EFFECTS OF ANDROGENS ON REPRODUCTION IN FEMALE PIGS

DISSERTATION

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By

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Various investigations were performed to examine how androgens might influence reproduction in gilts. The first investigation was designed to begin to address the endocrinology of how androgens increase ovulation rate in gilts when administered during the follicular phase. Gilts were injected with androgen receptor agonists (5α-dihydrotestosterone, DHT or testosterone), antagonist (flutamide) or a combination of both from day 13 of the estrous cycle (day 0= onset of estrus) until the onset of estrus. Hourly blood samples were collected when treatments were initiated until the onset of estrus. Testosterone or DHT treatments increased (P<.05) mean concentrations of FSH in serum and this effect was blocked by co-treatment with androgens and flutamide (an androgen receptor antagonist). This augmentation of FSH secretion occurred without altering the rate of luteolysis (as determined by progesterone concentrations) or induction of consistent changes on luteinizing hormone. Estradiol-17ß and androstenedione concentrations augmented (P<.05) after 2 hours of injections of testosterone even when flutamide was present. Treating gilts with androgens decreased embryonic survival and co-treating gilts with androgens and flutamide reversed these effects. Results of this experiment indicate that androgen actions, mediated by the androgen receptor, increased FSH concentration and this increase might be related, in part, to the previously observed increase in ovulation rate.
The second investigation comprised two experiments and was conducted to examine the effects of androgens on neonatal development of the uterus. A total of sixty gilts were utilized. In Experiment 1, twenty four newborn gilts were assigned randomly to increasing dosages of DHT (0, 6, 60 or 600 µg per kg of body weight) from birth (postnatal day, PND 0) to PND 13. Treating gilts with increasing dosages of DHT did not alter any of the components of uterine development examined on PND 14 (wet weights of the uterus, liver, heart or longissimus dorsi, endometrial thickness, height of luminal epithelium, glandular penetration nor myometrial thickness). In Experiment 2, thirty six gilts were utilized to investigate up to 14 days of age the effects of androgens on estrogenic-induced stimulation of uterine development. Gilts were randomly assigned at birth to receive daily injections of vehicle, 50 µg of estradiol valerate, 60 µg of DHT, 10 mg of flutamide, estradiol valerate plus DHT or estradiol valerate plus DHT plus flutamide, per kg of body weight, from PND 0 to PND 13. Treatment of neonatal gilts with estradiol valerate increased uterine wet weight, endometrial thickness, glandular penetration and myometrial thickness but only the estradiol valerate-induced effects on myometrial thickness were blocked by co-treatment with DHT. Results of these experiments indicate that within the first two weeks of age, androgen treatment failed to influence most of the estrogenic-induced stimulatory effect as has been observed in the adult uterus.
To my wife Yadira Malavez

To my parents Nemesio Jiménez and Carmen Cabán

To my sisters Yajaira and Carmencita Jiménez

To my niece Kiana M. Pérez
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CHAPTER 1

INTRODUCTION

Litter size (number of piglets born per farrowing) is a major economic trait in the swine industry. It is obvious that obtaining a greater number of pigs born alive from a single farrowing would increase production efficiency. Subsequently, production efficiency reduces fixed costs for the swine producers such as labor, facilities and equipment.

Ovarian and uterine function can influence litter size. In turn, ovarian and uterine function(s) are highly controlled by hormones produced by these same organs and by hypothalamic and pituitary hormones. Androgens, which historically were regarded as male hormones, can influence ovarian and uterine function. For example, androgen treatment during the follicular phase increases ovulation rate (number of follicles that ovulate) in adult gilts. Although advantages are obtained in ovulation rate after treatment with androgens at relatively high dosages, many of the resulting embryos are lost after 3 days of pregnancy, indicative of androgenic disruption of uterine function(s). As a consequence litter size would be reduced and not augmented by high doses of androgen. However, litter size can be increased by one pig per litter with low doses of testosterone. Therefore, to utilize the advantages in ovulation rate with higher dosage of androgens it is
important to investigate the mechanisms involved in those androgen-stimulated actions on ovaries and the uterus.

The porcine uterus, which is undifferentiated at birth, undergoes rapid development early during postnatal life. Theoretically, by promoting uterine development, specifically uterine gland invasion and branching, these effects could be beneficial to supporting an increase in litter size. Treatment with hormones such as estrogens and relaxin are known to alter uterine development at these early stages. The androgen receptor was recently detected in uteri of newborn gilts but the implications of these receptors in the development of the neonatal uterus have not been investigated to date.

This dissertation will approach the effects of androgens in neonatal and adult gilts. For this thesis, knowledge will be expanded about how exogenous androgens affect ovulation rate and uterine function in adult gilts. In addition, for the first time in pigs, this dissertation provides insight about the effects of androgens in neonatal development of the porcine uterus.
CHAPTER 2

REVIEW OF THE LITERATURE

The reproductive system consists of various organs such as the hypothalamus, pituitary, ovaries and uterus. Communication between these organs is important for the function and control of the reproductive system. The communication between these organs is through endocrine secretions referred as hormones. Hormones are substances released from one tissue; travel through the blood to its target, another tissue. The next section will address some of the hormones implicated in female reproduction. Following that discussion, how hormones affect the development and function of the uterus will be reviewed. As this dissertation will investigate how hormones affect the reproductive system of female pigs, a discussion of the factors affecting reproductive performance, specially litter size, will be presented. Finally, with an understanding of the reproductive system in female pigs, and its economic relevance established, this review will describe the effects of exogenous androgens in females. The potential effects of androgens on female pigs are the focus of my dissertation.

2.1 Endocrine control of female reproduction

All the organs mentioned above synthesize and secrete different hormones. Hormones have different structures and mechanisms of action. Structurally, hormones
consist of four groups of chemicals; amines, polypeptides, steroids and eicosanoids. Functionally, amines, polypeptides and eicosanoids differ from steroids on how their actions are mediated in target cells. Water-soluble hormones and eicosanoids bind to membranous receptors provoking a cascade of events for the synthesis of second messengers within the cell (the first messenger being the hormone). In contrast, steroidal hormones bind primarily (membranous is rare) to nuclear receptors. The steroid-receptor complex dimerizes and binds to specific regions of the DNA to allow gene transcription. This mechanism of steroidal hormone action is commonly referred to as the genomic pathway.

Control of the reproductive system by hormones is complex. This complexity includes positive and negative feedbacks among the hypothalamus, pituitary, ovaries and uterus. These organs synthesize and secrete the following hormones and each will be discussed in the initial portion of this review; gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol-17β (estradiol), progesterone, relaxin and androgens.

2.1.1 Hormones of the hypothalamus

All hypothalamic hormones affect reproduction to some extent. As the hypothalamus-pituitary-ovarian axis is primarily regulated with the initial/sequential release of GnRH, the discussion of hypothalamic hormones will focus on GnRH secretion.

Gonadotropin-releasing hormone is a decapeptide synthesized by neurons of the hypothalamus, specifically the tonic and surge centers. To date, more than 20 isoforms
of GnRH have been identified in different species (Millar, 2005). These isoforms differ in their sequence of amino acids but the amino and carboxyl terminals has been conserved (Millar, 2005). Of all these isoforms, GnRH1 (number 1 because it was the first identified) is the prominent secretory isoform of GnRH in most mammals including the pig (Okubo and Nagahama, 2008). For convenience, in this dissertation GnRH1 will be referred simply as GnRH.

Secretion of GnRH into the median eminence of the hypothalamus is then directed to the adenohypophysis by the hypophyseal portal system. Secretion of GnRH into the first capillary bed in the median eminence of the portal system is important because of its short half life (Doble and Liptrap, 1983) and might allow local concentration of this hormone after diffusing out of the secondary capillary bed in the adenohypophysis. It has been known for years that the gonadotropes, in response to GnRH, synthesizes LH and FSH, respectively. Collectively, LH and FSH are known as gonadotropins.

In females, secretion of GnRH can be controlled by higher centers of the brain and steroidal feedback pathways. Ovarian steroids have a complex feedback on GnRH release. A biphasic effect of estradiol on GnRH has been observed in gilts. Cox and Britt (1982) observed that estradiol treatment of ovariectomized gilts decreased GnRH concentrations in the hypothalamus after 24 hours but at 36 hours, concentrations of this hormone were increased. During the luteal phase, which is characterized by high concentrations of progesterone, GnRH pulsatility in ewes decreased (Moenter et al., 1991). When luteolysis occurs and consequently progesterone concentrations decrease, the negative feedback of progesterone on the hypothalamus is reduced, allowing GnRH
pulsatility to increase (Clarke, 1995; Moenter et al., 1991). In addition, estradiol
treatment of ovariectomized ewes decreased total GnRH secretion regardless of an
increase on its pulsatility (Evans et al., 1994). It is now relatively accepted that if
luteolysis has occurred, the transient increases in estradiol during the follicular phase will
ultimately result in a GnRH surge. In contrast, presence of corpora lutea and
consequently high concentrations of progesterone decreases GnRH pulsatility.

The receptor for GnRH (GnRH-R) belongs to the family of G-protein coupled
receptors with seven transmembrane domains. In mammals, it is widely known that the
necessary surge of LH for ovulation is a consequence of increased secretion (surge) of
GnRH. Apparently to aid luteotrope function at the time of ovulation, Wise et al. (1984)
observed increased number of GnRH-R in the anterior pituitary of heifers making the
pituitary more responsive to the increased secretion of GnRH. Throughout the estrous
cycle the number of GnRH-R can be altered by at least three different mechanisms; 1)
GnRH increases its own receptors (female rats, Yasin et al., 1995), 2) synergistically
between estradiol and GnRH to increase GnRH-R (ewes, Kirkpatrick et al., 1998) and 3)
progesterone decreases GnRH-R (ewes, Laws et al., 1990; Nett et al., 2002).

Receptors for GnRH also exist in non-hypophyseal tissues as reviewed by
Ramakrishnappa et al. (2005). In addition to being located in gonadotropes, receptors for
GnRH have also been observed in tissues of the reproductive tract such as the ovaries and
endometrium of domestic animals. Moreover, GnRH-R have been also detected in
tissues of the mammary glands and immune cells (Tanriverdi et al., 2003). These
observations suggest an extra-hypophyseal role of GnRH. For example, GnRH has been
implicated with direct inhibitory or stimulatory actions on steroidogenesis in the ovaries
(Ramakrishnappa et al., 2005). In the endometrium and mammary gland, GnRH might have anti-proliferative action in cancerous cells. Furthermore, increases on T-cells and other immune cells have been observed with GnRH treatment (Tanriverdi et al., 2003).

Collectively, GnRH regulation of pituitary function can be influenced by ovarian steroids by many different pathways: affecting GnRH secretions per se or responsiveness of gonadotropes by altering receptors for GnRH. In addition, the presence of GnRH-R in other tissues suggests that GnRH might be involved in more than its currently known functions on the reproductive system.

2.1.2 Hormones of the adenohypophysis

FSH

The structure of FSH is two proteinaceous subunits: α and β subunits. The α subunit is conserved among two other hormones; LH and thyroid stimulating hormone. The β subunit is unique for each of these hormones. Secretion of FSH during the estrous cycle of pigs is variable. On days 1 or 2 (day 0 = onset of estrus), FSH concentrations increase to around 4.5 ng/ml in peripheral blood (Knox et al., 2003). Those concentrations decrease slightly around day 5 to around 3.5 ng/ml when they remain more or less constant until day 15 to 16; afterward concentrations gradually decreases to around 0.5 ng/ml on day 19 (Knox et al., 2003).

As mentioned before, secretion of FSH occurs from folliculotropes of the adenohypophysis and it is controlled by GnRH and various feedback mechanisms. For example, ovariectomized gilts treated with a GnRH agonist every 180 minutes secreted more FSH than when treated every 30 or 60 minutes (Jayes et al., 1997). Therefore, it
appears that FSH secretion depends on slow rather than fast pulsatility of GnRH secretions. Regarding how feedback pathway affect FSH, serum or pituitary concentrations of FSH decreased with estradiol treatment (ewes, Di Gregorio and Nett, 1995; mares, Garza, Jr. et al., 1986; gilts, Tilton et al., 1994). Furthermore, inhibin, another peptide hormone synthesized by granulosa and theca cells, is known to reduce FSH secretion (Welt et al., 2002, review). In addition, activin is a peptide hormone secreted by the granulosa and theca cells known to stimulate FSH secretion (Welt et al., 2002, review). Moreover, follistatin, a third hormone secreted by granulosa and theca cells, inhibits FSH secretion by neutralizing activin (Welt et al., 2002). Dr. Jerry Reeves involved in the laboratories where GnRH was discovered and a guest speaker in our department speculated that the reason GnRH controls the release of two hormones, LH and FSH, is that the negative feedback of inhibin effectively and uniquely allows control of one of these two hormones, FSH.

Follicle stimulating hormone stimulates cell proliferation of ovarian follicles. The following will be examples of experiments supporting this proliferative effect of follicle stimulating hormone. Nitric oxide, which was observed to inhibit proliferation of cumulus cells obtained from pigs, was reduced by approximately 50% when these cells received FSH treatment (Hattori et al., 2000). In addition, treatment of pig follicles with FSH increased DNA synthesis (Hickey et al., 2004) which is indicative of cell proliferation. Furthermore, it has been demonstrated that cell proliferation in the ovaries might result from synergism between FSH and other hormones. Granulosa cells obtained from rats and further treated with a combination of FSH and activin increased protein and
receptors for proliferating cell nuclear antigen and cyclyn D2 (El-Hefnawy and Zeleznik, 2001), both of which are markers of cell proliferation.

In addition to the proliferative effects of FSH on follicles, this hormone reduces follicular atresia. Estradiol is related to follicular health, as will be discussed later, and increases of estradiol secretion by granulosa cells were related to FSH stimulation of aromatase activity (bovine cells, Silva and Price, 2000). Moreover, ovaries of rats co-treated with equine chorionic gonadotropin and FSH had increased expression of myeloid cell leukemia-1 (an anti-apoptotic factor, Leo et al., 1999). In the pig, increased atresia coincides with decreased secretions of FSH (Guthrie and Garrett, 2001) suggesting an involvement of this hormone in anti-apoptotic events. In addition, small follicles (1 to 2 mm) from pigs when treated with FSH had reduced atresia which perhaps was consequence of decreased activity of caspase-3 (Asahara et al., 2003). The latter effects were augmented by co-treatment with FSH and thyroid-stimulating hormone.

Collectively, these investigations implicate FSH with survival of follicles by acting alone or in combination with other hormones.

Another function of FSH is enhancement of oocyte maturation and it has become a common ingredient in media for oocyte maturation. In vitro maturation of porcine oocytes was enhanced by FSH treatment (Ding and Foxcroft, 1994). In addition, in vivo treatment with FSH increased number of oocytes from monkeys (*Macaca fascicularis*) developing to metaphase II when further cultured in vitro (Younis et al., 1994). Furthermore, oocyte maturation by FSH treatment might be related to increases in glucose metabolism (mice, Downs et al., 1998) which was speculated to provide the oocyte with phosphoribosyl-pyrophosphate a compound needed for purine synthesis.
Previously, it has been demonstrated that FSH positively influences cell proliferation and follicular health. These traits influence ovulation rate, the number of follicles that actually ovulate. Increases in ovulation rate were achieved by treating gilts with FSH on days 15 and 16 of the estrous cycle (Phillippo, 1968). In addition, increases in ovulation rate in gilts treated with anti-inhibin serum were related to around 27% increase in preovulatory secretions of FSH (area under the curve, King et al., 1993). Moreover, concentrations of FSH in the blood of pigs have been considered as a selection method to increase ovulation rate in pigs as positive correlations exist between FSH concentrations and number of ovulations (Cassady et al., 2000). Collectively, it is relatively safe to suggest that FSH is one of the major determinants of ovulation rate in pigs (review, Cárdenas and Pope, 2002a).

As FSH is the primary “driver” of antral follicular growth, a brief review of follicular dynamics will be discussed in this paragraph. Porcine follicles develop up to the antral stage independent of gonadotropins. However by day 14 to 16 of the estrous cycle, follicular recruitment, which refers to formation of a pool of antral follicles from which the ovulatory follicle(s) is subsequently selected (Cárdenas and Pope, 2002a), occurs in pigs (Clark et al., 1982; Foxcroft and Hunter, 1985). Follicular recruitment appears to be controlled mainly by follicle-stimulating hormone (Foxcroft and Hunter, 1985; Guthrie et al., 1988; Knox and Zimmerman, 1993). Ovulation rate, therefore, depends on FSH-induced follicular recruitment and survival of follicles.

The receptor for FSH (FSHR), as GnRH-R, belongs to the family of G-protein coupled receptors with seven transmembrane domains. The importance of FSHR was demonstrated when in FSHR knocked out mice which had impaired folliculogenesis
(antral follicles did not form, Dierich et al., 1998). The FSHR has been observed in granulosa cells from different mammals including the pig (Cárdenas and Pope, 2002b), cattle (Xu et al., 1995) and rats (Camp et al., 1991). In pigs, FSHR in granulosa cells increased after exposure to its own ligand (Sites et al., 1994). However, although FSHR mRNA increased as follicles grew during the follicular phase, by day 19 of the estrous cycle of pigs, the amounts of this message were undetectable or reduced (Cárdenas and Pope, 2002b; Yuan et al., 1996). The decrease on FSHR by day 19 might be related to decreased secretions of FSH that are observed at the end of the estrous cycle of pigs. Moreover, the health status of the follicle was related to mRNA expression of FSHR (decrease in atretic follicles, Tilly et al., 1992). As will be discussed in a subsequent section, one aspect of androgen treatment of gilts to increase ovulation rate might be related to increased expression of FSHR mRNA.

LH

Luteinizing hormone, similar to FSH, is a heterodimer with unique β subunit produced by luteotropes in response to GnRH stimulation. Secretion of LH, in contrast to FSH, relies on increased number of GnRH pulses (Jayes et al., 1997; Padmanabhan and McNeilly, 2001). In pigs, basal concentrations of LH (around 1 ng/ml) in the blood remain almost constant during the estrous cycle except during the preovulatory surge when they can increase around 4 to 5 ng/ml (Guthrie and Bolt, 1990; Knox et al., 2003). Pulses of LH secretion were negatively influenced by progesterone implants probably by an effect of progesterone on GnRH secretion (Skinner et al., 1998). This effect appears to be mediated by the progesterone receptor (PR) as treatment with RU486 (a PR
antagonist) negated the suppression of LH pulsatility by progesterone treatment (Skinner et al., 1998).

One of the known functions of LH is stimulation of testosterone production in thecal cells (Fortune and Armstrong, 1977). In thecal cells, LH promotes synthesis of androgens by inducing the expression of 17α-hydroxylase/17-20 lyase (Lund et al., 1997; Zhang et al., 1996). Androgens, in turn, are important for estradiol synthesis in granulosa cells (two-cell two-gonadotropin theory). Furthermore, a positive feedback from estradiol concentrations induces the preovulatory surge of luteinizing hormone (Andrews et al., 1981; Weiss et al., 1976).

The ovulatory surge of LH results in different actions in the ovary such as inhibition of aromatase, resumption of meiosis in the oocyte, prostaglandin synthesis and early synthesis of progesterone during the luteal formation (luteinization). Inhibition of aromatase by the LH surge shifts the production by follicular cells from estradiol to progesterone; consequently estradiol concentrations decrease and progesterone concentrations increase. Furthermore, it is widely accepted that LH increases progesterone secretion even before ovulation occurs (Juengel and Niswender, 1999). In addition the LH surge aids oocyte resumption of meiosis by degradation of the gap junctions between cumulus cells and the oocyte. Luteinizing hormone induces expression of cyclooxygenase-2 (Sirois et al., 1992) an important enzyme in prostaglandins synthesis. Prostaglandin E\textsubscript{2} in turn is known to increase blood flow to the ovary while prostaglandin F\textsubscript{2α} induces contractions of muscle cells in the ovary.

Ovariectomy, but not adrenalodectomy, resulted in initial increases of basal and frequency of LH peaks in prepubertal gilts (Fonda et al., 1983) suggesting that gonadal
factors are involved in the control of LH secretion. Control of LH secretions by estradiol appears to have a biphasic nature. Concentrations of LH in serum of ovariectomized gilts injected with estradiol initially decreased by 12 hours (Cox and Britt, 1982) but 26 hours later, LH concentrations return to concentrations similar to those observed in non-treated gilts (Cox and Britt, 1982). Furthermore, treatment of ovariectomized gilts with estradiol decreased LH concentrations by 60% within 12h and further increased by 72 hours (Ford et al., 2000). Similar effects of estradiol have been observed in other mammals (female rats, Libertun et al., 1974; ewes, Scaramuzzi et al., 1971). Collectively, these observations clearly suggest that in ovariectomized gilts, the initial effect of estradiol is to decrease LH secretions followed by a subsequent increase. In vivo, as mentioned earlier, increasing concentrations of estradiol during the follicular phase eventually provoke an LH surge (GnRH surge).

Some hormones other than estradiol synergize with luteinizing hormone. For example, Cortvrindt et al. (1998) observed that combination of LH and FSH was more effective for survival of mice follicles than when each hormone was supplemented individually. Furthermore, synergistic effects of LH and FSH in gilts have been observed to influence follicular growth after antrum formation (Wu et al., 2007). In addition to having synergistic effects with FSH, LH can also synergize with insulin. Luteinizing hormone and insulin treatment increased progesterone secretion of granulosa cells obtained from pigs when compared to supplementation of individual hormones (Sekar et al., 2000).

Structurally, the receptor for LH (LHR) is similar to the receptor for follicle stimulating hormone. In pigs, expression of LHR mRNA has been detected in thecal and
granulosa cells (Yuan et al., 1996). The importance of LH receptors (LHR) was demonstrated in mice which were knocked out for the receptor of luteinizing hormone (LHRKO). Ovaries from LHRKO mice did not have preovulatory follicles nor corpora lutea, rendering these animals infertile (Huhtaniemi et al., 2002). In normal (wild type) pigs, as follicular diameter increases during the follicular phase, the amount of LHR mRNA increases (Yuan et al., 1996). More specifically, Liu et al. (2000) observed that LHR expression in porcine follicles was dependent on size; 6 mm follicles had greater expression than 2 or 4 mm follicles. Expression of LHR in granulosa cells obtained from rats was induced by FSH treatment (Zeleznik et al., 1974). Increased expression of LHR related to size of the follicle is perhaps a consequence of FSH stimulation on these cells. Recently, our laboratory demonstrated that DHT treatment from day 13 to 16 of the estrous cycle decreased LHR in follicles on day 17 when compared to non-treated gilts (Cárdenas et al., 2008). These androgenic effects appear to be temporal as DHT treatment did not alter follicular LHR on day 19.

Existence of LHR in parts of the reproductive tract other than the gonads such as the oviducts, endometrium, myometrium and cervix has been recently reviewed (Fields and Shemesh, 2004). In these tissues LHR have been related to secretory activity (oviducts, endometrium and cervix) and muscle relaxation (myometrium and blood vessels of the broad ligament). Therefore, the presence of LHR on extragonadal tissues of the reproductive system of women, cows and pigs might be also important for successful reproduction.
2.1.3 Hormones of the ovary

_Estradiol-17β_

Estradiol-17β (estradiol) is a 18-carbon steroid produced primarily by granulosa cells and to a lesser extent by theca cells. Concentrations of estradiol in the blood are relatively constant from day 1 to around day 13 of the estrous cycle (around 2 to 5 pg/ml, Knox et al., 2003). Thereafter, estradiol concentrations increase and peak up to 70 pg/ml one day before the onset of estrus (Knox et al., 2003). As mentioned earlier, FSH increases aromatase activity in ovarian cells. The enzyme aromatase can convert testosterone to estradiol (Daniel and Armstrong, 1980) and androstenedione to estrone (an estradiol precursor). In pigs, aromatase is primarily expressed in the granulosa cells of large follicles (>5mm, Garrett and Guthrie, 1997).

In addition to FSH and LH, estradiol appears to be required for follicular antrum formation. In vitro co-treatment with estradiol and FSH increased the number of follicles developing an antral cavity when compared to FSH treatment alone (rats, Gore-Langton and Daniel, 1990). Furthermore, in vivo investigations utilized hypophysectomized rats and co-treatment with estradiol and FSH increased number of antral follicles when compared to hormones injected individually (Wang and Greenwald, 1993). An additional synergistic effect between estradiol and FSH might be related to synergism in cAMP production (Knecht et al., 1984).

As mentioned earlier, estradiol influences LH secretion. The effects of estradiol on LH secretion might be through feedback systems to the hypothalamus or adenohypophysis. Almond and Dial (1990) inserted an estradiol implant in sows and observed a reduction on pulsatility and mean concentrations of LH even when sows were
given GnRH injections. These results suggest that estradiol might directly inhibit secretion of LH from luteotropes. Gilts with their hypophyseal stalk transected and treated with various estrogens failed to induce an LH surge (Ford et al., 2000; Kesner et al., 1989) suggesting that actions of estrogens on this process are mediated directly at the hypothalamus and not at the level of the pituitary. Perhaps estradiol might promote the LH surge by increasing hypothalamic secretion of GnRH or GnRH-R in luteotropes (ewes, Kirkpatrick et al., 1998).

Health status of ovarian follicles might be related to estradiol concentrations in the follicle. In pigs, higher concentrations of estradiol in follicles classified as non-atretic versus atretic has been observed (Guthrie and Cooper, 1996; Tilly et al., 1992). Similar associations were determined in ewes (Carson et al., 1981), goats (Yu et al., 2004) and heifers (Ireland and Roche, 1982) and many other species. In pigs, Grant et al. (1989) observed heterogeneity within a cluster of preovulatory follicles as some follicles were more developed than others, closer to ovulation.

Estradiol has a pronounced effect on the adult uterus of pigs. Treatment with estradiol influence uterine secretions and development of neonates, and elongation of the uterus during early pregnancy. For example, sows treated with estradiol on days 12 and 13 of the estrous cycle or pregnancy had longer uterine horns than vehicle-treated sows (Pope and First, 1985). In ewes, estradiol treatment increased uterine weight which was related to hypertrophy and hyperplasia (Reynolds et al., 1998). Increase in blood flow to the uterus (ewes, Magness et al., 1998), formation of gap junctions and myometrial contractility (sows, Langendijk et al., 2002) have been observed after estradiol treatment. Secretion of estradiol by the pig embryo has been suggested to signal maternal
recognition of pregnancy (Bazer and Thatcher, 1977) by redirecting uterine secretions of
PGF$_{2\alpha}$ into its lumen. Moreover, Pope et al. (1982) demonstrated that embryonic
synthesis of estradiol advances uterine secretions; this advancement might explain a
mechanism of embryo survival in swine. Collectively, these investigations demonstrate
the various pronounced effects of estradiol on uterine physiology.

To date, two receptors for estradiol (ER) have been identified; ER$\alpha$ and ER$\beta$. In
pigs, expression of both receptors has been detected in granulosa, theca cells and corpora
lutea (Cárdenas and Pope, 2005; Lavoie et al., 2002), and in the uterus (Cárdenas and
Pope, 2005). Greater immunostaining of ER$\alpha$ was observed in granulosa than thecal cells
from pigs (Cárdenas and Pope, 2005) perhaps explaining how estradiol can influence
antrum formation. Expression of ERs is related to different events during the estrous
cycle; 1) control of progesterone synthesis as ER is expressed in corpora lutea and the
activity of 3$\beta$-hydroxysteroid dehydrogenase, an enzyme involved in this process, can be
influence by estradiol treatment (Tonetta et al., 1987), 2) aid in myometrial contractions
for delivery of sperm to the oviducts as in gilts, ER increases in the endometrium of gilts
at the onset of estrus (Deaver and Guthrie, 1980), and 3) luteolysis as peak ER on day 10
(Deaver and Guthrie, 1980).

**Progesterone**

Progesterone is a 21-carbon steroid produced from the conversion of
pregnenolone by the enzymes 3$\beta$-hydroxysteroid dehydrogenase/ delta 5,4 isomerase.
Concentrations of progesterone increase from day 2, can reach values of 40 ng/ml around
days 10 and 12 (Hendricks et al., 1972). Thereafter, progesterone concentrations steadily
begin to decrease and can reach very low concentrations from days 18 to 20 of the estrous cycle (Hendricks et al., 1972).

In pigs, progesterone, the “pro-gestational” hormone is required for maintenance of pregnancy to term. Secretion of progesterone is mainly by corpora lutea in pigs and is induced by luteinizing hormone. Spies et al. (1967) observed that treatment of gilts with LH antiserum resulted in decreased progesterone production by the corpora lutea which resulted in total embryonic loss. Administration of progesterone reversed these anti-LH effects suggesting the requirement of progesterone throughout pregnancy for embryo survival. Some actions of progesterone in the uterus, which might relate to maintenance of pregnancy, include induction of pregnancy-specific secretions from endometrial glands and quiescence of myometrial tissue. Progesterone attenuates myometrial activity by reducing gap junctions between myocytes and reducing secretions of nitric oxide (rats, Buhimschi et al., 1996). Furthermore, Kurowicka et al. (2005) observed that blocking oxytocin actions under progesterone treatment resulting in myometrial quiescence. During the early establishment of pregnancy progesterone is also very important as uterine secretion of essential proteins for the developing concepti are requisite for their survival. Progesterone treatment increased protein content in uterine flushes of pigs (Vallet et al., 1998). In support, treatment of gilts with the PR antagonist, mifepristone, resulted in decreased secretion of uterine proteins and concepti diameter (Vallet and Christenson, 2004). In addition, secretion of prostaglandin F\(_2\alpha\) from the uterus of ovarietomized sows was increased with progesterone treatment (Edgerton et al., 2000).

Progesterone can influence the length of the estrous cycle although its effects appear to be species dependent. Woody et al. (1967) observed that progesterone injection
decreased length of the estrous cycle in guinea pigs, cattle and sheep but did not alter this variable in gilts. Similar effects of progesterone on duration of the estrous cycle were further demonstrated in cattle (Garrett et al., 1988) and sheep (Ottobre et al., 1980). These priming effects of progesterone on uterine responsiveness to oxytocin and secretion of prostaglandins are now rather well accepted. It appears that progesterone might also play a role in ovulation as Hibbert et al. (1996) observed that injecting rhesus monkeys with progesterone inhibitor, trilostone, blocked ovulation but ovulation was restored by injecting progesterone. In support, Robker et al. (2000) observed that mice lacking PR failed to ovulate.

Progesterone receptors (PR) have been detected in the ovaries, uterus and mammary gland of pigs. Slomczynska et al. (2000) detected PR in granulosa cells from early-antral follicles and thecal cells of medium antral follicles of pigs. Furthermore, in preovulatory follicles of pigs, both granulosa and thecal cells have progesterone receptors (Slomczynska et al., 2000). In addition, endometrial PR increased by day 5 of the estrous cycle (Geisert et al., 1994) which might be involved in the control of myometrial contractility. Endometrial PR normally decrease on day 10 and reach a minimum by day 12 of the estrous cycle (Geisert et al., 1994). Geisert et al. (2006) suggested that downregulation of PR is involved in maternal recognition of pregnancy in pigs as it 1) precedes endometrial secretions, 2) trophoblast expansion and 3) estradiol secretion by the concepti. This process might be common in mammals as PR decreases in the endometrium during attachment in sheep (Spencer and Bazer, 1995) and mice (Tan et al., 1999).
In addition to the reproductive tract, PR has been observed in mammary tissues. Lin and Buttle (1991) observed the presence of PR in mammary glands throughout pregnancy and was observed to increase on day 75. This increase in binding of progesterone might be related to increased development of the mammary gland near the end of pregnancy. Curiously, when PR was analyzed on day 21 of lactation it was undetectable in mammary tissue which is common with other species (Lin and Buttle, 1991, and references therein).

**Relaxin**

Relaxin is a peptide with A (pig, 22 amino acids) and B (pig, 32 amino acids) chains held together by disulfide bonding. Secretion of relaxin has been observed by different tissues of the reproductive tract. In the pig, sources of relaxin are corpora lutea (Fields and Fields, 1985), thecal cells (Bagnell et al., 1987) and the uterus (Knox et al., 1994). Concentrations of relaxin in peripheral blood when examined from day 13 to 17 of the estrous cycle, period when corpora lutea of pigs are regressing, averaged 0.59 ng/ml (Kotwica et al., 1991). As will be discussed in the next paragraph relaxin concentration increase during late pregnancy to aid parturition.

It has been demonstrated that supplementation with porcine relaxin decreased contractions of electrically-stimulated uteri obtained from rats (Sarosi et al., 1983). In vivo investigations have demonstrated that injections of relaxin to ovariectomized mini-pigs inhibited almost completely any myometrial activity (Porter and Watts, 1986). In addition, Hall et al. (1990) observed greater cervical distensibility in prepubertal gilts treated with relaxin. Two days before parturition relaxin concentrations in pigs increased to around 75 ng/ml (Sherwood et al., 1977). It is widely accepted that relaxin is needed
during parturition. For example, prolonged parturition in gilts treated previously with progesterone was related to undetectable concentrations of relaxin in plasma (Nara et al., 1982). Moreover, treating gilts with anti-relaxin serum was observed to decrease relaxin concentrations in plasma and increase duration of delivery (Cho et al., 1998). Collectively, the latter investigations demonstrate that relaxin influences muscle physiology in pigs and these effects are important during parturition.

Relaxin might also influence the ovary during the estrous cycle. Relaxin has been observed in the follicular fluid of cyclic pigs (Bryant-Greenwood et al., 1980; Matsumoto and Chamley, 1980). Receptors for LH are greater in thecal cells than other cells in the ovaries and both protein and gene expression of relaxin were determined exclusively in thecal cells of immature pigs (Ohleth et al., 1998). Moreover, granulosa cells obtained from preovulatory follicles of pigs were observed to increase relaxin secretion when treated with luteinizing hormone (Loeken et al., 1983). Furthermore, Zhang and Bagnell (1993) observed that relaxin treatment increased DNA synthesis of granulosa cells obtained from cyclic pigs. Collectively, the latter investigations suggest that relaxin secretion, probably controlled by LH, might be an additional mechanism to control proliferation of granulosa cells.

Relaxin has demonstrated to have uterotrophic effects in gilts. Cross-sectional area of the endometrium and myometrium of ovariectomized gilts was increased with relaxin treatment (Vasilenko et al., 1986). In addition, Hall et al. (1990) observed that treating prepubertal gilts with relaxin increased wet and dry weight, protein content and amounts of DNA of the uterus. In addition, Zaleski et al. (1995) observed synergistic effects of relaxin and estradiol on uterine weight. It can be inferred that relaxin promotes
uterine development during the estrous cycle. Recently, relaxin treatment was observed to alter neonatal development of the pig uterus but this topic will be discussed in a further section.

Although relaxin was discovered in the early 1900’s, it was not until recently that its receptor (LGR7) was identified (Hsu et al., 2002). For that reason, information on LGR7 is still limited. Relaxin receptor belongs to the family of G-protein coupled receptors which contain leucine-rich repeated sequences. To date, some tissues in which LGR7 has been detected are uteri of neonate pigs (Yan et al., 2005), women (during proliferative and secretory phase of the menstrual cycle, Bond et al., 2005), and pregnant mice (Novak et al., 2006), in the rat brain (Ma et al., 2006) and human heart (Samuel et al., 2006). Not surprisingly, it was demonstrated that mice with LGR7 knocked out had impaired parturition (Krajnc-Franken et al., 2004).

2.2 Uterine growth and development

As the physiology of follicular growth and development has been incorporated into the previous discussion of ovarian hormones, this section will specifically focus on uterine function. The forthcoming discussion of uterine physiology will be partitioned into prenatal, neonatal and adult physiology.

The uterus is one of the most essential organs in the reproductive system. Oocytes can be fertilized in vitro and embryos can be grown in cultures for days but eventually embryos need to be transferred into a uterus for completion of development. The importance of the uterus is unquestionable as it nurtures and hosts the conceptus throughout pregnancy, is involved in maternal recognition of pregnancy, maintenance of
estrous cycles and aids the parturition process. It is unlikely we will ever produce an artificial uterus. Uterine differentiation, growth and development begins prenatally and continues after birth; and in some species it continues for a number of years after birth. The following review will present our current knowledge on the development of the uterus with emphasis on the pig.

2.2.1 Pre-natal development of the uterus

The genotype of the embryo influences the secretions from the bipotential gonad. Absence of the Y chromosome, and consequently absence of the testis-determining factor, will permit the differentiation into ovaries from the bipotential gonads.

The reproductive tract develops close and simultaneously as the renal system. In fetal life there is three different pair of kidneys: pronephric, mesonephric and metanephric. The metanephric kidney develops into the kidney of the adult and in the process, develops its own separate collecting duct. The collecting ducts of the mesonephric kidneys, mesonephric ducts, develop into the epididymis, vas deferens and part of the urethra of the male tract. This differentiation occurs in the presence of the Y chromosome, the testis-determining factor, the anti-Müllerian hormone and testosterone. The paramesonephric ducts develop as neomorphs now called Müllerian ducts and will form the oviducts, uterus, cervix and vagina. Ovaries do not produce anti-Müllerian hormone nor significant amounts of testosterone and therefore allow degeneration of the wolffian ducts (old mesonephric collecting ducts). The latter process permits early development and continued fusion of the Müllerian ducts.
In the embryo, uterine development initiates with differentiation of, and further fusion of, the Müllerian ducts. The type of uterus (simplex, duplex or bicornuate) depends on the point where the two Müllerian ducts fuse. For example, the bicornuate uterus of the pig is a consequence of the caudal fusion of the Müllerian ducts resulting in a small uterine corpus and two long uterine horns. In humans, the simplex uterus is a consequence of the cranial fusion of the Müllerian ducts, resulting in a disproportionately large uterine corpus and absence of horns. Finally, the duplex uterus of the rat is a consequence of the absence in fusion of the two Müllerian ducts cranial to the vagina.

The adult uterus consists of two major components: the endometrium and the myometrium. The endometrium contains glandular and luminal epithelium plus stroma. The myometrium is the muscular component of the uterus with two distinct layers; an inner circular and outer longitudinal. In the embryo, the endometrium and both muscular layers develop from the mesenchyme of the Müllerian ducts. In domestic animals, this development continues during fetal life but is not completed until after the animal is born (Bartol et al., 1993). More specifically, the circular layer of the myometrium develops from the intermediate layer of the mesenchyme while the outer layer develops from subperimetrial mesenchyme (Bartol et al., 1993).

### 2.2.2 Neonatal development of the uterus

Neither endometrial nor myometrial tissues are completely developed at birth. At birth, the infantile endometrium consists of a single layer of luminal epithelium and dispersed stroma (Tarleton et al., 2001), and the myometrium appears single layered (Christenson et al., 1997). This primitive neonatal uterus does not contain endometrial
glands (Spencer et al., 1993a) and lacks detectable amounts of ER (Tarleton et al., 1998). In gilts, complete transformation of the uterine tissues from an infantile histoarchitecture to that of an adult takes around 120 days after birth (Gray et al., 2001). Rapid development of the uterine glands occurs after birth with increased DNA synthesis in epithelial and stromal cells (Spencer et al., 1993a). The different stages composing growth and development of the uterine glands are illustrated in Figure 2.1. In the pig, bud formation occurs from postnatal day 0 (PND 0; day of birth) to PND 7, tubulogenesis from PND 7 to 14, coiling from PND 14 to 28 and branching neo-morphogenesis from PND 28 to 120 (Bartol et al., 1993). Initial glandular development occurs from PND 0 to 14 when epithelial invaginations develop into the shallow stroma (Figure 2.1, Spencer et al., 1993a). By PND 28 to 56 these initial epithelial invaginations progress deeper into the stroma. The histoarchitecture of the uterine glands is considered mature and completed by 120 days of age (Bartol et al., 1993).

During the early period of epithelial invagination, expression of the ER occurs in a tissue specific manner. Appearance of detectable ER was not observed in significant amounts in the uterine glands of gilts until PND 15, in the stroma until PND 60, and in the luminal epithelium until PND120 (Tarleton et al., 1998).

Many events in the early development of the uterus and uterine glands are apparently independent of the ovary. Tarleton et al. (1998) observed glandular morphogenesis progressing normally to PND 60 in gilts ovariectomized at birth. Expression of ER in the neonatal uterus was also independent of the ovary, which might be common to the effects of ovariectomy on continued expression of ER in the adult uterus (Cárdenas and Pope, 2004).
The architecture of the neonatal uterus can be affected by hormones. Estrogen and relaxin are hormones known to influence the development of the neonatal uterus. For example, neonatal gilts treated with estradiol had precocious formation of glandular epithelium and increased uterine weight (Spencer et al., 1993b). In support, ICI 182,780 (an ER antagonist) treatment inhibited genesis of uterine glands in neonatal gilts (Tarleton et al., 1999). Similarly, in neonatal rats, treatment with tamoxifen (an ER antagonist) inhibited formation of uterine glands (Branham et al., 1985a). Branham et al. (1985b) observed that treating neonatal rats with estradiol increased uterine genesis by PND 5 but decreased between 15 and 26 days of age. Therefore it appears that estradiol can mediate development of uterine tissues in neonates acting through its receptor. Recently, relaxin administration to neonatal gilts was observed to increase height of the luminal epithelium and uterine weight by PND 14 (Bagnell et al., 2005). However, it remains unknown how this early treatment with relaxin affects uterine function on to the

Fig 2.1. Endometrial morphogenesis in the uterine wall in pigs (modified from Gray et al., 2001)
adult stages. Finally, the potential role of androgens, such as testosterone and DHT, in the development of uterine glands is completely unknown. In gilts, presence of AR on uterine tissue at birth (Slomczynska et al., 2008) and at PND 14 (E. Jiménez, H. Cárdenas and W.F. Pope, unpublished data) suggests a possible role of receptivity to androgenic effects might exists in the neonate.

2.2.3 Adult development of the uterus and its secretions

Uterine changes in the adult pig are indicative of the reproductive status of the animal; cyclic versus pregnant. Changes during the follicular phase of the estrous cycle are different to those of the luteal phase because the hormonal patterns governing each phase are different. For example, during the follicular phase there is a transient increase in estradiol concentrations while during the luteal phase progesterone is high. Changes during the follicular phase prepare the uterus to receive the ejaculate and fight uterine microflora introduced at mating. On the other hand, changes during the luteal phase, which are very similar to up to day 12 of pregnancy (Kaeoket et al., 2001), for example the myometrium is quiescent and the endometrium is becoming prepared to receive and ensure embryo survival.

All mammalian uteri contain endometrial cells which synthesize and secrete substances as part of the normal function of the uterus. In non-pregnant pigs, the uterus secretes prostaglandin F\textsubscript{2\alpha} to induce regression of corpora lutea (Bazer and Thatcher, 1977) around day 12 of the estrous cycle. The decline in progesterone concentrations due to regression of corpora lutea allows the beginning of a new follicular phase and initiates events that will allow the animal to subsequently display estrus.
During the follicular phase blood flow to the pig uterus increases (Ford, 1982) perhaps in relation to a transient increase in estradiol secretions from the developing follicles. The increase in blood to the uterus during this phase induces edema and lymphocyte infiltration, preparing the uterus to fight infections that might result from copulation. Note that in pigs, boars penetrate deeper into the female tract than other species, ejaculate into the cervix and uterine lumen. Likewise, the long proestrus period in the pigs, allow additional time to prepare the uterus for the events at mating. As probably expected, endometrial expression of enzymes involved in the synthesis of prostaglandin decrease during this follicular phase of pigs (Ashworth et al., 2006; Blitek et al., 2006), as the luteolytic events are completed by this time. Regarding specific morphological changes during the follicular phase, Kaeoket et al. (2001) observed that height of surface epithelium of pigs increased from around 38 µm at proestrus to 57 µm at estrus. Height of the glandular epithelium or number of endometrial glands did not change during proestrus or estrus (Kaeoket et al., 2001). Similarly, Okano et al. (2007) found that height of epithelium increased from days 15 to 18 and further increased on day 20. Although there is an increase in height of uterine tissues, apoptosis can also occur in uterine cells at this phase. Okano et al. (2007) observed that on days 15 and 18 some cells in the surface epithelium were undergoing initial stages of apoptosis but when analyzed on day 20 most of the cells stained positive for active apoptosis. Not only the surface epithelium but also some stromal cells were apoptotic on days 15, 18 and 20. Curiously, no apoptotic cells were detected in the glandular epithelium when analyzed on days 15, 18 and 20 of the estrous cycle (Okano et al., 2007).
During the luteal phase, on days 1 and 2 of the estrous cycle there is an increase in blood flow but then decreases and reaches a minimum around days 10 to 12 (Ford and Christenson, 1979). In contrast to the follicular phase, during the luteal phase of pigs increasing concentrations of progesterone results in a flow reduction of uterine blood (Ford and Christenson, 1979). Regarding morphological changes, during early stages of luteal phase height of surface epithelium increased as compared to follicular phase but then decreased by late luteal phase (Kaeoket et al., 2001). This latter decreased on late stages of luteal phase might be consequence from decreased PR after long exposure of progesterone concentrations (Geisert et al., 1994). Similar results were obtained by Okano et al. (2007) who observed that the height of epithelial cells during the entire cycle was greater on day 4 of the estrous cycle, decreased on day 8 and further decreased on day 12. Furthermore, the number of glands per mm$^2$ in the upper half of the endometrium increased during the luteal phase as compared to follicular phase (Kaeoket et al., 2001).

In contrast to the follicular phase, no cells stained positive for apoptosis in the surface epithelium during the luteal phase when examined on days 4, 8 or 12 of the estrous cycle (Okano et al., 2007). Intriguingly, most of the stromal cells stained positive for apoptosis on day 8 but only some on days 4 and 12 (Okano et al., 2007) suggesting some changes might be occurring around day 8 for these cells to die. As in the follicular phase, no cells on the glandular epithelium stained positive in the glandular epithelium when examined on days 4, 8 and 12 (Okano et al., 2007). The observations that apoptosis was not observed in glandular epithelium during both follicular and luteal phase suggests a unique role of these cells. Moreover, downregulation of PR during the luteal phase
from luminal and glandular epithelium is associated with decreased mucin-1 in the luminal epithelium (Bowen et al., 1996). The decrease on this mucin-1 protein from luminal epithelium seems to be required for trophoblast attachment by exposing integrins (Bowen et al., 1996).

The density of oxytocin receptors increases in the endometrium and myometrium during the luteal phase of pigs (Okano et al., 1996). Secretion of PGF$_2\alpha$ from the pig endometrium is apparently stimulated by oxytocin binding (Carnahan et al., 1996). Oxytocin is produced by both the hypothalamus and corpora lutea of pigs. It has been proposed that PGF$_2\alpha$ secretions during the luteal phase might be related to the increase in oxytocin receptors which in turn will cause luteolysis if no concepti are present. If concepti is present in the uterus, as mentioned earlier, their secretion of estradiol (Geisert et al., 1982) will cause an exocrine secretion of PGF$_2\alpha$; therefore corpora lutea and pregnancy are maintained.

In pigs, gestational length is around 114 days. Stroband et al. (1986) determined that luminal epithelium is similar in pregnant and cyclic gilts from estrus up to 10 days. In addition, similarly to the luteal phase, height of surface epithelium was also greatest on day 4 and decreased on day 8 of pregnancy. In contrast to uteri in nonpregnant gilt, on day 12 of gestation, height of surface epithelium remains similar to day 8, decrease on day 15, but on days 18 and 21 increase to height similar to values observed on days 8 and 12 (Okano et al., 2007). Perry and Crombie (1982) observed that by mid-, and continuing during the last third of pregnancy, endometrial glands appeared dilated indicative of a high secretory activity. Blair et al. (1991) administered estradiol to gilts on day 9 and 10 of pregnancy and observed a breakdown of the glycocalyx on the
luminal epithelium. This loss of glycocalyx is probably needed for conceptus attachment and therefore might be another function of conceptus estradiol secretion during maternal recognition of pregnancy. Noteworthy, Garlow et al. (2002) observed glandular expression of osteopontin throughout pregnancy and as will be discussed later, this protein is involved in attachment of placenta with uterine epithelium. Furthermore, vascular permeability in the endometrium increased by day 19 of pregnancy (Keys and King, 1988) which probably is necessary to facilitate transport of nutrients to the conceptus.

Uterine physiology also changes dramatically after parturition. Subsequent to parturition, uterine glands undergo rapid involution (Perry and Crombie, 1982). Involution perhaps is induced by a lack of support from ovarian steroids because progesterone and estradiol concentrations drop within 24 hours after parturition in pigs (Robertson and King, 1974). Specifically, diameter and length of uterine horns decreased from day 1 to 13 post partum (Ogawa et al., 2001). The latter authors also observed that on day 12 post partum the luminal epithelium has a flattened appearance. On days 1, 13 and 20 post partum, height of epithelial cells did not change appreciably but by day 31 post partum epithelial growth had occurred (Okano et al., 2007). Regarding apoptosis, on day 13 post-partum half of the cells in surface epithelium and stroma stained positive for apoptosis. In contrast, on days 20 or 31 post partum none or just some cells of the surface epithelium or stroma stained for apoptosis, respectively (Okano et al., 2007). Once more, no cells in the glandular epithelium were positive for apoptosis when analyzed up to day 31 post partum (Okano et al., 2007).
After weaning, luminal epithelium was highly columnar in shape (Ogawa et al., 2001). Glandular epithelium also began to develop after weaning (Ogawa et al., 2001). In total, endometrial thickness was observed to increase after weaning (Palmer et al., 1965). Nursing sows have been observed to have low concentrations of steroid hormones in their blood because the ovaries lack large follicles or corpora lutea (Ash and Heap, 1975). Therefore, it appears that during lactation, support to uterine tissues might be absent and consequently, it is not until after weaning that these tissues begin developing again.

Collectively, the latter observations demonstrate different changes that occur in the histology of uterine tissues during the estrous cycle, pregnancy and post partum. In the following paragraphs will be discussed some specific uterine secretions produced by the cells described above and how these secretions change during the estrous cycle and pregnancy.

The placenta of the pig is diffuse which implicates uniform distribution of chorionic villi attaching the placenta to the uterus. In addition the placenta of the pig is classified as epitheliochorial which implicates six layers of separation between maternal and fetal blood making the transport of nutrients from the sow to the concepti more difficult. With those barriers, not surprisingly uterine secretions have a significant role as nutrients and as mechanisms to allow attachment of the embryo. Furthermore, uterine secretions vary in response to hormonal changes during the estrous cycle and pregnancy. The following review will discuss the specific proteinaceous secretions of the uterus known to date.
Uteroferrin

Uteroferrin is a protein utilized for the embryonic and placental transport of iron (Ducsay et al., 1982; Renegar et al., 1982). The glandular epithelium secretes uteroferrin (Raub et al., 1985) under the primary control of progesterone (Knight et al., 1973) and estradiol (Simmen et al., 1991). The presence of concepti is correlated with increased production of uteroferrin (Vallet et al., 1998) and secretions can reach up to 2 g/day during pig pregnancy and is over half the secretions by the uterus at this time. Uteroferrin can be found throughout pregnancy (Renegar et al., 1982) and it can reach 40 or 80 ng/ml in umbilical arterial or venous blood, respectively (Renegar, 1982). This protein travels from the aerola to chorioallantoic capillaries and then on to the fetus through the umbilical vein. Uteroferrin can be found in the liver, which supply iron for early embryonic hematopoiesis. Excess uteroferrin can enter and accumulate in the allantoic sac which might serve as iron storage for the embryo. Uteroferrin concentrations were relatively low on day 7 of the estrous cycle but they increase greatly by day 13 (Vallet et al., 1996). During pregnancy it has been observed an increase of uteroferrin secretions at the time of maternal recognition of pregnancy (Geisert et al., 1982; Zavy et al., 1984) and by day 30 (Basha et al., 1979). Peak secretions of uteroferrin occur around day 60 of pregnancy (Basha et al., 1979). In addition to transport of iron, uteroferrin has been implicated with catalysis of lipid peroxidation (Vallet et al., 1996).

Retinol binding protein

Excessive peroxidation of lipids can result in cell damage or death (Vallet, 1995). Uteroferrin treatment induced lipid peroxidation in endometrial membranes obtained
from pigs; therefore the uterus requires mechanisms to alleviate lipid peroxidation. Lipid peroxidation of endometrial membranes, caused by uteroferrin supplementation, was decreased by supplementation with retinol binding protein (RBP, Vallet, 1995). In contrast, retinol has been observed to inhibit lipid peroxidation in rat liver (Vile and Winterbourn, 1988). Moreover, RBP might serve to transport retinol (a form of vitamin A) to the pig concepti (Fazleabas et al., 1982; Harney et al., 1994). Furthermore, high correlations were observed between uteroferrin and RBP in uterine flushing from pigs and this relationship exists throughout pregnancy (Vallet et al., 1996). Retinol-binding protein was detected in glandular epithelium and areolae of the pig suggesting RBP is secreted from endometrial glands (Johansson et al., 2001). Furthermore, uterine secretion of RBP appears to be mediated by progesterone although estradiol secretions from concepti might also trigger secretion of this protein at critical times during early gestation (Adams et al., 1981; Trout, 1992).

*Uterine plasmin/trypsin inhibitor*

The pig embryo has been demonstrated to have an invasive nature when transplanted to kidney capsule, oviduct or wall of a non-pregnant uterus (Samuel, 1971; Samuel and Perry, 1972). The placenta of the pig, however, is epitheliochorial which implies a non-invasive nature of the trophoblast in utero. Pig blastocysts were observed to secrete plasminogen activator (Fazleabas et al., 1983), which activates plasmin, a protein that degrades tissue. Therefore, the pig uterus should secrete substances to protect itself against the invasive nature of the embryo. *Uterine plasmin/trypsin inhibitor* (UPTI), a protein first purified by Fazleabas et al. (1982) in the pig uterus, inhibits actions of plasminogen activator by inhibiting plasmin to prevent damage (invasion) to
the maternal endometrium. This protein secretion is induced by the presence of periimplantation concepti and suggested an embryonic estrogen signal pathway (Fazleabas et al., 1983). In addition to uterine origin, UPTI can be secreted by the trophoblast and placenta throughout pregnancy in the pig. Expression of UPTI mRNA in trophoblast was detected as early as day 10 of pregnancy in the pig (Duffy et al., 1997). Following day 10, secretion of UPTI is maximal during the first 30 days of gestation and later decreases throughout gestation (Stallings-Mann et al., 1994). In addition to the protective function of UPTI, it has been demonstrated to increase DNA synthesis of day 12 epithelial cells in culture (Badinga et al., 1999), which might suggest that UPTI is also involved in promoting growth of the uterus tissues in preparation for pregnancy.

**Osteopontin**

Osteopontin (OPN) is a protein found in many tissues of the body including the uterus where it is involved with cell-adhesion. Osteopontin production from the uterus during periimplantation of different species, including the pig, suggests that this protein is involved in the adhesion of the chorion-endometrium during implantation (Johnson et al., 2003). In the pig, expression of OPN mRNA is first observed at least on day 9 in the stroma of cyclic and pregnant gilts, on day 15 of pregnancy in the luminal epithelium and on day 35 on glandular epithelium (Garlow et al., 2002). Production of OPN was induced by progesterone supplementation of endometrial cells obtained from sheep (Johnson et al., 2000). In the pig, the glandular epithelium secretes OPN again in response to estradiol production by the embryo (White et al., 2005).
**Bikunin**

Bikunin is a protease inhibitor that has been observed in uterine flushings from day 12 to 18 of the estrus cycle of gilts (Hettinger et al., 2001). Expression of bikunin gene can be observed in glandular epithelium and is low on day 10 but increase abruptly on day 15 of the estrous cycle or pregnancy (Hettinger et al., 2001). After day 15, if there is no pregnancy, expression of bikunin decreases by day 18 but if pregnancy is present then its expression continues throughout pregnancy (Hettinger et al., 2001). Moreover, Geisert et al. (2003) suggested that bikunin may be involved in the conceptus attachment in pigs, by keeping it from invading the endometrium.

**Integrins**

Integrins are subunits of proteins involved in placenta-endometrium attachment during placentation. Different subunits of integrins were observed localized to attachment sites between trophoblast and endometrium from day 12 to 15 of pig pregnancy (Bowen et al., 1996). When supplemented with estradiol, uterine cultures secreted greater amounts of integrins than when untreated or treated with progesterone (Bowen et al., 1997) suggesting that estradiol is its major modulator. In pigs, mRNA expression for different integrins was observed not only in the endometrium but also in blastocysts on placental tissues and cultured cells from trophoblast.

**2.3 Some factors affecting litter size**

It is obvious that the importance of the reproductive system in maintenance of a species; if a species cannot reproduce it will disappear. The efficiency of reproduction of farm animals has an additional importance to food production. Some species are
monotocous and others polytocous meaning that they produce one or more than one offspring, respectively. In polytocous species, food production might be enhanced by improving the efficiency of how these animals reproduce. A major trait that should be considered about these animals is litter size, the number of offspring produced per farrowing.

Average of litter size in pigs for the year 2006-2007 in the United States was 9.2 (NASS, USDA). Increasing litter size potentially will increase profitability for the swine industry (Rothschild, 1996; Tomes and Nielsen, 1982). Although litter size might be increased by selection, its heritability is low (Johnson et al., 1999). In addition, birth weight of offspring decreased in pigs selected for increased litter size (Johnson et al., 1999; Mesa et al., 2003). Low weight at birth was related to increased death of piglets (Damgaard et al., 2003); therefore selection for litter size might not be beneficial as other traits are influenced by this method. Other factors could potentially influence litter size. This review will discussed some of these factors including ovulation rate (OR), fertilization rate, embryonic/fetal losses, uterine capacity and placental efficiency.

2.3.1 Ovulation and fertilization rate

Ovulation rate represents the upper limit for litter size. Increasing ovulation rate might increase the probability of having oocytes of better quality for fertilization or to have embryos better synchronized with the uterine environment. Researchers have utilized ovulation rate in different models as a criteria to increase litter size.

Genetic selection has been utilized to increase ovulation rate in pigs (Bennett and Leymaster, 1990; Lamberson et al., 1991; Rosendo et al., 2007; Ruiz-Flores and Johnson,
In some of these investigations, selection for ovulation rate increased litter size up to 2 pigs (Lamberson et al., 1991). Although genetic selection for increasing ovulation rate seems promising it can take up to 14 generations to see an increase on 1.1 pig per litter and can result in detrimental effects such as increase in number of stillborns and decreased number of weaned pigs (Johnson et al., 1999). Therefore, as selection for litter size, it appears that selection for ovulation rate alters other traits in female reproduction resulting in poor benefits.

Treatment with an analogue for GnRH, Hoe766, increased ovulation rate in ewes (Findlay and Cumming, 1976) probably by increasing FSH secretion. In addition, immunization of ewes against inhibin increased ovulation rate (Henderson et al., 1984) perhaps by removing inhibition for FSH release. Furthermore, ewes treated with anti-LH serum also demonstrated increased ovulation rate as a result increase in FSH secretion (Fitzgerald et al., 1985). Moreover, FSH treatment has been observed to increase ovulation rate in gilts (Guthrie et al., 1997; Hunter, 1979). Interestingly, gilts treated with androgens (DHT or testosterone) during the follicular phase had increased number of corpora lutea (Cárdenas et al., 2002; Cárdenas and Pope, 1994). Collectively, these results prove that manipulation of the endocrine system can increase ovulation rate in different species. Interestingly a more recent survey suggests questioning the importance of ovulation rate in improving litter size in swine, as it is suggested that modern sows have ovulation rates that supply more than enough the amount of eggs to improve litter size (Vonnahme et al., 2002).

Fertilization rates are high in pigs as 95 to 100% of the ovulated oocytes are recovered as blastocysts between 7 to 12 days after mating (Pope, 1994). In beef heifers,
fertilization rates are greater than 85% and slightly lower in dairy heifers (Bridges, 2007). Therefore, fertilization rate in good management systems does not appear to be a critical aspect in determining reproductive performance of farm animals given that good practices are followed.

### 2.3.2 Embryonic/ Fetal losses

After fertilization, the zygote initiates a rapid process of cellular division without transcription of the embryonic genome. The 8-cells embryo then slows down cell division and the cell cycle, proceeding on to form a blastocyst. The resulting conceptus goes through a process of attachment which occurs around days 15 to 18 of gestation. Death of embryos from fertilization until attachment is complete are considered embryonic losses (Jainudeen and Hafez, 1987) and losses thereafter are termed as fetal losses. An estimated 30 to 50 % of the oocytes ovulated are lost during gestation (Pope, 1994). Some factors implicated in embryonic/fetal losses are oocyte or sperm quality, uterine-embryo asynchrony, uterine capacity and placental efficiency.

Embryonic losses can occur naturally by reproductive tract asynchrony with respect to embryo development. Pope et al. (1982) observed embryonic losses in pigs due to uterine-asynchrony. These authors utilized gilts on day 6 of the estrous cycle as recipients for day 5 and day 7 embryos. The different age-embryos were separately transferred into one uterine horn. No differences on embryo survival were observed when determined on day 11, however, by day 60 of pregnancy fewer fetuses that developed from the transferred day-5 embryos were recovered (only 8%) as compared to those that developed from day-7 embryos transplants (63%). In addition, Wilson et al.
(2001) transferred 2.5 days old blastocysts to gilts on day 3.5 of the estrous cycle and observed heavier fetuses and larger placentas than when blastocysts were transferred to gilts on day 2.5. Collectively, these observations suggest that a difference of simply one day on uterus-embryo asynchrony can have influence embryonic survival. Pope (1992) went on to discover that this natural, utero-embryo asynchrony is the result of asynchrony in ovulation.

Hormones can also influence embryonic/fetal survival. For example, inanition of sows reduced embryo survival but progesterone and estradiol supplementation reversed this negative effect (Anderson, 1975). Afterward, progesterone was suggested to mediate nutrition-related increases in embryonic survival (Jindal et al., 1997). In addition, peak concentrations of estradiol closer to the onset of estrus were greater in high (3 hours after) versus low (13 hours before) embryo survival gilts (Blair et al., 1994); therefore, estradiol concentrations appear to be related to embryonic survival. Furthermore, sows treated with ACTH every 4 h from onset of estrus to 48 h had significant loss of oocytes or embryos 60 hours after ovulation (Brandt et al., 2007). Moreover, Pope and First (1985) suggested that ovarian steroids such as progesterone and estradiol might influence embryonic survival by altering uterine secretions.

2.3.3 Uterine crowding/capacity

Litter size can also be influenced by embryonic/fetal losses from uterine crowding/capacity. Uterine crowding/capacity can limit litter size by affecting the number of concepti that the pig uterus can successfully carry to term (Ford et al., 2002). Probably one of the first investigations on uterine crowding was done in mice, where
superovulation increased embryo mortality at 18 days of pregnancy (McLaren and Michie, 1959). Other pioneer investigations utilized pigs where ligation of the uterus to restrict the uterine space decreased embryo survival by day 27 (Dhindsa and Dziuk, 1968; Dziuk, 1968). By day 25, unilateral hysterectomy of gilts did not influence the number of concepti but they decreased by day 105 of gestation (Fenton et al., 1970). Uterine capacity appears to be critical around 30 days of gestation (Fenton et al., 1970; Webel and Dziuk, 1974) when the number of embryos was reduced significantly. Furthermore, Wu et al. (1989) observed that length of the uterus was positively correlated with embryo survival. In addition, Chen and Dziuk (1993) determined a high correlation between prenatal survival of embryos and uterine length assigned initially. Regarding pigs, losses due to crowding commonly occur and insufficient space within the uterus can occur from day 30 to term.

### 2.3.4 Placental efficiency

Meishan gilts compared to Yorkshire had similar uterine size and length, however, Meishan gilts had larger litters (Ford, 1997). When placenta size was compared, it was determined to be smaller in Meishan gilts than other breeds at different times during pregnancy (Biensen et al., 1998; Hunter et al., 1994; Wilson et al., 1998). Furthermore, birth weight is lower on Meishan than Yorkshire offspring (Wilson et al., 1998). These scientists suggested that Meishan gilts had greater “placental efficiency”. Placental efficiency (PE) is defined as the ratio of fetal weight: placental weight. Placental area and weight were determined to be highly correlated (Biensen et al., 1998). Subsequently, PE was utilized as a selection method and demonstrated to increase litter
size in Yorkshire pigs (Wilson et al., 1999). The latter authors observed larger litters in gilts selected for increased PE as soon as the first parity. Birth weight of offspring was 20% lower for these animals and their placentae were 40% lighter. The smaller placenta of Meishan pigs have greater density of blood vessels (Biensen et al., 1998). Perhaps related, Yorkshire gilts selected for increased PE had increased expression of vascular endothelial growth factor mRNA on their placenta (Vonnahme and Ford, 2004). No negative effects were observed on production traits of offspring from gilts selected for increased PE (Wilson et al., 1999) therefore selection in favor of this trait might be a good method to increase litter size. The reverse was not true; selection for increased litter size was not related to placental efficiency (Mesa et al., 2003).

2.4 Effects of androgen on females

The word androgen was historically implicated with hormones influencing primarily the physiology of males. This perception has changed as numerous investigations have demonstrated effects of androgens such as androstenedione, testosterone, and 5α-dihydrotestosterone (DHT) on female physiology. As androgens are not historically consider “female” hormones, a complete section will be dedicated to discussing androgens as they are the basis of the investigations presented in this dissertation.

2.4.1 General actions of androgens on females

Androgens can influence many non-reproductive and reproductive organs and tissues in females. This section of the review is intended to present the literature explaining the effects of androgens effects on non-reproductive organs of females.
**Mammary tissues**

Prenatal treatment with testosterone suppressed nipple formation in female rats (Goldman et al., 1976). In contrast, dehydroepiandrosterone- or DHT-stimulated growth of lobuloalveoles and ducts in the mammary gland of rats and this effect was reduced by treatment with an AR but not ER antagonist (Sourla et al., 1998). Furthermore, Zhout et al. (2000) observed that co-treatment with testosterone and estradiol reduced proliferation and ERα expression of mammary epithelium in ovariectomized rhesus monkeys as compared to estradiol treatment. Moreover, Zhang et al. (2004) observed that DHT induced growth and secretion of mammary tissues in female rats when administered from 2 to 7 months of age. Therefore, androgens might influence mammary development depending on the species or stage of development.

Aromatase activity and proliferation of cancerous cells from mammary tissue are significantly correlated (Lu et al., 1996). In contrast, the high concentrations of androgens in pre-menopausal (Secreto et al., 1984) and post-menopausal women (tissue, Recchione et al., 1995; serum, Secreto et al., 1991), which have developed breast cancer, suggests a possible involvement of androgens on this disease. Androgen-induced proliferation of HC11 cells (cancerous cells obtained from mammary tissue of female rats) was negated when cells were co-treated with AR antagonists, cyproterone acetate or flutamide (Baratta et al., 2000). In contrast, Ortmann et al. (2002) observed that androgen treatment inhibited proliferation of different lines of cancerous cells from mammary tissue of women. Collectively, these results implicate androgens with mammary cancer although their actions on this disease can be confounded. Currently one of the treatments for women with breast cancer is androgen therapy.
Nervous tissues

The enzyme 5α-reductase has been found in all major divisions of the brain (Callard et al., 1978). Neurons and glial cells have 5α-reductase but neurons have more activity than glial cells (Celotti et al., 1991). These same authors suggested that neurons also have, but glial cells lack, the ability to convert testosterone to estradiol. Two isoforms of 5α-reductase are present in the brain of female rats and it appears that DHT is the primary regulator of 5α-reductase1 expression in the brain (Torres and Ortega, 2006). Moreover, ovariectomized mice treated with DHT in absence or presence of estradiol increased frequency of postsynaptic currents on gamma-aminobutyric acid neurons obtained from mice (Sullivan and Moenter, 2005). Perhaps related to the previous results, androgens were observed to increase the firing activity of GnRH-producing neurons even in the presence of progesterone (Pielecka et al., 2006). Female rats treated with androgen implants (Raber et al., 2002) experienced improved memory suggesting possible roles of androgens in function of nervous tissue. Collectively, these investigations suggest that androgens can alter function of nervous tissue.

In addition to influencing general functions of the brain, androgens can also influence the growth of neurons. Rats treated with testosterone during pregnancy had female offspring with reduced number of axons in the mammary nerve trunk (Huerta-Ocampo et al., 2005). In addition, Sullivan and Moenter (2005) observed that DHT treatment increased size of GnRH neurons obtained from mice. Similarly, treatment of ovariectomized rats with androgens increases the synaptic density in the hippocampus (Leranth et al., 2004). As androgens affect neuronal outgrowth, neurons in turn, may influence androgen synthesis in the ovary. Extracts of the superior ovarian nerve of rats
added to luteal cells of gilts, in vitro, inhibited LH stimulated androgen synthesis by
inhibition of the 17α-hydroxylase:C_{17-20} lyase complex (Morley et al., 1990).

Liver

Androgens influence hepatic physiology different between the sexes. In humans,
two different genes encode for 5α-reductase-1 and -2 (5α-R-1 and 5α-R-2, respectively
(Russell and Wilson, 1994) and although expressed throughout the body, they exhibit
different functions. The isozyme 5α-R-1 is expressed primarily in the liver (Deplewski et
al., 1997) and has been hypothesized to have a catabolic role, such as inactivation of
testosterone (Mahendroo et al., 1997). While the other isozyme, 5α-R-2, is more
prevalent in reproductive tissues (Russell and Wilson, 1994). In rats, the liver of females
has greater 5α-reductase activity than that of males (Yates et al., 1958). These authors
also observed that female liver metabolized more delta4-3-keto-steroids than that of
males. Less AR has been detected in the liver of female rats than males (Tejura et al.,
1989) suggesting sexual dimorphism in liver function. These differences between
gender, which might be androgen-related, perhaps influenced success of organ
transplantation in humans (Marino et al., 1995). Intriguingly, in gilts liver weight was
positively correlated with ovulation rate (Wise and Ford, 1998) by as yet an
undetermined relationship.

Exposure to androgens can alter hepatic development. Expression and activity of
male-specific enzymes in the liver (16α- and 6β- hydroxylases) were programmed by
androgen treatment during neonatal life of female rats (Dannan et al., 1986). Perhaps, of
significance to this thesis, androgens also alter uterine development.
Kidney

The activity of the enzyme ornithine decarboxylase is higher in male than female mice and treatment of female mice with testosterone propionate increased the activity of this enzyme (Sanchez-Capelo et al., 1999). In addition, the expression of the “kidney androgen-regulated protein” is greater in males than females and administration of testosterone to female rats increased expression of this protein mRNA in the epithelial cells of proximal tubules (Ding et al., 1997). The function(s) of this protein is still unknown but is usually utilized as a positive control when investigating androgens effects in the kidney. In contrast, DHT treatment decreased the expression of 16α-hydroxylase in the kidney of female mice when compared to untreated females (Melia et al., 1998). Collectively, these investigations suggest sexual dimorphism in the kidney, as well as the liver (above), that might be androgen-related.

Bone

Androgens have dramatic effects on building and maintaining bone mass in females. Evidence for this effect is available regarding different bones of rats. Ovariectomy in rats reduced the volume of cancellous bone from the proximal metaphysis, while androstenedione treatment of these rats increased bone formation (Lea et al., 1998). The latter observations were AR-dependent because antiandrogen treatment (Casodex) and not anti-aromatase treatment (Arimidex) negated the effects on bone formation (Lea et al., 1996; Lea and Flanagan, 1998). Furthermore, Martel et al. (1998) observed that mineral density in the femur of ovariectomized rats decreased by 11% (1 year after surgical procedure) and dehydroepiandrosterone or DHT treatment increased mineral density in this bone. In a contradictory experiment, cortical bone of tibial
diaphysis from ovariectomized rats responded differently to ovariectomy as androgen treatment (testosterone or DHT) suppressed, instead of augmented bone formation as observed in the ovariectomized group (Turner et al., 1990). Co-treatment with dehydroepiandrosterone and flutamide decreased mineral density in the femur suggesting again effects mediated by the androgen receptor. Flutamide treatment of females rats induced osteopenia by reducing bone formation (Goulding and Gold, 1993). Lea and Flanagan (1999) reinforced the idea that androgens have a skeletal-protective influence when they failed to detect aromatase mRNA in the bone of female rats and observed that reduction on cancellous bone from proximal metaphysis caused by ICI 182,780 (antagonist of estrogen receptor) was further enhanced with co-treatment of the antiandrogen, Casodex. Therefore, the response to androgens might depend on the bone tissue affected.

In women, androgenic effects on bone mass are similar to those of rats. Androgen receptors are present in all cells of human bone but primarily in osteoblasts (Colvard et al., 1989). Watts et al. (1995) observed that orally co-treatment of estrogens and androgens increased mineral density of the lumbar spine and hip of women even more than estrogen only. Similar results on the same bones have been observed with implants, instead or oral administration, of estrogen and testosterone versus estrogen alone (Davis et al., 1995). While administration to postmenopausal women with oral estrogens decreased markers of bone formation in the lumbar spine, administration of estrogens with androgens increased these markers by at least from 3 weeks after treatment (Raisz et al., 1996). Therefore, it can be concluded that androgen treatment is favorable to
postmenopausal women as many suffer from osteoporosis and presently is an accepted treatment for this disease.

Premenopausal women as well can benefit from androgen therapy on bone tissues. Buchanan et al. (1988) determined a positive correlation between androgen concentrations in the serum and density of lumbar bone in premenopausal women. Young women who experience anorexia nervosa suffer significant loss of bone tissue (Gordon et al., 2002). Dehydroepiandrosterone treatment of young women experiencing bone loss increased mineral density in the hip and markers for bone formation in the serum (Gordon et al., 2002). Collectively, androgens appear to be beneficial to treat women of all ages suffering from some form of osteoporosis.

2.4.2 Androgens and female reproduction

Androgens are produced in the ovary primarily by theca cells in response to LH stimulation. These androgenic steroids can in turn serve as substrates for the synthesis estrogenic compounds. As mentioned earlier, in the ovary, testosterone or androstenedione are converted to estradiol or estrone, respectively, by aromatase (Fortune and Armstrong, 1977). In addition, testosterone can be converted to a more potent androgen (higher affinity for the AR for DHT versus testosterone), DHT, by the enzyme 5α-reductase. Although DHT cannot be aromatized to estradiol and for years it was thought to act exclusively through AR, recent evidence demonstrated that its metabolite, 3ßdiol, binds to both ERs and more preferentially than to androgen receptor (Kuiper et al., 1997; Weihua et al., 2002b). The following discussion will review effects of
androgens in female reproduction and attempt to partition those effects through the AR from those as substrate for estrogenic compounds.

Concentrations of testosterone and DHT in the plasma of gilts, every other day during the estrous cycle, have been determined in our laboratory (Figure 2.2; E. Routman, E. Jiménez, H. Cárdenas and W.F. Pope, unpublished data). The concentration of DHT in systemic blood is low and does not change during the cycle. However, testosterone increased during the luteal phase, transiently decreased from days 14 to 16 (day 0 = first day of estrus; gilts averaged a 19.5 day cycle) and increased again before estrus.

Perhaps the rise in systemic testosterone late in the luteal phase is from the CL, as has been observed in cattle (Shemesh and Hansel, 1974). In rats, the activity of 5α-reductase peaks coincident with corpora lutea formation (Lephart et al., 1992). Perhaps related, Haning et al. (1996) observed that within the human ovary, 5α-reductase is greater in the corpus luteum than stroma or follicle tissues. In addition, activity of 5α-reductase within the follicle was observed to be mostly in stroma and theca and in these cells it is stimulated by LH (Payne et al., 1992). The amount of 5α-reductase activity observed in granulosa cells was relatively low and inhibited by FSH treatment (Payne et al., 1992). Furthermore, in rat granulosa cells, insulin growth factor-1 slightly elevated 5α-reductase activity whereas, FSH did not alter the activity of the enzyme (deMoura et al., 1997). Our laboratory examined differences in arterial versus venous plasma from the ovarian vessels (E. Routman, E. Jiménez, H. Cárdenas and W.F. Pope, unpublished data). In that experiment it was demonstrated that the ovary of pigs is a source for testosterone but not for DHT. Thecal and granulosa cells from humans (Jakimiuk et al.,
1999), cattle (Wise and Fields, 1978) and rats (Payne et al., 1992) have demonstrated 5α-reductase activity. Interestingly, 5α-reductase activity was observed to be around four fold in follicles obtained from women with polycystic ovarian syndrome when compared to women with normal menstrual cycles (Jakimiuk et al., 1999). The latter investigations suggest that endogenous 5α-reductase activity influences proliferation of ovarian cells. Since differences on DHT concentrations between the ovarian artery and vein did not exist in plasma of gilts, the ovary cannot be consider an active source of endogenous DHT. Perhaps the adrenal cortex might be the source of these low amounts of DHT observed in gilts (Figure 2.2).

2.4.2.1 Androgens and ovarian follicles

Androgens might be involved in follicular health. The number of granulosa cells demonstrating apoptosis in monkeys demonstrating apoptosis was reduced with DHT treatment (Vendola et al., 1998). In women, Otala et al. (2004) observed reduced apoptosis in ovarian tissue in the presence of DHT versus the lack of DHT in the medium. Furthermore, our laboratory recently demonstrated that DHT treatment for 4 or 6 days increased number of healthy follicles in gilts treated on day 17 and 19 of the estrous cycle, respectively (Cárdenas et al., 2008). Moreover, testosterone or DHT treatment during the follicular phase of pigs increased ovulation rate (Cárdenas et al., 2002; Cárdenas and Pope, 1994).

Follicular growth can also be influenced by androgens in species other than pigs. Cellular proliferation and diameter of preantral follicles increased when female mice were treated with various androgens (Wang et al., 2001). These effects were negated
when animals were co-treated with testosterone and flutamide (an AR antagonist) but not when co-treated with testosterone and fadrozole hydrochloride hydrate (an aromatase inhibitor) suggesting an AR response. Murray et al. (1998) observed slower growth of

![Graph showing concentrations of testosterone and dihydrotestosterone in blood samples.](image)

**Figure 2.2** Concentrations of testosterone and dihydrotestosterone in blood samples obtained every other day during the estrous cycle of gilts.

mice follicles when cultured in the presence of an anti-androgen serum or androgen receptor antagonist and this effect was reversed when androstenedione or DHT were added to the medium. In pregnant ewes, Steckler et al. (2005) observed that testosterone treatment decreased number of primordial follicles but increased number of follicles at
other stages of development in their offspring. Furthermore, an increase in the number of
primary follicles (Vendola et al., 1999) and, preantral and small antral follicles (Vendola
et al., 1998) was observed in the ovaries of rhesus monkeys treated with DHT or
testosterone. Treatment of ovarian follicles from cattle with testosterone increased the
number of secondary follicles and this was negated when co-treated with testosterone and
flutamide or was not observed when cells were treated with estradiol (Yang and Fortune,
2006) suggesting androgenic actions. Collectively, the results in pigs and other species
suggest positive effects of androgens in follicular growth.

The cell cycle can be disrupted by androgen treatment. For example, Pradeep et
al. (2002) observed 70% of granulosa cells obtained from immature rats at G1 phase and
26% at S phase of the cell cycle when cultured for 48 hours in the presence of DHT in
comparison to 42 and 55% on G1 and S phase, respectively, in non-treated cells. The
latter results suggest slowing of the cell cycle after DHT treatment. This attenuation of
the cell cycle might have occurred by a decreased expression of cyclin D2 that was
observed both in vitro and in vivo (Pradeep et al., 2002).

Localization of the AR in follicles has been well characterized. Expression of AR
has been demonstrated in granulosa cells of various mammals (pigs, Cárdenas and Pope,
2002b; cattle, Hampton et al., 2004; Hillier and Tetsuka, 1997; human, Horie et al., 1992;
rats, Tetsuka et al., 1995; non-human primates, Vendola et al., 1998). Within the porcine
follicle, greater amounts of AR have been detected in the granulosa cells of preantral,
small and medium than larger follicles (Cárdenas and Pope, 2002b; Garrett and Guthrie,
1996; Slomczynska and Tabarowski, 2001). During late development of follicles, the
amount of AR was observed to further decrease in these preovulatory follicles (Cárdenas
and Pope, 2002b; Garrett and Guthrie, 1996; Tetsuka et al., 1995). However, intensity of staining for the AR protein in cells of pre-antral and small follicles remained rather constant during the estrous cycle and only decreased just before the onset of estrus (H. Cárdenas and W.F. Pope, unpublished data). Specifically, theca cells contain 40 to 60% less AR than granulosa cells and, unlike granulosa cells, AR in theca are not influenced by day of the cycle (H. Cárdenas and W.F. Pope, unpublished data). Curiously, in the sheep ovary, AR has been observed as early as day 55 of fetal life in stroma, granulosa and thecal cells (Juengel et al., 2006). Horie et al. (1992) localized AR in the ovaries of women during most stages of the menstrual cycle. Hence although it is suggested that DHT acts through the AR, identification of the existence of the AR in follicles at these various stages support the earlier conclusions.

Probably the best model to confirm AR effects is the knock-out animal. Female mice in which the AR was knocked out (ARKO) were fertile but litter size was reduced in comparison to wild type mice (Yeh et al., 2002). In addition, Hu et al. (2004) observed longer estrous cycles and reduced number of corpora lutea in ARKO mice. These authors also observed increased apoptosis in the various follicular stages in ARKO mice when they were triggered to ovulate by treatment with human chorionic gonadotropin. More recently, it was observed that ARKO mice had about half offspring at 8 weeks of age which continue decreasing up to 34 weeks (Shiina et al., 2006). At 36, 38 and 40 weeks of age, these mice were infertile. Serum concentrations of estradiol, FSH, T, progesterone, and LH were not altered at 8-10 weeks of age. In addition, these authors observed that branching and elongation of mammary ducts were reduced on ARKO
female mice. Furthermore, follicular atresia at 8 weeks of age was increased and the number of corpora lutea reduced in ARKO females (Shiina et al., 2006).

Androgens have been associated with infertility in females. Hillier et al. (1979) observed a decreased ovarian weight when rats were treated for 4 days with testosterone. In addition, Bagnell et al. (1982) observed that a single injection of DHT to rats previously treated with equine chorionic gonadotropin, also decreased ovarian weight. This DHT treatment negatively influenced ovulation as it reduced the number of oocytes collected from the oviducts. Somewhat related, a single injection of 1 mg of DHT increased the duration of diestrus, decreased number of large follicles and impaired fertility (reduced pregnancy rates and number of fetuses) in female rats (Nandedkar and Munshi, 1981). Furthermore, Conway et al. (1990) observed a decreased ovulation rate in rats treated with 1 mg of DHT, apparently a direct effect on the ovary as FSH concentrations and the LH surge were not altered. Lower dosages of DHT did not have any effects. As observed by the latter investigations many of the negative effects of androgens in fertility have been demonstrated in rats, but the positive effects of androgens in ovarian function in pigs merit further investigation as to fully take advantage of this treatment.

Androgens effects have also been observed in females of non-mammal vertebrates. Rangel et al. (2006) observed that treating laying hens with flutamide reduced egg laying as soon as 24 hours and ovulation was blocked in all hens after two days of treatment. Flutamide treatment reduced progesterone, estradiol and LH surges related to preovulatory events. Passive or active immunization against testosterone blocks ovulation in laying hens and induces atresia of pre-ovulatory follicles (Rangel et
al., 2005) even when follicles reach pre-ovulatory size. Therefore, androgen treatment should be investigated in these animals as it might be beneficial to increase egg production.

### 2.4.2.2 Androgens interactions with other hormones

Androgens might influence gonadotropin actions in follicles. Androgen treatment of FSH-stimulated granulosa cells, obtained from rats, reduced the amounts of LH receptor (Jia et al., 1985). However, in another experiment the effects of androgens on FSH was more evident as, Weil et al. (1999) observed increased expression of FSHR by 50 to 100% in granulosa cells of rhesus monkeys treated with testosterone. Hillier and De Zwart (1981) demonstrated that granulosa cells obtained from rats and co-cultured with recombinant human FSH and testosterone, had more aromatase activity in comparison when cells were co-cultured with hFSH and DES (an estrogen) or hFSH and progesterone. In the first experiment of this type with pigs, Cárdenas et al. (2002) observed increased expression of FSHR in pre-ovulatory follicles treated with DHT but recently this effect was not repeated (Cárdenas et al., 2008).

Androgens can also influence the amounts of gonadotropins. When ewes (Campbell et al., 1990; Martensz et al., 1979) and gilts (McKinnie et al., 1988) were immunized against androstenedione, secretion of LH increased. In contrast, exogenous testosterone increased FSH secretion in female rats (Naqvi and Johnson, 1969) and ewes (Radford and Wallace, 1971). Recently, Burger et al. (2007) observed that female rats implanted with DHT had increased concentrations of FSH in their serum as compared to vehicle or testosterone-treated animals.
Androgens can also affect hormones other than gonadotropins. Vendola et al. (1999) treated female rhesus monkeys with T and DHT and observed a three to four fold increased expression of insulin-like growth factor 1 and its receptor on granulosa and thecal cells in follicles ≤ 1 mm of diameter. In addition, androgens also appear to stimulate progesterone secretion. Schomberg et al. (1978) observed that granulosa cells obtained from pigs and cultured in the presence of flutamide had decreased progesterone secretion. Moreover, androgen treatment (DHT or methyltrienolone) increased progesterone secretions from granulosa cells obtained from rats (Welsh et al., 1982). Hillier et al. (1977) observed approximately 3 to 11 times greater concentrations of progesterone in the medium of granulosa cells cultured in the presence of increasing dosages of testosterone and this stimulus was blocked when cells were co-treated with testosterone and flutamide.

2.4.2.3 Androgens and the uterus

Although utilizing multi-cell type (whole) cultures of endometrial tissues, 5α-reductase activity has been known to exist in gilts for a considerable period of time (Fischer et al., 1985; Henricks and Tindall, 1971) it was not until recently that cell specific localization of this enzyme had been determined. In the human endometrium, 5α-reductase was localized in epithelial, but not stromal (Ito et al., 2002) nor myometrial (Bulun et al., 1994) cells.

Specific localization of AR has been observed in tissues of the pig uterus. Cárdenas and Pope (2003) observed greater immunostaining of AR in luminal epithelium and glandular epithelium versus myometrium of cyclic gilts. Interestingly, these authors
increased AR mRNA in whole endometrium with estradiol treatment of ovariectomized
gilts when compared to the non-treated group. In that experiment, progesterone co-
treatment with estradiol blocked the estradiol-induced increase in AR mRNA. Similarly,
Weihua et al. (2002a) also observed increased AR immunostaining in uterus of immature
rats treated with estradiol. The AR has also been localized in the uterus of other species
for example in humans (endometrium and myometrium, Kimura et al., 1993) and dogs
(epithelium, stroma and myometrium, Vermeirsch et al., 2002). The presence of the AR
in the uterus and observations of its regulation (partial) by estradiol suggests a role of the
AR in normal physiology of the uterus.

Endometrial expression of ERß mRNA was augmented while ERα expression
remained unchanged after DHT treatment (Cárdenas and Pope, 2005) but the implications
of the increase in ERß are still unknown. In that investigation, it was also observed that
DHT treatment reduced complement C3 mRNA (a gene responsive to estrogens).
Expression of complement C3 is implicated in immune responses (Mastellos et al., 2003;
Suresh et al., 2003) and perhaps DHT treatment can compromise the “sterile” nature of
the uterus. Moreover, treating ovariectomized rats with flutamide disrupted the ability of
estradiol to increase PR in the uterus (Chandrasekhar and Armstrong, 1991). Collectively
it can be suggested that hormonal actions within the uterus, by controlling receptor
expression, can be affected with androgens.

Uterine anatomy is also influenced by androgens. Ovariectomized rats treated
with DHT or mibolerone (AR agonist) had increased uterine weight and cross-sectional
area of the endometrium and myometrium (Nantermet et al., 2005). Although androgen
treatment also increased epithelial height, estradiol-induced increases of this variable
were suppressed with co-treatment of mibolerone suggesting different mechanisms of action of these hormones. In pigs, Cárdenas and Pope (2005) observed a tendency to decrease in uterine wet weight with DHT treatment from day 13 to 18 but not from day 13 to 16 of the estrous cycle. Perhaps by the end of the estrous cycle, uterine tissues are more sensitive to androgens. Treatment with 17α-methyltestosterone increased uterine weight and epithelial height from mice which were blocked by co-treatment with flutamide (Papaconstantinou et al., 2002) suggesting once more effects mediated by the androgen receptor in the uterus. In addition, treating rats with flutamide decreased fetal weight on day 19 of pregnancy while placental weight was not influenced (Chandrasekhar and Armstrong, 1991), therefore the AR might be involved in placental efficiency.

On day 3 of the estrous cycle, recovery of embryos was similar between gilts treated with DHT or vehicle but the recovery of day 11 embryos was reduced with DHT treatment (Cárdenas et al., 2002). On the other hand, Chandrasekhar et al. (1990) observed that flutamide treatment delayed the time of implantation by at least 2 days and delayed day of delivery by 1 day but no effects were observed on litter size or birth weight. As a result, it can be concluded that androgens might be involved in uterine support of embryonic development and attachment.

2.5 Nongenomic effects of androgens

To date, the most recognized mechanism of action for steroidal hormones, such as androgens, is the genomic pathway. This concept is being expanded as increasing evidence is accumulating about other mechanisms of action for steroids. Newer, so
called nongenomic pathways are being proposed suggesting mechanisms mediated by other than stimulation of gene transcription directly and occurring within seconds or a few minutes. As will be discussed below, nongenomic effects of androgens have been demonstrated in many cells types of mammals and they can be ligand only, receptor only or ligand-receptor dependent. The following review will present some newly discovered nongenomic effects of androgens.

2.5.1 Nongenomic effects of androgens on reproductive cells

Granulosa cells obtained from preovulatory follicles and treated with androstenedione had increased concentrations of intracellular calcium (Ca\(^{2+}\)) that occurred within 5 seconds of treatment (Machelon et al., 1998). These fast responses were blocked when flutamide was administered suggesting an involvement of the androgen receptor. Furthermore, these membranous responses appeared to involve phospholipase C activation and pertussis toxin-sensitive G-proteins as inhibitors for these proteins blocked the androstenedione effects. Perusquia et al. (2005) observed that DHT treatment of myometrium, from pregnant or nonpregnant women, induced relaxation of this muscle but flutamide or actinimycin D (transcription inhibitor) treatment failed to alter these effects. Collectively, these observations suggest that DHT mediated these actions through mechanisms other than genomic pathways; perhaps nongenomic pathways.

2.5.2 Nongenomic effects of androgens on immune cells

Androgen treatment of immune cells can influence intracellular calcium. For example, testosterone treatment increased Ca\(^{2+}\) concentrations in T-cells obtained from
female mice within a few seconds (Benten et al., 1997). Similar to the nongenomic effects discuss above with granulosa cells, the fast response with immune cells was not altered by cyproterone (an AR antagonist). Benten et al. (1997) did observe that cells treated with Ni$^{2+}$ (inhibitor of Ca$^{2+}$ channels) blocked the effects of testosterone suggesting that these androgenic actions were mediated by Ca$^{2+}$ channels. Furthermore, macrophages obtained from ARKO mice had increased Ca$^{2+}$ when treated with testosterone (Benten et al., 1999). In contrast to the earlier investigation on T-cells, this increase appears to involve release of Ca$^{2+}$ from intracellular storage (Benten et al., 1999). Furthermore, Popova et al. (2007) observed that human lymphocytes treated with testosterone or DHT increased Ca$^{2+}$ concentrations in these cells. Addition of cyproterone did not alter these results suggesting AR-independent actions of these androgens. All the above observations support the androgenic effect of DHT or testosterone on immune cells to increase Ca$^{2+}$ were a membrane associated event.

2.5.3 Nongenomic effects of androgens on muscle cells

Androgens can also induce nongenomic effects in muscle cells. Testosterone treatment of skeletal muscle obtained from rats increased intracellular amounts of Ca$^{2+}$ and inositol triphosphate through a G-protein receptor mechanism (Estrada et al., 2003; Vicencio et al., 2006). Furthermore, Gonzalez-Montelongo et al. (2006) observed that AR induced contractility of ileal tissue continued even when transcription or protein synthesis were blocked suggesting nongenomic actions. Reduced contractility of aortic tissue was observed within 2 minutes of DHT treatment (Perusquia et al., 1996). In addition, Ceballos et al. (1999) observed that testosterone treatment increased vascular
resistance and blocked vasodilatation. Therefore, people that expose themselves to high concentrations of androgens might suffer clinical implications.

2.6 Statement of the problem

The latest survey from the US Department of Agriculture determined the average litter size (number of piglets born per farrowing) in the nation was currently 9.2 pigs. The average litter size can be potentially increased and this would be important to profitability of the swine industry. Increasing litter size would potentially allow better usage of space and facilities. Although many efforts have been put to increase litter size, domestic breeds still average less piglets per farrowing as compared to Chinese Meishan pigs.

As shown in this review of the literature, litter size can be influenced by the function of hormones on the ovaries and uterus. Previous investigations from our laboratory have increased ovulation rate with androgen treatment. Although androgen treatment increases ovulation rate in gilts, embryonic survival is decreased. The endocrine implications of androgen treatment on gilts are currently unknown.

Litter size in adult pigs, might also be improved by augmenting development of the endometrium and myometrium during the first 14 days in the life of the female. As discussed in this literature review, the neonatal uterus is responsive to estradiol and relaxin. To date, there is no information on the neonatal effects of androgens in uterine development of the pig. Therefore, the objectives of the investigations presented in this dissertation were to examine the effects of androgen treatment; 1) on the concentrations
of gonadotropins and ovarian steroids in the serum of adult gilts during the follicular phase and 2) on uterine development in neonatal gilts up to 14 days of age.
CHAPTER 3
EFFECTS OF ANDROGENS ON SERUM CONCENTRATIONS OF 
GONADOTROPINS AND OVARIAN STEROIDS IN GILTS

ABSTRACT

To examine how androgens increased ovulation rate, gilts were injected with androgen receptor agonists, antagonist or a combination of both. Blood samples were collected hourly from day 13 to estrus (day 0= onset of estrus) coincident with gilts (n=6) receiving daily treatments of; vehicle (corn oil), 10 mg of testosterone, 10 mg of 5α-dihydrotestosterone (dihydrotestosterone), 1.5 g of flutamide (an androgen receptor antagonist), testosterone plus flutamide or dihydrotestosterone plus flutamide. Treatment of gilts with testosterone or dihydrotestosterone alone increased (P<.05) mean concentrations of FSH in serum and this effect was blocked by co-treatment with flutamide. Mean concentrations of estradiol-17β and androstenedione were increased (P<.05) at two hours after injection of testosterone or testosterone plus flutamide but not after dihydrotestosterone treatment; probably due to the role of testosterone as a substrate for estradiol-17β and androstenedione synthesis. There were no effects of the six treatment regimens on serum concentrations of progesterone during luteolysis, but
treating gilts with testosterone shortened (P<.05) the proestrous period. Treating gilts with dihydrotestosterone increased the subsequent ovulation rate (P=.06) but decreased embryonic survival and these effects were negated when co-treated with flutamide. Results of this experiment indicate that androgen actions, mediated by the androgen receptor, increased FSH concentrations which might be related to the observed increase in ovulation rate.

**INTRODUCTION**

Ovulation rate (OR), a factor important to litter size in swine (Pope and First, 1985), is affected by the number of follicles recruited and the number of follicles that undergo atresia. Follicular recruitment appears to be mainly controlled by FSH (Foxcroft and Hunter, 1985; Guthrie et al., 1988) while FSH and LH are necessary for the final growth and steroidogenesis of pig follicles (Quesnel et al., 1998). Testosterone (T) and androstenedione serve as substrates for the synthesis of estradiol-17β in pig granulosa cells (Evans et al., 1981) while estradiol-17β is related with the health of the follicle (Guthrie et al., 1993) and induces estrous behavior. These hormones, alone with other signals, interact in a complex manner to regulate the growth and ovulation of follicles.

Although androgens in females have been associated with altering gonadotropins and ovarian steroids, their effects were confounded. For example, secretion of LH and progesterone were increased by immunizing ewes (Campbell et al., 1990; Martensz et al., 1979) and gilts (McKinnie et al., 1988) against androstenedione. In contrast, exogenous
T increased FSH secretion in female rats (Naqvi and Johnson, 1969) and ewes (Radford and Wallace, 1971) hypothetically through the conversion of T to estradiol-17β. Recently, Burger et al. (2007) observed that female rats implanted with 5α-dihydrotestosterone (DHT) had increased concentrations of FSH in their serum as compared to vehicle or T-treated animals. However, treating rats with DHT decreased OR without altering FSH or LH secretions (Conway et al., 1990). Conversely, our laboratory has demonstrated that androgens can increase OR (Cárdenas et al., 2002; Cárdenas and Pope, 1994) and litter size (Cárdenas and Pope, 2002a) in pigs.

The increase in OR observed in gilts might be due in part to androgen enhancement of follicular development through proliferation of granulosa cells and/or augmenting responsiveness of follicular cells to gonadotropins (Vendola et al., 1999; Vendola et al., 1998; Weil et al., 1999). Alternatively, the increase in OR after androgen treatment of gilts might be related to changes in gonadotropin or ovarian steroid secretion. In this experiment the latter was investigated; how exogenous androgens affect changes in serum concentrations of gonadotropins and ovarian steroids. In addition, the specific involvement of the androgen receptor (AR) in increasing OR in gilts was investigated since androgens can bind to nuclear receptors (AR; genomic pathway), membranous receptors (Falkenstein et al., 2000) or be metabolized into estrogen ligands (Pak et al., 2005).

**MATERIALS AND METHODS**

Crossbred gilts obtained from the university herd were observed in pens for estrus, twice daily with boars. Animals were included in the experiment if the duration of two consecutive estrous cycles was between 19.5 and 20.5 days (day 0= onset of estrus).
Thirty six gilts (n=6 per treatment) were assigned randomly to one of six treatments; vehicle (corn oil), T (10 mg/day), DHT (10 mg/day), flutamide (F, 1.5 g/d), T plus F and DHT plus F daily injections from day 13 to onset of the estrous cycle. Similar dosages of androgens were previously observed in our laboratory to increase OR in gilts (Cárdenas et al., 2002; Cárdenas and Pope, 1994). The dosage of F to block the AR was similar, on a per kg of body weight basis, to that used in rats (Kellogg and Lundin, 1999). Testosterone and DHT were dissolved, and F suspended, in vehicle before injection into the muscular tissue. Animals treated with F did not enter the food chain.

A cannula was placed into a jugular vein of each gilt on day 9 of the estrous cycle. Following cannulation procedures, a period of 4 days was allowed for the gilts to adapt to being moved into individual stalls. On day 13, blood was sampled hourly (5 to 10 ml depending on how many hormones were assayed at that time) and continued until the onset of estrus. Injections of the hormones were conducted at 8:00 AM each day and continued until the onset of estrus. For gilts due to be sampled at 8:00 AM, blood samples were collected immediately before the daily hormone treatments. Blood was allowed to clot at 4 °C and resulting serum stored at -20 °C until analysis.

Gilts were temporarily removed twice-a-day from their stalls for estrous detection utilizing boars. When gilts came into estrus, the cannulas were removed and gilts mated to different boars, 12 and 24 hours later. Subsequently, an ovariohysterectomy was performed on day 11 for purposes of counting the number of corpora lutea and blastocysts. Blastocysts were recovered by flushing the uterus with a saline solution (0.9%, w/v, sodium chloride). All procedures were approved and supervised by The
Ohio State University, College of Food, Agriculture and Environmental Sciences Animal Care and Use Committee.

Gonadotropins in serum were quantified by double antibody RIA (modification of Acosta et al. (1983). Purified FSH (AFP-10640B) and LH (AFP-11043B) from pigs, and antiserums (AFP-2062096 and AFP-15103194 for FSH and LH, respectively) were purchased from A.F. Parlow, National Hormone and Peptide Program, Torrance, CA. For FSH determination the standard curve consisted of 1.0 to 40.0 ng/ml while LH consisted of 0.25 to 20.0 ng/ml. Antiserums were diluted to 1:400,000 and 1: 3,000,000 (final dilutions in assay tubes) for FSH and LH, respectively. The second antibody (goat-anti rabbit IgG obtained from Lampire Biological Laboratories, Pipersville, PA) was diluted 1:32 and 1:20 for FSH and LH, respectively. The sensitivity was 0.20 and 0.17 ng/ml for FSH and LH, respectively. The inter- and intra-assay coefficients of variation (CV) for a sample containing 3.5 ng FSH/ml were 5.5 and 7.8%, respectively; for a sample containing 0.90 ng/ml these values were 14.5 and 14.7%, respectively. The inter- and intra-assay CV for a sample containing 4.6 ng LH/ml were 7.2 and 15.7%, respectively; for a sample containing 1.6 ng/ml these values were 6.2 and 9.4%, respectively.

The concentrations of estradiol-17β were determined by a single antibody RIA (modification of Anderson et al., 1996). The standard curve ranged from 2.0 to 50.0 pg/ml. The sensitivity was 1.1 pg/ml. The inter- and intra-assay CV for a sample containing 10.5 pg/ml were 7.4 and 13.6%, respectively; for a sample containing 23.5 pg/ml these values were 10.0 and 11.5%, respectively.
Progesterone, T and androstenedione were analyzed using commercial kits (Coat-A-Count Progesterone, Coat-A-Count Total Testosterone and Coat-A-Count Direct Androstenedione, all obtained from Diagnostic Products Corporation, DPC; Los Angeles, CA). For the analysis of progesterone, a parallelism test indicated that serum samples needed to be diluted, therefore 50 µl of PBS containing 0.1% gelatin (PBS-Gel) were added to 50 µl of serum. Subsequently, manufacturer procedures were followed. The sensitivity (given by the manufacturer) was 0.02 ng/ml. The inter- and intra-assay CV for a sample containing 18.6 ng/ml were 4.1 and 3.4%, respectively; for a sample containing 1.1 ng/ml these values were 4.3 and 15.1%, respectively. For the analysis of T, 500 µl of serum samples were extracted as previously described for estradiol-17β analysis (Kojima et al., 1992). Samples were reconstituted with 60 µl of testosterone matrix calibrator (Total Testosterone Matrix Calibrator, DPC). Subsequently, manufacturer procedures were followed. The sensitivity (given by the manufacturer) was 0.04 ng/ml. The inter- and intra-assay CV for a sample containing 14.1 ng/ml were 8.8 and 16.5%; for a sample containing 0.2 ng/ml these values were 8.4 and 12.9%, respectively. Androstenedione was analyzed similar to T with the modification that standards were also extracted and 110 µl of PBS-Gel were utilized for reconstitution of the samples. Subsequently manufacturer procedures were followed. The sensitivity (given by the manufacturer) was 0.04 ng/ml. The inter- and intra-assay CV for a sample containing 11.4 ng/ml were 4.8 and 3.7%, respectively; for a sample containing 0.6 ng/ml these values were 8.5 and 3.1%, respectively.

The concentrations of FSH in serum were analyzed in every 2 hour (even hour) samples because of its slower pulsatile nature than LH which was analyzed in each
hourly sample. Progesterone and T were analyzed at 0 and +12 hours (0 hours = 8:00 AM), estradiol-17β at 0, +2 and +12 hours and androstenedione at +2 hours each day. Gilts began exhibiting estrus on day 18 and to avoid the increase in LH concentrations associated with preovulatory surge, hormonal changes were analyzed from days 13 to 17. One of the gilts treated with T was removed from the experiment because her serum concentration of progesterone on day 13.0 were significantly lower (9.8 ng/ml) than the rest of the animals (32.0 ± 6.9 ng/ml) and was determined to be undergoing luteolysis prematurely.

Heteroscedastic data (determined by use of Levene’s test) were logarithmically transformed and untransformed means are presented. Hormonal observations were subject to repeated measures analysis of variance. The model included treatment, day and hour as main effects and the respective interactions. For illustration purposes, and because there were no hour x day interactions, daily means of FSH and LH concentrations are presented. Differences in length of the estrous cycle, length of proestrus (from day when progesterone concentrations were less than 2 ng/ml until onset of estrus), and number of corpora lutea were determined by utilizing one-way ANOVA and means compared by utilizing LSD tests. No statistical analysis was conducted for embryonic survival data as no embryos were recovered from gilts in one treatment group. The data analyses for this paper were generated using SAS/GLM software, Version 9.1 of the SAS System for Windows, SAS Institute, Inc., Cary, NC, USA.

RESULTS

Treatment of gilts with either DHT or T increased (P<.05) the mean concentrations of FSH in serum (Figure 3.1). This increase represented a 31 and 38%
elevation in FSH concentrations, across days (no interactions), for gilts treated with DHT (1.7 ± 0.03 ng/ml) and T (1.8 ± 0.03 ng/ml), respectively, above gilts treated with vehicle (1.3 ± 0.03 ng/ml). Treatment with DHT plus F resulted in FSH concentrations that were not different than gilts treated with vehicle and were less (P<.05) than gilts in the DHT treatment group. This antagonistic effect of F was also evident with treatment of gilts with testosterone.

Treatment with DHT increased the mean concentrations of LH on day 13 when compared to T and DHT plus F treatments (Figure 3.2; treatment x day interaction; P<.03 and P<.08, respectively). No other differences were observed in LH concentrations after day 13.

The above changes in FSH and LH were considered from days 13 to 17 to allow comparing how the various treatments affected gonadotropin secretion in a chronological manner. Analyzing FSH and LH data backwards, relative to estrus (from -5 to -1 days before the onset of estrus, data not shown), failed to alter the interpretation of how androgens affected gonadotropin secretion.

Changes in estradiol-17β secretions were considered in both a forward (chronological) and backward orientation (with estrus as the end point) because each has a meaningful interpretation. Analyzing these data in the forward direction, from day 13 to 17, it was observed that the mean concentrations of estradiol-17β were increased (P<0.05) in samples taken +2 hours after injecting T or T plus F (6.5 ± 0.8 or 6.9 ± 0.8 pg/ml, respectively) as compared to gilts treated with vehicle, F, DHT or DHT plus flutamide (2.3 ± 0.8, 3.0 ± 0.8, 3.8 ± 0.8 or 3.4 ± 0.8 pg/ml, respectively). Thereafter, within each day at the 0 or +12 hours samples, treatments had no effect on serum
concentrations of estradiol-17β. As expected, estradiol-17β concentrations increased (P<.01) as gilts came into estrus (Fig. 3.3; backward orientation relative to estrus). Estradiol-17β concentrations the day before estrus tended (P=.10) to be greater in gilts treated with DHT when compared to vehicle treatment (Figure 3.3).

Androstenedione concentrations were increased by T or T plus F treatments, similar to changes in estradiol-17β concentrations, in samples collected at +2 hours (Figure 3.4). On days 15 and 16 androstenedione concentrations of gilts treated with T plus F were lower than T (P<.05) but both were still greater than the other treatments.

Progesterone, like estradiol-17β, was considered in a forward (chronological) and backward orientation. Chronologically, the rate of luteolysis was apparently not affected by treatments as the serum concentrations of progesterone did not differ (Figure 3.5). In a backward orientation, changes in progesterone concentrations were utilized to determine the length of proestrus in individual gilts. Proestrus was defined as the interval from when progesterone concentrations in the serum dropped below 2 ng/ml during luteolysis, to the onset of estrus. Treatment of gilts with T or T plus F resulted in a shorter (P<.05) proestrus (Table 3.1) and declining concentrations of progesterone that were higher (P<.05) in those treatment groups from days -5 to -3 than the other treatments (Figure 3.6). Conversely, gilts treated with DHT had a longer proestrus (P<.05) and declining concentrations of progesterone that were lower (P<.05) than gilts treated with vehicle.

Duration of the estrous cycle was shortened (P<.05) in gilts treated with T or T plus F (Table 1) as compared with gilts treated with vehicle. Regarding OR, treating gilts with DHT increased (P=.06) the number of corpora lutea compared with gilts receiving
vehicle (Table 1). However, embryonic survival was negatively affected in gilts that were treated previously with DHT or testosterone. None or only 26.6% of the potential embryos were recovered on day 11 post-mating in gilts receiving DHT or T from day 13 to estrus, respectively. This negative effect on embryonic survival was eliminated when gilts were co-treated with flutamide (Table 3.1).

Verification that gilts actually had increased concentrations of T was validated when serum concentrations of T were elevated 12 hours after injection of T or T plus flutamide (Figure 3.7). At 24 hours, the concentrations in the serum had not returned to the previous day’s concentrations. Likewise, in a preliminary trial with additional gilts treated with 10 mg of DHT daily, concentrations of DHT peaked at 4 hours and thereafter decreased but were still elevated at 24 hours post injection. Therefore, it can be suggested that the exogenous androgens, given at the dosages in this investigation, were not completely cleared from these animals at the time of the next day’s injection.

DISCUSSION

In the present experimentation, we demonstrated that serum concentrations of FSH were increased by injecting gilts with DHT or testosterone. The increase in FSH might explain the increase in OR observed in the present and previous investigations (Cárdenas et al., 2002; Cárdenas and Pope, 1994). In female rats, treatment with DHT, but not T, increased serum concentrations of FSH (Burger et al., 2007) but decreased ovulation rate (Conway et al., 1990). In support of the effect of androgens on FSH secretion, gonadotropes obtained from female rats had increased FSH secretion when treated with androgens (Drouin and Labrie, 1976). Likewise, T and DHT have been demonstrated to induce the promoter of mouse FSHβ gene in Lβt2 cells (immortalized
gonadotropes, Thackray et al., 2006), to increase FSHβ mRNA in the adenohypophysis of female rats (Burger et al., 2007) and to increase the activity of GnRH neurons in ovariectomized mice (Pielecka et al., 2006). Utilizing the female pig as a model, androgens were observed to increase FSH secretions and also increase in ovulation rate.

Results of the present experiment also demonstrated that neither DHT nor T, when co-treated with F, were able to alter concentrations of FSH in serum. The inability of androgens to effect FSH concentrations in presence of F support the involvement of the AR in this endocrine pathway affecting OR in gilts.

Concentrations of LH were increased with DHT treatment as compared to T or DHT plus F on day 13 but were subsequently unchanged thereafter. Although androgens have a strong negative feedback on LH secretion in males (Tilbrook et al., 1991; Tilbrook and Clarke, 2001), such effects have not been observed in mares (Thompson, Jr. et al., 1986), female rats (Conway et al., 1990), nor gilts (present experiment). Androgenic effects on LH secretion might be dose-related as in ovariectomized ewes treated with 5 mg of DHT (once or twice daily) did not alter LH concentration but when treated with 20 mg of DHT daily decreased concentrations of LH within 4 days (Clarke et al., 1982). In the present investigation, LH was not affected by androgen treatment as compared to gilts treated with vehicle. However, our laboratory recently demonstrated that the role of LH might be related to the LH receptor as the amount of LHR mRNA in follicles of gilts decreased with DHT treatment (Cárdenas et al., 2008).

Concentrations of estradiol-17β were increased in the serum 2 hours after injecting T, even with co-treatment with F, and were back to similar concentrations of estradiol-17β in gilts of the vehicle group by 12 hours post-treatment. Exogenous T
apparently has to be elevated above the already high intra-follicular amounts of endogenous T (Smith et al., 1992) to cause detectable increases in systemic estradiol-17β secretion and this only occurred, at least, for the first 2 hours post-injection.

Concentrations of androstenedione in serum also increased when gilts were injected with T or T plus F, consistent with more substrate (T) for androstenedione synthesis. Increased exposure to androstenedione could also have increased aromatase mRNA in the granulosa cells, as has been observed with cattle cells (Hamel et al., 2005). Curiously, on day 15 and 16, concentrations of androstenedione were increased further with T as compared to T plus F treatments. Corture et al. (1993) suggested a role of the AR to stimulate the activity of 17β-hydroxysteroid dehydrogenase and perhaps by day 15 to 16 exogenous T increased both the activity of this enzyme and served as additional substrate. In the present investigation, the transient increase in androstenedione concentrations in the serum failed to be related to ovulation rate. Similarly, OR failed to increase by injecting gilts, during the same period of this investigation, with 1 mg of androstenedione (Cárdenas and Pope, 1997).

Serum concentrations of progesterone decreased from days 13 to 17 similarly among all treatment groups. As examined under the conditions of the present experiment, androgens apparently have no role in the rate of luteolysis in pigs. By contrast, in ewes, Cooke and Benhaj (1988) suggested a role of T in luteolysis as they observed an increase on plasma concentrations of T at the beginning of luteolysis and a delay of luteolysis when T was suppressed. These differences on androgens might again represent dosage and species differences in luteolysis.
Duration of the estrous cycle was shortened when treatment of gilts included T, alone or in combination with flutamide. Treating gilts with T increased estradiol-17β and shortened proestrus suggesting that perhaps relatively small increases in estradiol-17β in the systemic circulation, or direct aromatization of T within the brain (Lephart, 1996), enhanced an earlier display of estrus. Although exogenous estrogens can lengthen or shorten the estrous cycle of gilts (Geisert et al., 1987; Kidder et al., 1955), the mechanism by which exogenous estrogens shortens the estrous cycle has not been extensively investigated.

Ovulation rate was increased in gilts treated with dihydrotestosterone. The inability of DHT to increase OR in presence of F suggests the involvement of the AR in this mechanism. Knox et al. (2003) selected gilts for high versus low OR and failed to detect, throughout the estrous cycle, a relationship between OR and estradiol-17β concentrations. However, we observed that concentrations of estradiol-17β increased the day before estrus in gilts treated with DHT, perhaps related to the increased FSH secretion and ovulation rate.

Similar to previous investigations (Cárdenas et al., 2002), treatment of gilts with DHT negatively affected embryonic survival. In the present experiment, the presence of F reversed the embryological effects of DHT treatment suggesting a role of the androgen receptor. Fertilization rate does not appear to be affected in gilts treated with androgens as Cárdenas et al. (2002) observed a similar number of embryos recovered 3 days after the onset of estrus from gilts treated with or without androgens. Sridaran and Gibori (1981) observed that rats implanted with DHT had decreased concentrations of progesterone in the ovarian vein and that 75% of these rats aborted by day 15. To our
knowledge, it is unknown how exogenous androgens, given during the follicular phase, affect early gestation in pigs.

In the female pig, treatment with androgens failed to substantially alter LH, estradiol-17β, progesterone, T and androstenedione from day 13 to 17 of the estrous cycle. However, results of this investigation suggest that exogenous androgens, binding to AR, increased FSH concentrations in the serum in association with an increase in ovulation rate.
Table 3.1. Effect of androgen receptor agonists, antagonists and combination of both on duration of proestrus, length of estrous cycle, number of corpora lutea and embryo survival in gilts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Length of proestrus (days)</th>
<th>Duration of Estrous Cycle (days)</th>
<th>Number of Corpora Lutea</th>
<th>Embryo Survival (%)(^i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>3.0 ± 0.2(^b)</td>
<td>20.3 ± 0.3(^c)</td>
<td>21.0 ± 1.4(^g)</td>
<td>90.5 ± 7.7</td>
</tr>
<tr>
<td>DHT</td>
<td>4.0 ± 0.2(^d)</td>
<td>20.3 ± 0.3(^e)</td>
<td>25.0 ± 1.4(^h)</td>
<td>0</td>
</tr>
<tr>
<td>T</td>
<td>2.1 ± 0.2(^c)</td>
<td>18.8 ± 0.4(^f)</td>
<td>18.4 ± 1.6(^g)</td>
<td>26.6 ± 7.7</td>
</tr>
<tr>
<td>F</td>
<td>3.3 ± 0.2(^b)</td>
<td>20.0 ± 0.3(^e)</td>
<td>18.8 ± 1.4(^g)</td>
<td>88.8 ± 7.7</td>
</tr>
<tr>
<td>DHT plus F</td>
<td>3.2 ± 0.2(^b)</td>
<td>20.1 ± 0.3(^e)</td>
<td>20.0 ± 1.4(^g)</td>
<td>92.0 ± 7.0</td>
</tr>
<tr>
<td>T plus F</td>
<td>2.2 ± 0.2(^c)</td>
<td>18.7 ± 0.3(^f)</td>
<td>21.7 ± 1.4(^g)(^h)</td>
<td>85.0 ± 7.0</td>
</tr>
</tbody>
</table>

\(^a\) Vehicle, corn oil; DHT, 5α-dihydrotestosterone; T, testosterone; F, flutamide (an androgen receptor antagonist); n= 6 per treatment expect for DHT (n=5)

\(^b, c, d\) Means (± SE) within a column lacking a common superscript differed (P<.05)

\(^c, f\) Means (±SE) within a column lacking a common superscript differed (P<.05)

\(^g, h\) Means (± SE) within a column lacking a common superscript differed (P=.06)

\(^i\) Means (± SE) were not statistically analyzed because no embryos were recovered from gilts on the DHT treatment group.
Figure 3.1. Daily concentrations of FSH in serum of gilts treated with vehicle (corn oil), androgen receptor agonists (5α-dihydrotestosterone, DHT; testosterone, T), antagonist (flutamide, F) and combination of both from day 13 to day 17 of the estrous cycle. Means presented were obtained from untransformed values. Standard errors from the ANOVA are not shown because data were heterogeneous. n=6 per treatment except for DHT (n= 5); * Treatments differ (P<.05) when compared to vehicle
Figure 3.2. Daily concentrations of LH in serum of gilts treated with vehicle (corn oil), androgen receptor agonists (5α-dihydrotestosterone, DHT; testosterone, T), antagonist (flutamide, F) and combination of both from day 13 to day 17 of the estrous cycle. Means presented were obtained from untransformed values. Standard errors from the ANOVA are not shown because data were heterogeneous. n=6 per treatment except for DHT (n=5); Treatment X day (P<.05)
Figure 3.3. Estradiol-17β concentrations in serum of gilts treated with vehicle (corn oil), androgen receptor agonists (5α-dihydrotestosterone, DHT; testosterone, T), antagonist (flutamide, F) and combination of both from -5 to -1 days before estrus. Means presented were obtained from untransformed values. Standard errors from the ANOVA are not shown because data were heterogeneous. n= 6 per treatment except for DHT (n=5); * Treatment X day, P<.05
Figure 3.4. Androstenedione concentrations in serum each day at 2 hours post-treatment of gilts treated with vehicle (corn oil), androgen receptor agonists (5α-dihydrotestosterone, DHT; testosterone, T), antagonist (flutamide, F) and combination of both from day 13 to day 17 of the estrous cycle. Means presented were obtained from untransformed values. Standard errors from the ANOVA are not shown because data were heterogeneous. n= 6 per treatment except for DHT (n=5); * T and T plus F greater (P<.05) than vehicle ** T greater (P<.05) than T plus F and vehicle
Figure 3.5. Progesterone concentrations in serum of gilts treated with vehicle (corn oil), androgen receptor agonists (5α-dihydrotestosterone, DHT; testosterone, T), antagonist (flutamide, F) and combination of both from day 13 to day 17 of the estrous cycle. Means presented were obtained from untransformed values. Standard errors from the ANOVA are not shown because data were heterogeneous. n= 6 per treatment except for DHT (n=5)
Figure 3.6. Progesterone concentrations in serum of gilts treated with vehicle (corn oil), androgen receptor agonists (5α-dihydrotestosterone, DHT; testosterone, T) antagonists (flutamide, F) and combination of both from -5 to -1 days before to estrus. Means presented were obtained from untransformed values. Standard errors from the ANOVA are not shown because data were heterogeneous. n= 6 per treatment except for DHT (n=5); * T and T plus F greater (P<.05) than vehicle
Figure 3.7. Testosterone concentrations in serum of gilts treated with vehicle (corn oil), androgen receptor agonists (5α-dihydrotestosterone, DHT; testosterone, T), antagonist (flutamide, F) and combination of from day 13 to day 17 of the estrous cycle. Means presented were obtained from untransformed values. Standard errors from the ANOVA are not shown because data were heterogeneous. n= 6 per treatment except for DHT (n=5); * T and T plus F greater (P<.05) than vehicle.
CHAPTER 4

INFLUENCE OF ANDROGEN TREATMENT ON UTERINE DEVELOPMENT OF NEONATAL PIGS

ABSTRACT

Sixty gilts were utilized to investigate the effects of androgens on the neonatal development of the uterus. In Experiment 1, twenty four newborn gilts were assigned randomly to receive daily injections of vehicle (corn oil), 6, 60 or 600 µg of 5α-dihydrotestosterone (DHT), per kg of body weight, from birth (postnatal day, PND 0) to PND 13. On PND 14 weights of the uterus, liver, heart or longissimus dorsi were not altered by DHT treatment. In addition, treating gilts with DHT failed to influence endometrial thickness (ET), height of luminal epithelium (HLE), glandular penetration (GP) nor myometrial thickness (MT). In Experiment 2, thirty six additional gilts were utilized to investigate the effects of androgens, and the involvement of the androgen receptor (AR), on estrogenic-induced stimulation of uterine development up to 14 days of age. Gilts were randomly assigned at birth to receive daily injections of vehicle, 50 µg of estradiol valerate (EV), 60 µg of DHT, 10 mg of flutamide (F, an AR antagonist), EV
plus DHT (EVD) or EV plus DHT plus F (EVDF), per kg of body weight, from PND 0 to PND 13. Treatment of neonatal gilts with EV stimulated uterine development by PND 14. All aspects of early uterine development; uterine wet weight, endometrial and myometrial thickness and glandular penetration, except height of luminal epithelium, were stimulated (P<.05) with this EV treatment. Of these stimulated tissues, only the increase on MT observed after EV treatment was attenuated with EVD treatment.

Relative amounts of mRNA for relaxin receptor (LGR7), and mRNA and protein for androgen receptor (AR) and estrogen receptor α (ESR1) were not altered in whole uteri of gilts treated with EV or 5α-dihydrotestosterone. The AR and ESR1 were relatively abundant in the uterus, as stromal cells had less, and glandular epithelium had more, of these receptors than the other uterine tissues. Treatment with F alone decreased (P<.05) endometrial thickness coincident with increasing (P<.05) mRNA for LGR7. Results of these experiments indicate that although the neonatal uterus has AR, within the first two weeks of age, DHT treatment failed to alter uterine development or to inhibit development of most of the estrogenic-stimulated cells, except for MT, as previously has been observed in uteri of adult pigs.

**INTRODUCTION**

The uterus of gilts is immature at birth (postnatal day, PND 0) as the endometrium and myometrium are undifferentiated (Bartol et al., 1993). The primitive endometrium begins active synthesis of DNA on PND 7, coincident with the initial development of endometrial glands (Bartol et al., 1993; Spencer et al., 1993a). By PND 30, the inner and outer layers of the porcine myometrium appeared differentiated to resemble that of the adults (Bal and Getty, 1970; Spencer et al., 1993a). The earliest the
entire uterus is considered mature is at PND 120 because at this age the uterus can sustain a pregnancy (Bartol et al., 1993).

Development of the neonatal uterus in gilts can be altered with estradiol valerate (EV) and relaxin (RLX). Treating neonatal gilts beginning at birth with EV advanced adenogenesis, increased uterine wet weight (UW) and endometrial thickness (ET) by PND 7 (Spencer et al., 1993b), however EV treatment subsequently compromised fertility in adult pigs (Tarleton et al., 2003). Relaxin treatment beginning at birth or PND 12 increased height of luminal epithelium (Bagnell et al., 2005) and cell proliferation in the glandular epithelium of the uterus in gilts (Masters et al., 2007). However the ability of other hormones to modify these effects remains unknown.

Androgens could be one of those hormones that modulate uterine development in neonatal pigs. The presence of AR was recently detected in uteri and ovaries from newborn gilts (Burek et al., 2007; Slomczynska et al., 2008). In adult pigs, high dosages of androgens inhibit the actions of estradiol in the uterus (Cárdenas and Pope, 2004), and might have similar effects in the neonate. Therefore, the objectives of this investigation were to examine the effects of androgen alone on uterine development and the effects of androgens on estrogenic-stimulation of the neonatal uterus up to PND 14.

**MATERIALS AND METHODS**

The approach of the first experiment was to compare increasing doses of 5α-dihydrotestosterone (DHT) on uterine development. Subsequently, in the second experiment, specific effects of DHT on the estrogenic-induced effects on uterine development were examined. All procedures with animals were approved and supervised.
Experiment 1

Twenty four gilts (cross of domestic breeds) were assigned randomly at birth to receive (n=6 per treatment) intramuscular injections of vehicle (200 µl per kg of body weight of corn oil), 6, 60 or 600 µg of DHT, per kg of body weight, from PND 0 to PND 13 to include the proliferative stages of the neonatal development of the uterus (Spencer et al., 1993b). Injections were given daily at 8:00 AM. The range of dosages of DHT utilized in this experiment, increased ovulation rate in a dose-dependent manner in adult gilts (Cárdenas et al., 2002). Gilts were euthanized on PND 14 to determine the weight of the uterus, heart, liver and longissimus dorsi. In addition, the heart, liver and skeletal muscle can respond to androgen treatment (Brown et al., 1976; Florini, 1987; Marsh et al., 1998), these organs and tissues were designed to be positive controls.

The middle portion of one uterine horn was fixed in 4% paraformaldehyde for 18 h at 4 to 5 °C, dehydrated in sequential dilutions of ethanol (50 and 70%; 3 times, 1 h each and then 95 and 100%; 3 times, 45 minutes each). Following dehydration, tissues were cleared with xylene (3 times, 45 minutes each) and embedded in paraffin (3 times, 1 h each). Transversal sections of the uterus (6 µm) were obtained non-sequentially and mounted on positive-charged slides. Tissues were stained with hematoxilyn and eosin. Pictures of three uterine sections per animal were taken utilizing a microscope fitted with a digital camera (MicroPublisher 5.0, QImaging, Surrey, BC, Canada V3S 6K3). Endometrial thickness (ET), glandular penetration (GP) and myometrial thickness (MT) were measured at 40X magnification while height of luminal epithelium at 100X.
magnification. Endometrial thickness and MT (including perimetrium) were measured as described by Spencer et al. (1993b). Endometrial thickness was considered from the basement membrane of the luminal epithelium to the interface with the inner myometrial layer. From the latter interface to the apical border of the perimetrium was considered the myometrial thickness. Glandular penetration was measured as described by Tarleton et al. (1999) from the lumen to the deepest interface between adjacent stroma and glandular epithelium. Height of luminal epithelium was measured from the border of the lumen to the surface adjacent to stroma (Yan et al., 2006). A picture of the bar width (35µm) of a G200-Cu grid (Electron Microscopy Sciences, Hatfield, PA), at each magnification considered, served as a scale for ImageJ software (http://rsb.info.nih.gov/ij/) analysis of the histological measurements mentioned above. All measurements were made by a person unaware of treatment assignment.

Experiment 2

Thirty six additional gilts (same crossbreed as Experiment 1; n=6 per treatment) were randomly assigned to receive vehicle (same as Experiment 1), 50 µg of estradiol valerate (EV), 60 µg of DHT, 10 mg of flutamide (F, an AR antagonist), EV plus DHT (EVD) or EV plus DHT plus F (EVDF), per kg of body weight, from PND 0 to PND 13. The dosage of EV has been utilized previously to stimulate postnatal development of the uterus (Spencer et al., 1993b) while the dosage of DHT attenuated some effects on the uterus of adult, ovariectomized gilts (Cárdenas and Pope, 2004; Jellinck and Newcombe, 1983). Similarly, the amount of exogenous F utilized in this experiment was previously observed to block androgenic effects on the uterus (E. Jiménez, H. Cárdenas and W.F. Pope, unpublished data). Gilts were euthanized on PND 14 to collect the uteri which
were immediately trimmed of connective tissue and weighed. The middle portion of a uterine horn was collected and snap frozen in liquid nitrogen for further RNA isolation. The middle portion of the other uterine horn was fixed, dehydrated and embedded in paraffin as described in Experiment 1. Embedded tissues were sectioned and histological measurements were obtained as described in Experiment 1.

Immunohistochemistry

Immunohistochemical staining for the AR and estrogen receptor α (ESR1) were performed using the Vectastain ABC and diaminobenzidine kit (both from Vector Laboratories, Burlingame, CA) following previously reported procedures (Cárdenas and Pope, 2002b; Cárdenas and Pope, 2005, respectively). Briefly, for antigen retrieval tissues were deparaffinized, rehydrated and then incubated for 30 minutes at 95 °C in 0.01M sodium citrate solution, pH 6.0. The concentrations of antibodies were 8 µg/ml and 4 µg/ml for AR (PA-111A) and ESR1 (PA1-309), respectively. Negative controls were run in the same assay in which solutions of antibody and immunizing peptide, pre-incubated for one hour at room temperature, replaced the antibody. The immunizing peptides were PEP-006 and PEP-037, for AR or ESR1 respectively. Antibodies and immunizing peptides were purchased from Affinity Bioreagents, Golden, CO. The nickel solution from the diaminobenzidine kit was utilized (8 minutes incubation) to obtain gray/black staining. The relaxin receptor (LGR7) could not be examined because an antibody was not available.

Pictures of stained tissues were taken as in Experiment 1 and converted to 8-bit utilizing ImageJ software. Immunohistochemical staining in the luminal epithelium,
glandular epithelium, myometrium and stroma cells were determined as previously described (Cárdenas and Pope, 2004).

**Determination of mRNAs**

Ribonucleic acid was isolated with TRI reagent (Molecular Research Center, Cincinnati, OH) following procedure from the manufacturer. The concentration and purity of RNA were determined by spectrophotometry, 260 nm and 260/280 nm ratio, respectively, utilizing the Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Integrity of RNA was verified by running an aliquot of each sample on 1.2% formaldehyde agarose-gel electrophoresis and then estimating the 28s to 18s ratio for ribosomal RNA.

Real time RT-PCR (standard curve method) was utilized to determine the relative amounts of mRNA for the AR, ESR1 and relaxin receptor (LGR7). Reverse transcriptions were performed utilizing a kit (TaqMan Reverse Transcription Reagents, Applied Biosystems, Foster City, CA). Random hexamers were selected as primers in reverse transcriptions. For all samples, additional reactions in which water substituted reverse transcriptase served as negative controls. For standards preparation, aliquots from animals of each treatment were combined and 1 µg of total RNA per 50 µl reaction was reverse transcribed. Resulting cDNA was diluted to create five standards ranging from 1:1 up to 1:10,000. For sample preparation, 200 ng of total RNA per 50 µl reaction were utilized. Resulting cDNA was diluted 1:5 with 1X reverse-transcription buffer. Individual reactions (25 µl total) for real time RT-PCR were prepared utilizing Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 300 µM of primers. Primers for detection of ESR1, AR, LGR7 and cyclophilin (normalizer gene) are
presented in Table 4.1. Each cDNA was run in triplicate. Amplification of cDNA was performed incubating reactions in 96-well plates for 10 minutes at 95 °C, followed by 40 cycles of 95 °C for 15 seconds and 60°C for 1 minute in Applied Biosystems 7500 Real Time PCR. Dissociation curves to verify the specific amplification of the expected products were obtained at the end of each run utilizing default parameters (one cycle of 95 °C for 15 seconds, 60°C for 1 minute and 95 °C for 15 seconds).

Statistical analysis

One of the gilts from DHT treatment in Experiment 2 was removed from the analysis because the gross development of her uterus was abnormal. Data were analyzed by analysis of variance. Weights, histological and real time RT-PCR data were analyzed as a completely randomized design. Immunohistochemical data were analyzed by use of a split-plot design. Data for ESR1 mRNA were logarithmically transformed to correct for heteroscedasticity (determined by Levene test) and untransformed means presented. Means for all variables investigated were compared utilizing least significant difference tests. Analyses were performed using SAS/GLM® software, Version 9.1 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA).

RESULTS

Experiment 1

Wet weights of the uterus, heart, liver and longissimus dorsi were not different among treatment groups (Table 4.2). Likewise, ET, GP, HLE and MT were unaffected by treatments (Table 4.3).
**Experiment 2**

Similar to experiment 1, in experiment 2, DHT treatment alone did not alter uterine wet weight (UW), ET, GP nor myometrial thickness (Table 4.4) relative to tissues of neonates receiving vehicle. Treatment of neonatal gilts with EV increased (P<.05) UW, ET, GP and myometrial thickness (Table 4.4). This estrogenic augmentation was not inhibited with DHT (except in myometrial tissue, P<.05) nor with DHT plus F treatment (Table 4.4). Height of luminal epithelium by PND 14 was unaffected by any of the treatments. Endometrial thickness was decreased (P<.05) or tended to decrease (P=.07) in gilts treated with F when compared to vehicle or DHT treatment, respectively (Table 4.4). In addition, F treatment did not affect MT when compared to vehicle, DHT and EVD, but the MT was less (P<.05) when compared to EV or EVDF treatments.

The relative amounts of ESR1 and AR mRNA were not affected by any of the treatments (Table 4.5). Treatment of gilts with F alone increased (P<.05) relative amounts of LGR7 mRNA when compared to the other treatments (Table 4.5). Relative amounts of immunohistochemical staining for the AR and ESR1 proteins (Table 4.6 and 4.7, respectively) in luminal epithelium, glandular epithelium, stromal or myometrial cells were not different among treatments. The amounts of immunohistochemical staining of AR in the stroma were less (P<.05) than the other tissues examined (Table 4.6). The glandular epithelium had more staining (P<.05) for the ESR1 protein than other uterine tissues (Table 4.7). Finally, in stroma, immunohistochemical staining for the ESR1 protein was less (P<.05) than luminal epithelium and myometrial cells (Table 4.7).
DISCUSSION

Uterine wet weight, in experiment 1, was unaffected by varying doses of exogenous DHT and was unaffected by the 60 µg dosage in experiment 2. Treatment of five gilts with 10 mg of DHT tended to decrease uterine wet weight in adult gilts (Cárdenas and Pope, 2005). In contrast, treating nine ovariectomized, adult rats with 3 mg/kg of DHT increased uterine wet weight (Nantermet et al., 2005). Perhaps, the effects of DHT on uterine wet weight are age, dosage or species dependent.

Androgen receptors exist in almost all mammalian tissues in adults. The heart, liver and skeletal muscle of adults were stimulated to growth with exogenous androgens (liver, Brown et al., 1976; skeletal muscle, Florini, 1987; heart, Marsh et al., 1998). However, failure of the heart, liver or longissimus dorsi to grow (weight) after 13 days of androgen treatment in neonate gilts might be real or due to a short duration of the treatment.

Results of experiment 2 on the effects of EV on uterine development to PND 14 confirm previously observed effects of exogenous EV treatment on uterine wet weight, endometrial thickness, and glandular penetration by PND 14 (Spencer et al., 1993b; Tarleton et al., 1999). Neither synergistic nor antagonistic effects of EV with DHT, or with DHT plus flutamide, were observed regarding uterine wet weight, endometrial thickness, glandular penetration or height of luminal epithelium. The myometrium was different as antagonistic actions of DHT on the effects of EV were observed in myometrial thickness, suggesting tissue specific responses to androgens at this young age.
The effects of flutamide were unexpected. The experimental design included flutamide as a control for the effects of DHT. As flutamide did not block the effects of DHT, interpretation of the effects of this drug, in the present experiment, is limited.

Relative amounts of AR mRNA and protein were not altered by any of the treatment regimens utilized in experiment 2. Slomczynska et al. (2008) first observed the presence of AR in the uterus of day-90 fetuses and newborn gilts. Our laboratory observed that immunohistochemical staining for AR was less in uterine stroma than the other uterine cells, consistent with adult uteri (Cárdenas and Pope, 2003). Estradiol-17β treatment increased AR mRNA in the uterus of adult gilts (Cárdenas and Pope, 2003) and AR protein in rats (Weihua et al., 2002a) but not in the present experiment. Collectively, these observations suggest that estrogen-induced AR expression is evident in adult, but not neonate gilts.

Immunohistochemical staining for ESR1 was greater in glandular epithelium than in stromal cells. Various investigations have observed similar results in neonatal and adult mammals. In neonates, Tarleton et al. (1998) observed strong immunohistochemical staining for ESR1 in glandular epithelium and weaker in stroma on PND15 in ovariectomized or intact gilts. In addition, similar patterns of estrogen receptor expression in the uterus have been observed in immature ewes (Meikle et al., 2000) and adult llamas (Bianchi et al., 2007).

In the present experiment, gene expression of the estrogen receptor α was not affected by DHT, EV, flutamide or their combination treatments. In cyclic gilts, exogenous DHT did not alter estrogen receptor α mRNA or immunohistochemical staining for the receptor protein in different cell types of the uterus (Cárdenas and Pope,
Therefore, DHT treatment does not seem to be related with the expression of estrogen receptor in intact gilts. Regarding estrogen receptor α, similarly to the present investigation, other investigators did not observed changes in this receptor with estrogen treatment in the uterus of gilts (Tarleton et al., 2001).

The present investigation confirmed the uterotrophic effects of estradiol valerate on neonatal gilts. Unlike adult uteri, exogenous androgens failed to antagonize estrogen-induced stimulation of most uterine cells; only myometrial thickness was antagonized by co-treatment with estradiol valerate and 5α-dihydrotestosterone. The latter observations suggest that the uterus of neonatal and adult gilts respond differently to androgen treatment. Obviously at some time between PND 14 and adulthood, this androgenic antagonism of uterine function matures and warrants further investigation.
<table>
<thead>
<tr>
<th>Gene (GenBank accession or reference)</th>
<th>Primer type</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen receptor α (ESR1; Z37167)</td>
<td>Sense</td>
<td>CTTGTCTGGCGCTCCATGG</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>ATCATACGGAAGCGAGATG</td>
<td></td>
</tr>
<tr>
<td>Androgen receptor (AF161717)</td>
<td>Sense</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>TCGGAATTATATCAATGGGTGCAA</td>
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</tr>
<tr>
<td>Relaxin receptor (LGR7, Vinall et al., 2006)</td>
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<td></td>
<td>Antisense</td>
<td>AAGAAACCGATGGAACAGC</td>
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<tr>
<td>Cyclophilin (AY266299)</td>
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<td>GGGTTCTGTCTTTACACAGA</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>AGGACCCTGTGCTTCAGGA</td>
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Table 4.1. Primers utilized for real-time RT-PCR.
Table 4.2. Uterine (UW), hepatic (HW), cardiac (CW) and longissimus dorsi wet weights (LDW) (means ± SE) of gilts treated with 5α-dihydrotestosterone (DHT) from birth to postnatal day 13.\(^a\)

\(^a\) Gilts were euthanized and tissues collected on postnatal day 14.

\(^b\) Dosages were based on µg per kg of body weight. n=6 per dosage of DHT.

<table>
<thead>
<tr>
<th>DHT(^b)</th>
<th>UW (g)</th>
<th>HW (g)</th>
<th>CW (g)</th>
<th>LDW (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1.2 ± 0.2</td>
<td>134.8 ± 13.7</td>
<td>29.5 ± 2.6</td>
<td>69.1 ± 10.6</td>
</tr>
<tr>
<td>6</td>
<td>1.5 ± 0.2</td>
<td>169.8 ± 13.7</td>
<td>31.6 ± 2.6</td>
<td>92.0 ± 10.6</td>
</tr>
<tr>
<td>60</td>
<td>1.5 ± 0.2</td>
<td>167.4 ± 13.7</td>
<td>31.9 ± 2.6</td>
<td>83.9 ± 10.6</td>
</tr>
<tr>
<td>600</td>
<td>1.4 ± 0.2</td>
<td>153.5 ± 13.7</td>
<td>30.8 ± 2.6</td>
<td>79.6 ± 10.6</td>
</tr>
</tbody>
</table>
Table 4.3. Endometrial thickness (ET), height of luminal epithelium (HLE) and myometrial thickness (MT) of gilts treated with 5α-dihydrotestosterone (DHT) from birth to postnatal day 13.\textsuperscript{a}

\begin{tabular}{lllll}
\textbf{DHT}\textsuperscript{b} & \textbf{ET} & \textbf{GP} & \textbf{HLE} & \textbf{MT} \\
 & (µm) & (µm) & (µm) & (µm) \\
Vehicle & 583.6 ± 50.7 & 425.8 ± 77.3 & 23.2 ± 1.5 & 252.1 ± 10.3 \\
6 & 561.6 ± 50.7 & 409.5 ± 77.3 & 20.3 ± 1.5 & 257.1 ± 10.3 \\
60 & 557.2 ± 50.7 & 382.7 ± 77.3 & 20.7 ± 1.5 & 235.0 ± 10.3 \\
600 & 555.2 ± 50.7 & 446.0 ± 77.3 & 19.4 ± 1.5 & 230.6 ± 10.3 \\
\end{tabular}

\textsuperscript{a} Gilts were euthanized and tissues collected on postnatal day 14.

\textsuperscript{b} Dosages were based on µg per kg of body weight. n= 6 per dosage of DHT.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>UW</th>
<th>ET</th>
<th>GP</th>
<th>HLE</th>
<th>MT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g)</td>
<td>(µm)</td>
<td>(µm)</td>
<td>(µm)</td>
<td>(µm)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.4 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>601.3 ± 56.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>412.8 ± 72.0&lt;sup&gt;h&lt;/sup&gt;</td>
<td>21.9 ± 1.5</td>
<td>220.5 ± 21.9&lt;sup&gt;l&lt;/sup&gt;</td>
</tr>
<tr>
<td>DHT</td>
<td>1.3 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>600.2 ± 68.6&lt;sup&gt;g&lt;/sup&gt;</td>
<td>392.8 ± 80.5&lt;sup&gt;h&lt;/sup&gt;</td>
<td>18.1 ± 1.8</td>
<td>198.5 ± 24.5&lt;sup&gt;l&lt;/sup&gt;</td>
</tr>
<tr>
<td>EV</td>
<td>3.0 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>921.8 ± 56.0&lt;sup&gt;f&lt;/sup&gt;</td>
<td>813.1 ± 72.0&lt;sup&gt;i&lt;/sup&gt;</td>
<td>19.1 ± 1.5</td>
<td>322.6 ± 21.9&lt;sup&gt;k&lt;/sup&gt;</td>
</tr>
<tr>
<td>EVD</td>
<td>2.4 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>882.7 ± 56.0&lt;sup&gt;f&lt;/sup&gt;</td>
<td>769.5 ± 72.0&lt;sup&gt;i&lt;/sup&gt;</td>
<td>18.1 ± 1.5</td>
<td>245.4 ± 21.9&lt;sup&gt;l&lt;/sup&gt;</td>
</tr>
<tr>
<td>EVDF</td>
<td>3.0 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>867.0 ± 56.0&lt;sup&gt;f&lt;/sup&gt;</td>
<td>774.9 ± 72.0&lt;sup&gt;i&lt;/sup&gt;</td>
<td>18.4 ± 1.5</td>
<td>261.4 ± 21.9&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>F</td>
<td>1.1 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>427.0 ± 56.0&lt;sup&gt;g&lt;/sup&gt;</td>
<td>246.3 ± 72.0&lt;sup&gt;h&lt;/sup&gt;</td>
<td>18.4 ± 1.5</td>
<td>190.0 ± 21.9&lt;sup&gt;l&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 4.4. Mean values (± SE) of uterine wet weight (UW), endometrial thickness (ET), glandular penetration (GP), height of luminal epithelium (HLE) and myometrial thickness (MT) of 14-day-old gilts treated with vehicle (corn oil), estradiol valerate (EV), 5α-dihydrotestosterone (DHT), flutamide (F), EV plus DHT (EVD) and EV plus DHT plus F (EVDF).<sup>a</sup>

<sup>a</sup> Daily treatments were given from birth to post natal day 13. Animals were euthanized on post natal day 14 for collection of uterine tissue.

<sup>b</sup> n= 6 per treatment except for DHT (n=5); EV= 50 µg/kg body weight, DHT= 60 µg/kg body weight and F= 10 mg/kg body weight.

<sup>c-d; e-g; h-i; j-l</sup> Means within a column with different superscripts differed (P<.05).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>ESR1&lt;sup&gt;c&lt;/sup&gt;</th>
<th>LGR7&lt;sup&gt;c&lt;/sup&gt;</th>
<th>AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1.5 ± 0.4</td>
<td>0.9 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>DHT</td>
<td>2.0 ± 0.5</td>
<td>0.9 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>EV</td>
<td>0.9 ± 0.4</td>
<td>1.3 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>EVD</td>
<td>1.1 ± 0.4</td>
<td>1.2 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>EVDF</td>
<td>1.1 ± 0.4</td>
<td>0.9 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>F</td>
<td>1.2 ± 0.4</td>
<td>2.0 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

Table 4.5  Relative amounts (means ± SE) of estrogen receptor α (ESR1), relaxin receptor (LGR7) and androgen receptor (AR) mRNA in uterine tissues of 14 days old gilts treated with vehicle (corn oil), estradiol (EV), 5α-dihydrotestosterone (DHT), flutamide (F), EV plus DHT (EVD) and EV plus DHT plus F (EVDF).<sup>a</sup>

<sup>a</sup>Relative amounts are ratios to cyclophilin. Daily treatments were given from birth to post natal day 13. Animals were euthanized on post natal day 14 for collection of uterine tissue.

<sup>b</sup>n= 6 per treatment except for DHT (n=5); EV= 50 µg/kg body weight, DHT= 60 µg/kg body weight and F= 10 mg/kg body weight.

<sup>c-d</sup>Means within a column with different superscripts differed (P=.001).

<sup>c</sup>Data was logarithmically transformed for analysis. Raw means ± SE are presented.
<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Luminal epithelium</th>
<th>Glandular epithelium</th>
<th>Stroma</th>
<th>Myometrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>59.0 ± 18.9</td>
<td>81.1 ± 20.3</td>
<td>35.9 ± 7.9</td>
<td>63.9 ± 16.7</td>
</tr>
<tr>
<td>DHT</td>
<td>58.9 ± 26.7</td>
<td>69.5 ± 24.9</td>
<td>28.9 ± 9.7</td>
<td>60.0 ± 20.4</td>
</tr>
<tr>
<td>EV</td>
<td>88.1 ± 18.9</td>
<td>111.0 ± 20.3</td>
<td>50.4 ± 7.9</td>
<td>100.5 ± 16.7</td>
</tr>
<tr>
<td>EVD</td>
<td>62.5 ± 18.9</td>
<td>81.0 ± 20.3</td>
<td>38.7 ± 7.9</td>
<td>65.1 ± 16.7</td>
</tr>
<tr>
<td>EVDF</td>
<td>72.9 ± 18.9</td>
<td>103.0 ± 20.3</td>
<td>37.7 ± 7.9</td>
<td>69.5 ± 16.7</td>
</tr>
<tr>
<td>F</td>
<td>67.3 ± 18.9</td>
<td>67.2 ± 20.3</td>
<td>35.4 ± 7.9</td>
<td>60.3 ± 16.7</td>
</tr>
<tr>
<td>Mean</td>
<td>68.9 ± 6.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>86.4 ± 6.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38.4 ± 6.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>70.5 ± 6.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 4.6. Immunohistochemical staining (mean gray values ± SE) of androgen receptor in uterine tissues of 14 days old gilts treated with vehicle (corn oil), estradiol (EV), 5α-dihydrotestosterone (DHT), flutamide (F), EV plus DHT (EVD) and EV plus DHT plus F (EVDF).<sup>a</sup>

<sup>a</sup> Daily treatments were given from birth to post natal day 13. Animals were euthanized on post natal day 14 for collection of uterine tissue.

<sup>b</sup> n= 6 per treatment except for DHT (n=5); EV= 50 µg/kg body weight, DHT= 60 µg/kg body weight and F= 10 mg/kg body weight

<sup>c-d</sup> Means within a row with different superscripts differed (P<.05).
Table 4.7. Immunohistochemical staining (mean gray values ± SE) of estrogen receptor α in uterine tissues of 14 days old gilts treated with vehicle (corn oil), estradiol (EV), 5α-dihydrotestosterone (DHT), flutamide (F), EV plus DHT (EVD) and EV plus DHT plus F (EVDF).\(^a\)

<table>
<thead>
<tr>
<th>Treatment(^b)</th>
<th>Luminal epithelium</th>
<th>Glandular epithelium</th>
<th>Stroma</th>
<th>Myometrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>47.2 ± 14.4</td>
<td>79.4 ± 18.8</td>
<td>25.2 ± 4.2</td>
<td>48.3 ± 12.6</td>
</tr>
<tr>
<td>DHT</td>
<td>50.0 ± 15.8</td>
<td>67.8 ± 20.6</td>
<td>23.3 ± 4.6</td>
<td>47.3 ± 13.8</td>
</tr>
<tr>
<td>EV</td>
<td>66.2 ± 14.4</td>
<td>99.5 ± 18.8</td>
<td>26.9 ± 4.2</td>
<td>72.5 ± 12.6</td>
</tr>
<tr>
<td>EVD</td>
<td>55.9 ± 14.4</td>
<td>84.6 ± 18.8</td>
<td>20.7 ± 4.2</td>
<td>45.8 ± 12.6</td>
</tr>
<tr>
<td>EVDF</td>
<td>59.3 ± 14.4</td>
<td>97.2 ± 18.8</td>
<td>27.3 ± 4.2</td>
<td>49.3 ± 12.6</td>
</tr>
<tr>
<td>F</td>
<td>52.7 ± 14.4</td>
<td>67.6 ± 18.8</td>
<td>27.2 ± 4.2</td>
<td>50.1 ± 12.6</td>
</tr>
<tr>
<td>Mean</td>
<td>55.4 ± 5.4(^c)</td>
<td>83.2 ± 5.4(^d)</td>
<td>25.2 ± 5.4(^e)</td>
<td>52.4 ± 5.4(^e)</td>
</tr>
</tbody>
</table>

\(^a\) Daily treatments were given from birth to post natal day 13. Animals were euthanized on post natal day 14 for collection of uterine tissue.

\(^b\) n= 6 per treatment except for DHT (n=5); EV= 50 µg/kg body weight, DHT= 60 µg/kg body weight and F= 10 mg/kg body weight.

\(^c-e\) Means within a row with different superscripts differed (P<.05).
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