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FOLLICULAR GROWTH AND DEVELOPMENT AND GONADOTROPIN RESPONSE OF MARES TREATED WITH DIHYDROTESTOSTERONE AND ESTRADIOL BENZOATE

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirement for the degree of Master of Science in The Interdepartmental Program in Animal, Dairy and Poultry Sciences

by

Scarlett Lynn McMeen
B.S., University of Tennessee at Martin, 1999
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ABSTRACT

An experiment was conducted to determine the effects of dihydrotestosterone (DHT) and estradiol benzoate (EB) given simultaneously on concentrations of FSH and LH and the associated follicular activity, and to monitor the subsequent recovery of the gonadotropes and follicular growth following treatment with either DHT or EB. Twelve mares were assigned to daily treatments of DHT (150 g/kg BW) plus EB (22 g/kg BW) from d 0 through 30 (Phase I). Beginning on d 31, mares were randomly assigned to one of three treatment groups: 1) oil, 2) DHT (150 g/kg BW), or 3) EB (22 g/kg BW). On d 30, 44, and 58, GnRH was administered to determine the response of FSH and LH. In phase I, DHT plus EB decreased (P = 0.0001) concentrations of LH and FSH. In phase II (d 31 to 66), DHT decreased (P = 0.0001) LH and FSH concentrations. Estradiol benzoate decreased (P= 0.0001) concentrations of FSH but increased (P = 0.0001) concentrations of LH. There was a significant treatment x day x minute interaction of FSH (P = 0.0001) and LH (P = 0.0002) concentrations in response to administration of GnRH on d 30, 44, and 58. The number of medium follicles (P = 0.0001), large follicles (P = 0.0012) and the size of the largest follicle per day (P = 0.0001) decreased during phase I. During phase II, there was a treatment x day interaction (P = 0.0001) for medium follicles and large follicles. Control mares had more large follicles on d 57 through d 63 whereas EB treated mares had more large follicles on d 45, 49, and 51. Control mares had increased size of the largest follicles at d 57 to 63. Mares in the DHT and EB groups had prolonged (P = 0.01) ovulation intervals as compared with controls. It is concluded that the combination of DHT plus EB suppresses gonadotropin concentrations and the associated follicular activity in mares. Also, concentrations of both gonadotropins must be restored to normal for growth of an ovulatory size follicle and the subsequent ovulation of that follicle.
INTRODUCTION

The estrous cycle of the mare has been a point of extensive research. It is generally accepted that FSH and LH are needed for normal growth and ovulation of ovarian follicles in mares. Concentrations of FSH and LH fluctuate throughout the estrous cycle. High concentrations of FSH are needed during diestrus for the growth of the preovulatory follicle. Increases in LH concentration are required in mares for the maturation and ovulation of the preovulatory follicle. Steroid hormones, produced from the ovaries, regulate the gonadotropins and the ovarian response to them. Garza et al. (1986b) reported that estradiol benzoate (EB) plus dihydrotestosterone (DHT) administered in combination to ovariectomized mares results in increased plasma concentrations of LH and increased LH response to exogenous GnRH. In contrast, these two steroids decreased plasma FSH concentrations and the FSH response to exogenous GnRH. Intact mares administered DHT alone were reported to have increased plasma FSH concentrations and no effect of LH concentrations (Thompson et al., 1983b), yet in ovariectomized mares, plasma FSH and LH concentrations decreased (Thompson et al., 1991). The secretion of FSH is increased in response to GnRH in DHT-treated mares, but LH secretion is decreased (Thompson et al., 1983b; 1991). Treatment with EB alone in intact mares is reported to decrease FSH concentrations and increase LH concentration after luteolysis (Burns and Douglas, 1981). Evans et al. (1982) reported that EB-treated mares had variable follicular growth and ovulation. The present experiment was conducted 1) to determine if the combination of DHT and EB given simultaneously would decrease the levels of LH and FSH and follicular activity to mimic that of an anestrous mare, and 2) to monitor the subsequent recovery of the gonadotropes and follicular growth following treatment with oil, DHT, or EB.
Hypothalamic-Pituitary-Gonadal Axis

Guyton and Hall (1996) give a good general description of the hypothalamic-pituitary-gonadal axis. The hypothalamus exerts its control on the anterior pituitary via releasing hormones, which travel down the primary capillary plexus of the median eminence via the hypothalamic-hypophysial portal system. The anterior pituitary produces gonadotropins that are then carried into the general circulation (Alexander and Irvine, 1992; Ginther, 1992). In the pituitary, GnRH binds to receptors, and the number of free receptors is the limiting factor in the response. Additional receptors can be induced by estradiol or low concentrations of GnRH (Alexander and Irvine, 1992). The hypothalamus secretes GnRH in pulses lasting 5 to 25 min occurring every 1 to 2 h (Guyton and Hall, 1996). It is suggested that the pulse frequency of GnRH in mares may control the differential release of the gonadotropins (Ginther, 1992). Pulses of GnRH occurring every 45 min cause predominantly LH secretion, whereas GnRH pulses every 6 h increases FSH secretion. Thus, during estrus, GnRH pulses are increased to a frequency of two per hour and LH is high while FSH is low, whereas in diestrus, GnRH pulses are decreased to a frequency of two per day and FSH is increased while LH is low (Alexander and Irvine, 1992). In intact mares during diestrus, FSH and LH have synchronized, pulsatile secretions. In ovariectomized mares, FSH and LH peaks are tightly coupled in both summer and winter (Ginther, 1992). In mares immunized against GnRH, there was a greater reduction in LH concentrations than FSH concentrations (Garza et al., 1986a). The lack of effect of GnRH immunization on FSH secretion maybe an indication that FSH stores in the pituitary are relatively independent of GnRH activity (Ginther, 1992). Mares infused with GnRH lose the
stimulatory effects on LH while FSH concentrations were decreased 55% (Porter et al., 1997). This could be due to inadequate dose and frequency of GnRH administration.

**The Estrous Cycle**

Daels and Hughes (1992) define the mare’s estrous cycle as the repetitive sequence of events that prepares the mare for conception. The duration of the mare’s estrous cycle normally ranges from 19.1 to 23.7 days (Ginther, 1992). The mare’s seasonal cycle consists of the breeding or ovulatory season and the anestrous or non-ovulatory season. The estrous cycle is under seasonal control, and daylight is the environmental signal for this regulation (Ginther, 1992). The cycle responds to changes in the light:dark relationship; mares ovulate more consistently in the spring and summer when the days are longer (Ginther, 1992). Reproductive seasonality can be manipulated in mares by altering the duration of light (artificial or natural). The increased daylight stimulates the hypothalamus to increase secretion of GnRH, thus stimulating the activity of the gonadotropes, which results in the mare cycling (Sharp and Davis, 1992). The cycle is also under a control of factors from the ovaries. The ovaries produce hormones, estradiol and progesterone, which through negative or positive feedback control the release of FSH and LH.

**Ovulatory Season.** Nonpregnant mares are polyestrus during the breeding season. That is, if not bred, they display repeated periods of estrus (Sharp and Davis, 1992). The ovulatory season of the mare consists of two phases. The phases are estrus, or the follicular phase, and diestrus, or luteal phase (Daels and Hughes, 1992). Concentrations of FSH and LH vary throughout the cycle, and drive the activity of the ovaries. The estrous cycle is normally longer early in the ovulatory season, becomes shorter and more stable as summer approaches, and then increases in length again during the fall (Ginther, 1992). The estrous cycle is the period in which
the mare is sexually receptive to a stallion and normally ranges 4.5 to 8.9 days in duration (Ginther, 1992). During this period, FSH concentrations are normally decreased while LH is rising to a peak 1 or 2 d after ovulation (Evans et al., 1975).

Evans et al. (1982), in a study of the estrous cycle in mares, reported that FSH concentrations declined while LH concentrations rose as estrus approached. Miller et al. (1980) compared the concentrations of FSH and LH in horse and pony mares. They found in both types of mares FSH started decreasing several days prior to ovulation, while LH concentrations began to slowly rise (approximately d 14) until 4 days before ovulation when levels rose quickly to a peak 1 d after ovulation of the dominate follicle. The decrease in FSH is thought to be due to a negative influence from the ovaries, while the increase in LH is due to a positive influence from the ovaries (Freedman et al., 1979).

Diestrus is the period in which the mare is not receptive to the stallion. The diestrus period normally lasts 12.1 to 16.3 d after ovulation (Ginther, 1992). Concentrations of FSH begin to rise in early diestrus with highest concentrations being during mid-diestrus (Evans et al., 1975). Irvine et al. (1998) reported that concentrations of FSH exceeded those of estrus values by d 4 or 5 and the period of highest amplitude of pulses preceded ovulation by 10.2 ± 0.7 days. They also reported that concentrations of LH decrease between d 4 and 10 to low diestrus values. The decrease in LH is thought to be due to a negative influence (progesterone) from the ovaries, while the increase in FSH is due to the lack of a negative influence (estradiol and inhibin) from the ovaries (Freedman et al., 1979).

Anestrous season. Ginther (1992) defines the anestrous season as the period from the last ovulation of the ovulatory season to the first ovulation of the subsequent ovulatory season. He divides the anestrous season into three phases: receding, inactive, and resurging. The receding
phase, also known as autumnal transition, begins after the last ovulation of the ovulatory season and is defined as the gradual retrenchment into the inactive phase. During this phase, the mare may develop large follicles that fail to ovulate. Ginther (1992) suggests that, even though there are fluctuations in FSH concentration, the failure to ovulate is due to the lack of an ovulatory surge of LH. Silvia et al. (1986) demonstrated that the pituitary content of LH decreases progressively from the middle of the ovulatory season to the middle of the anestrous season. Mares may exhibit inconsistent behavioral estrus during this time.

The inactive period is the time in which the ovaries are inactive and is normally characterized by a lack of sexual behavior. The hypothalamic-pituitary axis becomes less active, and GnRH content and secretion are greatly reduced (Sharp and Davis, 1992). Thompson et al. (1987) described suppression of both FSH and LH concentrations in a study of the gonadotropin profile of anestrous mares. Concentrations of LH during this period are comparable to the low levels of mid-diestrus (Garcia et al., 1979). Unlike LH, there are easily-measured circulating levels of FSH during anestrous, but they are highly variable among mares (Ginther, 1992). Pituitary content of FSH does not change throughout the year, yet LH content is greatly reduced during anestrous (Thompson et al., 1986a,b).

The resurging phase, also known as the spring or vernal transition, is the gradual return to the ovulatory season. It is characterized by increasing FSH concentrations, but the length of time between the onset of increased FSH and the first ovulation can be quite long (Sharp and Davis, 1992). Fluctuations in FSH levels decrease as ovulation approaches, and levels of LH remain minimal until the ovulatory surge a few days before the first ovulation similar to that of diestrus (Hines et al., 1991). Mares may exhibit periods of erratic, prolonged estrous behavior with days in between when mares are unresponsive to a stallion (Hines et al., 1991).
Folliculogenesis and The Estrous Cycle

The follicle is the fundamental structural and functional unit of the ovary and has both endocrine (produces estrogens and nonsteroidal hormones) and exocrine (release of the oocyte) functions (Pierson, 1992). The activities of the ovaries are controlled by hormones released into the circulation (endocrine), local intercellular diffusion of substances (paracrine), and autoregulation by release of substance that binds to the cell’s own receptors (autocrine; Pierson, 1992). It is thought that the mare, like other species, has a finite number of follicles that are not replenished (Ginther, 1992). Small follicles (≤ 10 mm) are continuously growing and regressing regardless of the hormonal status or reproductive state of the mare, and these small follicles provide a reservoir for larger follicles (Ginther, 1992). Pierson (1992) gives a good general description of the importance of FSH and LH in the development of growing antral follicles. Follicles develop to the antral stage in the absence of the gonadotropins, but become atretic thereafter without the influence of FSH and LH. Pre-antral follicles acquire receptors in the granulosa cell membrane for FSH and in the thecal cell membrane for LH. Under influence from LH, the thecal cells produce androgens that pass through basal lamina of the follicle to the granulosa cells where they are aromatized to estrogens under the influence of FSH. The rising estrogen concentrations stimulate LH secretion, which in turn induces greater synthesis of estrogens. The increase in estrogen also induces LH receptors in the granulosa cells, and this facilitates the transition from the small antral stage to preovulatory stage. This is a critical point for the life of a follicle, for it must be able to respond to the preovulatory increase in both gonadotropins in order to enter the final phases of maturation.

Follicular Phase. Follicular activity fluctuates over the estrous cycle of the mare in association with changes in gonadotropin concentrations. Ginther (1992) classifies ovarian
follicles into three categories, small (2 to 10 mm), medium (11 to 24 mm) and large (>25 mm). The numbers of small follicles decrease during the preovulatory period (late diestrus to early estrus) and increase during the postovulatory period (estrus to early diestrus). Ginther and Bergfelt (1993) reported that changes in FSH concentrations were not associated with the increased numbers of small follicles in the follicular phase, but increased FSH concentrations did correspond to the increase in small follicles during the luteal phase. This is possibly due to small follicles becoming gonadotropin dependent and then recruited for follicular growth. Medium follicles seem to remain relatively constant throughout the estrous cycle. It is likely that this is due to small follicles growing to replace those that move to the larger categories. Large follicles increase in mid-diestrus, due to the mid-diestrous surge of FSH, and reach maximum numbers 5 to 7 d before ovulation, which corresponds with the decrease in FSH concentrations (Pierson, 1992).

Waves of follicular growth have been observed to correspond with the bimodal and unimodal surges of FSH, respectively (Pierson, 1992). The emergence of all follicular waves are associated with increased concentrations of FSH (Ginther and Bergfelt, 1993). Ginther (1992) describes a working hypothesis for follicular waves in the mare. All mares have at least one major follicular wave while some may have two follicular waves. He defines the major follicular wave as the emergence of several follicles. These follicles grow in synchrony until dissociation into dominant (ovulatory) follicle and the subordinate (anovulatory) follicles approximately 7 d before ovulation. Similar to the modes of FSH peaks, early in the ovulatory season mares may have two major follicular waves while later in the season they may only have one follicular wave. In mares that have two follicular waves, the first wave that emerges is the secondary wave. The secondary wave occurs in late estrus or early diestrus. The dominant follicle in this
wave normally becomes static and regresses; however, it sometimes ovulates, even under progesterone dominance. The second wave to emerge is the primary wave and it occurs in mid-diestrus. This wave will provide the primary follicle for ovulation and is independent of the secondary wave. The ovulatory follicle must be physiologically selected from the other follicles in the ovary for continued development. The dominate follicle is the most steroidogenically active follicle and most responsive to stimulation from the gonadotropins. This is thought to be due to increased receptors for FSH and LH in the granulosa and theca cells (Pierson, 1992).

During this time of selection of the dominant follicle, LH concentrations are beginning to increase and continue to rise concurrently with the growth of the follicle until ovulation, while FSH concentrations are decreasing (Ginther, 1992). While the prevailing dominant follicle is still present, it will prevent the emergence of small follicles into the next major wave. The dominant follicle begins to produce factors that cause regression of the other follicles. The two factors are estrogen and inhibin, produced by the dominant follicle, which feedback negatively on the pituitary. This feedback causes the pituitary to decrease secretion of FSH, thus resulting in atresia of the other follicles (Ginther, 1992; Pierson, 1992).

Ginther et al. (2001) reported an FSH: follicular-coupling mechanism that is the essence of follicular selection. The growing follicle, when it is about 13 mm in diameter, causes the decline in FSH concentrations after the peak of the wave-stimulating surge. Although the follicle causes the decrease in FSH, it still requires some FSH for development. The most developed follicle is able to utilize the low levels of FSH to continue to develop. Even though the largest follicle secretes increased estradiol that feeds back on the pituitary to decrease the secretion of FSH, apparently both estradiol and inhibin are needed for FSH suppression. Ginther et al. (2001) suggest that the first 2 d of the FSH decline is due to inhibit in based on the
corresponding relationship between the retained follicles, increasing levels of inhibin and decreasing levels of FSH. Yet, estradiol concentrations were reported not to increase until the day before selection of the dominant follicle. Inhibin levels remain elevated around the expected day of deviation.

This mechanism of follicle divergence has been supported by several methods. Bergfelt et al. (2001) administered progesterone at the time of deviation, which resulted in increased FSH concentrations, and decreased inhibin and estradiol. Ablation of the largest follicle resulted in increased concentrations of FSH, while concentration of FSH still decline after ablation of the next largest follicle. Administering inhibin antibodies when the largest follicle was 20 mm resulted in increased number of ovulatory size follicles (Briant et al., 2000). Also, administration of pituitary extracts has resulted in the recovery of some follicles from atresia, which were stimulated to grow (Woods and Ginther, 1985). The administration of FSH to mares has also been reported to retrieve some follicles from atresia and to allow them to grow to preovulatory size (Rosas et al, 1998).

**Ovulation and Luteal Phase.** Once the dominant follicle is selected, it will continue to grow and develop. The LH receptors in the granulosa cells of the dominant follicle, or preovulatory follicle, allow the follicle to respond to the preovulatory surge of LH (Ginther et al., 2001). In mares, the trigger for ovulation may simply be the increase in LH per se because the peak in LH does not occur until 1 d after ovulation (Pierson, 1992). The preovulatory follicle increases in diameter (with a mean of 3 mm per day), changes shape from spherical to non-spherical, and has an increased thickness of the follicular wall (Ginther, 1992).

The size of the preovulatory follicle at the onset of estrus has an effect on the size of the follicle at ovulation. The time of the year also affects the size of follicle at ovulation. Early in
the ovulatory season, the follicles that ovulate are larger than those later in the season (Ginther, 1990). By some mechanism, not clearly understood in the mare, the follicle reaches the ovulation fossa where it ovulates (Ginther 1992). The increase in the size of the preovulatory follicle causes it to bulge from the ovary. The wall of the follicle ruptures at the ovulatory fossa expelling the oocyte and follicular fluid (Pierson, 1992).

Niswender and Nett (1992) give a good description of the luteal phase of the mare. After ovulation, the granulosa cells begin to luteinize, and by 3 d following ovulation, a corpus luteum (CL) will be completely formed. Luteinizing hormone supports the lifespan of the CL during the estrous cycle in the mare. The CL produces progesterone, which increases receptors for LH in the CL. Progesterone concentrations increase and reach maximal secretion by d 9, which corresponds to the maximal growth of the CL. If the mare is not pregnant, the uterus secretes \( \text{PGF}_2\alpha \) on approximately d 14, which causes the regression of the CL. Progesterone levels then decrease along with regression of the CL and the cycle begins again.

**Steroidal Feedback**

Steroids modulate the secretion of the gonadotropins and thus their effect on the ovaries. As mentioned before, estrogens, progesterone, and inhibin are produced from the ovaries and feed back on the pituitary, which alters the secretion of the gonadotropins. Steroids can also be used exogenously to manipulate the gonadotropins and follicular activity in mares.

**Estradiol.** In the mare, estrogens are produced from the dominant follicle and feed back to the pituitary resulting in the decrease in FSH and the increase in LH concentrations. The estrogens produced from the ovaries also are responsible for behavioral estrus in the mare in the absence of progesterone (Brinsko et al., 1992). Estradiol has been used exogenously to manipulate the secretion of FSH and LH. Administration of estradiol caused plasma FSH
concentrations to decrease in intact mares (Burns and Douglas, 1981; Evans et al., 1982; Thompson et al., 1983b). In ovariectomized mares, it has been reported that plasma FSH concentrations decreased after exposure to estradiol (Garza et al., 1986b; Wiest et al., 1987; Thompson et al., 1991). Miller et al. (1981) reported that plasma FSH concentrations in ovariectomized mares decreased for 8 h, had a surge at 12 h, and then decreased again 48 h after initiation of estradiol. Estradiol decreases plasma FSH concentrations in geldings as well (Thompson et al., 1979). Administering estradiol to stalk-sectioned, ovariectomized mares did not re-establish the concentrations FSH or LH (Porter et al., 1997).

Estradiol increases plasma LH concentrations over that of controls in intact cycling mares after luteolysis, when progesterone concentrations are low (Woodley et al., 1979; Burns and Douglas, 1981). Yet, some researchers have reported no effect of estradiol on LH concentrations in intact mares (Evans et al., 1982; Thompson et al., 1983b). In ovariectomized mares, plasma concentrations of LH are increased in response to treatment with estradiol (Garcia and Ginther, 1978; Garcia et al., 1979; Garza et al., 1986b; Wiest et al., 1987; Sharp et al., 1991; Thompson et al., 1991). In geldings, low doses of estradiol increase LH concentration, but higher doses do not stimulate LH further (Thompson et al., 1979). Estrogen treatment also resulted in a greater response of LH to GnRH injection in ovariectomized mares (Garza et al., 1986b; Wiest et al., 1987; Sharp et al., 1991; Thompson et al., 1991). Follicle stimulating hormone response to GnRH in estrogen-treated mares has been shown to increase (Garza et al., 1986b; Wiest et al., 1987), to not respond (Thompson et al., 1983b, 1991), and to decrease (Sharp et al., 1991) relative to controls. Treatment with estradiol also results in increased LH and FSH content in the pituitary (Sharp et al., 1991; Thompson et al., 1991).

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Treatment with estradiol also results in prolonged length of behavioral estrus and interovulatory interval (Burns and Douglas, 1981). Mares treated with estradiol had increased numbers of follicles over that of controls. However, these numbers were variable among mares, and all but one failed to ovulate (Evans et al., 1982). Follicular growth in mares treated with high doses of estradiol is suppressed, resulting in a smaller largest follicle than controls and delayed ovulation (Woodley et al., 1979; Burns and Douglas, 1981; Loy et al., 1981; Taylor et al., 1982).

**Dihydrotestosterone.** Dihydrotestosterone (DHT) is an androgenic metabolite of testosterone that is non-aromatizable in the mare (Garza et al., 1986b). In the granulosa cells of the mare, DHT seems to be the most potent competitive inhibitor of the aromatase activity (Amri et al., 1993). Dihydrotestosterone seems to have both androgenic and estrogenic effects (Thompson et al., 1983b). Administering DHT to intact mares during estrus caused FSH concentration to increase above the control mares by the end of treatment (Thompson et al., 1983b). In anestrous mares, DHT had no effect on plasma concentrations of FSH (Thompson et al., 1986a). Ovariectomized mares administered DHT had decreased plasma concentrations of FSH (Wiest et al., 1987; McNeill-Wiest et al., 1988; Thompson et al., 1991).

Plasma concentrations of LH were decreased in response to DHT in ovariectomized mares (Wiest et al., 1987; McNeill-Wiest et al., 1988; Thompson et al., 1991), whereas in intact mares administered DHT, plasma LH concentrations were similar to control mares (Thompson et al., 1983b; 1986a). The response of FSH to GnRH is greatly increased in DHT-treated intact estrous (Thompson et al., 1983b) and ovariectomized (Wiest et al., 1987; Thompson et al., 1991) mares. Due to its androgenic effect, administering DHT following treatments of dexamethasone or progesterone also caused an increased FSH response to GnRH (McNeill-Wiest et al., 1988).

Dihydrotestosterone increases FSH content but decreases LH content in the pituitary (Thompson et al., 1991). The effect of DHT on pituitary content is characteristic of the androgenic effect while decreasing FSH concentrations is characteristic of the estrogenic effect.

**Estradiol and DHT in Combination.** Together, estradiol and DHT work synergistically. The combination decreases plasma concentrations of FSH to an extent greater than either steroid alone (Garza et al., 1986b). Mares administered DHT following a period of estradiol treatment had decreased FSH concentrations to a greater extent than those administered estradiol alone (Wiest et al., 1987). Concentrations of LH were increased in mares that received both steroids (Garza et al., 1986b). Administration of DHT to mares following treatment with estradiol resulted in a rapid decrease in the elevated LH levels (Wiest et al., 1987). The combination also caused a greater response of both FSH and LH to exogenous GnRH (Garza et al., 1986b; Wiest et al., 1987).

**Testosterone.** Testosterone, along with other androgens, is found naturally in the mare during the estrous cycle. The androgens function to bring follicles into the antral stage, and it is speculated to be involved in atresia or regression of follicles (Ginther, 1992). Testosterone levels have been reported to be higher during estrus when FSH concentrations are low and LH concentrations are high (Ginther, 1992). Testosterone, like DHT, exhibits both androgenic
(increase in pituitary FSH content) and estrogenic (decrease plasma FSH concentrations) effects. Administration of testosterone results in decreased plasma concentrations of FSH in ovariectomized mares (Thompson et al., 1983a, b, 1991; Reville-Moroz et al., 1984). Testosterone-treated mares in diestrus have been reported to have increased FSH concentrations (Thompson et al., 1983c). Plasma concentrations of LH are decreased in testosterone-treated ovariectomized mares (Thompson et al., 1983b, 1991; Reville-Moroz et al., 1984). In cycling mares, testosterone is reported to have no effect on plasma LH concentration (Thompson et al., 1983a,c, 1986a). Testosterone enhances the FSH response to GnRH in mares (Reville-Moroz et al., 1984; Thompson et al., 1984, 1986a, 1991; Garza et al., 1989), whereas the LH response to GnRH is decreased in testosterone-treated ovariectomized mares (Reville-Moroz et al., 1984; Thompson et al., 1984, 1991). Intact mares’ LH responses to GnRH were reported to not be effected by testosterone (Thompson et al., 1983c, 1986a). Testosterone increases FSH content but decreases LH content in the pituitary (Reville-Moroz et al., 1984; Thompson et al., 1991). Similar effects of testosterone treatment on FSH and LH concentrations have been reported for geldings (Thompson et al., 1979). Immunization against androgens in the mare results in increased concentrations of LH but no effect on FSH (Thompson et al., 1987b).

**Progesterone.** Progesterone is one of the key hormones controlling reproductive function in the mare (Squires, 1992). It is the main steroid product of the CL of the estrous cycle and is known as the hormone of pregnancy (Ginther, 1992). Progesterone concentrations are highest during diestrus and during pregnancy, when FSH concentrations are high and LH concentrations are low (Ginther, 1992). Progesterone is one of the most common steroids used in manipulation of the estrous cycle in the mare. It has many different uses such as estrous regulation, estrous suppression, and maintenance of pregnancy (Squires, 1992). When progesterone is administered

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to mares, it results in decrease of LH concentrations (Garcia et al., 1979; Evans et al., 1982; Thompson et al., 1991; Gastal et al., 1999). Progesterone from the CL causes LH levels to begin decreasing by d 2 to 3 with greatest suppression occurring approximately on d 13 (Garcia and Ginther, 1978; Sharp et al., 1991). The level of LH suppression is not to the extent as that of androgens (Thompson et al., 1991). Squires (1983) reported that the oral progestin, altrenogest, did not result in a significant decrease in LH concentrations, but they did tend to decrease. In anestrous mares, LH was not suppressed below that of basal levels.

The role of progesterone in control of FSH secretion is unclear. Progesterone has been reported to cause FSH levels to increase in intact cycling mares (Evans et al., 1982) and in ovariectomized mares (McNeill-Wiest et al., 1988; Thompson et al., 1991). Others have reported no effect on FSH levels in anestrous mares (Thompson et al., 1986a) and in ovariectomized mares (Garcia et al., 1979; Sharp et al., 1991). Gastal et al. (1999) reported that administering high doses (300 mg) of progesterone resulted in a decrease in FSH during mid-diestrus but increases during late diestrus; an intermediate dose (100 mg) resulted in similar FSH levels to that of controls until late diestrus, when it increased above control but not to the extent of the high dose. Progesterone has also been reported to decrease the LH response to GnRH (McNeill-Wiest et al., 1988; Sharp et al., 1991). Thompson et al. (1991) reported no effect of progesterone on the LH or FSH responses to GnRH, whereas McNeill-Wiest et al. (1988) reported that progesterone increased the FSH response to GnRH. Progesterone has no effect on either FSH or LH when administered in anestrous mares (Garcia et al., 1979; Garcia and Ginther 1978; Thompson et al., 1986a). Pituitary content of the gonadotropins are not affected in mares treated with progesterone (Sharp et al., 1991).
Ovarian activity in mares given progesterone is suppressed, as is ovulation (Squires, 1992). Progesterone treatment results in suppressed follicular growth and decreased diameter of the largest follicle in mares (Evans et al., 1982; Taylor et al., 1982; Squires et al., 1983). Administering progesterone before follicle deviation results in regression of the largest follicle and therefore delayed ovulation (Gastal et al., 1999; Bergfelt et al., 2001).

**Progesterone in Combination with Estradiol or DHT.** Estradiol and progesterone given in combination result in greater suppression of LH concentrations than progesterone alone (Garcia and Ginther, 1978; Garcia et al., 1979; Evans et al., 1982; Sharp et al., 1991). Evans et al. (1982) reported that the combination of progesterone and estradiol increased FSH concentrations similar to that of the controls, whereas Sharp et al. (1991) reported no effect of this combination on FSH concentrations. The progesterone and estradiol combination greatly reduced the LH content in the pituitary but did not affect FSH content (Sharp et al., 1991). Diestrous follicular activity and diameter of the largest follicle were suppressed in mares treated with estradiol and progesterone in combination (Loy et al., 1981).

Progesterone in combination with DHT resulted in increased FSH response to GnRH but did not affect the response of LH (Thompson et al., 1986a). Treatment with DHT following a period of progesterone treatment resulted in decreased plasma concentrations of FSH and LH, an increase in the FSH response to GnRH, and a decrease in the LH response to GnRH (McNeill-Wiest et al., 1988).

**Manipulation of the Ovarian Factor.** Removal of the ovaries eliminates the negative feedback of ovarian products on FSH and LH. Thompson et al. (1987) reported that ovariectomized mares in the summer had an increase in LH and FSH peaks per 24 h over those of intact mares, but intact mares in estrus had higher concentrations of LH than ovariectomized
mares. Garcia et al. (1979) reported that ovariectomizing mares in the breeding season resulted in increased LH and FSH concentrations compared to intact mares, but ovariectomizing mares during the non-breeding season resulted in no rise in LH concentrations and no change in FSH concentrations. Freedman et al. (1979) also reported stable elevated levels of FSH and increased concentrations of LH in the ovulatory season with lower concentrations in the anovulatory season in ovariectomized mares. Porter et al. (1997) removed both ovaries and the influence from the pituitary by pituitary- stalk- sectioning ovariectomized mares, which resulted in suppression FSH and LH concentrations to undetectable levels. They were able to maintain lower FSH and LH concentrations with pulsatile injections of GnRH in these pituitary stalk-sectioned ovariectomized mares.

**Rationale for Present Experiment**

Based on previous experimental results with horses, DHT appears to be the most potent inhibitor of LH production and secretion, whereas EB appears to be the most potent inhibitor of FSH. The present experiment was designed to test the hypothesis that DHT plus EB treatment would completely suppress gonadotropin secretion in the mare. Such mares would potentially be good models for studying follicular dependence on gonadotropins. Subsequently, removal of one or both steroids would hypothetically remove the pituitary from feedback, such that high LH vs high FSH secretion rates would be produced. The ovarian responses to these different gonadotropin conditions should provide further insight into the gonadotropin dependence of follicles in the mare.
MATERIALS AND METHODS

Twelve mature, cyclic, light horse mares were used. The mares were between 4 and 18 years of age and were of good body condition (6 to 9, Henneke et al., 1983). All mares were maintained on native grass pasture and supplemented with grass hay when needed to maintain body condition.

Beginning in early June, mares were checked each morning for behavioral estrus with a vigorous stallion. Based on ultrasonography, 15 mares were determined to be between d 6 and 15 post-ovulation with a visible CL. On day -1, all mares received an injection of prostaglandin F$_2$\alpha (Lutalyse) to induce luteolysis. After determination of complete lysis of the CL by progesterone measurement, 12 mares were randomly selected and assigned to daily treatments of DHT (150 mg/kg BW) plus EB (22 mg/kg BW) in vegetable oil from d 0 through 30 (Phase I). For Phase II (d 31 to 66), mares were randomly assigned to one of three daily treatment groups: 1) control (vegetable oil), 2) DHT (150 mg/kg BW) in oil, 3) EB (22 mg/kg BW) in oil. The treatment assignment of individual mares was unknown to personnel involved with data collection.

Daily samples of jugular blood were collected each morning beginning on d -1 of Phase I and ending 5 d after ovulation in Phase II. All blood samples were collected prior to estrus detection and ultrasound examination on a given day. In addition, GnRH challenges were performed on d 30, 44, and 58 to determine the responses of LH and FSH. Blood samples were drawn through indwelling jugular catheters at -10, 0, 10, 20, 30, 45, 60, 90, 120 min after the i.v. injection of GnRH (0.1 mg/kg BW). Blood samples were immediately centrifuged and heparinized plasma was harvested and stored frozen. Radioimmunoassay was performed on daily and GnRH-challenge samples to determine concentrations of FSH and LH as described
previously (Thompson et al., 1983a,c) and on selected samples to determine concentrations of progesterone (Diagnostic Laboratory Systems, Webster, TX).

Ovarian activity was evaluated every other day by transrectal ultrasonography until a follicle 25 mm or greater was detected, at which time the mare was examined every day until ovulation or regression of the follicle to less than 25 mm. Activity of the ovaries was characterized by follicle size and numbers. Follicles were classified into three groups, small (≤ 10 mm), medium (11-24 mm), and large (≥ 25 mm).

Data were analyzed using the GLM procedures of SAS (SAS Inst. Inc., Cary, NC) in a completely randomized design for single point variables and split plot design (Gill and Hafs, 1971) for repetitive sampling variables. Factors in the analysis for data in Phase I were horse and day. Phase II factors for the split plot analysis were treatment, horse within treatment, day, and treatment × day. Additional factors in the analysis for the split-split plot design were minute, treatment × minute, day × minute, and treatment × day × minute. Differences between treatment groups for each period were assessed by Tukey’s HSD test (Steele and Torrie, 1998).
RESULTS

In Phase I (d 0 to 30), treatment of all mares with the combination of DHT and EB decreased (P = 0.0001) plasma concentrations of both LH and FSH (Figure 1). The number of small follicles was not affected by the combination of DHT and EB (Figure 2), whereas the number of medium follicles decreased (P = 0.0001) over time during Phase I (Figure 2). The number of large follicles also decreased (P = 0.0001) over time in mares during Phase I (Figure 3), as did the size of largest follicle, (P = 0.0001), from 23.2 ± 7.4 mm to 13.4 ± 7.5 mm (mean ± SD; Figure 3).

Mean plasma FSH and LH concentrations for the three treatment groups during Phase II are shown in Figure 4. In Phase II, DHT decreased (P = 0.01) plasma FSH concentrations below that of the control mares. Plasma concentrations of LH were also suppressed (P = 0.01) in mares treated with DHT. Estradiol benzoate also decreased (P = 0.0001) concentrations of FSH during Phase II below that of control mares. In contrast, EB increased (P = 0.0001) concentrations of LH over those of control and DHT-treated mares.

Mean plasma FSH and LH responses to GnRH for all treatments are shown in Figure 5. There was a significant treatment x day x minute interaction for FSH (P = 0.0001) and LH (P = 0.0019) concentrations in response to GnRH on d 30, 44, and 58. The response of FSH in DHT treated mares was increased (P = 0.0001) in response to GnRH on d 44 and d 58. In EB-treated mares, the FSH response to GnRH was decreased (P = 0.0001) on d 44 but was similar on d 58 to the control mares. Mares treated with DHT had decreased (P = 0.0002) LH responses to GnRH on d 44 and d 58. The LH response to GnRH in EB-treated mares was similar to the response of the control mares on d 30, d 44, and d 58.
Figure 1. Phase I mean plasma FSH and LH concentrations. Concentrations decreased over time for both FSH ($P = 0.0001$) and LH ($P = 0.0001$). Pooled standard error of the means were 1.8 ng/ml and 0.1 ng/ml for FSH and LH, respectively.
Figure 2. Phase I mean number of small follicles and medium follicles. The number of small follicles was not affected by treatment. The number of medium follicles decreased ($P = 0.001$) over time. Pooled SEM of the means were 2.0 and 0.8 for small follicles and medium follicles, respectively.
Figure 3. Phase I means of number of large follicles and size of the largest follicle. The number of large follicles decreased ($P = 0.001$) over time. The size of the largest follicle per day decreased ($P = 0.0001$) over time. Pooled SEM of the means were 0.2 and 2.5 mm for the number of large follicles and size of the largest per day, respectively.
Figure 4. Phase II mean plasma FSH and LH concentrations. There was a treatment effect ($P = 0.01$) and a treatment x day interaction ($P = 0.0001$) for FSH and LH. Pooled SEM were 1.6 ng/ml for FSH and 1.2 ng/ml for LH. Tukey’s honest significant difference (HSD) value ($P \leq 0.01$) for comparison of means is indicated by the vertical line located in the upper left hand corner of the graph.
Figure 5. Mean plasma response of FSH and LH to GnRH. There was a treatment x day x minute interaction (P = 0.0001) for FSH. The responses for all treatments were similar on d 30. On d 44 and d 58 DHT treated mares had a greater FSH response to GnRH. Estradiol treated mares FSH response was decreased below control mares on d 44 but was similar on d 58. There was an overall treatment effect (P = 0.05) and a treatment x day x minute interaction (P = 0.0002) for LH. DHT treated mares had decreased responses on d 44 and d 58. The EB treated and control mares had similar response on all days. The pooled SEM was 4.18 ng/ml and 0.62 ng/ml for FSH and LH, respectively. Tukey’s honest significant difference (HSD) value (P ≤ 0.01) for comparison of means is indicated by the vertical line located in the upper left hand corner of the graph.
During Phase II, there was no difference (P = 0.42) between groups for the number of small follicles (Figure 6). There was a treatment x day interaction for the number of medium follicles (P = 0.0001) during Phase II (Figure 6). Control mares and mares treated with EB had similar numbers of medium size follicles with the exception of d 39 where EB-treated mares had a greater number than did the control mares. The number of medium size follicles in EB-treated mares was greater than those in DHT treated mares on d 45, d 49, and d 51. There was also a treatment x day interaction (P = 0.0001) for the number of large follicles during Phase II (Figure 7). Mares treated with EB had larger numbers of large follicles on d 45 and d 47 than DHT treated mares. Mares treated with DHT had no large follicles throughout the duration of this study. There was a treatment x day interaction (P = 0.0001) for size of the largest follicle during Phase II (Figure 7). The size of the largest follicle in controls was greater on d 57 thru d 63 compared to EB and DHT treated mares, but was not different on d 65. Mares in the DHT (116.8 d) and EB (100.3 d) groups had a delayed (P = 0.01) day of ovulation compared with control mares (66.8 d).
Figure 6. Phase II mean number of small and medium follicles. The number of small follicles did not differ ($P = 0.42$) between groups or over time. There was a treatment x day interaction ($P = 0.0001$) for the number of medium follicles. Mares treated with EB had more medium size follicles on d 39 than control mares and on d 45, d 49, and d 51 than DHT treated mares. Pooled SEM were 8.1 and 2.7 for the number of small and medium follicles, respectively. Tukey’s honest significant difference (HSD) value ($P \leq 0.01$) for comparison of means is indicated by the vertical line located in the upper left hand corner of the graph.
Figure 7. Phase II mean number of large size follicles and size of the largest follicle per day. There was a treatment x day interaction (P = 0.0001) for the number of large follicles. Mares treated with EB had more large follicles on d 45 and d 47 than DHT-treated mares but were similar to control mares throughout the phase. There was a treatment x day interaction (P = 0.0001) for the size of the largest follicle. Control mares had an earlier emergence of large follicles by d 57 than either EB or DHT treated mares. Pooled SEM was 0.2 and 2.58 mm for number of large size follicles and size of the largest follicle per day, respectively. Tukey’s honest significant difference (HSD) value (P ≤ 0.01) for comparison of means is indicated by the vertical line located in the upper left hand corner of the graph.
DISCUSSION

During Phase I, concentrations of FSH and LH were decreased in response to treatment with the combination of DHT plus EB and appeared to be similar to those reported in anestrous mares (Thompson et al., 1986a). In contrast, LH concentrations increased in long-term ovariectomized mares treated with DHT and EB in combination (Garza et al., 1986b). Administering DHT following previous EB treatment resulted in a decrease in the elevated LH concentrations in ovariectomized mares (McNeill-Wiest et al., 1988).

During Phase II, plasma FSH concentrations decreased and remained constant with no apparent surges during the period of treatment with only DHT or EB, as it has been previously reported in ovariectomized mares (Garza et al., 1986b; Wiest et al., 1987; Thompson et al., 1991) and EB-treated cycling mares (Thompson et al., 1983b). These results differ from reports of DHT- (Thompson et al., 1983b) and EB- (Burns and Douglas, 1981; Evans et al., 1982) treated cycling mares having increased plasma FSH concentrations. There was a rise of FSH in the EB-treated mares on d 40, but this was due to one mare in this group having a surge of FSH resulting in the growth of a large follicle which ovulated 18 d later. Similar to other reports in the literature, DHT treatment in this experiment resulted in suppressed plasma LH concentrations (Wiest et al., 1987; McNeill-Wiest et al., 1988; Thompson et al., 1991). Further, the elevated plasma LH concentrations following treatment with EB (in the absence of high progesterone) observed in this experiment were similar to those reported previously (Woodley et al., 1979; Burns and Douglas, 1981; Evans et al., 1982). In ovariectomized mares, treatment with estradiol increased plasma concentrations of LH (Garcia and Ginther, 1978; Garcia et al., 1979; Garza et al., 1986b; Wiest et al., 1987; Sharp et al., 1991; Thompson et al., 1991).
The GnRH dose (0.1 µg/kg BW) used in this experiment elicited a physiological response of the gonadotropins similar to those reported by Alexander and Irvine (1986), where they used small doses of GnRH to elicit a response similar to the endogenous pulses of LH. Previous experiments have used a larger dose (1.0 µg/kg BW) of GnRH, which is believed to be saturating (high on the dose-response curve; Thompson et al., 1983b, 1991; Garza et al., 1985, 1986b). This could result in GnRH overcoming any inhibition from the steroids, therefore the lower dose was used.

Both intact (Thompson et al., 1983b, 1991) and ovariectomized (Wiest et al., 1987; McNeill-Wiest et al., 1988) mares treated with DHT alone had an increased FSH response to exogenous GnRH. This is likely due to the fact that the androgenic effect of DHT treatment increases the pituitary content of FSH (Thompson et al., 1991). The response of LH to exogenous GnRH in DHT-treated mares was decreased on d 44 and d 58. This could be the result of decreased pituitary content of LH in DHT-treated mares (Thompson et al., 1991). On d 30, the FSH and LH responses were similar between the EB, DHT, or control groups. This may be due to continued suppression of FSH and LH by both EB and DHT from Phase I. The FSH response to exogenous GnRH was similar in both control and EB mares on the d 44 and d 58 challenges. This similarity may be due to the fact that the control mares had large follicles that may have been producing estrogen. There was no LH response to exogenous GnRH in the EB-treated mares on d 44 and d 58 due to the fact that LH concentrations were already elevated. This lack of LH response to exogenous GnRH in EB-treated mares may support the idea that estradiol stimulates the synthesis of LH but does not alter the pituitary responsiveness to GnRH (Sharp et al., 1991). Control mares responded to exogenous GnRH with increased concentrations of LH on d 44. On d 58, the LH response in control mares was similar to that of
EB-treated mares, which maybe due to that fact the control mares were in estrus during this time, having a large, steroidogenically active pre-ovulatory follicle.

Ovarian activity was altered during this experiment depending on the gonadotropin concentrations present. However, the number of small follicles observed in either phase of this experiment did not differ. This suggests that small follicles are gonadotropin independent, which agrees with reports from Ginther (1992). During Phase I, no new follicles larger then 15 mm were observed, and any follicles greater than that size present at the start of treatment regressed. The decrease in the number of medium and large follicles and the size of the largest follicle during Phase I (treatment with the combination of DHT and EB) coincides with the decrease in the concentration of gonadotropins.

During Phase II, the number of medium follicles was similar between control and EB treatment groups with the exception of one day (d 39). This similarity could be due to the continued growth of small follicles, the failure of medium follicles to develop into large follicles, or the regression of large follicles (Pierson, 1992; Ginther and Bergfelt, 1993). The number of large follicles increased in EB-treated mares compared to control mares from d 41 to d 49, and DHT mares, d 41 through d 57, but they failed to ovulate. Woodley et al. (1979) and Burns and Douglas (1981) reported that estradiol treatment resulted in reduced follicular growth, decreased size of the largest follicle, and delayed ovulation. In the current study, the continued follicular development in EB-treated mares could be due to the high concentrations of LH. It is unclear why these follicles responded but did not ovulate when concentrations of LH were similar to levels that stimulate ovulation in an estrus mare.

Control mares had increased size of largest follicle compared to EB- and DHT- treated mares by d 59, which could be due to the recovery of the gonadotropins resulting in the growth
of the pre-ovulatory follicle. The decrease in the size of the largest follicle at the end of the experiment in the control mares was due to the fact all mares in this group had ovulated. Furthermore, concentrations of the gonadotropins had recovered in the control mares, and these mares ovulated by d 62. In contrast, ovulation was delayed in EB- and DHT-treated mares due to continued gonadotropin suppression.

Pregnant mares have suppressed gonadotropin concentrations late in gestation similar to the mares in this experiment. Yet, post-partum mares have an estrus and ovulation an average of 12 d after foaling (Irvine and Evans, 1975; Pope et al., 1987). During Phase II, the FSH concentrations did not begin to recover in the control mares for 10 days after conclusion of treatment while ovulation did not occur until an average of 36 days after conclusion of treatment. In pregnant mares, concentrations of FSH have been reported to surge before parturition with a large peak occurring at parturition, and LH concentrations increase a few days before ovulation (Irvine and Evans, 1975; Turner et al., 1979). This could be due to the rapid decrease in concentrations of progesterone and estrogen at parturition, removing the negative influence on the gonadotropins (Irvine and Evans, 1975; Lovell et al., 1975; Turner et al., 1979; Pope et al., 1987). The fetal-placental unit is the main source of progesterone and estrogen in late pregnancy, thus the expulsion of the foal and placenta is the reason for the rapid decrease in the steroid concentrations (Lovell et al., 1975; Pope et al., 1987). Turner et al. (1979) reported that removing the foal before parturition results in a shorter interval to estrus and to increased FSH and LH concentrations.

During this experiment, a common behavioral change in mares was observed. Some mares developed stallion-like behavior. This behavior consisted of increased aggression, herding, fleman responses, marking, and mounting other mares in estrus. Mares exhibiting this behavior
usually would not show behavioral estrus to a stallion and showed more aggression when teased with a stallion. This was noticed in some mares during the first week of treatment in Phase I and continued into Phase II. This change in behavior is thought to be due to the treatment with EB for the following reasons. First, treatment of geldings with estradiol results in restored stallion-like behavior and libido (Thompson et al., 1980). Second, during Phase II, the stud-like behavior was observed in 3 of the 4 EB-treated mares. Third, control and DHT-treated mares that exhibited this behavior during Phase I did not exhibit it during Phase II. Another observation was that the behavior appeared to occur more often in the dominant mares than in the mares lower in the social hierarchy. It is unknown why EB would cause male-like behavior in mares, and further research on this phenomenon is warranted.
Conclusions

It is concluded from this experiment that the use of DHT and EB can alter the synthesis and secretion of the gonadotropins in mares. These results support the hypothesis that estradiol and DHT work synergistically to decrease both FSH and LH to a greater extent than either steroid alone. Moreover, these results indicate that DHT in cycling mares results in decreased plasma FSH and LH concentrations while EB treatment results in decreased plasma FSH concentration and increased plasma LH concentrations. It also appears that ovarian activity is largely dependent on the concentrations of the gonadotropins with the exception of the small follicles. During Phase I, when DHT and EB were administered together and FSH and LH concentrations were decreased to anestrous levels, mares did not grow any follicles greater than 15 mm. This experiment also indicates that both gonadotropin levels must be restored for growth of an ovulatory size follicle and the subsequent ovulation of that follicle.
REFERENCES


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VITA

Scarlett Lynn McMeen, daughter of Billy and Linda Gourley, was born in Nashville, Tennessee, on November 7, 1976. Scarlett is the youngest of three children. She lived in Nashville until the age of one. At this time she moved to Dickson, Tennessee, where she lived and attended public schools until age eighteen, when she graduated from Dickson County High School in May, 1995. Scarlett attended the University of Tennessee at Martin, an agricultural college in Martin, Tennessee, from August 1995 until May 1999 when she received a bachelor’s degree in animal science. In July 2000, Scarlett married her husband, Joseph Wayne McMeen, in Burns, Tennessee. Scarlett moved to Baton Rouge, Louisiana, along with her husband, in August 2000, to attend Louisiana State University and pursue a master’s degree in animal science with an emphasis on equine reproductive physiology and endocrinology.