric oxide synthase (eNOS). Nitric oxide then leaves the endothelial cell where it diffuses into vascular smooth muscle cells of blood vessels. In the vascular smooth muscle cells, NO activates guanylate cyclase (GC) converting GTP to cGMP causing the muscle cell to dilate (Gornik and Creager, 2004). Therefore, NO is a major endothelium-derived relaxing factor, and plays an important role in regulating placental-fetal blood flows and, thus, the transfer of nutrients and

oxygen from the mother to the developing fetus (Bird et al., 2003). In the non-pregnant tract, NO acts on the endothelial cells of the blood vessels increasing their dilation allowing blood flow to increase.

Previous studies have shown that when arginine was supplemented into the maternal diet or injected intravenously, it caused a 22% increase in the number of piglets born alive (Mateo et al., 2007) and increased embryonic survival and litter size by 30% in rats (Zeng et al., 2008). In sheep, arginine treatment during late gestation increased the transportation of nutrients to the fetuses (Thureen et al., 2002) ultimately increasing the birth weights of lambs (De Boo et al., 2005). Luther et al. (2009) demonstrated that pregnant ewes that were given arginine gave birth to more lambs when compared to ewes that did not receive arginine. In summary, reproductive losses and altered fetal growth and development can be prevented, at least in part, by arginine treatment. Since we already observed that diet can negatively impact reproductive function and pregnancy outcome in many species, Lassala et al. (2010) demonstrated how supplementing arginine to undernourished ewes affected fetal development. Results from this study indicate that administration of arginine to undernourished pregnant ewes prevented fetal growth restriction from occurring and enhanced birth weights of lambs by 21%. Therefore, arginine treatment increased the availability of nutrients from the underfed mother to the fetus in order to support proper fetal growth and development. Unfortunately, there is little information regarding the effects of arginine treatment on reproductive function of non-pregnant ewes receiving different nutritional planes (see Chapter 3).

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CHAPTER 2: IMPACT OF MATERNAL PHYSICAL ACTIVITY DURING GESTATION ON PORCINE FETAL, NEONATAL, AND ADOLESCENT OVARIAN DEVELOPMENT

Abstract

To determine how exercise from mid to late gestation impacts offspring ovarian growth at three different developmental stages, Yorkshire gilts were either exercised by walking (EX) or not exercised (CON) from day 40 to 104 of gestation. In parity 1, neonatal ovaries and uteri were collected from the heaviest (H) and lightest (L) females after birth. All remaining gilts were raised until 6 months (i.e., adolescent) and ovaries and uteri were collected. In parity 2, mothers were assigned to the same treatment groups, and on day 94 of gestation, ovaries and uteri were collected from H and L fetuses. Body weight (BW) was greater ($P < 0.02$) for H than L fetuses and neonates; however, there were no effects of treatment on BW at any developmental stage. Neonatal ovarian weight was similar across treatments in H gilts, but in L gilts was greater ($P < 0.02$) in EX than CON. Labeling index (LI; percentage of proliferating cells) was greater ($P < 0.01$) in cortex than medulla regions of fetal and neonatal ovaries. In fetal ovaries, EX enhanced LI ($P < 0.01$), and LI was greater ($P < 0.01$) in H compared to L gilts. In adolescent ovaries, LI was greatest ($P < 0.01$) in healthy antral and least in atretic antral follicles, and LI was greater ($P < 0.01$) in granulosa than theca cells of healthy antral follicles. While exercise increased LI in fetal ovaries, LI was not affected in neonatal and adolescent ovaries of EX and CON sows. While maternal exercise during gestation influences fetal and neonatal ovarian development, impacts on offspring fertility are unknown.

Keywords: Exercise, Gestation, Ovarian development, Swine

Introduction

In the swine industry, goals of the producer include increasing growth, feed efficiency, and carcass quality, as well as reproductive traits such as litter size (Cole et al., 1994) to improve productivity of the herd. However, selection for greater total number of piglets born is associated with increased within-litter variation in piglet birth weight, and can cause an overall decrease in birth weight (Quiniou et al., 2007). Low birth weight can lead to delayed puberty and a decreased body weight at puberty, which can decrease overall lifetime performance (Foxcroft et al., 2009).

The events that occur during embryonic and fetal development can ultimately have long-lasting effects on the health and production outcomes of the developing offspring (Gluckman and Hanson, 2004), including reproductive performance and efficiency, as well as fertility (Foxcroft et al., 2009). Mammals such as cattle, swine, sheep, and humans are born with a highly variable number of follicles and oocytes in their ovaries that eventually dwindle during ageing and will never be replenished (Evans et al., 2012). It has been previously demonstrated that nutrition, stress, and environmental factors that occur during gestation can have both positive and negative effects on reproductive performance of female offspring, as well as effects on neural, immune, cardiovascular, and other functions (Rhind et al., 2003; Vonnahme and Lemley, 2012).

Litter size in litter bearing species has also been demonstrated to have an effect on the reproductive performance of the female. In sows, as the size of the litter increases, blood flow to each fetus decreases (Reynolds et al., 1985). Reduction of blood flow ultimately can cause detrimental effects by reducing the amount of nutrients and oxygen going towards development to lightweight fetuses, thereby reducing the reproductive efficiency and fertility. However, blood flow can be enhanced by physical exercise (Francescomarino et al., 2009; Harris et al., 2013). In pigs, Harris et al. (2013) demonstrated that overall blood flow to the fetus of exercised mothers was greater throughout gestation compared to non-exercised mothers housed in individual stalls. Fetuses receiving less blood flow under control conditions may experience a greater adaptive increase in blood flow when mothers exercise. Thus, regular exercise may lead to enhanced placental and umbilical blood flow at rest, and potentially increase overall delivery of oxygen and nutrients to developing fetuses (Clapp, 2003). Yet in mice and goats, it has been demonstrated that walking or running on a treadmill caused greater embryonic/fetal loss and lower birth weights compared to non-exercised controls (Terada, 1974; Dhindsa et al., 1978).

Effects of maternal physical exercise on puberty attainment and fertility have been investigated using several animal models including pigs (Soede and Schouten, 1991), humans (Negro-Vilar, 1993), and horses (Sairanen et al., 2011). But information concerning the effects of maternal physical exercise on reproductive measurements in offspring of any species is very limited. Nevertheless, it has been demonstrated that exercise did not have any effects on the total number born or number of piglets born alive (Hale et al., 1981; Harris et al., 2013). However, increasing intensity of exercise regimens has been

shown to decrease mortality and increase live litter birth weights of those exercised offspring, with high intensity exercise having a greater number of piglets making it to their weaning age (Schenck et al., 2008). So it seems that exercise, whether low or high intensity, did not have any detrimental effects on the reproductive performance of the sow, and benefited the surviving piglets to their weaning age. However, variation of experimental conditions such as duration and intensity of exercise, as well as stage of pregnancy, likely contributed to conflicting and variable results.

In livestock species in particular, the number and quality of oocytes determines the reproductive efficiency of that female (Ireland et al., 2011). Quality of oocytes is associated with the rate of cell proliferation within the follicles at different stages of folliculogenesis (Durlej et al., 2012). However, the growth and differentiation, as well as cellular proliferation, of ovaries can be affected by many factors (Grazul-Bilska et al., 2009). In sheep, it has been demonstrated that maternal underfeeding caused a decrease in fetal ovarian cell proliferation (Grazul-Bilska et al., 2009) and umbilical blood flow (Lemley et al., 2012). Thus, it seems that decreasing umbilical blood flow to the fetus could potentially decrease the cell proliferation in fetal ovaries during development; therefore, likely decreasing the reproductive efficiency and fertility of the offspring. In fact, it has been reported that environmental factors can affect the growth, development, and physiology in fetal as well as postnatal life of mammals (Wu et al., 2006; Barker, 2007; Fowler et al., 2008).

We hypothesize that an increase in blood flow to the fetus caused by maternal exercise (Harris et al., 2013) would enhance ovarian and uterine development measured by organ weight and ovarian cell proliferation in the offspring of exercised mothers. Therefore, the objective of this study was to determine how maternal exercise from mid to late gestation in the sow will impact offspring body weight, ovarian and uterine weight, and cell proliferation in ovarian compartments at fetal, neonatal, and adolescent stages of development.

Materials and Methods

All animal procedures were approved by the North Dakota State University (NDSU) Animal Care and Use Committee (# A0927). Animal procedures were performed as previously described by Harris et al. (2013).

Animal Procedures

Experiment 1. At the onset of estrus (day 0), Yorkshire gilts (parity 1; $n = 15$) derived from the same sire were bred to a common boar (Hampshire x Duroc) at the NDSU Swine Research Center. On day 28 of gestation, 10 gilts were confirmed pregnant by ultrasonography (Aloka 500 SSD Ultrasound, Corometrics Medical Systems Inc., Wallingford, CT) and transported 1.6 km to the NDSU Animal Nutrition and Physiology Center. All gilts were individually housed in farrowing stalls (57 x 176.5 cm) in the same room. On day 36.1 ± 0.3 of gestation, gilts were assigned to treatment with a littermate pair (i.e., four pairs of littermate gilts). Within a pair, one gilt was assigned to the control group (CON), which remained in the stall for the duration of gestation except for times when BW was determined, and the other gilt was removed from her stall for exercise 3 times per week for 30 min (EX).

The EX treatment was initiated on day 40 of gestation, after the time when maximum uterine capacity had occurred and the majority of embryonic and early fetal loss passed (Webel and Dziuk, 1974; Foxcroft et al., 2006). On day 40 of gestation, duration of exercise was gradually increased with 10 min of exercise on day 40 and 41, 20 min of exercise on day 42 and 43, and 30 min of exercise on day 44 and every subsequent Monday, Wednesday, and Friday. Gilts were exercised individually to remove any pig-to-pig social interactions. On day 104 of gestation, gilts were then taken off their treatments and allowed to finish the remainder of gestation individually penned.

Within 15 h of farrowing completion, piglets were weighed, and the lightest (L; $n = 8$) and heaviest (H; $n = 8$) female piglets in each litter were subject to CO₂ asphyxiation until confirmed unconscious. The ovaries and uterus were removed and weighed. One ovary was fixed in Carnoy's solution and another was fixed in formalin solution. The remaining piglets were allowed to grow until approximately 6 months of age. Then, all remaining piglets were harvested at the NDSU Meat Science Laboratory. One female from each litter was selected based on the adolescent status of her ovaries (a lack of CL; $n = 8$) and her ovaries and uterus were collected, weighed, and fixed as described above.

Experiment 2. After 22 ± 3 days lactation, gilts that were used in parity 1 had piglets weaned, were rebred to a common boar, and placed on the same treatment as they were assigned in Experiment 1. One pair of females was removed from Experiment 2 as one female did not get pregnant after weaning (parity 2; EX; $n = 3$; CON; $n = 3$). Similar to Experiment 1, exercise adaptation began on day 40 of

gestation, and 30 min of exercise was initiated on day 44 and every subsequent Monday, Wednesday, and Friday until day 94 of gestation. On day 94 of gestation, sows were euthanized, and the H (n = 6) and L (n = 6) female fetuses were selected and her ovaries and uterus were collected, weighed, and fixed as described for Experiment 1.

Immunohistochemistry

Since ovarian morphology was better preserved by fixation in formalin than in Carnoy's solution, only formalin fixed tissues were used for immunohistochemical procedures as described before (Grazul-Bilska et al., 2009). Ovarian tissue sections were cut to 4 μ m, placed on individual slides, and then submerged in Histo-Clear to remove paraffin. Tissue slides were rehydrated through an ascending series of alcohols before undergoing antigen retrieval in citrate buffer (10 mM; pH 6.0; Onions et al., 2008) and use of a microwave set at 100% power for 15 min (3 intervals at 5 min each). After each 5 min interval, citrate buffer was added to the dish to ensure all slides were submerged. Tissue sections were then incubated for 20 min using a blocking buffer (Tris-buffered saline [TBS] containing normal horse serum; 2%, vol/vol) followed by incubation with mouse monoclonal primary antibody (1:250; Vector Laboratories, Burlingame, CA, USA) against Ki-67 (a marker of proliferating cells), for 1 h at room temperature (25°C). A secondary anti-mouse antibody coupled to peroxidase (ImPress Kit; Vector Laboratories) was then applied for 1 h at room temperature followed by incubation with Vector SG (Vector Laboratories) for 10 min and counterstained with nuclear fast red (Sigma).

Image Analysis

Image analysis was performed similarly to that described by Grazul-Bilska et al., (2009). For each ovary, 8-10 images of the randomly chosen cortex (COR) and medulla (MED) regions of the fetal (n = 12) and neonatal (n = 16) ovaries were generated using an Eclipse E600 Nikon microscope and digital camera (Nikon Instruments Inc., Melville, NY, USA). For the adolescent ovaries (n = 8), images of stained sections were taken for each of the three types of follicles, including secondary (SF), healthy antral (HA), and atretic antral (AA; total 5-10 images/follicle/ovary). Follicles chosen for image analysis were selected based on the following criteria as described by Senger (2003), SF containing the oocyte surrounded by two or more layers of granulosa cells, however, no fluid filled cavity, or antrum. Healthy antral follicles containing an antrum housing the oocyte surrounded by many layers of granulosa and theca cells, and

AA classified as an antral follicle with degenerated basement membrane, pycnotic nuclei, and some detached granulosa cells. The images were then used for quantitative image analysis using the Image Pro-plus software (Media Cybernetics Inc., Silver Spring, MD, USA) to determine labeling index (LI; the proportion of proliferating cells within specific tissue area). The number of analyzed SF, HA, and AA follicles is provided in Table 2.1.

Table 2.1: Number of ovarian follicles used for image analysis from adolescent ovaries collected from mothers that remained individually penned (CON) or exercised (EX) from d 40-104 of gestation.				
Treatment	No. Sows	Secondary	Healthy Antral	Atretic Antral
CON	4	19	19	18
EX	4	21	19	20
Total	8	40	38	38

Statistical Analysis

Measurements that were recorded for fetal, neonatal, and adolescent offspring data (e.g., BW, ovarian weight, ovarian weight/kg BW, uterine weight, uterine weight/kg BW, and LI in the COR and MED regions of the fetal and neonatal ovaries) were analyzed using the MIXED procedure (SAS Institute Inc., Cary, NC) with random effect being sow within treatment and fixed effects being treatment (CON vs. EX) and weight (H vs. L; fetal and neonate data). The model for the fetal and neonatal data included the effects of treatment, weight, and their interaction. Statistical analysis for LI of adolescent ovarian follicles (e.g., granulosa and/or theca layer of SF, HA, AA) were analyzed using the GLM procedures of SAS with fixed effects being treatment (CON vs. EX). Since, there were no treatment effects of the LI of adolescent ovarian follicles; therefore, the mean value was calculated for each follicle type. Means were separated using the LSMeans option of SAS and were considered significant when $P \leq 0.05$. LSMeans \pm SEM are presented.

Results

Weight Measurements

At day 94 of gestation and birth, the H fetuses and neonates were heavier ($P \leq 0.02$) than the L offspring (Table 2.2). However, fetal or neonatal BW was not affected by maternal exercises. Since the H and L females from each litter were already selected at birth for neonatal analysis, adolescent females at 6 months of age had similar ($P = 0.60$) BW regardless of maternal treatment.

Table 2.2: Body weight (BW) and reproductive organ weights of female offspring born to mothers housed in an individual stall for the duration of gestation (CON) or exercised (EX) 30 min, 3 times per week from d 40 to 104 for neonates and adolescent gilts, and d 40 to 94 for fetal gilts.

	Treatment			Weight			P-values		
	CON	EX	SEM	H	L	SEM	Trt	Wt	Trt x Wt
Fetal (n = 12)									
BW (g)	768.60	674.93	79.62	836.47 ^a	607.07 ^b	63.17	0.45	0.02	0.38
Ovary (g)	0.17	0.21	0.03	0.21	0.16	0.03	0.48	0.30	0.96
Ovary/BW (g/kg)	0.22	0.30	0.04	0.25	0.27	0.03	0.20	0.65	0.71
Uterus (g)	0.62	0.70	0.10	0.84	0.48	0.10	0.61	0.24	0.57
Uterus/BW (g/kg)	0.87	1.03	0.28	1.06	0.84	0.18	0.68	0.07	0.08
Neonatal (n = 16)									
BW (g)	1271.25	1415.50	165.27	1578.50 ^a	1108.25 ^b	124.36	0.58	0.01	0.50
Ovary (g)	0.08	0.11	0.007	0.10	0.09	0.007	0.01	0.28	0.02*
Ovary/BW (g/kg)	0.04 ^a	0.04 ^b	0.004	0.03	0.04	0.003	0.05	0.10	0.08
Uterus (g)	0.51	0.53	0.05	0.54	0.49	0.05	0.76	0.50	0.08
Uterus/BW (g/kg)	0.04	0.04	0.004	0.03 ^a	0.04 ^b	0.003	0.37	0.01	0.08
Adolescent (n = 8)									
BW (kg)	116.36	113.98	3.06	-	-	-	0.60	-	-
Ovary (g)	9.28	9.45	0.87	-	-	-	0.89	-	-
Ovary/BW (g/kg)	0.08	0.08	0.008	-	-	-	0.74	-	-
Uterus (g)	52.10	112.68	23.35	-	-	-	0.12	-	-
Uterus/BW (g/kg)	0.45	1.10	0.235	-	-	-	0.14	-	-

^{a,b}LSMeans ± SEM within a measure differ; *P* < 0.05; *interactive means are depicted in Figure 1

H = Heavyweight

L = Lightweight

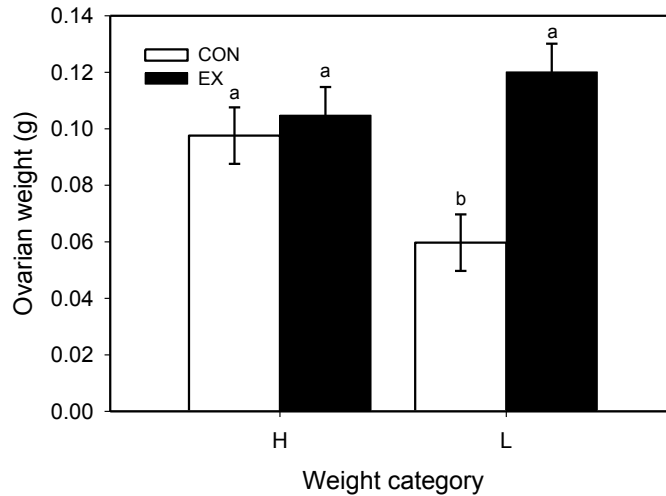


Figure 2.1: Ovarian weights of heavyweight (H) and lightweight (L) neonates collected from control (CON; open bars) and exercised (EX; black bars) gilts within 15 h after birth. ^{a,b}LSMeans \pm SEM with different superscripts differ; $P < 0.02$.

Uterine weight at any age was not affected ($P > 0.08$) by weight or maternal treatment. When expressed per unit BW, neonatal uterine weight was greater ($P < 0.01$) for L vs. H piglets (Table 2.2).

Ovarian weight (g or g/kg) was not influenced ($P > 0.45$) by maternal exercise in fetuses or adolescent pigs (Table 2.2). There was a treatment by weight interaction ($P < 0.02$; Figure 2.1) in neonatal gilts where ovaries from the L neonates of CON dams were significantly lighter than ovaries from all other groups. When expressed per unit BW, there was a main effect of treatment, with piglets from EX gilts having heavier ovaries than piglets from CON gilts (Table 2.2).

Morphology and Ovarian Cell Proliferation

Fetal and neonatal ovaries contained primordial and primary follicles in COR and MED regions, but the adolescent ovaries contained primordial, primary, SF, HA, and AA follicles in the COR (Figure 2.2). Expression of Ki-67 was detected in several compartments including COR and MED regions in stromal cells and granulosa cells of primordial and primary follicles of fetal and neonatal ovaries, and in stromal cells and primary, primordial, SF, HA, and AA follicles of adolescent ovaries (Figure 2.2). In all ovaries, Ki67 was also detected in surface epithelium (Figure 2.2). In fetal ovaries, LI was greater

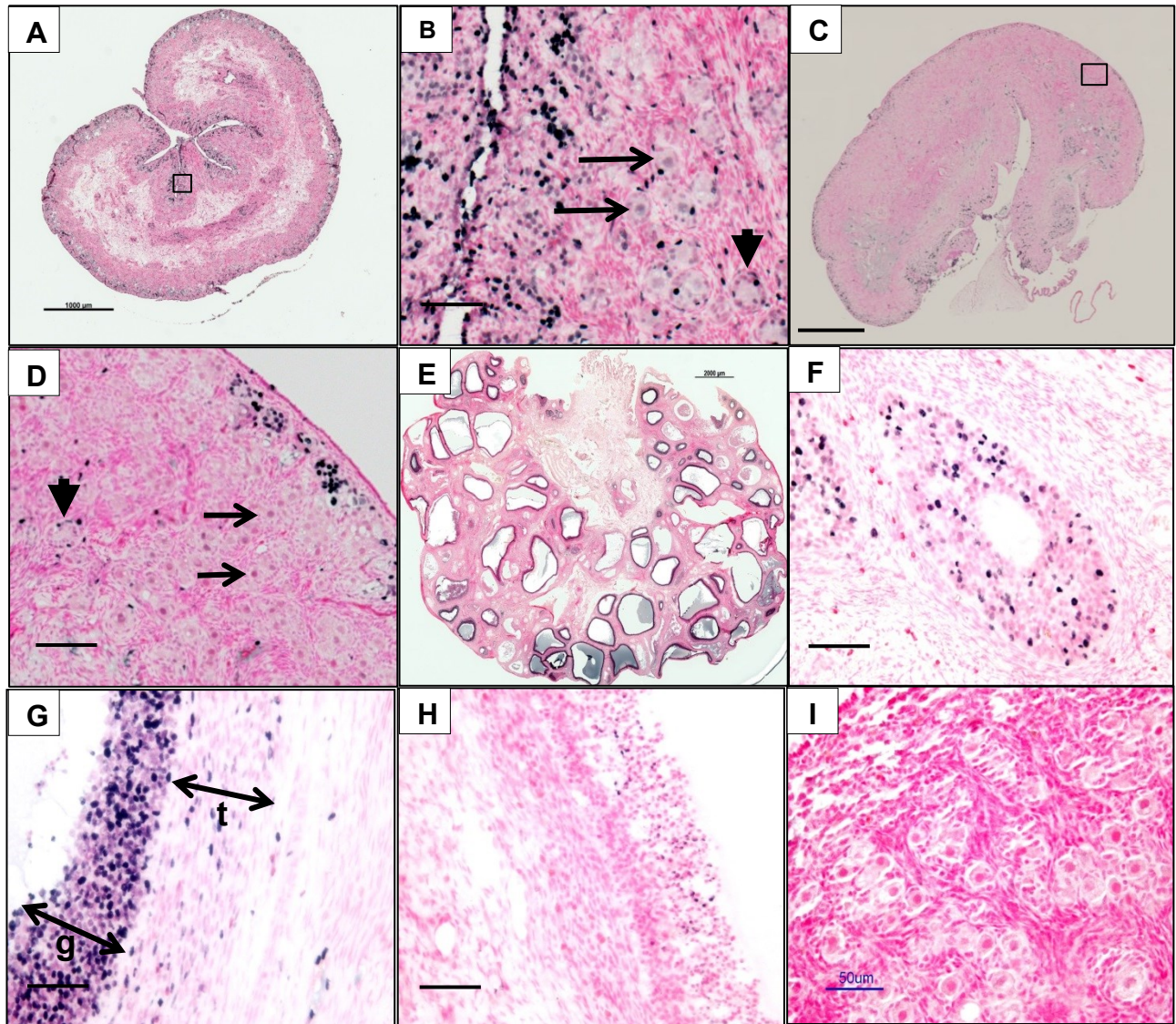


Figure 2.2: Representative micrograph of Ki67 staining in the porcine fetal ovary collected at day 94 of gestation (A), neonatal ovary collected within 15 h after birth (C), and an adolescent ovary collected at 6 months of age (E). Dark staining indicates proliferating cells, and pink staining indicates cell nuclei of non-proliferating cells in both follicular and stromal tissues. Figure (I) represents control staining (no primary antibody). Note that (B) is a zoomed in micrograph of the box in (A) and demonstrates at the fetal stage of development, primordial (arrow) and primary (arrowhead) follicles are present throughout the medulla and cortex regions. At the neonatal stage of development, primordial (arrow) and primary (arrowhead) follicles are present throughout the medulla and cortex regions (D), note that (D) is a close up micrograph of the box in (C). The adolescent ovary contains primordial, primary, secondary (F), antral (G), and atretic (H) follicles. Note more proliferating cells in the granulosa (g) than theca (t) layer of the antral follicle (G); whereas in the atretic follicle (H) the basement membrane is degenerating and pycnotic nuclei are present. Bar (A and C) = 1000 µm, (E) = 2000 µm, and (B, D, F- I) = 50 µm.

($P < 0.01$) in the COR than the MED region (Figure 2.3A), and LI was greater ($P < 0.05$) in EX H than CON H, CON L, and EX L ($P < 0.02$; Figure 2.3B). In neonatal ovaries, LI was greater ($P < 0.01$) in COR than the MED region (Figure 2.4), and LI was not affected by maternal exercise, weight, or their interaction. In adolescent ovaries, LI in granulosa and/or theca cells in follicles from three stages of follicular development was not affected by maternal exercise; therefore data for EX and CON groups are combined. Thus, overall in adolescent ovaries, LI in the granulosa layer was greater ($P < 0.05$) in HA than SF ($8.4 \pm 1.7\%$ vs. $20.1 \pm 1.4\%$). In HA follicles, LI was greater ($P < 0.01$) in the granulosa than the theca layer ($20.1 \pm 1.4\%$ vs. $5.6 \pm 0.5\%$), and LI was greater ($P < 0.01$) in the granulosa plus the theca layer of the HA than AA follicle ($12.9 \pm 0.9\%$ vs. $2.4 \pm 0.7\%$).

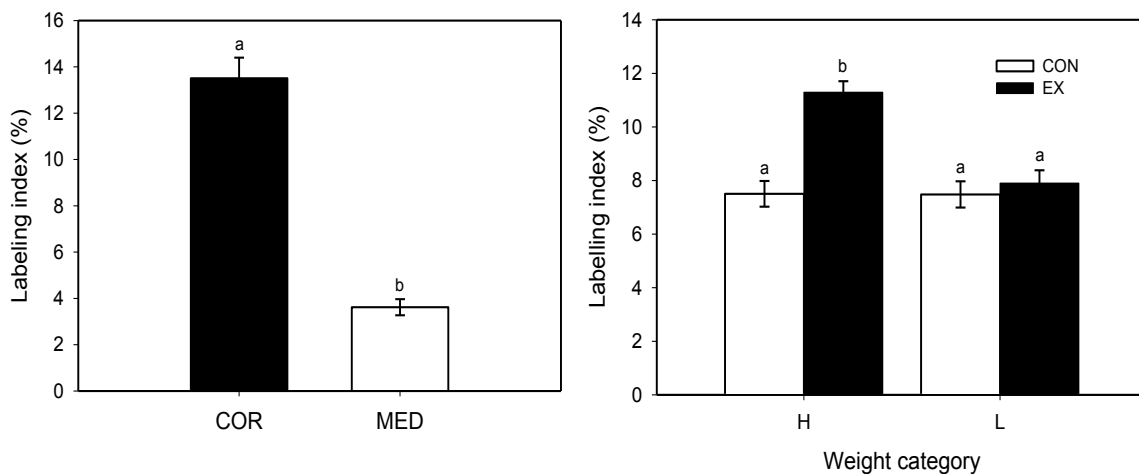


Figure 2.3: Labeling index in the ovarian cortex (COR) and medulla (MED) regions (A), and in combined COR and MED(B) in the H and L fetal ovaries from control (CON) or exercised (EX) sows from day 40 to 94 of gestation. Since exercise did not have any effect on LI in the ovarian regions, data are combined for CON and EX. There was a weight effect ($P < 0.02$; B) with the heavyweight (H) piglets born to EX sows having greater labeling index than lightweight (L) fetal ovaries of CON or EX sows. ^{a,b}LSMeans \pm SEM with different superscript differ; $P < 0.02$.

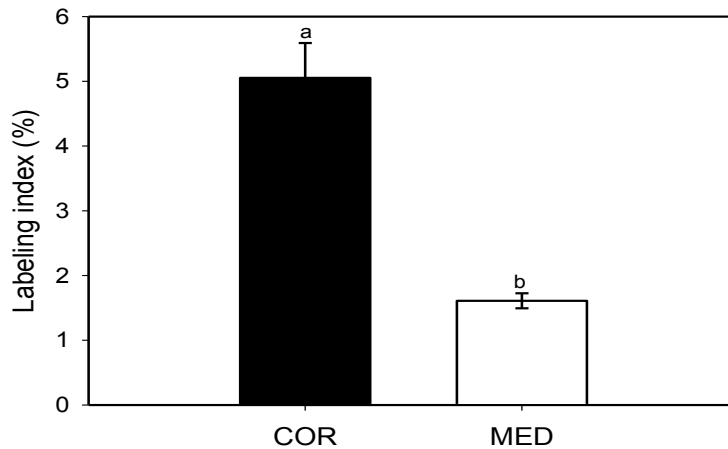


Figure 2.4: Labeling index of the ovarian cortex (COR) and medulla (MED) of neonatal ovaries from control (CON) or exercised (EX) gilts from day 40 to 104 of gestation. Since exercise did not have any effect on LI, data are combined for CON and EX. ^{a,b}LSMeans \pm SEM with different superscripts differ; $P < 0.01$.

Discussion

Current results demonstrated that maternal exercise did not affect female offspring BW but affected selected measurements of reproductive function including ovarian weight and cell proliferation, but this effect depended on the stage of offspring development. In rats, it has been shown that overall growth of fetuses from mothers that exercised during the entire pregnancy showed a markedly decreased size and BW compared to the control (Nagai et al., 1993). In the same study, fetal size and BW from dams that had exercised only during late gestation were similar to non-exercised dams. In humans, fetal growth was similar and growth rate appropriate between mothers that initiated exercise at 13 or 20 weeks of pregnancy with a non-exercised control (De Oliveria Melo et al., 2012). This demonstrates that exercise starting in mid-gestation does not have any negative effects on fetal growth and development measured by BW or growth rates.

During pregnancy, blood flow to the uterus increases exponentially throughout gestation causing an increase in nutrients and oxygen delivery to the fetus to support normal growth and development. Dziuk (1985) reported that pig fetuses located next to the ovary occupied a greater amount of space and have greater BW than fetuses located in the middle region of the uterus, which could cause the differences seen in BW. Exercise, in general, causes an increase in blood flow to working muscles and visceral tissues. However, exercise during pregnancy in sheep results in an increase in blood flow to respiratory muscles, nonrespiratory limb muscles and adipose tissue while decreased flow was observed

in the umbilical and uterine arteries while exercise was being performed (Bell et al., 1986). Since the reproductive tract is not needed during exercise, blood flow is shunted to other areas of the body that have increased demand for blood flow. This results in decreased uterine and umbilical blood flow. During pregnancy, Harris et al. (2013) demonstrated that sows that had increased physical activity had elevated umbilical blood flow at rest. However, umbilical blood flow was determined on two randomly selected fetuses by Doppler ultrasonography so uterine horn location and sex of the fetuses were not determined in that study. Therefore, we are unaware if our ovarian measurements could be directly related to their individual umbilical blood flows. Body weights of neonates were similar for EX and CON in female piglets selected for this study. However, as seen with the fetuses, and as designed, there was an effect of weight, with the H being heavier than L neonates. Similarly, there were no differences in BW between the offspring of sows that exercised on the treadmill for 15 min/day for a distance of 0.48 km compared to those born to control sows (Hale et al., 1981). However, a high intensity exercise regime (increasing distance from 122 m/day to 427 m/day for 3 days/week from day 35 to 110 of gestation) resulted in enhanced piglets' BW at birth compared to both a low intensity (exercised for 122 m/day for 5 days/week from day 35 to 110 of gestation) and no exercise (Schenck et al., 2008). In contrast, the litters from exercised rat dams grew slower and the BW of pups at birth was 10% lower than that of the sedentary dams (Pinto and Shetty, 1995). In humans, there has been controversial data demonstrating decreased, increased, or similar birth weights of mothers that exercised during gestation compared to those that did not (Clapp et al., 2000). These conflicting data are likely due to species, type of exercise performed, duration of exercise, and stage of pregnancy when the mother was exercising.

In our current study, adolescent BW did not differ between CON or EX treatments; however, the H and L neonates were already selected from each litter. Clapp (1996) and Clapp et al. (1998) found that infants born to exercising mothers weighed less than mothers that did not exercise, this difference in weight was not evident at one year of age, but was again present at five years of age. Currently, it remains unclear why such differences appeared. These results are not consistent with the results from our study as we did not observe an effect of exercise on BW at birth or six months of age. However, unlike Clapp (1996) and Clapp et al. (1998), we followed the offspring to adolescent age. Thus, it seems

that exercise does not have any effects on BW at the fetal, neonatal, or adolescent stages of development in pigs.

The mammalian uterus differentiates from the Müllerian ducts during early fetal development (Cooke et al., 2013), so any environmental or nutrition factors early in gestation could positively or negatively affect development of the uterus, along with other organs. Our results show that uterine weight was similar between CON and EX treatments as well as between H and L fetuses. Uterine development is complete by birth, as shown by the differentiation of the mesenchyme into endometrial and myometrial tissue layers (Bartol et al., 1993). It has been previously demonstrated that IUGR piglets had significantly lighter body and internal organ weights when compared to their normally developed 1 day old littermate piglets (Xu et al., 1994). However, when comparing relative organ weight, there were no differences between normal and IUGR piglets. Unfortunately, these results did not take into account any reproductive organs, such as the ovary or uterus. In our study, uterine weight did not differ between either EX or CON treatments, or between H and L neonates, opposing the results observed from normal and IUGR neonates. Interestingly, our results show that uterine weight relative to BW was greater in L than H neonates; however reasons as to why still remain unclear.

The onset of puberty, as designated by the first ovulation, includes changes in reproductive tract development, such as increasing the endometrial thickness of the uterus (Monteiro et al., 2013). Endometrial thickness and uterine weight increase with age, as we observed between neonate and adolescent offspring in our study. However, we did not observe any effects of exercise on uterine weight or relative uterine weight in adolescent pigs. Similar to our results, Estienne and Harper (2010) demonstrated that there was no effect of housing (individual stall or group pen), and thus sow movement throughout gestation, on uterine weight of gilts. This demonstrates that maternal physical activity does not have an effect on uterine weight in adolescent offspring.

Ovarian weight of fetal offspring was affected by maternal physical activity, but there were no differences seen between H or L fetuses in our study. It has been reported that fetal runts (< 600 g) had significantly lighter ovaries when compared to normally developed fetuses in the sow (Da Silva-Buttkus et al., 2003). Thus, it seems that exercise could have a positive effect on compromised fetuses to reach similar ovarian weights as the rest of the litter in this study. Furthermore, ovarian weight relative to BW

was similar in runts and normally developed fetuses (Da Silva-Buttkus et al., 2003). Our results also showed that ovarian weight relative to BW were similar between the H and L fetuses, regardless of treatment indicating a lack of maternal exercise on ovarian development at the fetal stage.

Maternal physical activity caused neonatal ovaries to be heavier than in controls. Interestingly, the L neonates born to EX mothers were significantly heavier than the L neonates born to CON mothers in our study. This suggests that maternal activity during mid to late gestation could benefit ovarian growth in L neonates when it comes to ovarian weight. In bulls, it has been previously demonstrated that as testicle size increases, the fertility of the bull also increases (Coulter and Foote, 1979; Ireland et al., 2011). This would imply as ovaries increase in size and weight, more oocytes are available to ovulate, which overall will enhance fertility (Ireland et al., 2011). Reasons as to why we see an increase in ovarian weight of the L neonates of EX mothers still remains unclear, especially since we did not detect enhanced cell proliferation. However, in rats, it has been demonstrated that blood flow decreased to the ovaries of offspring in both trained and untrained dams while running on a treadmill for 1 h/day, which was equivalent to running 30 m/min (Jones et al., 1990). In pre-exercised dams, ovarian blood flow at rest was greater than in those that were already accustomed to training compared to those that were untrained. Even though there was a decrease in blood flow during exercise in the rat, overall blood flow increased in uterine and umbilical vessels of those exercised dams at rest; it is likely that this increase could cause differences in ovarian weight between the EX and CON neonates in our study. Also, it is interesting to note when comparing ovarian weight relative to BW of the neonate, the EX offspring had greater relative ovarian weight than those born to the CON, suggesting that exercise benefits neonates.

At the adolescent stage of development, ovarian weight did not differ between treatments, nor did ovarian weight relative to BW in our study. From birth to 90 days of age, body and ovarian weights increased in a proportional manner; thus, there was no change in relative ovarian weight between neonates and adolescent gilts (Oxender et al., 1979). Dyck and Swierstra (1983) found that relative ovarian weight declined from birth to 70 days of age in gilts. Our results also demonstrate a decrease in relative ovarian weights as age increases. So it seems that maternal physical activity does not have an impact on ovarian weight at the adolescent stage of development; however, exercise did affect ovarian weight at the fetal and neonatal stage of development with exercise benefiting the L neonates.

The formation of ovarian follicles in the deepest part of the COR, next to the MED, was first observed at day 56 of gestation in the pig fetus, and as pregnancy progresses, the primordial follicles migrate towards the outer COR from the MED (Bielańska-Osuchowska, 1989, 2006). In our study, fetal and neonatal ovaries contained primordial and primary follicles in the inner COR and also in MED regions. Only about 5% of the follicles present on the neonatal ovary are primary follicles (Oxender et al., 1979). However, unlike sheep (Grazul-Bilska et al., 2009), no other follicle types (e.g., secondary or antral) were present at fetal and neonatal stages of development.

Fetal ovaries present a type of tissue that expresses high cellular proliferation (Juengel et al., 2002; Grazul-Bilska et al., 2009). Our results show that the outer COR region of the ovary had significantly greater LI than the MED region in fetal and neonatal ovaries. This is likely due to the COR housing developing follicles; whereas the MED contains less follicles than COR, and contains the blood and lymph vessels and stromal tissues. While not many studies have looked at exercise and the effects on ovarian cell proliferation, previous studies looking at stem cells (Macaluso and Myburgh, 2012) and intervertebral discs (Sasaki et al., 2012) in humans have found that by increasing exercise cell proliferation was enhanced. In our study, greater LI occurred in the ovaries of EX H fetuses than the EX L, CON L, and CON H, indicating that exercise increases LI in H fetuses, and, interestingly, elevated LI was not observed in neonatal or adolescent ovaries of EX mothers.

In the present study, maternal physical activity affected cell proliferation in the fetal but not neonatal and adolescent ovaries. The lack of treatment or weight effects on cell proliferation at the neonatal or adolescent stages in this experiment could be due to ceasing exercise ~10 days before parturition or insufficient room for movement throughout gestation. However, we observed increasing cell proliferation as folliculogenesis progressed from secondary to antral follicles, as reported by others for sheep (Jablonka-Sharif et al., 1994, 1996; Grazul-Bilska et al., 2009). During follicular development, granulosa cells replicate and the rate of proliferation changes with size of the follicle and oocyte (Pedersen, 1970; Cupps, 1991). We also observed greater LI in the granulosa than theca layer in HA follicles, similar observations were reported for sheep (Jablonka-Shariff et al., 1994, 1996; Grazul-Bilska et al., 2009). This is likely due to the differences in function of the two follicular layers.

Atretic antral follicles were present in adolescent but not in fetal or neonatal ovaries in our experiment. Maternal activity during gestation did not have an impact on LI of atretic follicles in this study. When compared to the combined layers of the HA follicles, as expected, LI was less in AA follicles. These results are consistent with results for other species including pigs, cows, and sheep which report reduced proliferation of granulosa and theca cells in atretic follicles compared with healthy follicles (Jablonka-Shariff et al., 1994, 1996; Guthrie, 1996; Isobe and Yoshimura, 2000; Garrett and Feranil et al., 2004).

Conclusion

In conclusion, these data demonstrate that exercise during mid to late gestation in the sow resulted in enhanced cell proliferation in fetal ovaries as well as ovarian weight in L neonates. Thus, the observed increase in umbilical blood flow to the developing offspring (Harris et al., 2013) may have an impact on ovaries from the fetal and neonatal stages of development. While exercise did not have any effects on ovarian weight or cell proliferation at the adolescent stage of development, we demonstrated that granulosa cell proliferation increases during growth from secondary to antral follicles, cell proliferation in AA follicles is very low, and that granulosa cell proliferation is greater than in theca cells in pigs. Since exercise during mid to late gestation in the sow enhanced fetal but not neonatal or adolescent ovarian cell proliferation, we hypothesize that ceasing exercise at day 104 of gestation withdraws cell proliferation stimulus, and this process comes back to the rate similar in non-exercised sows. In order to test this hypothesis, allowing the sows to exercise until parturition would be warranted. Exercise also increased ovarian weight of the compromised lightweight neonates. This increase in ovarian weight could be due to the increase in cell proliferation that was seen in fetal development, and this increase could also potentially increase fertility of those neonates. Thus, exercise seems to benefit ovarian development in the L fetuses into their neonatal life. Results from this study help to provide preliminary insights onto the effects of maternal exercise during mid to late gestation and on body weight, ovarian weight, uterine weight, and ovarian and follicular cell proliferation at the fetal, neonatal, and adolescent stages of development. Increased ovarian weight in the L neonates of EX mothers was likely caused by the increase in cell proliferation seen in the fetal ovaries of EX mothers. In spite of the increase in fetal cell proliferation, there were no effects of exercise on neonatal ovarian cell proliferation or adolescent follicle

proliferation. The effect of maternal exercise on offspring fertility remains to be elucidated. Further research is warranted to determine if increased maternal activity can impact reproductive efficiency.

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CHAPTER 3: EFFECTS OF ARGININE SUPPLEMENTATION ON SERUM METABOLITES AND REPRODUCTIVE AND METABOLIC HORMONE CONCENTRATIONS OF NON-PREGNANT EWES OF DIFFERENT BODY CONDITIONS DURING THEIR ESTROUS CYCLE

Abstract

The aim of this study was to determine the effects of diet and arginine (Arg)-treatment on peripheral reproductive and metabolic hormones, and selected metabolites. Non-pregnant, non-lactating Rambouillet ewes ($n = 82$) were randomly assigned to receive a maintenance control (C; $n = 24$; 100% NRC requirements) diet, or were overfed (O; $n = 27$; 2xC) or underfed (U; $n = 31$, 0.6xC) 60 days prior to the onset of estrus (day 0) which was synchronized using a CIDR device. At day 0, ewes from each nutritional group were randomly assigned to one of two treatments: saline (Sal) or Arg (155 $\mu\text{mol/kg BW}$ L-Arg HCl), which was administered three times per day for 21 or 26 days. Blood samples were collected at days 0, 6, 10, 12, 16, 21, and/or 26 of Arg-treatment for evaluation of arginine, nitric oxide (NO) metabolites, cholesterol, glucose, insulin like growth factor 1 (IGF1), insulin, leptin, and progesterone (P4). During 10-weeks of the study, C maintained BW and BCS, O gained 5.7 ± 0.7 kg, and U lost 14.7 ± 0.1 kg; for O, BCS increased by 1.10 ± 0.02 , but U decreased by 0.51 ± 0.10 . Overall, serum concentrations of arginine, glucose, IGF1, insulin, leptin, and P4 were greater (P in O than C and U ewes). There were no effects of Arg-treatment ($P > 0.15$) on any serum hormone or metabolite concentrations. These data reinforce the importance of dietary effects on endocrine function, and indicate that Arg-treatment does not alter serum hormone or metabolites in ewes fed various nutritional planes.

Keywords: Sheep, Diet, Arginine, Estrous Cycle, Serum Hormone, Body Condition

Introduction

Environmental influences, such as changes in dietary intake and body condition, can have a profound effect on ovarian function and thus fertility (Robinson et al., 1999; Crosignani et al., 2002; Adamiak et al., 2005). Previous research demonstrated that poor nutrition in cattle resulted in delayed puberty attainment, aberrant estrous cycles, lowered conception rates, and reduced birth weights of offspring (Armstrong et al., 2001). It has also been reported that sheep fed a restricted diet expressed reduced ovulation rates compared to sheep that received a maintenance diet (Smith, 1991). Similarly, overfeeding can affect ovarian function and productivity in female livestock species. In sheep, increased

dietary intake stimulated folliculogenesis and ovulation rates (Scaramuzzi et al., 2006; Naqvi et al., 2012) but decreased oocyte quality measured by in vitro fertilization (IVF) rates and early embryonic development (Grazul-Bilska et al., 2012), and pregnancy rates (Parr et al., 1987). The effects of diet on ovarian function were associated with changes in circulating concentrations of various metabolic and reproductive hormones, such as IGF1, insulin, leptin, E2, and/or P4 (Webb et al., 2004).

Nutritionally-induced changes in the concentrations of circulating metabolic hormones such as IGF1, insulin, and leptin, have the potential to interact directly with gonadotropins to regulate follicle growth and steroidogenesis, since gonadotropins provide the primary drive for antral follicle development (Webb et al., 2004). When ewes were subjected to feed flushing by providing enhanced energy in the diet, ovulation rates and serum concentrations of IGF1, insulin, and leptin was increased compared to controls (Scaramuzzi et al., 2006; Naqvi et al., 2012). Therefore, overfeeding, in both pregnant and non-pregnant females, has been shown to cause an increase in the concentrations of IGF1, insulin, and leptin, (Keisler et al., 1999; Armstrong et al., 2001), whereas underfeeding in sheep and cattle has demonstrated to decrease the concentrations of insulin and leptin (Caldeira et al., 2007; Delavaud et al., 2007; Tsiplakou et al., 2012).

Progesterone is an important steroid hormone in reproductive function because it is responsible for preparing and maintaining pregnancy, and for regulation of reproductive cyclicity (Ashworth et al., 1989; Stronge et al., 2005; McNeill et al., 2006). In heifers fed a high energy diet, P4 concentrations in plasma were significantly increased (Armstrong et al., 2001). However, in non-pregnant sheep, Rhind et al. (1989) and McEvoy et al. (1995) demonstrated a negative relationship between dietary intake and systemic P4. Ewes fed an excess diet had lower P4 concentrations in circulation than ewes on an underfed diet (O'Callaghan et al., 2000). Altered reproductive and metabolic hormones can cause changes in ovarian and reproductive function such as embryo development and fertilization (Armstrong et al., 2001), oocyte growth and quality (McCaffery et al., 2000), and fertility (Brannian et al., 2002) in livestock species.

Ovarian and reproductive functions are dependent on the diet the female is receiving, such as the different levels of energy, dietary components, or supplements, which have been investigated for several species (O'Callaghan et al., 2000; Webb et al., 2004; Scaramuzzi et al., 2006). Arginine (Arg), which is a

diet component as well as a supplement, has been demonstrated to enhance selected reproductive functions in several species such as sheep and pigs (Mateo et al., 2007; Luther et al., 2009; Li et al., 2010; Lassala et al., 2010). Since Arg is a precursor for nitric oxide (NO), it may affect ovarian function acting through the NO system (Wu, 2009). The NO system is involved in the regulation of oocyte development, angiogenesis, steroidogenesis, ovulation, and luteolysis in several species (Rosselli et al., 1998; Gregg, 2003, Gao et al., 2009; Wu, 2009; Kim et al., 2011; Zeng et al., 2013). Previous research of Arg-treatment in pregnant females has demonstrated an increase in embryonic survival and litter size in rats (Zeng et al., 2008) and increased the transportation of nutrients and proteins as well as enhanced P4 secretion in sheep (Thureen et al., 2002; Lassala et al., 2010). However, the role of body condition and the effects of Arg-treatment on endocrine and ovarian function of the non-pregnant animal remain to be elucidated.

We hypothesize that Arg-treatment will affect reproductive and metabolic hormones, and selected metabolite serum concentrations in non-pregnant overfed (O) and underfed (U) ewes. To investigate the role that the NO system plays in regulation of endocrine and ovarian function, our objective was to determine if Arg-treatment impacts serum hormones (IGF1, insulin, leptin, and P4), and selected metabolites (arginine, NO metabolites, cholesterol, and glucose) concentration in non-pregnant ewes with different body conditions.

Materials and Methods

All animal procedures performed were approved by the North Dakota State University (NDSU) Institutional Animal Care and Use Committee (#A12013).

Animal Procedures

Non-pregnant, nonlactating Rambouillet ewes (n = 82) between 3-5 years of age and of similar genetic background, were individually penned at the Animal Nutrition and Physiology Center on the NDSU campus. Ewes were stratified by weight and randomly assigned into three dietary treatments: control (C; n = 24) received a maintenance diet, overfed (O; n = 27) received 200% NRC requirements, and underfed (U; n = 31) received 60% NRC requirements 60 days prior to the onset of the estrous cycle (day 0). Table 3.1 shows diet composition and Figure 3.1 presents our experimental design.

Table 3.1: Composition of the diet¹.

Amount as fed in mix	Diet DM %	% DM	Amount As Fed (lbs)	Diet % As Fed
Beet Pulp	36.5	91	40.1	36.2
Alfalfa Meal	22.3	90	24.8	22.3
Corn	18.2	88	20.7	18.7
Soy Hulls	20.0	91	22.0	19.8
SBM	3.0	90	3.3	3.0

¹To provide 2.4 Mcal of metabolizable energy (ME)/kg of diet and contain 14% crude protein (dry matter basis)

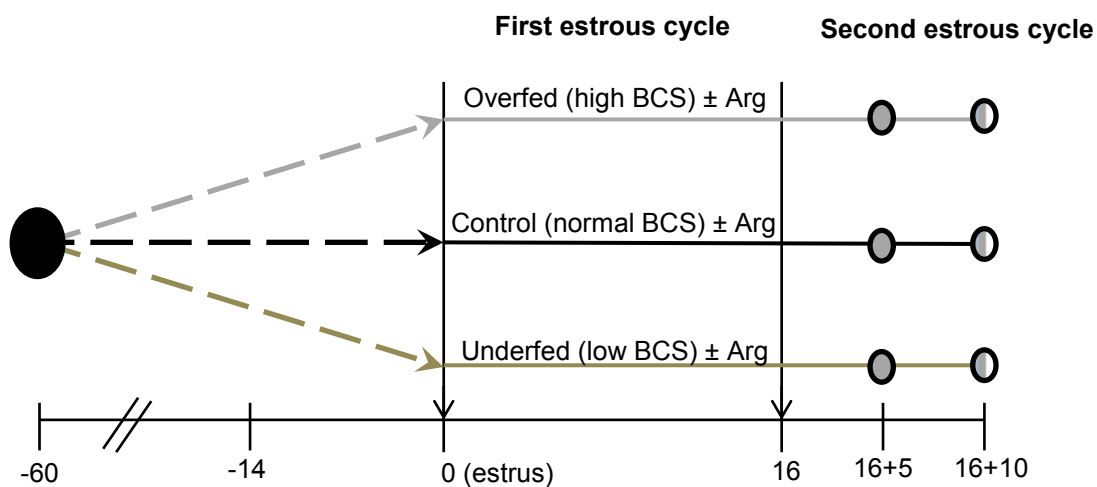


Figure 3.1: Nutritional groups were initiated 60 days prior to the onset of estrus (day 0) to obtain overweight and underweight models. A CIDR was inserted vaginally into each ewe to synchronize estrus 14 days prior to day 0. At estrus, nutritional groups continued to maintain the three different body conditions, and ewes were randomly assigned to either an arginine (Arg) or saline (Sal) treatment that lasted the end of the experiment (either early- or mid-luteal phase of the second estrous cycle or day 21 or 26 of Arg- or Sal-treatment) to obtain early and mid-luteal phase of the estrous cycle. Blood collection was also initiated at day 0 and occurred every other day the end of the experiment.

Ewes were fed their individual diets once daily at 0800 h. For the duration of the experiment, every week, ewes were weighed, and BCS was evaluated on a 5-point scale (1 = extremely thin and 5 = obese) by the same individual. Diets were adjusted weekly for each ewe to ensure the proper BW and BCS was achieved at day 0, and maintained throughout the estrous cycle. Estrus was synchronized by insertion of a controlled internal drug release (CIDR) device for 14 days. Based on previous results (Grazul-Bilska et al., unpublished), approximately 36 h after removal of the CIDR, ewes were considered

in estrus, and treated as day 0 of the estrous cycle. Thus, at day 0, ewes were randomly assigned into Arg- or saline-treatment groups, and blood collection and treatment injections were initiated at 0700 h.

Jugular Vein Cannulation

Jugular vein cannulations were conducted 5-7 days prior to day 0 of the estrous cycle. Wool on the neck of the sheep was removed by shearing, then disinfected with 70% ethyl alcohol and wiped with sterile gauze. Approximately 10 minutes before the cannulation process, a local anesthetic (lidocaine HCl, 2%, sterile, Phoenix Scientific Inc., St. Joseph, MO) was injected subcutaneously into two sites (approximately 1 mL/site) along the jugular vein. The sheep was restrained manually and an incision was made into the neck. After the incision was made, a sterile cannulation needle (12-gauge, Popper & Sons Inc., New Hyde Park, N.Y.) was inserted into the jugular vein, parallel to the trachea near the middle of the neck. Immediately after inserting the needle, a sterile jugular venous cannula (outer diameter [O.D.] 2.16 mm, inner diameter [I.D.] 1.02 mm, Silastic Laboratory Tubing, DOW Corning Corporation, Midland, MI) was inserted through the needle into the jugular vein and the needle was carefully removed. The length of the cannula was 25 cm (20 cm was inserted inside the jugular vein and 5 cm was allowed to remain outside). A sterile syringe filled with sterile saline solution was attached to the cannula and flushed to make sure the cannula was functional. The cannula was then filled with a sterile solution of heparin (sodium salt from porcine intestinal mucosa; Sigma, St. Louis, MO) in saline (300 IU of heparin/mL) and sealed with a sterile stopper. The outside portion of the cannula was attached to the skin via surgical tape, staples, and silicon glue. The skin around the cannula was washed to remove traces of blood using sterile water, wiped with sterile gauze, and washed with 70% ethyl alcohol.

Blood Collection

Blood collection was initiated at day 0 (or ~36 h after CIDR removal) and occurred at 0700 h from day 0 to tissue collection (day 21 [early luteal phase of the second estrous cycle] or 26 [mid luteal phase of the second estrous cycle] of Arg-treatment). Blood was collected via Luer Monovette 9 mL blood collection tubes (Sarstedt, Newton, NC) by removing the stopper and attaching the monovette to the cannula via a sterile blunt needle. After blood was collected, 1 cc of heparin solution was added to the cannula to prevent blood from clotting. Collected blood samples were allowed to clot for 15-20 min at room temperature then centrifuged (Allegra 6R Centrifuge; Beckman Coulter Inc., Indianapolis, IN) at

room temperature for 20 min at 3,500 rpm. Serum was then poured into two 2 mL serum tubes and stored at -20° C until further analysis.

Treatment Injection

Ewes in the Sal-treatment group received ~10 mL of sterile saline solution, while ewes in the Arg-treatment group received a dose of 155 µmol Arg/kg BW (Sigma, St. Louis, MO) as previously described by Lassala et al. (2010) in ~10 mL injections. Arg- or Sal-treatments were initiated on day 0 (~2 days after CIDR removal) of the estrous cycle after blood samples were collected and occurred three times daily (0700, 1400, 2100 h) until the end of experiment. As with blood collection, after injection, heparin solution was placed into the cannula to prevent clotting.

Arginine, Metabolite, and Hormone Analysis

For Arg, NO metabolites, cholesterol, glucose, IGF1, insulin, and leptin, serum samples collected on days 0, 10, 21, and/or 26 of Arg- or Sal-treatment were analyzed. For P4 analysis, serum collected on days 0, 6, 10, 12, and 16 of Arg- or Sal-treatment were analyzed, which corresponded to days 0, 6, 10, 12, and 16 of the first estrous cycle. For each treatment group, samples from 6-8 ewes were analyzed.

Arginine Analysis

Arginine concentration was determined using Ultra Performance Liquid Chromatography (UPLC) for serum collected on days 0, 10, and day 26 of Arg-treatment as previously described by Lemley et al. (2013).

Nitric Oxide Metabolite Analysis

For serum deproteinization, centrifugal filters (10K pore, 500 µL, VWR, Chicago, IL) for deproteinization were pre-rinsed with 0.5 mL of MilliQ water and spun in a centrifuge for 10 min at 10,000 rpm. Filters were then rid of all water and 400 µL of serum was added and centrifuged for 40 min at 10,000 rpm. The serum samples were prepared as follows: 1) 0.1 M Nitrite Standard (Griess Reagent Assay Kit, Promega, Madison, WI) was diluted 1:1000 in dH₂O to prepare 1 mL of 100 µM nitrite solution; 2) a 6 serial two-fold dilution (50 µL/well) was performed to generate the nitrite standard reference curve (100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0 µM); 3) 100 µL of deproteinized serum was added to the wells in duplicate in 96-well plate; 4) 100 µL vanadium (III) chloride was added to the wells containing serum, and mixed by pipetting; and 5) to each well, 50 µL sulfanilimide and 50 µL of NED (Griess Reagent Assay Kit,

Promega) were added and mixed by pipetting. The plate was then incubated at 60°C for 20 min, and read at an absorbance of 540 nm (Epoch Microplate Spectrophotometer, BioTek). The intra-assay CV was 3.65%.

Cholesterol and Glucose Analysis

The Infinity Cholesterol Reagent (TR 13421, Thermo Electron Corporation, Pittsburgh, PA) and the Infinity Glucose Reagent (TR 15221, Thermo Electron Corporation) were used to determine serum cholesterol and glucose concentrations, respectively. For cholesterol and glucose, samples were run in duplicate in a 96-well plate.

For cholesterol, in each well, 297 μ L of the cholesterol reagent was pipetted and 3 μ L of sample was added to each well and mixed, except two wells for the cholesterol standard. Next, plates were incubated for 5 min at 37°C and absorbance was measured at 500 nm (Epoch Microplate Spectrophotometer, BioTek, Winooski, VT). Cholesterol concentrations (mg/dL) were calculated by taking the average absorbance for each cell and dividing it by the average absorbance for the standard then multiplying by 200 mg/dL (stock reagent). The intra-assay CV was 4.86%.

For glucose, 298 μ L of glucose reagent was pipetted into each well. Next 2 μ L of serum was added to each well, except for the two wells for the standard, and mixed. Plates were then incubated for 10 min at 37°C and absorbance was measured at 500 nm (Epoch Microplate Spectrophotometer, BioTek). Glucose concentrations (mg/dL) were calculated by taking the average absorbance reading for each sample and dividing by the average absorbance for the standard of that plate and multiplied by 300.5 mg/dL (stock reagent). The intra-assay CV was 6.69%.

IGF1, Insulin, and Leptin Analysis

Serum concentrations of IGF1 were determined using a competitive, liquid-liquid phase, double-antibody IGF-1 radioimmunoassay procedure as described previously by Lalman et al., (2000). The intra-assay CV was 6.27%.

Insulin concentrations were determined using chemiluminescence immunoassay using the Immulite 100, utilizing components of commercial kits (Diagnostic Products Corp., Los Angeles, CA; Vonnahme et al., 2013). The intra-assay CV was 13.2%.

Leptin serum concentrations were quantified using a competitive, liquid-liquid phase, double-antibody leptin radioimmunoassay procedure described previously (Delavaud et al., 2000) with one modification whereby the primary antiserum reported by Delavaud et al., (2000) was substituted with rabbit anti-ovine leptin primary antiserum # 7105. The intra-assay CV was 5.9%.

Progesterone Analysis

Progesterone concentrations were determined using the chemiluminescence immunoassay (Immulite 1000, Siemens), as described by Galbreath et al. 2008. The intra-assay CV was 11.7%.

Statistical Analysis

Statistical analysis was performed using the MIXED procedures of SAS 9.2 (SAS Institute Inc., Cary, NC) for BW, BCS, and P4. The model included diet, ARG-treatment, week, and their interactions. Statistical analysis for arginine, cholesterol, glucose, IGF1, insulin, leptin, P4 area under the curve, and NO metabolites were analyzed using the GLM procedures of SAS 9.2 (Cary, NC). The model included diet, ARG-treatment, day, and their interactions. Means were separated using the LSMeans option of SAS and were considered significant when $P \leq 0.05$.

Results

Body Weight and Body Condition Score

At the time when dietary treatment was initiated, BW was similar for all ewes (61.8 ± 1.2 kg). When compared to the initial BW, O ewes gained 5.7 ± 0.7 kg, U ewes lost 14.7 ± 0.1 kg, and C ewes maintained their BW during the 11-wk experiment. During their estrous cycles (weeks 8 through 10), O ewes were significantly heavier ($P = 0.02$) than both the C and U ewes, and C ewes were significantly ($P = 0.02$) heavier than the U ewes (Figure 3.2).

At the beginning of dietary treatment, BCS was similar for all ewes (2.74 ± 0.06). When compared to the initial BCS, BCS increased by 1.10 ± 0.02 for the O ewes, decreased by 0.51 ± 0.10 for the U ewes, and was maintained for the C ewes during the 11-wk treatment. During their estrous cycles (weeks 8 through 10), O ewes had greater ($P < 0.01$) body condition than both the C and U ewes, and C ewes had greater ($P < 0.01$) body condition than the U ewes (Figure 3.2).

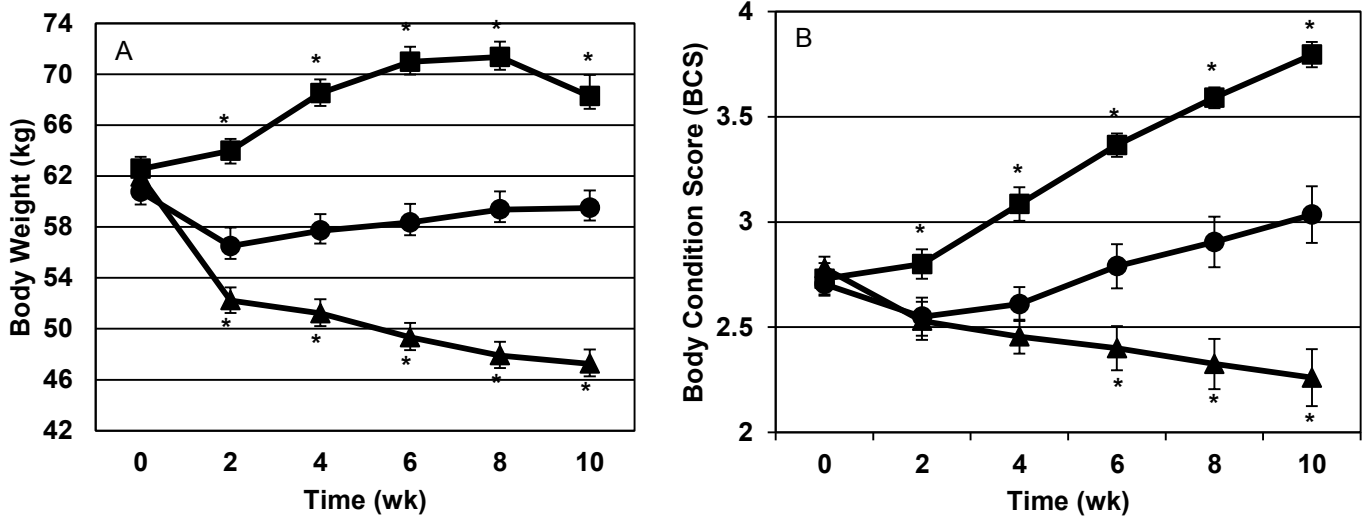


Figure 3.2: Body weight (BW; A) and body condition score (BCS; B) in control (circle), overfed (square), and underfed (triangle) ewes during a 10-wk period. A diet effect ($P < 0.01$) and diet by week interaction was detected ($P < 0.01$) for both BW and BCS. By the time ewes reached estrus (week 8), three different body conditions were achieved: control, over- and under-condition. *Means \pm SEM differ from control at specific point in time ($P < 0.01$).

Arginine, Metabolite, and Hormone Concentrations

Serum Arginine

Overall, serum Arg concentrations were affected by diet ($P < 0.01$) but not Arg- or Sal-treatment ($P = 0.1$; Fig. 3.3). In the U group, Arg concentrations were greater in Arg-treated ewes on day 10 than on day 0 (just before Arg or Sal injections) and on day 26 in Sal-treated ewes, and values were similar to Sal-treated ewes on day 10 and 26 of Arg-treatment (Figure 3.3).

Serum Nitric Oxide Metabolites

Serum concentrations of NO metabolites were unaffected by diet ($P > 0.1$), Arg-treatment ($P > 0.1$), or day of Arg- or Sal-treatment ($P > 0.1$).

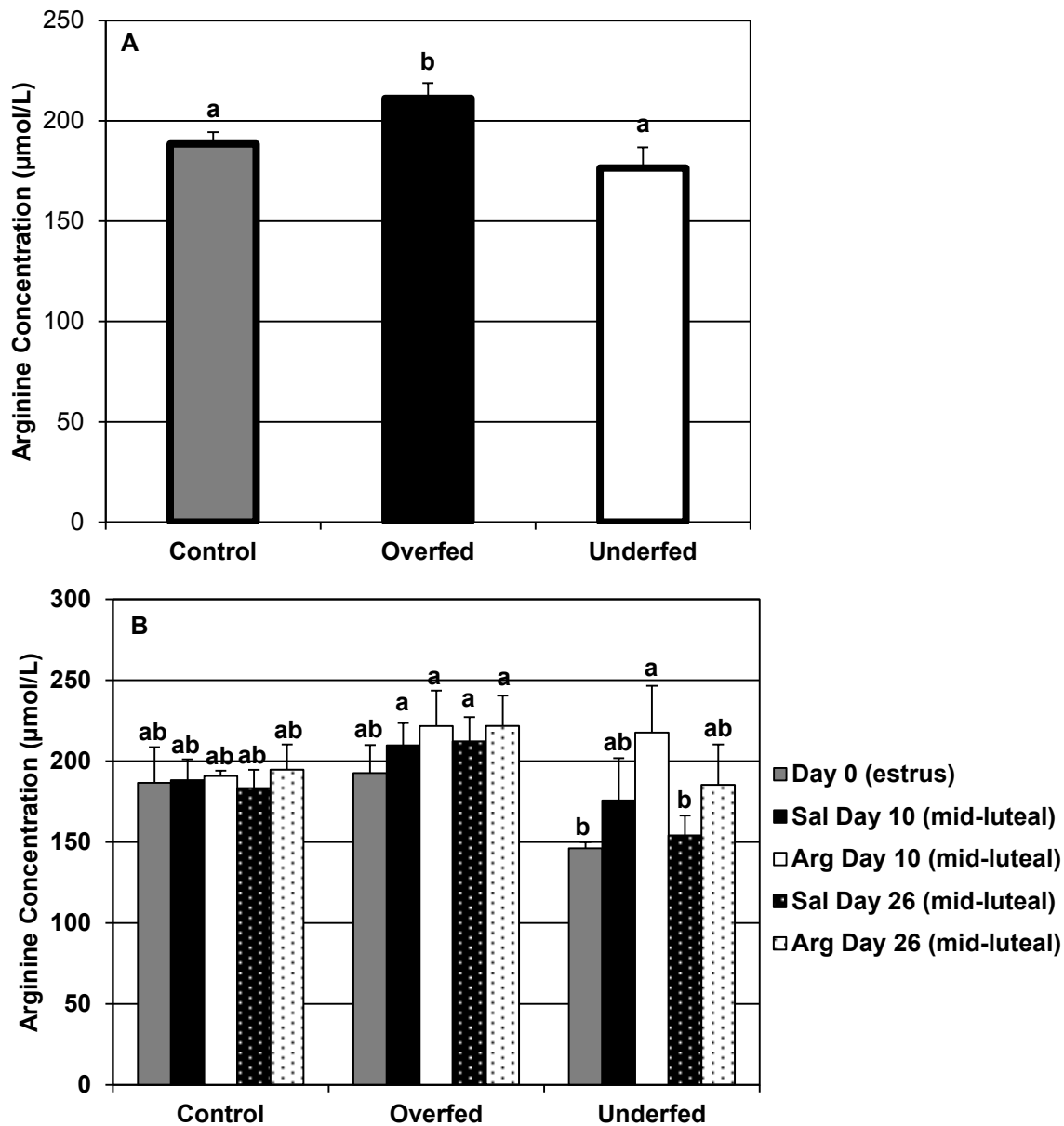


Figure 3.3: (A) Arginine serum concentrations ($\mu\text{mol/L}$) in control, overfed, and underfed ewes. (B) Arginine serum concentration ($\mu\text{mol/L}$) in control, overfed, and underfed ewes treated with arginine (Arg) or saline (Sal) on first day of expected estrus (day 0; before Arg-treatment initiation), day 10 (mid-luteal phase of the first estrous cycle), and day 26 (mid-luteal phase of the second estrous cycle) of Arg-treatment. ($^{a,b}P < 0.01$, means \pm SEM with different superscripts differ.

Serum Cholesterol and Glucose

Serum concentrations for cholesterol were unaffected by diet ($P = 0.1$) with the average concentrations in C, O, and U groups were 44.4 ± 2.1 mg/dL, 52.4 ± 2.4 mg/dL, 53.5 ± 2.5 mg/dL, respectively. Glucose concentrations were greater ($P < 0.01$) in O than U ewes, with C ewes being intermediate (Figure 3.4). Both serum concentrations of cholesterol and glucose were unaffected by Arg-treatment, or day of Arg- or Sal-treatment ($P > 0.1$).

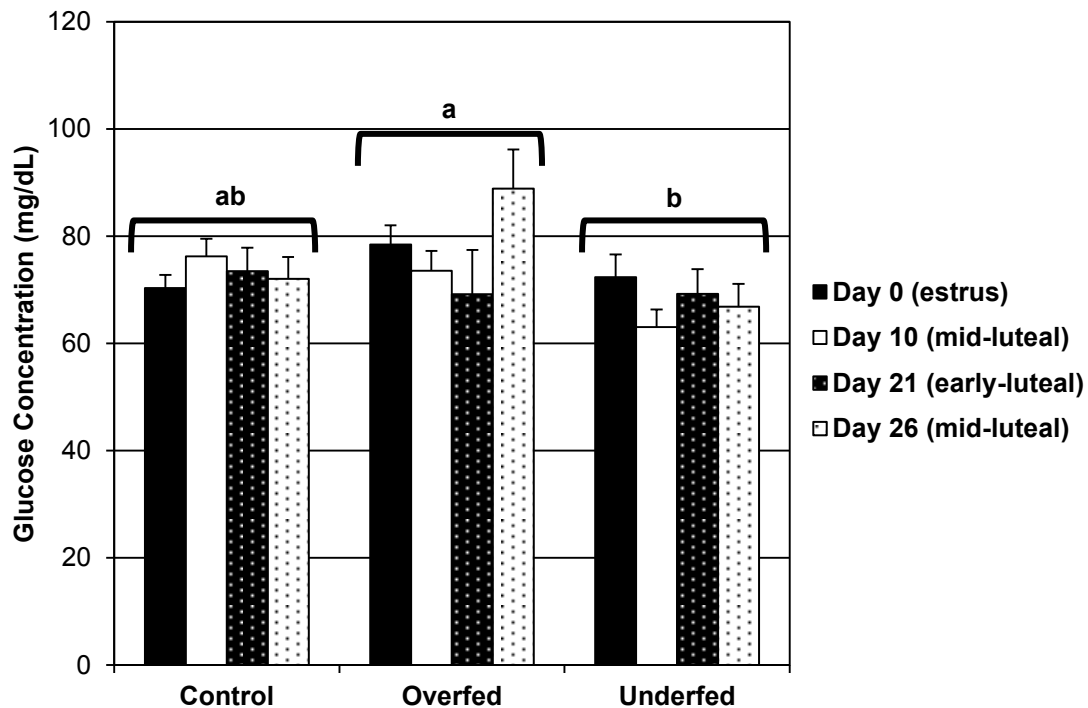


Figure 3.4: Glucose serum concentrations (mg/dL) collected on days 0, 10, 21, and 26 of Arg-treatment. A diet effect was observed with overfed (O) ewes having greater concentrations of glucose in serum than underfed (U) ewes, with control (C) ewes being intermediate. ^{a,b} $P < 0.01$, means \pm SEM with different superscripts differ.

Serum IGF1, Insulin, and Leptin

Serum concentrations of IGF1 were 1.3- and 1.4-fold greater ($P < 0.01$) in both the C and O ewes compared to the U ewes, respectively (Figure 3.5). IGF1 was unaffected by Arg-treatment or day of Arg- or Sal-treatment ($P > 0.1$).

Serum concentrations of insulin were affected by dietary treatment ($P < 0.01$), but not Arg-treatment or day of Arg- or Sal-treatment ($P > 0.1$). Insulin concentrations in O ewes were 1.5- and 3.3-

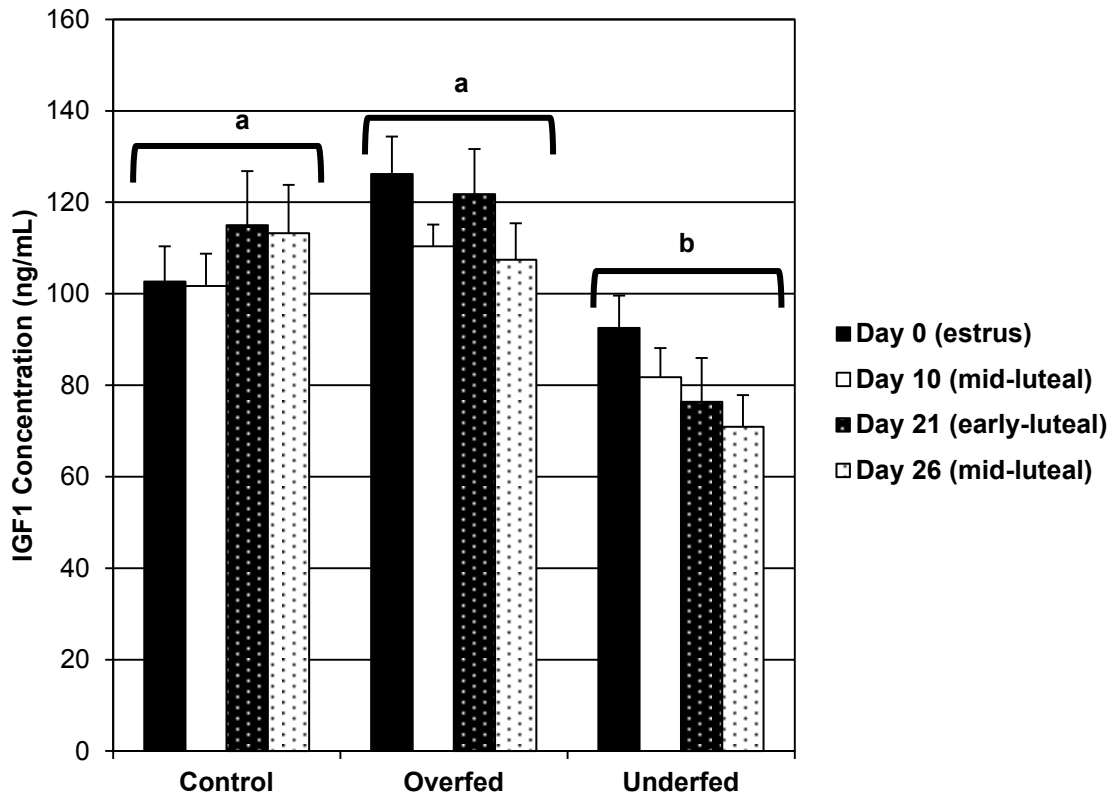


Figure 3.5: IGF1 serum concentrations in control, overfed, and underfed ewes on days 0 (first day of expected estrus; blood collection before treatment initiation), day 10, day 21, and day 26. A diet effect ($P < 0.01$) was observed with overfed and control ewes having greater concentrations of IGF1 than underfed ewes. ^{a,b} $P < 0.01$, means \pm SEM with different superscripts differ.

fold greater than C and U ewes, respectively, and concentrations in C ewes were 2-fold greater than in U ewes (Figure 3.6).

Concentrations of leptin were affected by diet, day of Arg- or Sal-treatment, and a diet by day interactions were detected ($P < 0.01$). Serum concentrations of leptin were 2.3- and 4.2-fold greater ($P < 0.01$) in O than C and U ewes, respectively, and was 1.8-fold greater in C than U ewes. In O, but not C or U groups, during the first estrous cycle, concentrations of leptin were less ($P < 0.01$) in mid-luteal phase than at estrus, and during the second luteal phase, leptin concentrations were less ($P < 0.01$) in mid- than early-luteal phase (Fig. 3.7).

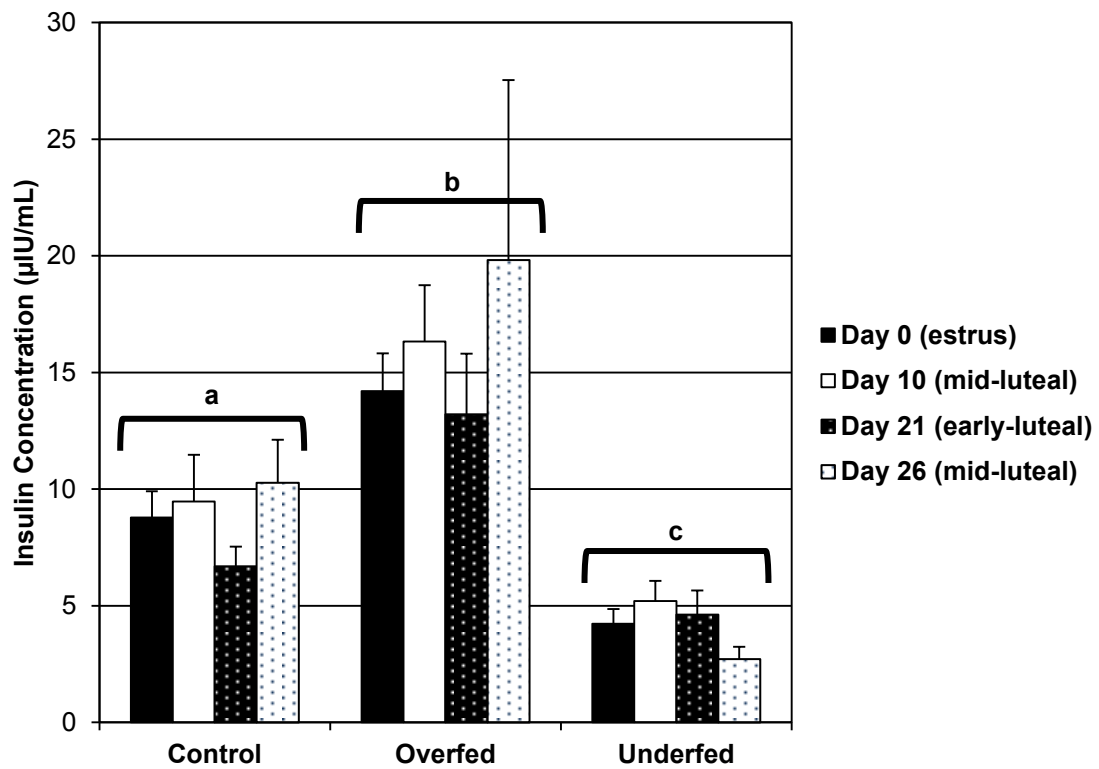


Figure 3.6: Insulin serum concentrations in control, overfed, and underfed ewes on days 0 (first day of expected estrus; blood collection before treatment initiation), day 10, day 21, and day 26. A diet effect ($P < 0.01$) was observed with overfed and control ewes having greater concentrations of insulin than underfed ewes. ^{a,b,c} $P < 0.01$, means \pm SEM with different superscripts differ.

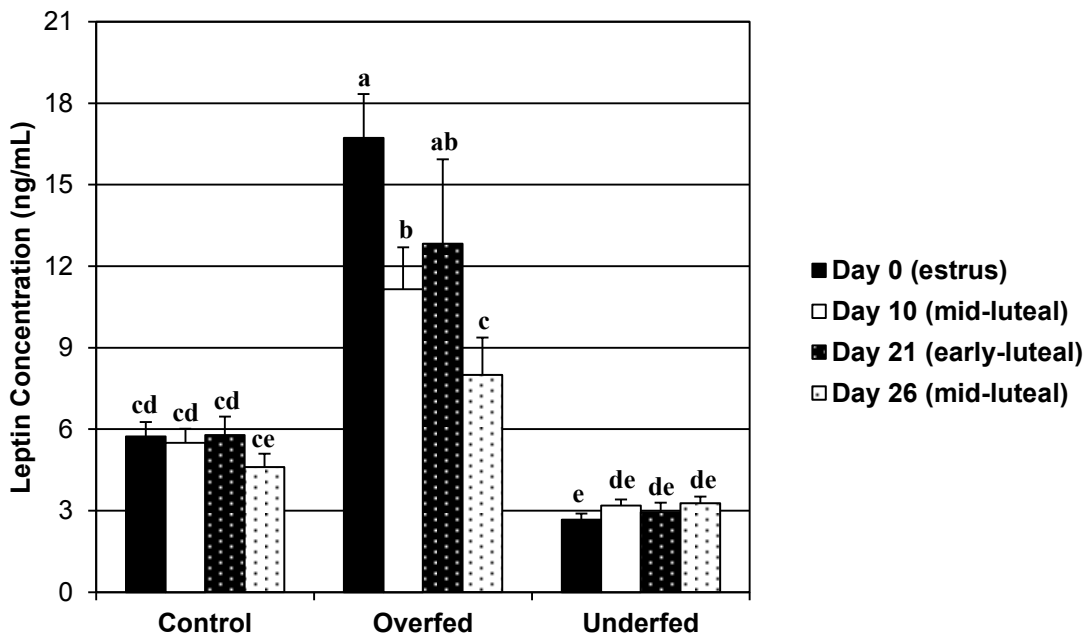


Figure 3.7: Leptin serum concentrations (ng/ml) in control, overfed, and underfed ewes on days 0 (first day of expected estrus; blood collection before treatment initiation), day 10, day 21, and day 26. There was a diet effect ($P < 0.01$) with overfed ewes having greater leptin concentrations than control ewes, and control ewes having greater concentrations than underfed ewes. There was also a day effect ($P = 0.01$) and a diet by day interaction ($P < 0.01$). ^{a,b,c,d,e} $P < 0.01$, means \pm SEM with different superscripts differ.

Serum Progesterone

Serum concentrations of P4 during the first estrous cycle were affected by diet ($P < 0.05$), and day of Arg- or Sal-treatment (which corresponded to a day of the estrous cycle; $P < 0.01$; Fig. 3.8).

Progesterone concentrations were greater in O compared to the C and U groups, and concentrations increased from day 0 to days 6 and 12, and then decreased at day 16 of the estrous cycle (Figure 3.8A).

Serum concentrations of P4, measured as area under the curve (AUC), was affected by diet ($P < 0.05$) in O than C or U groups (Figure 3.8B).

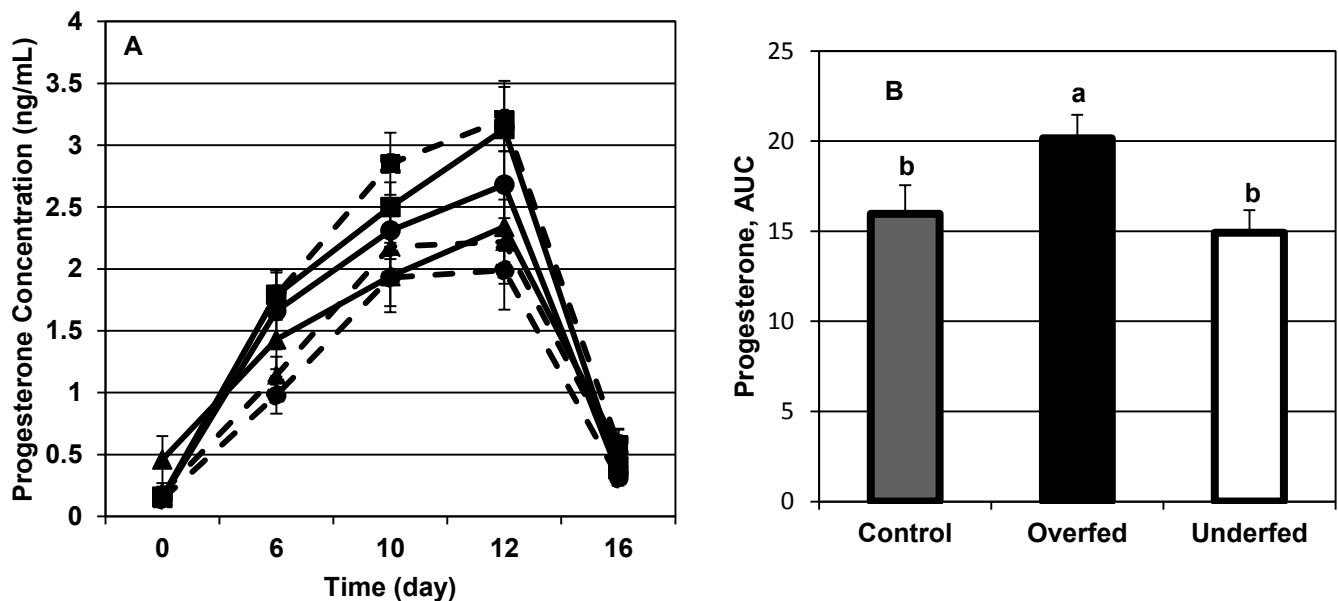


Figure 3.8: (A) Progesterone (P4) concentrations (ng/mL) in control (circle), overfed (square), underfed (triangle) ewes receiving saline (solid line) or arginine (dashed line) during their estrous cycle (day 0 to day 16). There was a day effect ($P < 0.01$) with P4 concentrations peaking at day 12 of the estrous cycle for each dietary group. (B) P4 measured as area under the curve (AUC). There was a diet effect ($P < 0.03$) with overfed ewes having greater P4 concentrations throughout their estrous cycle than both the control and underfed ewes. ^{a,b} $P < 0.03$, means \pm SEM with different superscripts differ.

Discussion

Prolonged overfeeding and underfeeding of non-pregnant ewes over a 10-11-wk period resulted in altered BW and BCS, as expected, and as reported by us before (Borowczyk et al., 2006, Grazul-Bilska et al., 2012). Furthermore, nutritional treatment altered arginine, glucose, IGF1, insulin, leptin, and P4 concentration in peripheral blood. In most domestic animals, such as cattle, pigs, and sheep, reproductive function is dramatically influenced by plane of nutrition (Dunn and Kaltenbach, 1980; Randel, 1990; Brow, 1994; Armstrong and Benoit, 1996). Many animal models have previously demonstrated that over- or undernutrition can alter BW and BCS (Grazul-Bilska et al., 2012), affect ovulation rates (Hunter et al., 2004), alter mRNA expression of selected genes in reproductive tissues (Da Silva et al., 2002; Wrenzycki et al., 2000), impact oocyte quality (Borowczyk et al., 2006; Grazul-Bilska et al., 2012; Adamiak et al., 2005; Foulandi-Nashta et al., 2007), and impact serum and follicular hormone concentrations (Boland et al., 2001; McEvoy et al., 1995; Grazul-Bilska et al., 2012).

In order to reverse some of these possible alterations to reproductive function in compromised conditions, many researchers have been interested in supplementing arginine to the female animal (Li et al., 2010; Lassala et al., 2010; Luther et al., 2009; Mateo et al., 2007). In the present study, serum concentrations of Arg were not affected by Arg-treatment in any nutrition group. It has been previously demonstrated in pregnant ewes that Arg concentrations after Arg injection increased during the first few hours and reached baseline within 6-8 hours after injection (Luther et al., 2009). Therefore, it is likely that Arg concentrations in our study were at baseline levels at the time of collection (0700 h and just before Arg injection), hence why we did not observe any effects of Arg on serum concentrations of Arg. However, Arg-treatment to U ewes from mid-gestation to parturition increased the concentration of Arg in serum compared to U ewes that did not receive Arg-treatment (Lassala et al., 2010). In our study, Arg concentrations of U Arg-treated ewes were greater in O than C, which were similar. Greater Arg concentrations in O ewes is likely due to a greater intake of feed, which is a source of arginine. However, in other studies, serum Arg concentrations were decreased or similar in ewes that were restricted or overfed prior to and during gestation compared to control ewes (Kwon et al., 2004; Satterfield et al., 2012). These differences are likely due to a different duration or length of Arg-treatment, reproductive stage, and/or breed.

Nitric oxide (NO) is a highly reactive free radical gas that is synthesized via oxidation of L-arginine and has been implicated in a wide variety of physiological and pathological roles, including reproductive function (Masuda et al., 2001). One of the functions of NO in the reproductive system is vasodilation, which increases blood flow to the organ. This increase in blood flow could potentially increase the transportation of metabolites and hormones around the body. However, results from our study indicate that both diet, a source of exogenous arginine, and Arg-treatment do not have any effects on NO concentrations in circulation. These results are interesting due to the fact that NO is synthesized from arginine. In contrast, it has been previously demonstrated that feeding a 1% Arg diet to young rats increased plasma Arg concentrations and *in vivo* constitutive NO production by 105% and 73% respectively compared with rats fed an Arg-free diet (Omura et al., 2001). It was also demonstrated in the same study that feeding an Arg- or protein-deficient diet to the young rats reduced plasma Arg concentrations and prevented maximal inducible NO synthesis (Wu and Meininger, 2002), which would reduce the amount of NO produced. However, there is limited knowledge on the effects of Arg-treatment on the NO system in ruminant species. The lack of changes in NO metabolite concentrations in our study could be due to the fact that NO metabolites undergo high turnover in the blood, and NO being a free radical molecule has a very short half-life of <5 s (Tjalkens et al., 2011).

Cholesterol is an important metabolite in reproductive function since it is the precursor of sex steroids, such as P4 and E2, in mammals (Rabiee and Lean, 2000). Changes in the quantity of cholesterol present in circulation, due to changes in the diet, could play a major role in regulating steroid biosynthesis of the ovary (Henderson et al., 1981) and affect serum and follicular P4 concentrations (Hawkins et al., 1995; Lammoglia et al., 1996; Talavera et al., 1985). It has been previously shown when 15% sunflower seed was added to their diet, total cholesterol concentrations in serum of Holstein heifers increased by 70% compared to heifers that did not receive sunflower seeds (Talavera et al., 1985). This helps reinforce that changing the composition of the diet can impact cholesterol concentrations in circulation. In our study, serum cholesterol was similar in all groups indicating that diet or Arg-treatment did not affect peripheral cholesterol concentrations. Ying et al. (2013) observed that feed-restricted ewes had greater cholesterol concentrations than in C or O groups. Thus, the ovaries might receive sufficient cholesterol substrates during the restriction period for the biosynthesis of steroid hormones (Ying et al.,

2013). The different results obtained in our and Ying et al. (2013) studies are likely due to different time and length of diet restrictions and/or breed of sheep.

Glucose, another metabolite involved in the regulation of reproductive function, is essential for the quality of oocytes (Rato et al., 2012; Sutton-McDowall et al., 2010), development of embryos, and can play many roles in the hypothalamic-pituitary-gonadal axis (Dupont et al., 2013). Previous studies have demonstrated that providing restricted diets to heifers (Freret et al., 2006) and sheep (Ying et al., 2011; Lassala et al., 2010; Kiani, 2013) reduced the concentrations of glucose in circulation. In a study by Ying et al. (2011), the mean blood concentration of glucose for the restricted ewes was lower than that of the control and overfed non-pregnant ewes. Similarly, our results also demonstrated reduced glucose concentrations in serum of U compared to both C and O ewes. Therefore, the present data indicate that plane of nutrition affects concentrations of glucose but not cholesterol in peripheral blood.

Metabolic and endocrine changes associated with nutrient shortage play signaling roles that can prevent female livestock species from establishing pregnancy when conditions are suboptimal via inhibitory actions at the level of the brain, ovary, or reproductive tract. The metabolic hormone, insulin, has an influence on nutrient partitioning (Wathes, 2012), and it is involved in the regulation of several reproductive functions in livestock species including granulosa and thecal cell proliferation and steroidogenesis (Gutierrez et al., 1997; Stewart et al., 1995), ovulation rates (Harrison and Randel, 1986; Downing et al., 1999), and influences the release of LH from the pituitary gland (Monget and Martin, 1997). Since insulin is influenced by dietary intake, overfeeding and underfeeding can drastically impact insulin concentrations in circulation. Pruiner and Quesnel (2000) demonstrated a decrease in the responsiveness of the ovary as a result of reduced insulin concentrations due to diet restriction in non-pregnant pigs. On the other hand, overfeeding increased peripheral concentrations of insulin in pigs (Ferguson et al., 2003). Similarly, in overfed and underfed non-pregnant ewes fed 130% and 70% of their individual energy and crude protein requirements, respectively for 60 day, insulin levels in plasma were elevated in overfed compared to the underfed ewes (Tsiplakou et al., 2012). These results are consistent with those observed in our study demonstrating serum insulin to be enhanced in O, and reduced in U animals. Thus, plane of nutrition has a direct effect on circulating insulin levels.

Another metabolic hormone that works in conjunction with insulin is IGF1 (Clemmons, 2004). IGF1 plays an essential role in mammalian reproduction, as shown by the impaired ovarian activity and embryo development in gene knockout mouse models (Liu et al., 1993; Baker et al., 1996; Zhou et al., 1997; Kadakia et al., 2001). Similar to insulin, IGF1 has been demonstrated to stimulate granulosa cell proliferation and steroidogenesis (Silva and Price, 2002; Spicer and Fanciso, 1997), affect oocyte quality (McCaffery et al., 2000), and suppress apoptosis in follicles (Monget and Martin, 1997; Poretsky et al., 1999; Scaramuzzi et al., 1999; Williams et al., 2001). Plane of nutrition directly relates to IGF1 concentrations in circulation. In overfed gilts, higher IGF1 circulating concentrations were observed (Ferguson et al., 2003), whereas underfeeding resulted in reduced concentrations of IGF1 in plasma (Pruiner and Quesnel, 2000). In sheep, ewes with a low body condition (2.0) had reduced IGF1 concentration in plasma, and ewes with a high body condition (4.0) had increased concentrations of IGF1 (Caldeira et al., 2007). Results from our study demonstrate reduced levels of IGF1 in circulation in U animals, and similar concentrations in C and O ewes. This was in agreement to those results by Viñoles et al. (2005). Therefore, restricted diets can cause a decrease in IGF1 production and/or turnover.

Leptin, a product primarily of white adipose tissue (Wathes, 2012), plays a critical role in linking metabolic state with fertility, such that as body fat percentage and leptin levels increase due to overfeeding, fertility becomes impaired (Brannian and Hansen, 2002). The importance of leptin for fertility and normal reproductive function was clearly demonstrated using female mice phenotypically displaying obesity (*ob/ob*). These *ob/ob* female mice were determined infertile; however, when treated with exogenous leptin, the reproductive system was rejuvenated, which led to growth and development of the reproductive organs and these mice became fertile (Chegab et al., 1996; Barash et al., 1996; Malik et al., 2001). Results from our study demonstrated that U ewes had approximately 2-fold less leptin in serum whereas the O had 2.5-fold more leptin than C ewes. These results are consistent with other studies in cattle (Delavaud et al., 2002; León et al., 2004), women (Bützow et al., 1999), and ewes (Satterfield et al., 2012). We also observed that leptin concentrations were less in mid-luteal phase than at estrus or early-luteal phase of the estrous cycle in overfed ewes, indicating association of leptin with reproductive function. Therefore, leptin concentrations are directly related to metabolic state of the female; however, the effects of leptin concentrations in relation to stage of the estrous cycle remain to be elucidated.

As previously mentioned, the reproductive hormone that is essential for the establishment and maintenance of pregnancy is P4. In the non-pregnant female, peripheral P4 concentrations decline as a result of luteolysis, and reach baseline levels (< 1 mg/dL) at the onset of estrous (day 0 of the estrous cycle). After the ovulation, P4 concentrations begin to rise until the CL undergoes luteolysis again (Senger, 2003). During the estrous cycle, peripheral P4 concentrations have been previously proven to be effected by dietary intake of the female (Chesworth and Easdon, 1983; Ferguson et al., 2003; Williams and Cumming, 1982; McEvoy et al., 1995). In the non-pregnant ewe, it has been reported that the peak levels of P4 were encountered at days 11 and 12 of the estrous cycle; however, reduced concentrations were observed with ewes fed an O diet, or twice that of the C ewes (Lamond et al., 1972; Chesworth and Easdon, 1983). These results conflict with our study where non-pregnant Oewes had greater P4 concentrations than both the C and U ewes, which had the least concentration in circulation. These differences are likely due to during of dietary restriction or excess, as well as breed of sheep used.

Conclusion

In conclusion, these data demonstrate that diet rather than Arg-treatment have effects on serum concentrations of arginine, glucose, IGF1, insulin, leptin, and P4 but not NO metabolites or cholesterol. Thus, Arg supplementation may not improve compromised reproductive function due to body condition of the non-pregnant animal. However, studies looking at the local effects of Arg-supplementation on follicular development, luteal function, and oocyte quality are warranted. Although there were observed changes in peripheral blood for several metabolites and hormones, which are involved in the regulation of reproductive function, it is unclear if these changes may affect fertility and offspring outcome. Therefore, further study should be undertaken to study dietary effects on fertility, as well as fetal and offspring development.

Acknowledgements

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CHAPTER 4: GENERAL CONCLUSION AND FUTURE DIRECTIONS

Ovarian function is an important aspect in reproductive performance in both pregnant and non-pregnant females. Many environmental factors, such as physical activity and nutritional intake, can influence ovarian functions including cellular proliferation, oocyte growth and development, blood flow, hormone production, and fertility. Physical activity during gestation was demonstrated to have an effect on ovarian function measured by cell proliferation or ovarian weight at the fetal and neonatal, but not at adolescent stages of development. Exercise enhanced cell proliferation in fetal ovaries and ovarian weight in L neonates; cell proliferation was greater in fetal than neonatal ovaries; granulosa cell proliferation increased during growth from secondary to antral follicle; LI was very low in AA follicles; and LI was greater in granulosa cells than in thecal cells. While exercise of sows increased LI in fetal ovaries, ovarian LI was similar at birth in EX compared to CON sows. Therefore, these data indicate that maternal exercise benefits offspring reproductive systems; however, the effect of maternal physical activity on the fertility of offspring remains to be elucidated. Future studies to answer how exercise during gestation affects offspring fertility would be to allow the mother to exercise from initiation of pregnancy, and to be able to follow the offspring to their reproductive ages.

In the non-pregnant ewe, feeding different planes of nutrition (i.e. overfeeding or underfeeding) for a prolonged period of time resulted in different body condition at the time of synchronized estrus. Previous studies have indicated that feeding different nutritional treatments can alter reproductive function; however, supplementing the diet with arginine was shown to compensate for compromised reproductive function in pregnant ewes. Our study demonstrated that overfeeding and underfeeding of non-pregnant ewes altered both BW and BCS. Overfeeding increased serum glucose, IGF1, insulin, leptin, and P4 concentrations in general circulation, but the dose and duration of Arg supplementation used did not have any effects on circulating hormone and metabolite concentrations. Therefore, it seems that in the non-pregnant female, Arg-treatment does not influence secretion of metabolites or metabolic and reproductive hormones. However, more research is warranted to determine if arginine influences oocyte quality, vascularity and growth of the ovarian follicles and CL, and the expression of P4 receptors in ovarian and uterine tissues, and to determine if elevated progesterone concentrations may affect fertility.