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THE EFFECTS OF REPRODUCTIVE MANAGEMENT SYSTEMS ON LUTEINIZING HORMONE AND ESTRADIOL 17-β IN MARES

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THE EFFECTS OF REPRODUCTIVE MANAGEMENT SYSTEMS ON LUTEINIZING HORMONE AND ESTRADIOL 17-β IN MARES

By

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ABSTRACT

THE EFFECTS OF REPRODUCTIVE MANAGEMENT SYSTEMS ON LUTEINIZING HORMONE AND ESTRADIOL 17-β IN MARES

By

Colleen M. Brady

Cortisol and β-endorphin have been shown to inhibit secretion of estradiol and luteinizing hormone. A decrease in luteinizing hormone and estradiol could cause infertility in mares. Experiments were performed to determine if increased intensity of reproductive management before breeding affects estradiol 17-β, luteinizing hormone, cortisol and β-endorphin in mares. In Experiment 1, ovaries of six two year old Arabian mares were examined by rectal palpation and ultrasound, once per day until the dominant follicle had a diameter of 35mm (phase 1). Ovaries were then examined by rectal palpation and ultrasound four times per day until ovulation was detected (phase 2). Mares were examined, and blood was sampled at 0600h, 1200h, 1800h and 2400h through three successive periods of estrus. Serum cortisol concentrations were higher ($P<.01$) when mares were palpated four times daily than when mares were palpated once daily. Serum luteinizing hormone and estradiol 17-β concentrations were not different between phase 1 and phase 2 of estrus. In Experiment 2, twenty-four Arabian mares were blocked by age and lactation status, and assigned to one of three groups. Mares were pasture bred (C) , hand mated every other day from onset of estrus to end of estrus (N), or examined by ultrasonography every other

day until the largest follicle reached 35 mm in diameter, then inseminated artificially and examined by utrasonography daily until ovulation occurred (A). Mares were teased daily and mated as assigned by treatment group protocol for one estrus period. Blood was collected via jugular venipuncture daily at 0600h, 1200h, and 1800h from day 1 of estrus to the end of estrus. Plasma cortisol, β-endorphin, luteinizing hormone and estradiol 17-β were quantified by radioimmunoassay. β-endorphin concentrations were not different between treatment groups. There was a trend ($P<.07$) for plasma cortisol concentrations to be higher in hand mated and pasture bred mares than in artificially inseminated mares ($P<.0001$). Plasma luteinizing hormone concentrations were higher ($P<.0005$) in hand mated mares than in artificially inseminated or pasture bred mares. Plasma estradiol 17-β concentrations were higher ($P<.05$) in artificially inseminated than in pasture bred mares. Estradiol 17-β concentrations in hand mated did not differ from artificially inseminated or pasture bred mares. There was no correlation ($P<.08$) between cortisol concentrations, and luteinizing hormone or estradiol 17-β concentrations among treatment groups. In conclusion, although cortisol was increased in intensively palpated mares in Experiment 1, with a subsequent attenuation of the late estrus increases in luteinizing hormone and estradiol, this affect was not observed in Experiment 2, when mares were managed with different reproductive management systems similar to those used in the industry.

DEDICATION

This dissertation is dedicated to my mother, Bette G. Brady, who has been an inspiration to me throughout my life, and without whose support this endeavor would have never been completed.

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INTRODUCTION

Among mammalian livestock, mares have the lowest reproductive efficiency. Pregnancy rate (number of mares pregnant/ number of mares mated), is estimated at 55% in a given ovulatory period, and 85% by the end of the breeding season (Ginther 1993). In other species, pregnancy rate is defined as the number of animals pregnant / number of animals in the breeding group, and number of animals pregnant / number animals mated is referred to as the conception rate. Differentiating between the number of animals in the breeding group and the number of animals mated accounts for failure to detect estrus. However, in mares, number of animals in the breeding group and number of animals mated are virtually identical. Mares are examined individually for signs of behavioral estrus, and mares not displaying behavioral estrus are examined by rectal palpation and ultrasound to detect physiological changes associated with estrus, and are then mated accordingly. These management tools result in virtually all mares in the breeding group being mated during the breeding season. But, the amount of human participation may have consequences.

The primary source of income for horse breeders is sale of offspring. Therefore, increased reproductive efficiency will increase income. There are several obstacles to overcome to increase reproductive efficiency in mares. Mares are seasonally polyestrous. In the Northern Hemisphere the spontaneous breeding season is from April to September and resulting foals are born from

May to August. From September to March, reproductive tracts of non-gravid mares become quiescent and the ovaries are non-ovulatory. Independent of biological birthdate, registered foals of all breeds are assigned an official birthdate of January 1. Horses perform within groups formed by age for showing and racing. Because the assigned birthdate is used to place horses into groups, there is a distinct advantage to foals born closer to January 1, but after December 31. To maximize the foal's chronological age at the time of the official birthdate, breeders strive to achieve conception in February and March. This desired time of conception precedes onset of the spontaneous breeding season by 2 to 3 months. Therefore, at the desired time for conception, many mares are anovulatory or cycling erratically.

A second obstacle to increased reproductive efficiency in mares is the age of mares at mating. Many cows are no longer part of a breeding herd and do not reproduce after seven years. In contrast, many mares are just beginning to produce offspring at that age. Mares are frequently over the age of five years, and sometimes greater than ten years of age, before they are mated or inseminated the first time. This delayed start of reproduction is especially prevalent in mares that engaged successfully in competitions. Although mares reach puberty at 12-18 months of age (Ginther 1993) they frequently enter the reproductive herd later than other species, and are retained in the reproductive herd for a longer time. Culling rates for reproductive failure are much lower in mares than in other species. Mares may remain in production up to and beyond the age of 20 years. As mares age, there is an increased tolerance by mare

owners for reduced efficiency. Many older mares will produce a foal only every other year yet still remain in the active breeding herd. It has been reported in mares(Carnevale 1991) as well as other species, including humans, that reproductive success decreases after an optimal age.

These obstacles could be overcome by changing the assigned birthdate and bringing it in alignment with the spontaneous breeding season, breeding mares at a younger age, and removing them from the breeding group when reproductive efficiency declined. However, these are drastic changes and are unlikely to occur. Therefore, work is needed to develop methods to reproductively manage mares more efficiently within the current industry constraints.

Over the last decade, reproductive efficiency in mares has increased by an estimated 9% (Ginther 1993), to approximately 85% at the end of the breeding season. This increased reproductive efficiency is associated with increased understanding of the reproductive physiology of mares and increased use of technology such as ultrasonography. Even with this improvement, the pregnancy rate for managed mares at the end of the breeding season (85%) is less than the pregnancy rate for feral horses (90 to 100%) at the end of the breeding season (Daels 1995). Although reproductive efficiency has improved through a greater understanding of physiology and the use of technology, opportunity exists for more improvement. Increased understanding of the effects of management on reproductive physiology could be an important link in

understanding why domestic mares have a lower pregnancy rate than feral mares.

Variations in hormonal secretion, and the coordinated changes in action of hormones largely control reproduction. The experiments in this study were designed to determine if human intervention in the equine reproductive process changes secretion of the hormones that exert primary control of estrus and ovulation in mares. If yes, this may help explain lower reproductive efficiency in domestic mares compared to feral mares. It is well known that many wild species have decreased reproductive efficiency in captivity, compared to non-captive. Therefore, our goals are to:

1) Determine whether intensity of reproductive management will alter estradiol and luteinizing hormone concentrations in blood.
2) Determine if secretion of cortisol and β-endorphin, physiological indicators of stress, are affected by management intensity.
3) Determine if there are associations between indicators of stress and hormones that regulate reproduction. Although this study may show a correlation between the physiological indicators of stress and reproductive hormones, it is not designed to determine a cause-effect relationship.

Figure 1: Seasonality of estrous cycles in mares in the Northern Hemisphere.
Mares are seasonally polyestrous. Within a calendar year there is a period of cyclicity bordered by two periods of anestrus. In the Northern Hemisphere, the resurging phase occurs in the spring, when mares return to cyclicity, and the receding phase occurs in the fall, when mares return to anestrus.

LITERATURE REVIEW

Reproductive Biology of Mares

Season

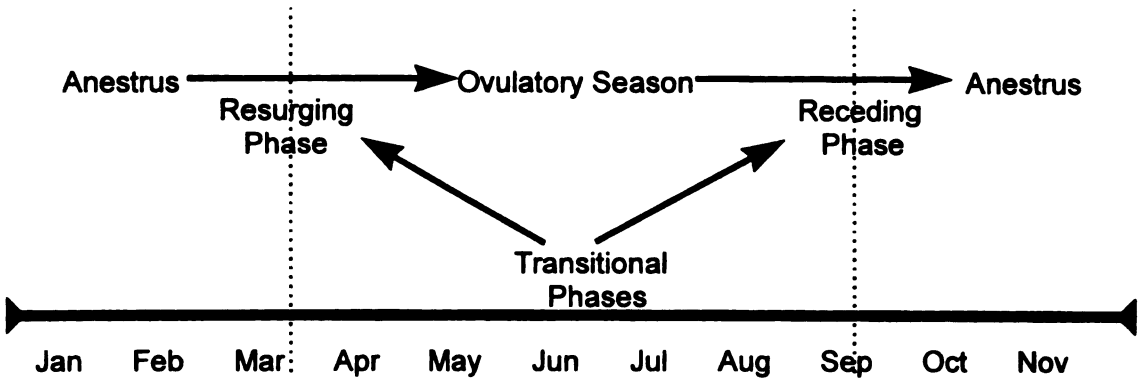


Figure 1.

Anestrus

Mares are seasonally polyestrus, with the spontaneous breeding season occurring from May to September in the Northern Hemisphere. During the fall and winter anestrus, the reproductive tract in mares is quiescent and the ovaries are inactive. The anestrus season is characterized by low concentrations of estradiol (Oxender 1977) and luteinizing hormone (Garcia 1976) in blood and low follicular activity (Ginther 1993). Concentrations of luteinizing hormone during anestrus are similar to concentrations during mid-diestrus (Garcia 1976), a time when spontaneous ovulation does not occur.

Transition

The resurging phase is the gradual resumption of activity in the reproductive tract before or after anestrus. Spring transition is characterized by

behavioral signs of estrus of variable length and intensity, increased ovarian activity, and the occurrence of three anovulatory follicular waves before the first ovulatory wave. Estradiol produced during the anovulatory follicular waves is presumed responsible for the erratic behavioral estrus typical of the transitional period. Follicles developing during the follicular wave produce estradiol, the hormone responsible for behavioral signs of estrus in mares. An ovulation has not occurred recently, so there is no corpus luteum (CL) present producing progesterone to prevent the estradiol from causing behavioral signs of estrus. Estrus and ovulation may be asynchronous during transition, decreasing fertility (Ginther 1993).

Increased hours of light per 24 hours instigates the onset of spring transition (Squires 1993). Exposure to light will decrease the secretion of melatonin by the pineal gland. This is important because melatonin inhibits secretion of luteinizing hormone releasing hormone (LHRH) and luteinizing hormone (LH). With less melatonin to suppress secretion of LHRH, LH and follicle stimulating hormone (FSH) increase, and the ovulatory season begins (Figure 2). Concentrations of LH in the pituitary are decreased during the anovulatory season, but with development of estrogen active follicles there is increased concentration of LH in the pituitary. The pituitary can then respond to estradiol and release a prolonged surge of LH to induce the first ovulation of the season.

Figure 2: The effect of photoperiod on gonadotropic hormones in mares. Increasing hours of daylight decreases melatonin secretion by the pineal gland. Melatonin inhibition of luteinizing releasing hormone (LHRH) is removed so increased secretion of LHRH stimulates increased secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH). Increased secretion of FSH and LH increases follicular activity. Decreasing hours of daylight increases pineal secretion of melatonin. Melatonin inhibits LHRH. LH and FSH decrease and follicular activity decreases. ⇧ Stimulatory ⬇ Inhibitory

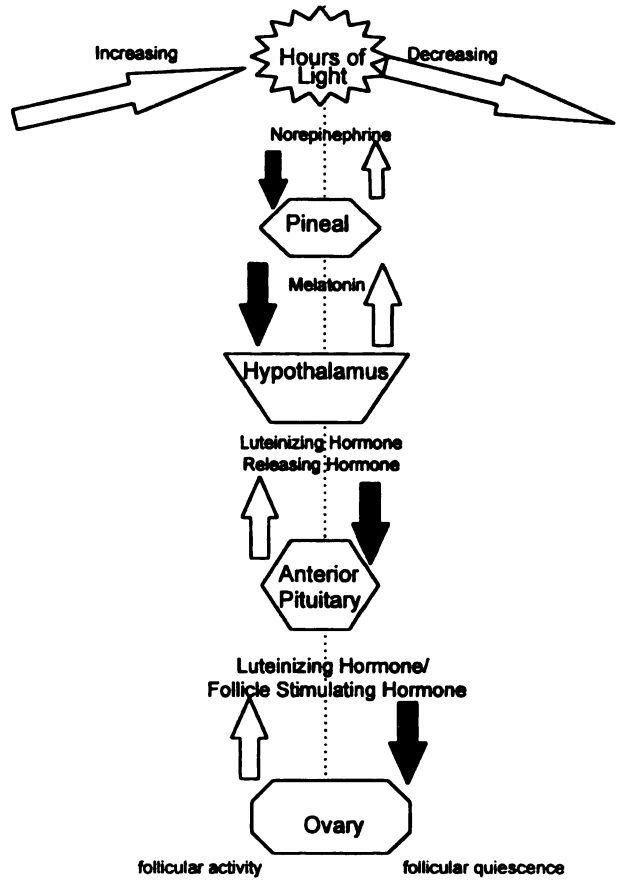


Figure 2

The ovulatory season begins spontaneously in May in the Northern Hemisphere. Attempts by managers to begin the ovulatory season in mares earlier and thus to start the breeding season earlier, have met with limited success. Exposure of mares to increased hours of light daily has been the most successful method for precocious induction of ovulatory season. Use of supplemental light to achieve 15 to 16 hours of light/day decreased melatonin and ovulation resumed, similar to when ambient photoperiod produced 15 to 16

hours of light per day (Loy 1968). To induce the ovulatory season, increased duration of daily light should begin 90 days prior to desired time of first ovulation. Exogenous progestins do not induce onset of the resurging phase, but can shorten the period of transition and increase regularity of early estrus periods (Squires 1979; Alexander 1990; Huszenicza 1990). Exogenous gonadotrophin releasing hormone (GnRH) induces ovulation in 75 to 100% of treated mares during the anovulatory season (Johnson, 1988; Fitzgerald, 1987; Allen, 1987). Variation in efficacy appears to be in part due to frequency of treatment, with more frequent treatment inducing more mares to ovulate than less frequent treatment (Palmer, 1988). However, this method is very expensive, and may not be cost-effective for some breeders.

The receding phase (Figure 1) occurs when the reproductive tract gradually becomes quiescent after the last ovulation of the ovulatory season. Decreased hours of light per twenty four hour period will increase melatonin secretion by the pineal gland. Increased melatonin will inhibit secretion of LHRH from the hypothalamus, and consequently there is decreased LH in the pituitary and decreased release of LH (Figure 2). Pituitary LH decreases progressively from the middle of the ovulatory season, to the middle of the anovulatory season (Silvia 1987). Failure to ovulate is associated with decreased LH in blood, absence of preovulatory "surges" of LH and stagnation of the growth of the largest follicle (Snyder 1979). Duration of the ovulatory phase is not fixed, it is controlled by day length. Therefore, early induction of the ovulatory season in the spring does not result in early onset of the anovulatory

season in the fall. The anovulatory season begins as daylength shortens regardless of when the ovulatory season began.

Estrous Cycles

After the first ovulation of a year, the ovulatory season continues with repeated estrous cycles ending in ovulation every 21 to 24 days. In mares the average duration of an estrous cycle is 21 days, with a 15 day (± 2 days) luteal phase (diestrus) and a 6 day (± 2 days) follicular phase (estrus). Duration of estrus is longest (up to 25 days) and most variable during transitions, resurgence and recession. In contrast, the duration of estrus is less variable in the middle of the ovulatory season(Ginther 1993).

Hormonal Profiles

Steroids

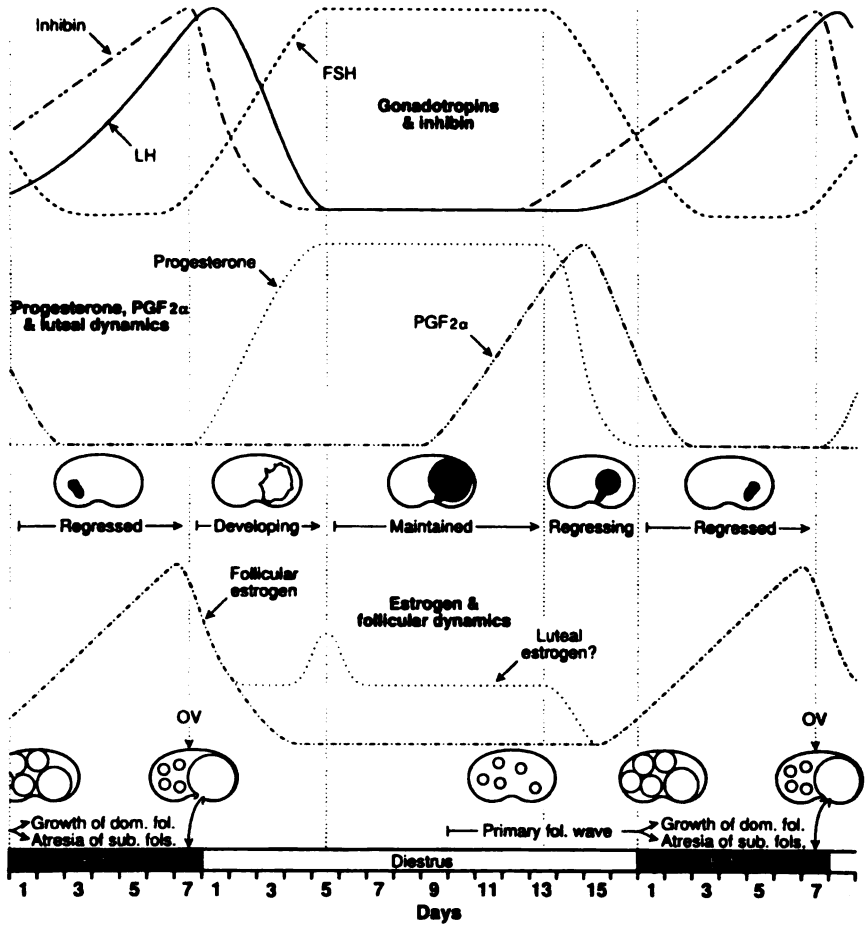
Estradiol 17-β and progesterone are produced by ovarian structures (Ginther 1993). These steroids control behavioral and physiological changes in mares throughout the estrous cycle. Cortisol is the most bio-active glucocorticoid in horses and is secreted by the adrenal cortex (James 1970).

Estradiol 17-β

Estradiol 17-β is produced by ovarian follicles during estrus. When progesterone secretion is low estradiol causes physical changes and behavioral signs of estrus in mares (Figure 3). As follicles grow and approach ovulation,

Figure 3: Temporal relationships among hormones in blood and ovarian events in mares.

(Ginther, 1993)


Inhibin
FSH
LH
Gonadotropins & inhibin
Progesterone
Progesterone, PGF2α & luteal dynamics
PGF2α
Regressed
Developing
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Follicular estrogen
Estrogen & follicular dynamics
Luteal estrogen?
OV
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Growth of dom. fol.
Atresia of sub. fols.
Primary fol. wave
Growth of dom. fol.
Atresia of sub. fols.
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1 3 5 7 1 3 5 7 9 11 13 15 1 3 5 7
Days

Figure 3

estradiol secretion by follicles increases. In fact, survival of follicles to ovulation is very dependent on secretion of estradiol. Increased concentrations of estradiol 17-β in blood stimulate increased secretion of LH from the pituitary. When a dominant follicle is present and a CL is absent, increased secretion of LH will cause ovulation (Figure 3 and Figure 4). Six to eight days before ovulation concentrations of estradiol 17-β in blood increase from a nadir in diestrus levels to a peak about 2 days before ovulation (Figure 3). Estradiol 17-β returns to diestrus levels within one to two days after ovulation, and signs of behavioral estrus disappear (Ginther 1993). Estrogens produced by the corpus luteum (CL), and from follicular waves, can increase concentrations in the blood during diestrus. Behavioral estrus does not accompany increased estradiol during diestrus because progesterone concentration in the blood is high during this time (Asa 1984).

Progesterone

Progesterone concentrations are high during diestrus and pregnancy in mares. The CL produces progesterone during the luteal phase of the estrous cycle, beginning 12 to 24 hours after ovulation (Townson 1989). Progesterone remains elevated for the life of the CL, either throughout diestrus in non-pregnant mares or throughout gestation in pregnant mares. Low progesterone, either from a small CL or luteal insufficiency, is diagnosed commonly in mares, and is

believed to be a source of infertility. Therapy for luteal insufficiency is exogenous progesterone.

Production of prostaglandin F2 α by the endometrium is a major stimulus for regression of the CL 12 days after ovulation in non-pregnant mares and in mares that have failure of maternal recognition of pregnancy. After regression of the CL, progesterone concentration in the blood declines to nearly undetectable levels during proestrus and estrus. The decline in progesterone removes the negative feedback effect on LH. With increased secretion of LH from the pituitary and estradiol from follicles, estrus and ovulation will occur.

Cortisol

Cortisol is secreted from the adrenal cortex in a diurnal pattern, or daily cycle. Within the diurnal pattern of cortisol, the nadir is in the morning and the zenith is in the evening (Irvine 1994). During an estrous cycle cortisol secretion is increased during diestrus, and lowest during estrus, and there is no effect of day of estrus on secretion of cortisol in mares (Asa 1983). Blood was sampled once daily to determine cortisol, so it is unclear if the diurnal pattern of cortisol is altered, or the magnitude of cortisol secretion. Noting that duration of estrus in mares is from 5 to 7 days, there is no effect of day of estrus on secretion of cortisol in mares.

Figure 4: Regulation of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in mares.

Secretion of luteinizing hormone releasing hormone (LHRH) from the hypothalamus stimulates release of FSH and LH from the anterior pituitary gland. FSH stimulates ovarian follicular growth and increased estradiol secretion. Estradiol stimulates LHRH secretion from the hypothalamus and stimulates LH secretion, and estradiol inhibits FSH secretion from the pituitary. LH causes ovulation, luteinization of the ovulatory follicle. Progesterone inhibits LH secretion from the pituitary. ——— Stimulatory - - - - Inhibitory

Gonadotropins

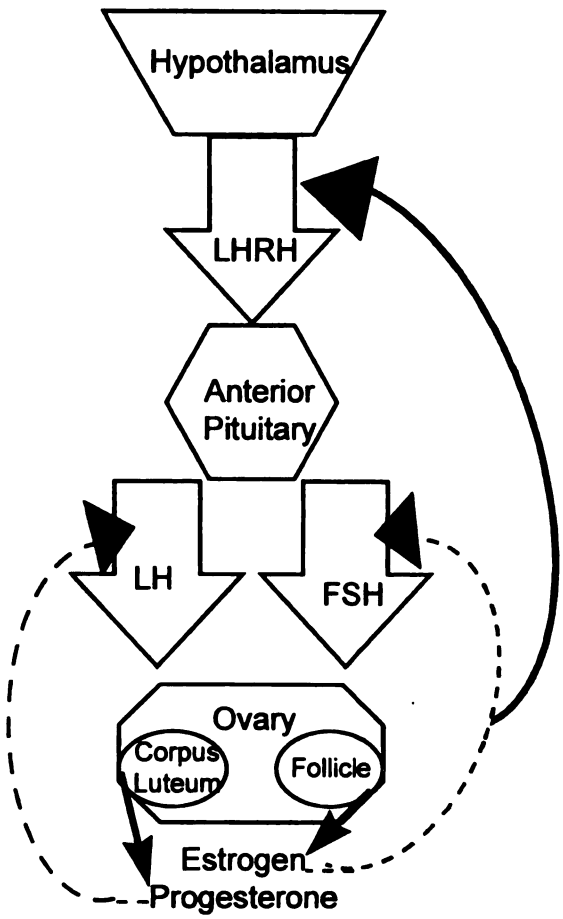

Figure 4

In response to neural activity in the hypothalamus, LHRH is released in pulses. Increased frequency of LHRH pulses causes increased secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) which are released in pulses from the anterior pituitary. (Figure 4). Pulses of LH and FSH are synchronous during diestrus, and asynchronous during estrus (Ginther 1993). The precise mechanism of control of LH and FSH pulses is unclear. It is

clear that LHRH affects the gondotrophs, but other secretagogues may also play a role.

Follicle Stimulating Hormone

In livestock , FSH is necessary for growth and function of follicles. FSH is released in a pulsatile manner from the pituitary in response to increased stimulus from hypothalamic LHRH. Secretion of FSH is related inversely to circulating concentrations of inhibin in blood. Concentrations of FSH in blood increase during early and mid diestrus, and peak about 8 days before estrus, with lowest concentrations during estrus. The frequency of FSH peaks in jugular blood is greater at day 0 than at days 7 to 9 or at day 15 after ovulation (Ginther 1993). The concentration of FSH in blood increases because FSH concentrations do not return to baseline between successive pulses. Secretory patterns of FSH vary among studies, with one (Miller 1980), or two (Nett 1979; Burns 1981), FSH surges being reported during an estrous cycle. Modality of FSH secretion may be affected by individual variation or by stage of breeding season (Miller 1980).

Luteinizing Hormone

Luteinizing hormone (LH) causes ovulation in all female domestic livestock. Except for mares, livestock females have an acute increase in luteinizing hormone concentration within 12 to 24 hours before ovulation. In mares increased LH concentration in blood is gradual and prolonged (Figure 3).

Concentrations of LH begin to rise during diestrus, 5 to 6 days before onset of estrus. The peak LH concentration occurs 24 hours after ovulation, then decreases over the first 4 to 6 days of diestrus and returns to basal levels (Miller 1980). In mares, the duration of increased LH is 8 to 10 days. Luteinizing hormone is released in a pulsatile manner. Increased concentration of LH in blood is caused by increased pulse frequency of LHRH and LH as ovulation approaches. The long half-life of equine LH (>1hour) produces an additive effect on concentration of LH in blood as subsequent pulses add to the concentration of LH already in blood.

It is curious why the preovulatory LH profile of mares is so different from other livestock. This difference was examined with attention to concentrations of bioactive and immunoactive LH (Alexander 1982). Blood concentrations of bioactive isoforms of equine luteinizing hormone increase acutely hours prior to ovulation in mares, with a second peak corresponding with the peak of immunoactive LH. So, the secretory pattern of bioactive LH in mares is similar to the preovulatory secretory pattern in other livestock females. In the same study, blood concentrations of immunoactive isoforms of LH increased gradually to peak after ovulation and decreased gradually over several days. This profile for immunoactive isoforms is similar to profiles commonly reported in mares. The decline in bioactive LH following the second peak is similar to that of immunoactive LH. This difference between secretory patterns of bioactive LH and immunoactive LH may explain why the LH profile in mares throughout the estrous cycle differs from the pattern in other livestock.

Hormonally Regulated Events

Ovary

Ovaries are very dynamic organs. Follicles and corpora lutea are two transient ovarian structures that produce the steroid hormones that control the cyclicity of estrous. Presence and function of follicles and a CL contribute to the dynamics of the ovary and are critical to regulation of estrous cycles. Ovaries are more active, with more follicular growth, early in the ovulatory season than late in the ovulatory season. Follicles produce many hormones but a major product is estradiol 17-β. Estradiol 17-β causes behavioral changes such as posturing, and physiological changes such as thickening of the endometrium, that are associated with estrus. Estradiol also stimulates secretion of luteinizing hormone (Figure 4), which is necessary to induce ovulation. Progesterone is the major hormonal product of corpora lutea. Progesterone is a steroid hormone responsible for physiological changes associated with diestrus and pregnancy, such as increased uterine tone and inhibition of estrus behaviors. Progesterone also inhibits secretion of luteinizing hormone (Figure 4).

Ovarian follicular activity is stimulated by increased hours of light per day. During the resurging phase of spring transition (Figure 2), follicles grow in waves with ovulation at the end of the third or fourth wave of follicular growth (LeBlanc 1998). Follicular waves continue throughout the ovulatory season and may occur during diestrus (secondary waves) or estrus (primary waves).

The specific mechanisms of follicular selection and atresia are unclear. In an ovulatory wave associated with estrus, the dominant follicle has increased LH receptors in the thecal cells (Fay 1987) and produces 30 to 50 times more estrogens than subordinate follicles (Ginther 1993). Ovulation occurs when intrafollicular proteolytic enzymes weaken the follicular wall. Thecal cells degenerate, and granulosal cells dissociate immediately before ovulation. The oocyte then enters the ovulation fossa, leaving remnants of the follicle as precursors to the corpus luteum.

The origin of a corpus luteum is the ovulatory follicle. There is cellular continuity between the ovulatory follicle and the CL. Granulosal cells of the ovulated follicle are luteinized in the presence of LH beginning 12 to 24 hours after ovulation. Unlike other livestock species, where luteal tissue is of thecal and granulosal origin, in mares, it appears that thecal tissue does not contribute to formation of the CL. Luteinization involves structural and functional differentiation of granulosal cells which is stimulated by LH, and is complete 4 to 5 days after ovulation (Ginther, 1993). With luteinization estradiol production ceases and production of progesterone is increased. Although luteinizing hormone concentrations decrease after ovulation, the number of LH receptors and the affinity of the luteal cells for LH increases from Day 1 to Day 13 (Roser, 1983).

The primary factor causing regression of the CL in mares is PGF2α from the endometrium. The CL is not responsive to PGF2α in the first four days

following ovulation (Kimball 1977), in part due to a lack of PGF2α receptors in the luteal cells (Vernon 1979). In non-pregnant mares, the CL is maintained until 14 days after ovulation when PGF2-α from the endometrium lyses the CL and progesterone concentrations in blood decrease to undetectable levels. Removal of the negative effects of progesterone on the brain and the pituitary, in conjunction with increased follicular growth and production of estradiol, causes the physical and behavioral signs of estrus. There are no direct adverse effects of estrogen on viability, function or life span of the CL in mares (Burns 1981; King 1990), so PGF2-α is required as a luteolysin to cause the regression of the CL necessary for repeated estrous cycles. In pregnant mares, the CL is maintained throughout pregnancy, and continues to secrete progesterone. After 40 to 50 days of pregnancy, endometrial cups are formed and secrete chorionic gonadotropin (eCG). In response to eCG, the CL of pregnant mares continues to secrete progestins and various estrogens throughout gestation.

Uterus

The equine uterus consists of two small uterine horns and a prominent uterine body. The internal bifurcation is marked by the uterine septum, and is less prominent than the bifurcation in cattle or sheep. The uterus consists of an external serosal layer, the myometrium, and the endometrium. The contractile activity of the myometrium changes throughout the estrous cycle. Myometrial activity is most intense during luteolysis, with short duration and high frequency periods of activity stimulated by PGF2-α. Exogenous PGF2-α increased

myometrial activity, in a pattern similar to that seen during spontaneous luteolysis. During estrus, contractility occurs in clusters, with highly active phases separated by extended periods of quiescence. This pattern occurs in response to increased concentrations of estrogen and concurrent very low levels of progesterone.

During diestrus, myometrial activity of low amplitude is interrupted by inactive periods of variable length. As luteolysis occurs and there is decreased progesterone in blood, uterine tone is decreased. In contrast, uterine tone is flaccid during estrus. With an embryo present, myometrial activity is increased by day 9 after ovulation, and is maximal from days 11 to 16 post ovulation. Increased myometrial activity is the basis for increased embryonic mobility which is vital for maternal recognition of pregnancy in mares (Mc Dowell 1987). Increased myometrial activity also causes turgid uterine tone typical of mares in diestrus or early pregnancy. Maximal uterine tone is present when the myometrium has been primed with progesterone and is exposed to low levels of estradiol. The low levels of estradiol needed for maximum tone of the endometrium are provided by the embryo (Ginther 1993). Uterine tone is vital to fix equine embryos 16 days after ovulation. Embryonic fixation occurs when increased uterine tone decreases the diameter of the uterine horn sufficiently to impinge on the walls of the growing embryonic vesicle, therefore restricting free movement of the embryo (Ginther 1983).

The endometrium is more edematous and has greater cellular activity during estrus than during diestrus (Kenney 1978; Hamer 1985). Edema is

present because vascularity increases in response to increased estradiol. Increased edema enlarges the uterus, disperses endometrial glands and the causes flaccid uterine tone. In contrast, under the influence of progesterone uterine edema is reduced and density of endometrial glands is increased (Kenney 1978; Hamer 1985). During diestrus, increased uterine tone decreases the diameter of the uterine lumen until movement of the growing embryo is restricted and fixation occurs. The chorion of the equine embryo does not invade the endometrium of the mare until day 38, when the endometrial cups begin to form. Presence of the endometrial cups is critical to impantation.

Sexual Behavior

The most dependable method to identify behavioral estrus is to expose mares to a stallion (teasing). In response to vocal, visual, and physical stimuli from a stallion, most mares in estrus will display the classic signs of estrus. Mares will assume a distinctive posture that includes squatting and tail raising. Frequent urination, and opening and closing of the vulva, exposing and projecting the clitoris (vulvar winking) accompany the estrus posture (Ginther 1993). When mares are not receptive, they will project ears posteriorly, kick, and try to escape the presence of the stallion.

Some domestic mares (about 7%) experience silent estrus(Cummings 1942; Nelson 1985). In silent estrus, the physiological changes of estrus, including ovulation, occur without overt expression of behavior. In contrast to ovulatory waves of follicles during diestrus, during silent estrus the cervix

becomes relaxed, and uterine tone is flaccid. These mares present a special challenge to managers because without a detected estrus it is difficult to determine the proper time to inseminate. To accurately determine time of insemination, mares must have the ovaries examined daily by palpation or ultrasonography throughout the estrous cycle.

Estradiol controls the display of behavioral estrus. In rats, high levels of estrogen increase the density of neuronal dendritic spines in the hippocampus, which is positively correlated with estrous behavior. The presence of progesterone reduces neuronal dendritic spine density, and blocks estrous behavior (Woolley 1993).

Feral mares have a very proactive, not passive, role in courtship and mating behavior (McDonnell 1998). Mares in estrus approach a stallion and will court his attention. The role of domestic mares in estrus detection varies with management system. Some domestic mares are not allowed an active role in estrus detection. In contrast to feral horses, domestic stallions are brought to the mares, which are usually restrained or confined. This difference in behavior at estrus of feral and domestic mares may be induced by management and may contribute in part to decreased reproductive efficiency in domestic mares compared to feral mares.

Reproductive Management

Pre-breeding Management

Teasing

Teasing is currently the most common, and effective, tool in pre-breeding management of mares (Squires 1993). Teasing is when mares are exposed to an active stallion and behavioral responses of the mare are documented. Mares respond to vocal, visual and physical stimuli from a stallion, and exhibit a wide range of behavioral and physical responses to teasing, both in estrus and diestrus. When exposed to stallions, mares in diestrus or that are anovulatory may have no overt response or may display violent aggression toward stallions. Mares in estrus are less resistant and display overt signs of estrus including, but not limited to, squatting, frequent urination, tail raising, and vulvar winking (Squires 1993). Optimal teasing efficiency is obtained when the operator knows the behavior of each individual mare well enough to detect subtle changes in behavior.

Several teasing methods have been developed, each with their own advantages and disadvantages (Evans 1990). Pen teasing is when mares are loose in a small corral or pasture with a non-restrained stallion in an enclosure within the pen. This style of teasing is very efficient because during teasing one person can observe a large number of mares at the same time. Humans do not restrict or handle mares or stallions so this method also allows the greatest range of instinctive behaviors for both genders. The stallion pen should be

10'x10' to 12'x12'. This allows some movement, but limits the stallion from running. The corral or pasture should be approximately 20 feet by 40 feet for 15 to 20 mares. It is important that the corral be large enough to allow non-estrus or timid mares to stay away from aggressive mares, but small enough to bring the mares in proximity to the stallion. Aggressive mares may prevent submissive mares that are in estrus from approaching the stallion, limiting the opportunity for interaction with the stallion. Mares will show estrus to just vocal and visual stimuli from the stallion without physical contact. It is also possible to lead a mare up to the fence for more intimate exposure to the stallion, or overly aggressive mares can be removed from the corral after observation.

Fenceline teasing is similar to pen teasing except the stallion is controlled on a lead shank. The mares remain loose in a pasture or pen, and the stallion teases them over the fence. There should be 8 to 10 feet of fenceline for each mare teased to ensure the safety of the mares. This method has the same disadvantages as pen teasing, because aggressive mares may keep submissive mares from the stallion. Fenceline teasing does offer a greater degree of control over the stallion, which may be safer for the stallion and people, but fenceline teasing may also require the use of two people, one to handle the stallion, and one to observe the mares, depending on the size of the pen, and the number of mares being teased.

The use of a tease chute is one of the most common teasing methods. Stallions are restrained on one side of a wall, and mares are brought into a chute on the other side of the wall. The stallion then teases the mare. This method

allows exposure of all mares to visual, vocal and tactile stimulation from the stallion. Some managers use twitches to restrain mares in the chute. This method allows the least expression of instinctive behaviors because both the mare and the stallion are restrained.

Stall teasing can involve bringing the mare to the stallion's stall, or bringing the stallion to the mare's stall. When the stallion is brought to the mare's stall, the mare is loose in her stall, and has the opportunity to display behavior with no intervention. The mare can also be led to the stallion's stall, where she is teased. This method requires more time, to lead each mare to the stallion's stall. Either version of this method usually requires only one person.

The use of a vasectomized stallion, or pony stallion, to tease mares is the least common of the teasing methods discussed. In this scenario, a stallion is turned to pasture with mares. Because of the vasectomy, or the size differential in the case of the pony, it is assumed that estrus can be detected with no pregnancy. This method has the advantage of allowing maximum instinctive behaviors by stallions and mares. Observation of the stallion and mares must be frequent to detect estrus behavior. This method has the potential for the same types of injuries to mares and stallions as pasture breeding. There is also an increased potential for sexually transmitted disease, and an increased opportunity for uterine infection is possible if copulation occurs.

As in other species, effective estrus detection is the cornerstone of a successful reproductive program that uses artificial insemination or hand mating. To maximize reproductive efficiency, mares should be teased a minimum of

every other day, and ideally daily (Evans 1990; Squires 1993). Timing of insemination is important in both artificial insemination and hand mating breeding programs. To minimize potential of uterine contamination, it is recommended that mares are inseminated or mated as few times as possible (American Association of Equine Practitioners 1999). In addition, minimal matings per mare decreases opportunity for injury to stallions or mares, and allows the stallion to breed more mares per year. To decrease the number of matings per pregnancy, it is important to maximize accuracy of timing of insemination.

Monitoring ovaries for ovulation

Mares have a long and variable follicular phase, with ovulation occurring approximately 24 to 36 hours before the end of behavioral estrus (Ginther 1993). Mares exhibit behavioral and physical signs of estrus throughout the follicular phase. Management techniques have been developed to estimate the time of ovulation so insemination or mating can be timed accordingly. Some of these techniques will be addressed below.

Palpation

Rectal examination of the reproductive tract is a valuable tool. Changes in the reproductive tract and ovaries that indicate pending ovulation can be assessed accurately by an experienced and skilled examiner. As mares progress through estrus, the cervix will relax until it is difficult to differentiate manually from the remainder of the tubular genitalia. During estrus, the uterus

becomes thick and edematous, but flaccid. The ovary with the most follicular activity will be enlarged and the presence of a dominant follicle can be determined. Within 24 hours before ovulation, the dominant follicle softens (Sertich 1998). Evacuation of the follicle results in a depression on the surface of the ovary which is palpable immediately after ovulation. Follicles in mares ovulate medially toward the center of the ovary, not laterally toward the surface. The ovulation depression becomes less distinct when the corpus hemorrhagicum and the CL form to fill the antral cavity of the antecedent follicle.

As with the effects of progesterone during diestrus, a gravid uterus will be turgid, with a tightly closed cervix early in pregnancy. The amniotic vesicle can be palpated per rectum at 28 days of pregnancy (Ginther 1983). As the pregnancy advances, the uterus migrates ventrally to the rim of the pelvis. Rectal examination of the reproductive tract is also useful to identify lacerations or adhesions in the reproductive tract (Sertich 1998) that may cause some infertility.

Ultrasonography

The use of ultrasonography, combined with accurate recordkeeping, is an effective method to estimate time of ovulation based on follicular size. Compared to mating among feral horses, palpation and ultrasonography are invasive and some herds use these techniques intensively. But, the effects of frequent palpation or frequent ultrasonography on reproduction in mares are not known.

Compared with rectal examination of reproductive organs alone, examination by ultrasonography has improved greatly the ability to evaluate the reproductive status of mares. Compared with palpation, ultrasonography allows more accurate inventory of ovarian structures and thorough examination of the tubular genitalia. In addition, ultrasonography can be used to identify the presence of endometrial cysts, pooling of fluid in the uterus, and the presence of early embryos (Sertich 1998).

Changes in the endometrium can be observed by ultrasonography. Early in estrus, the edematous endometrial folds are defined clearly in a cross sectional view. Twenty-four hours before ovulation, this pattern dissociates and no folds are clearly visible (Hayes 1985). In diestrus, the endometrial folds are not edematous and the uterus has uniform density (Sertich 1998).

Ultrasonography also allows accurate measurement of follicular growth. Within a mare, but among periods of estrus, the size of an ovulatory follicle is consistent. But among mares, the size of the ovulatory follicle can range from less than 35mm to greater than 65mm. With approaching ovulation, follicular shape changes from round to slightly pear-shaped. This change in shape can be detected by ultrasonography.

Pregnancy can be detected as early as 9 to 11 days after conception with ultrasonography (Ginther 1986). In mares, twin pregnancies frequently end in mid-term abortions. Pregnancies with twins that are carried to term generally produce foals that are small, weak and have high morbidity and mortality (Jeffcot 1973). Ultrasonography allows early detection of twins to support informed

management of twin pregnancies. If the amnion of one embryo is ruptured manually before 30 days gestation, 90% of mares will carry the remaining conceptus to term (Pascoe 1987). These single foals are similar to foals resulting from a single conception in birth size, morbidity and mortality rates.

Preparation for Breeding

Mares are commonly prepared for artificial insemination or hand mating by restraining the tail. Mares may or may not be restrained in palpation stocks at this time, depending on the facilities available. The tail is confined in some manner to prevent tail hairs from contaminating the perineum once it is cleaned, and to reduce contact with the stallion's penis during breeding. Gauze or a tail wrap may be applied from the base of the tail to the end of the tailbone, or the entire tail may be enclosed in a cotton stocking or tail bag. The perineum is then thoroughly washed with mild soap and water, and cotton or disposable toweling using minimum contamination technique (Evans 1990). The perineal area is cleaned laterally from the vulva outward toward the buttocks. After the area is clean, all soap residue is rinsed thoroughly, and excess water is wiped off as both soap and water are spermicidal. Use of these reproductive management techniques for domestic mares involves considerable human intervention at the time of mating. In contrast feral mares are mated with unrestrained tails, no pre-breeding cleaning, and no restraint. But reproductive success in feral mares is greater than in domestic mares. Though there may be several differences

between domestic and feral horses, management systems and extent of human intervention are major factors potentially explaining this difference in reproductive success.

Breeding Management

Hand mating

Hand mating is the most common method used to breed mares. Once the mare is prepared as described above, if she is to be hand mated she will be taken to the breeding area and restrained (Evans 1990). The most common restraints are a twitch applied to the upper lip or breeding hobbles. The stallion is then allowed to breed the mare. After the stallion has dismounted, the restraints are removed from the mare and she is returned to her stall or pasture.

Some breeds, such as Thoroughbred, will not register foals resulting from artificial insemination (The Jockey Club). Compared with pasture breeding, hand mating results in fewer injuries to mares and stallions and each stallion can breed more mares in a season. But, compared with pasture breeding, hand mating requires more labor to detect estrus and to prepare mares for mating. With hand mating, there is less instinctive interaction between horses and more human intervention.

Artificial Insemination

Artificially inseminated mares are restrained in the palpation stocks or other restraint where cleaning of the perineum occurred. The inseminator will

insert a hand coated with non-spermicidal lubricant and an insemination pipette past the vulva, through the vagina, and to the cervix. The pipette is advanced through the cervix and into the uterine body. After placement of the pipette 30 to 50 ml of semen is deposited into the uterine body. The pipette is then withdrawn from the uterus back through the cervix into the vagina and to the technician's hand. The hand and pipette are removed from the vagina. Lubricant is cleaned from the perineum, and the mare is returned to her stall or pasture.

Over the last 15 to 20 years there use of artificial insemination in horses has increased (Ginther 1993). Techniques have been developed to transport cooled semen and there are improved techniques that make frozen semen an option. Use of ultrasonography and rectal palpation is common in artificial insemination programs to minimize the number of breedings per mare. Except for Thoroughbreds, most equine breed registries in the United States will accept foals conceived from artificial insemination with fresh or cooled semen. Frozen semen is less widely accepted, and use is closely governed by different breed organizations.

Artificial insemination allows the largest number of mares to be bred to an individual stallion within a season. With AI there is reduced injury to mares and stallions and there is reduced transmission of disease. Semen samples can, and should, be evaluated before insemination, so the inseminator knows the concentration and motility of sperm deposited in the reproductive tract. Training is required to handle and deposit semen for AI, especially if cooled or frozen semen is used. In addition, equipment and facilities, such as an artificial vagina

and a breeding phantom are needed for semen collection to use fresh semen for artificial insemination. In an artificial insemination program it is likely that there is no social interaction between mares and the service stallion because a different stallion will likely tease the mare. In this type of breeding program, the frequent use of technologies such as ultrasonography and rectal palpation, combined with restraint and no social interaction between genders, increases further the degree of human intervention and decreases the innate behavioral elements of reproduction in horses.

Pasture Breeding

Pasture breeding requires the lowest management intensity of any of the breeding methods to be discussed. The mares and stallion are in a large pasture until the end of the breeding season. Where there is an estimated 7% occurrence of silent estrus in the breeding population, it is difficult to determine silent estrus in feral or pasture bred mares. High pregnancy rates in feral mares imply no major limitation to estrus detection by the service stallion. For mares with silent estrus it is recommended that mares are housed in close proximity or allowed regular physical contact with a stallion to facilitate estrus detection (McDonnell 1997). Compared to artificial insemination, pasture breeding does reduce the number of mares a stallion can breed per season and causes the highest number of injuries to stallions and mares.

Stress

Among different reproductive management systems, with increased human intervention and decreased innate behavior, is there increased stress? Before that can be addressed, what constitutes stress must be determined.

Definitions

Stress is defined as an influence of the external environment on an animal that has a negative effect on fitness (Broom 1993). Fitness describes the likelihood that the animal will survive and reproduce. Fitness is determined by age at first mating, interval between successive matings, survival from birth to first mating and survival of adults between successive mating attempts (Broom 1993).

Using this definition of stress, it is not practical to measure fitness as a tool to determine stress. Meaningful data would require a very large number of animals studied over a long period of time. Measures of stress that are sensitive to changes in the environment, but require fewer animals and shorter periods of time are practical and financially prudent for research. As animals attempt to maintain homeostasis, physiologic changes in animals as they respond to stimuli are much more useful measures of stress than is fitness.

In contrast to fitness as defined above, this research will focus on endogenous regulators of homeostasis that are responsive to changes in the external environment. For this research, the animal's environment includes both social and physical features. The environment is influenced by changes in the

physical environment such as weather and nutrition and by social interactions with other animals and humans.

Measures

In animals, measures of stress can be physiological or behavioral. It is important to recognize that the measures of stress used do not distinguish or categorize "good" or "bad" stress. Measurements are intended to provide empirical data to determine if the mares have made adjustments to maintain homeostasis.

Physiological

Cortisol

Cortisol is an endogenous glucocorticoid. Glucocorticoids stimulate key enzymes for gluconeogenesis. In addition, cortisol regulates cellular responsiveness of lymphoid tissues and causes immunosuppression. Glucocorticoids are primarily catabolic and divert metabolism from growth and storage toward increased physical activity and energy consumption. Chronic excess of glucocorticoids, such as in Cushing's syndrome, leads to muscle wasting, skin atrophy, and osteoporosis . Cortisol is used extensively as a measure of chronic stress. Stress increases secretion of corticotropin releasing factor (CRF) from the hypothalamus. CRF increases secretion of adrenocortictrophic hormone (ACTH) from the anterior pituitary. ACTH stimulates release of cortisol from the adrenal cortex (Figure 5). Habituation to

chronic stress can result in the return of cortisol to pre-stress levels, even if the stressor continues. Transportation of animals increased cortisol concentration in blood of sheep (Broom 1996), cattle (Nanda 1989), pigs, and horses (Baucus 1990). Restraint and isolation increased cortisol concentrations in blood of sheep (Hashizume 1994). In horses, twitching for 5 minutes increased cortisol concentration in blood of horses for 30 minutes (Thompson 1988; Colborn 1991). Transportation, restraint, and isolation are commonly associated with reproductive management of domestic mares.

β-endorphin

β-endorphin is an opioid released from the anterior pituitary in response to acute stress and has narcotic and analgesic effects (Broom 1993). Release of β-endorphin is stimulated by increased secretion of CRF by the hypothalamus. CRF stimulates release of proopiomelanocortin (POMC) in the pituitary, which is processed to release ACTH and β-endorphin into systemic blood (Figure 5).

Additional measures of stress

Stress can be detected by changes in heart and respiration rate, packed cell volume, basal body temperature, and immune function. In most mammals, heart rate and respiration rate increase in response to stress. Packed cell volume increases, especially in horses because of the splenic release of erythrocytes in response to exercise and other stressors. Body temperature is

challenging to use as a measure of stress, because changes are not specific to stress. Body temperature increases when stress is induced, and decreases when animals habituate to the stressor (Broom 1993). Body temperature also has a diurnal pattern, which must be considered when using it to assess stress. Immune function is reduced in response to chronic stress (Griffin 1989) and a glucocorticoid pathway possibly mediates this response. Stress effects on immune function can be measured by calculating the ratio of eosinophils to lymphocytes (Heller 1985). Stress will also decrease T-helper and T-suppressor lymphocytes (Baker 1985). Antibody production in response to vaccines or introduction of other antigens is reduced in animals that are restrained or confined (Broom 1993).

With so many physiological measures of stress available, it is necessary to identify which measures most accurately assess a specific research question. In this case, cortisol and β-endorphin were selected because samples could be taken with minimal human intervention. It was not necessary to introduce extra equipment, such as heart rate monitors, which could confound the primary question. In addition, cortisol is less responsive to subtle fluctuations in external environment than a measure such as body temperature. Finally, cortisol has been demonstrated to affect luteinizing hormone and estradiol in mares(Asa 1982), separate from its role as a stress response. Independent of whether cortisol is the most reliable measure of stress, if changes in cortisol affect LH and

estradiol, and this modulates reproduction, this would be the best test of our hypothesis.

Effects on Reproduction

Effects of management on reproduction are most likely mediated by the hypothalamic- pituitary axis. Cortisol and β-endorphin have deleterious effects on reproduction in livestock. Cortisol and β-endorphin decrease secretion of luteinizing hormone (LH) and estradiol, reducing display of behavioral signs of estrus (Asa 1980) (Figure 4).

Physiology

Incubation of pituitary cells in media containing cortisol decreased LH secretion, although the LH concentration in the cells did not change (Padmanabhan 1983). Exogenous cortisol and ACTH reduced estrous behavior, the LH surge, and ovulation in cattle (Stoebel 1982). Transport of cows within 30 days postpartum increased cortisol and decreased the magnitude and duration of the LH surge induced by exogenous estradiol. Cattle greater than 30 days post-partum did not experience an LH surge until cortisol returned to basal levels (Nanda 1989).

Increased cortisol in mares is associated with decreased estradiol, decreased follicular growth, and suppression of ovulation (Asa 1982). Mares transported in early pregnancy experienced increased concentrations of cortisol

Figure 5: Effect of increased β-endorphin and increased cortisol on estradiol 17-β and luteinizing hormone in mares.

Stress results in increased secretion of corticotropin releasing factor (CRF) from the hypothalamus. CRF increases secretion of pro-opiomelanocortin (POMC), the precursor for adrenocorticotropic hormone (ACTH) and β-endorphin, in the anterior pituitary. ACTH and β-endorphin are released from the anterior pituitary in an equimolar ratio. Increased ACTH concentration in circulation increases secretion of cortisol from the adrenal gland. Cortisol inhibits secretion of follicle stimulating hormone (FSH) and luteinizng hormone (LH) from the anterior pituitary gland. β-endorphin inhibits secretion of luteinizing hormone releasing hormone (LHRH) from the hypothalamus, decreasing LH and FSH secretion. Decreased LH and FSH secretion decreases follicular growth, ovulation, and circulating estradiol concentration. ——▶ stimulatory - - - - - - ▶ inhibitory

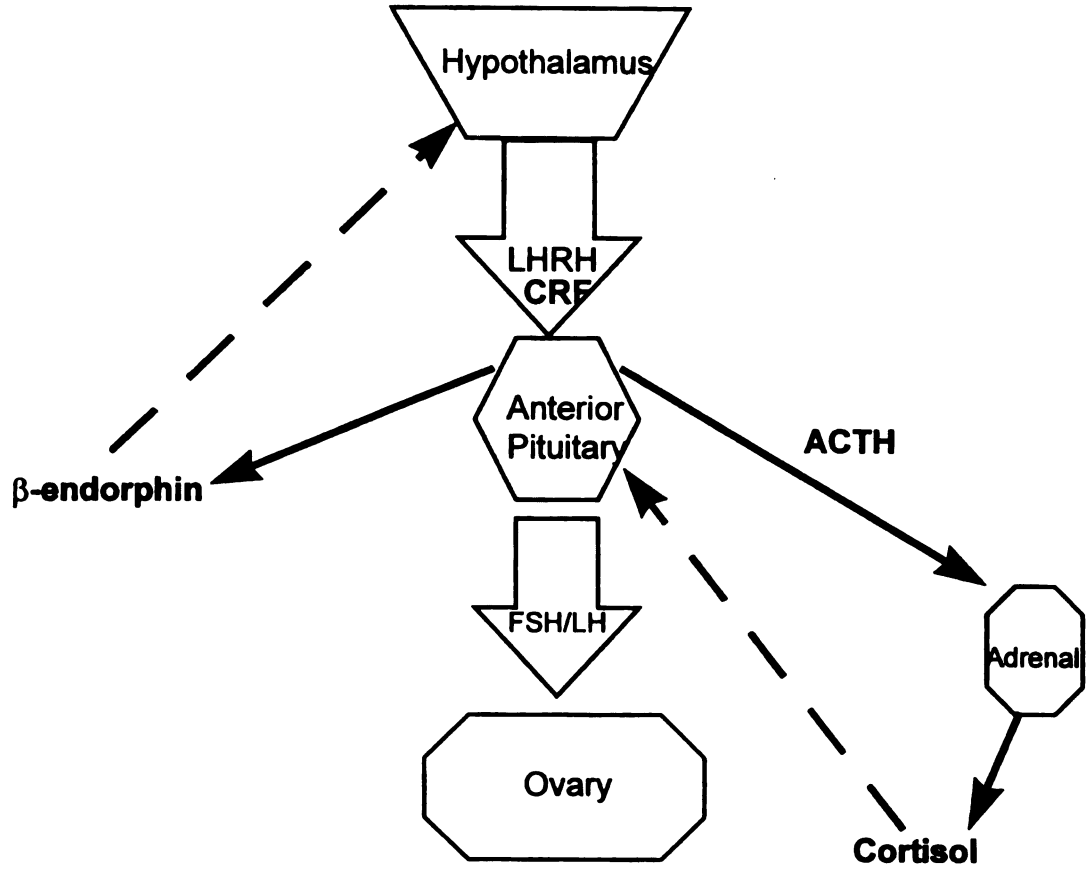

Figure 5

in blood and a concurrent increase in progesterone (Baucus 1990). It is clear that the equine adrenal gland is responsive to stress, but the subsequent effects on reproduction are unknown.

Gonadal steroids influence β-endorphin release from hypothalamic neurons. β-endorphin decreases secretion of LH by inhibition of LHRH. β-endorphin release is greatest when estradiol and progesterone are both present, such as during follicular waves of diestrus. Estrogen and progesterone increase the production of POMC, the hypothalamic precursor of β- endorphin (Whisnant, 1992). β-endorphin is increased during the luteal phase, when only progesterone

is present. Secretion of progesterone in cultured bovine luteal increases when cells are incubated with β-endorphin (Varsano, 1990). The combination of increased progesterone and increased β-endorphin decreases LH pulse frequency. Mares are often isolated from other mares, and their foals for breeding. Isolation increases β-endorphin secretion in sheep (Hashizume 1994). Other external factors, such as training and exercise, increase β-endorphin secretion in horses (Rivera 1998).

Behavior

The effects of stress on female sexual behavior are mediated primarily by effects on estradiol. In most mammals, estradiol is the hormone with primary control over behavioral estrus. In rats, increased estradiol increases density of dendritic spines in the ventromedial hypothalamus, which is the region of the brain that controls estrus behavior (Wade 1996). The synthetic glucocorticoid dexamethasone suppresses estrus behavior in ovariectomized mares treated with exogenous estradiol (Asa, 1980). Exogenous ACTH increases plasma cortisol and progesterone and suppresses estrous behavior in cattle (Hein, 1992). Although it is clear that exogenous glucocorticoids and opioids can alter reproductive events, it is unclear if common management procedures, though invasive, can increase endogenous cortisol and/or β-endorphin sufficiently to have effects similar to exogenous stress hormones.

Summary of review

Cortisol and β-endorphin can affect physiological and behavioral aspects of reproduction. There is a paucity of data on the effects of husbandry and management procedures on cortisol and β-endorphin in mares. Artificial insemination during estrus has no effect on cortisol in cattle (Macaulay 1986), but there is no equivalent research in other species, especially horses. This void is important because of the extensive use of invasive techniques. Some procedures for reproductive management in horses may be more stressful than other procedures, or more stressful than management in other domestic species. Reproductive management procedures that are generally accepted and commonly used for mares involve a much greater degree of manipulation and human intervention than is seen in other species. Except for transportation (Baucus 1990; Baucus 1990), the effects of other management procedures on stress and reproduction in mares have not been examined.

Procedures such as twitching (Colborn 1991) rectal palpation, and ultrasonography (Brady 1997) increase cortisol. But, it is not known if the magnitude or duration of increased cortisol is sufficient to affect reproductive events. Inhibitory effects of glucocorticoids and opioids on reproduction have been observed primarily after exogenous dexamethasone (cortisol analog), exogenous ACTH (cortisol precursor), exogenous morphine (β-endorphin agonist), and exogenous naloxone (β-endorphin antagonist).

Approximately 18% of pregnancies in mares are lost in the first 20 days (Ginther 1993), possibly due to changes in the hormonal milieu. Are the combined affects of common reproductive management procedures sufficiently stressful to increase cortisol and β-endorphin in mares? If so, is the increase in cortisol and/or β-endorphin associated with decreased secretion of estradiol or luteinizing hormone in mares during an estrous cycle?

This study was not designed to test a causal relationship between the stress hormones and the reproductive hormones in mares. The goal of this study was to determine if common practices for reproductive management of horses elicit stress and consequently decrease reproductive success of mares, and if different management systems affect reproductive hormone in mares. Very large numbers of mares would be required to measure reproductive success and to detect changes with confidence, so for this study hormones known to be important for reproductive success will be studied as markers for reproductive success. We hypothesized that increased human intervention in the reproductive process would increase β-endorphin and cortisol, and decrease estradiol 17-β and luteinizing hormone. The specific aims of this study were:

1) To determine if common procedures for reproductive management, and different reproductive management systems are stressful to mares,
2) To determine in mares if secretion of luteinizing hormone and estradiol 17-β are altered by stress, and/or different management systems

3) To determine in mares the correlation between measures of stress and secretion of hormones necessary for reproductive success.

THE EFFECT OF FREQUENT OVARIAN PALPATION ON SERUM LUTEINIZING HORMONE, ESTRADIOL 17-β AND CORTISOL IN MARES

Introduction

The follicular phase of the estrous cycle of mares is extended and irregular compared to females of other livestock species. Monitoring preovulatory follicular growth by ultrasonography and ovarian palpation is helpful to estimate time of ovulation and to schedule insemination. Thus, frequent ovarian palpation of mares is common in the horse industry. Mares may be palpated and examined by ultrasonography as frequently as every six hours. If mares are to be inseminated with frozen semen, it is recommended that semen be deposited within 6 hours of ovulation to maximize conception rates.

Feral horses, and pasture bred horses have greater reproductive success compared to more intensely managed mares (Daels 1995) (Ginther 1993). Foaling rates in feral mares are reported from 90-100% (Daes 1995), while foaling rates in domestic mares are reported in the range of 50-85% (Ginther 1993). Intensive monitoring of follicular growth may increase cortisol secretion in mares. Exogenous cortisol reduced estradiol concentration in blood, reduced follicular development, and blocked ovulation in mares (Asa 1982). Estradiol is a marker for follicular function, and secretion of estradiol increases as follicular growth progresses. Luteinizing hormone (LH) is required to cause ovulation, and

secretion of LH increases throughout estrus to peak 12 to 24 hours after ovulation (Ginther 1993).

We chose to quantify luteinizing hormone and estradiol 17-β because 1) LH and estradiol have numerous important roles in successful reproduction, 2) assays could detect small changes in secretion of estradiol and luteinizing hormone, and 3) because estradiol and luteinizing hormone are continuous variables so differences are detectable with relatively few experimental units. In contrast, to study conception rate very large numbers of mares would be needed because conception is a discrete variable.

We hypothesized that increased frequency of ovarian palpation would increase cortisol concentrations and decrease concentrations of estradiol and luteinizing hormone in blood.

Materials and Methods

Six Arabian mares, 2 years of age, were studied from May to August 1996. During the experiment the six mares were maintained together on approximately 20 acres of pasture from 0600 to 1800 hours, and in stalls from 1800 to 0600 hours during three successive periods of estrus. All mares were acclimated to the palpation stocks before the study by leading them into the stocks and feeding them small amounts of grain until they would enter the stocks readily, stand, and back out quietly.

Estrus was determined by daily fenceline teasing at 1200h with the same stallion for all mares on all days. Mares were not restrained for teasing. Mares

were determined to be in estrus when they performed one or more of the following behaviors: approached a stallion, raised their tails, squatted, and urinated. The end of estrus was identified as the time when the mares did not approach a stallion, or kicked, or squealed during teasing. Each estrus was divided into two phases. Phase 1 of estrus was from the onset of behavioral estrus until the diameter of the largest follicle was at least 35mm. During phase 1, ovaries were examined once daily by palpation and ultrasonograhpy with a 6.25 MHz linear probe (Pie Medical) at 1800h to determine size of the largest follicle. Phase 2 of estrus was the time the largest follicle developed from 35 mm to ovulation. During phase 2, ovaries were examined by palpation and ultrasonograpy four times daily at 0600h, 1200h, 1800h, and 2400h, until ovulation was detected. Occurrence of ovulation was determined by ultrasonography. The disappearance of the dominant follicle, accompanied by the presence of an ovulation depression, or corpus hemorrhagicum were the criteria for ovulation.

Blood was sampled via jugular venipuncture at 0600h, 1200h, 1800h and 2400h from the first day of behavioral of estrus to the end of behavioral estrus. Blood was collected in serum tubes, allowed to clot, and centrifuged at 2500g for 25 minutes at 4° C. Serum was harvested and stored at -20° C until assayed. Radioimmunoassays for cortisol (Diagnostic Products Corporation), luteinizing hormone (Whitmore 1973), and estradiol 17-β (Diagnostic Products Corporation) (Appendix I) quantified hormone in serum samples. Data were analyzed with the

Proc Mixed procedure of SAS. Estradiol 17-β and luteinizing hormone data were analyzed by auto-regression to adjust for heterogeneous variance. Quadratic regression analysis was performed to identify correlations between cortisol and luteinizing hormone, and cortisol and estradiol concentrations. Significance was defined as P<.05.

Results

All mares exhibited behavioral estrus while a dominant follicle was present, and ovulated during all periods of estrus studied. Among mares, duration of estrus ranged from 4 to 9 days and was consistent within mare. Although ovulation and estrous behavior occurred in all mares, other factors necessary for reproductive success could be changed by frequent palpation.

Endogenous cortisol concentrations in mares increased during Phase 2 of estrus (53.77 ± 1.02 ng/ml) when mares were palpated four times daily, compared Phase 1 when mares were palpated once a day (47.02 ± 1.27 ng/ml)(Figure 6). This increase in cortisol was attenuated over successive estrus periods (Figure 7). Analysis of the interaction of period of estrus and phase of estrus showed that serum cortisol concentrations were higher in phase 1 than phase 2 of estrus during the first period of estrus, but did not differ during the second or third period of estrus (Figure 8).

Luteinizing hormone concentrations were not different by phase, although there was a trend (P<.10) for LH to be lower during Phase 2 (11.51 ± 2.19 ng/ml) than Phase 1(12.07 ± 2.18 ng/ml) (Figure 9). Increased LH would be expected in

Phase 2 as mares approach ovulation, so this tendency to decrease could be due to the increased frequency of palpation and increased cortisol in Phase 2. Concentrations of LH did not differ among the three successive periods of estrus (Figure 10). Increased cortisol was not correlated with decreased luteinizing hormone ($P<.08$).

Estradiol-17β concentrations did not differ between phase 1 ($6.06 \pm .35$ pg/ml) and phase 2 ($5.35 \pm .41$ pg/ml) of estrus (Figure 11). In Phase 2, increased estradiol would be expected, as estradiol increases throughout estrus until ovulation (Ginther 1993). Estradiol 17-β did not differ among successive periods of estrus (Figure 12). There was no correlation ($P<.08$) between estradiol and cortisol in this study.

Figure 6: The effect of frequency of examination by rectal palpation and ultrasonography on serum cortisol concentration (ng/ml) in nulliparous mares during three successive estrus periods.

Mares were examined once daily at 1800h during Phase 1 of the estrus period, when the largest follicle was < 35 mm in diameter. Mares were examined four times daily at 0600h, 1200h, 1800h, and 2400h during Phase 2 of estrus, from the time the largest follicle was > 35mm in diameter until ovulation occurred. Bars with different superscripts are different (P<.05).

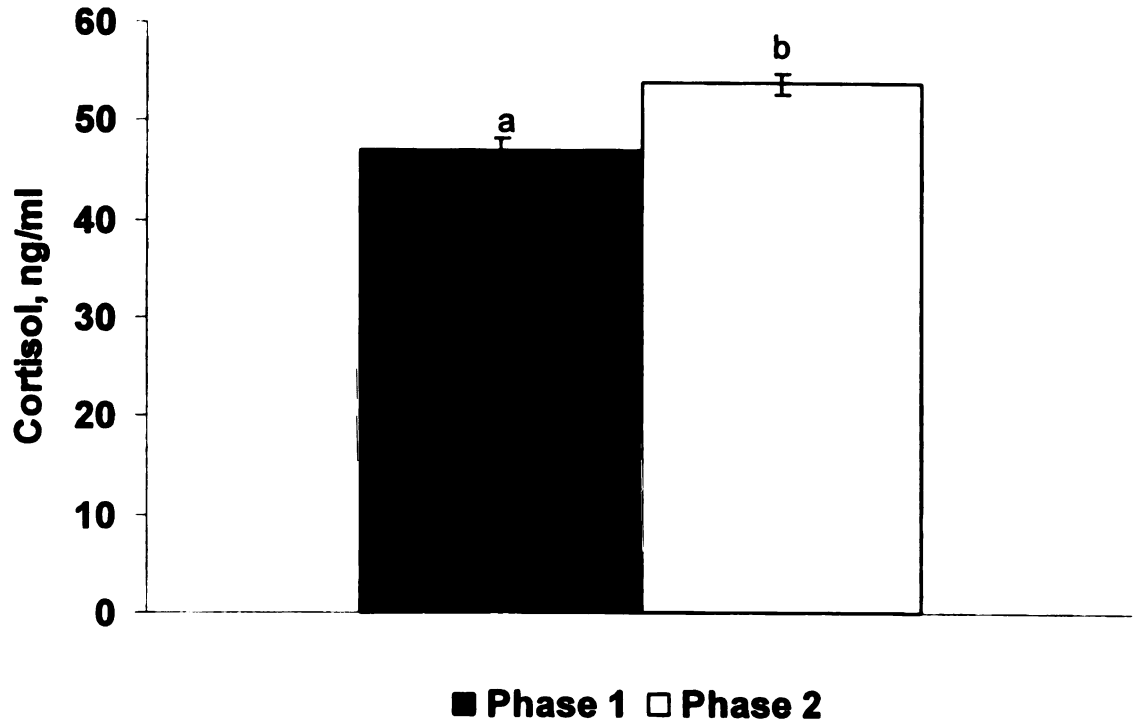

Figure 6

Figure 7: Serum cortisol concentration (ng/ml) in nulliparous mares that were examined by rectal palpation and ultrasonography over three consecutive estrus periods.

Mares were examined by palpation and ultrasonography once daily at 1800h until the largest follicle was > 35mm, and then four times daily at 0600h, 1200h, 1800h, and 2400h until ovulation occurred. Bars with different superscripts are different (P<.05).

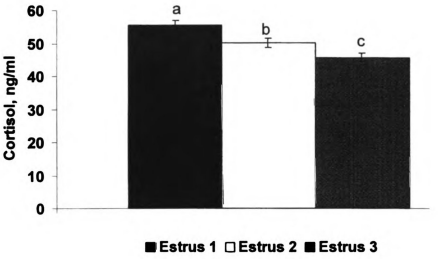

Figure 7

Figure 8: Interaction of period of estrus and phase of estrus on serum cortisol concentration (ng/ml) in nulliparous mares that were examined by rectal palpation and ultrasonography over three successive estrus periods.

Mares were examined once daily at 1800h during Phase 1 of the estrus period, when the largest follicle was < 35 mm in diameter. Mares were examined four times daily at 0600h, 1200h, 1800h, and 2400h during Phase 2 of estrus, from the time the largest follicle was > 35mm in diameter until ovulation. Bars with different superscripts are different (P<.05).

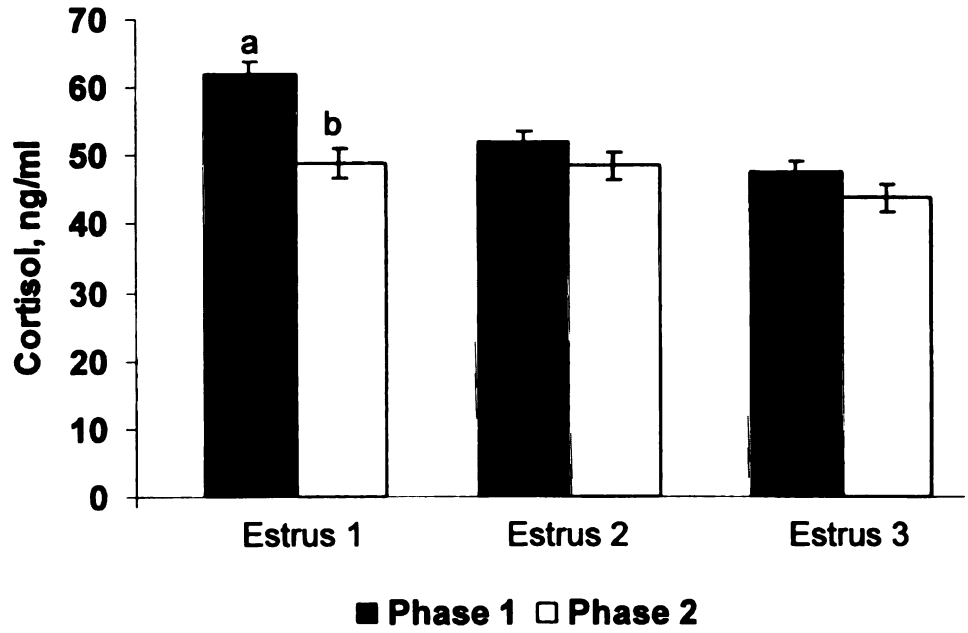
70
60
50
40
30
20
10
0
Cortisol, ng/ml
a
b
Estrus 1
Estrus 2
Estrus 3
Phase 1
Phase 2

Figure 8

Figure 9: The effect of frequency of examination by rectal palpation and ultrasonography on serum luteinizing hormone concentration (ng/ml) in nulliparous mares during three successive estrus periods.

Mares were examined once daily at 1800h during Phase 1 of the estrus period, and the largest follicle was < 35 mm in diameter. Mares were examined four times daily at 0600h, 1200h, 1800h, and 2400h during Phase 2 of estrus, from the time the largest follicle was > 35mm in diameter until ovulation occurred. Bars with different superscripts are different (P<.05).

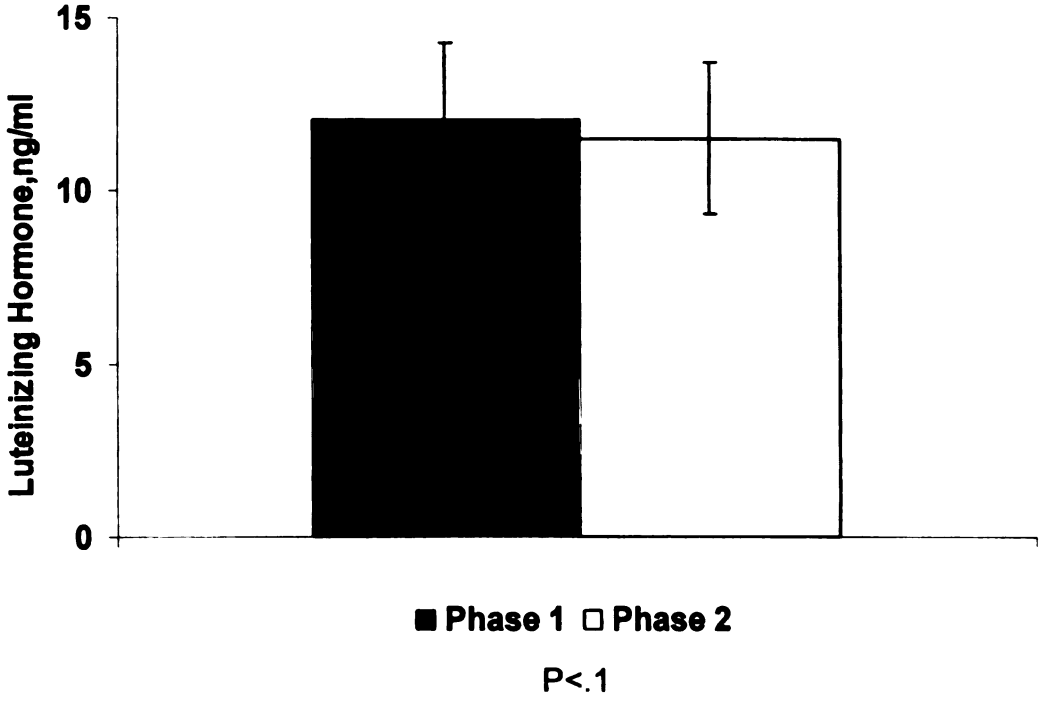

Figure 9

Figure 10: Serum luteinizing hormone concentration (ng/ml) in nulliparous mares that were examined by rectal palpation and ultrasonography over three consecutive estrus periods.

Mares were examined by palpation and ultrasonography once daily at 1800h until the largest follicle was > 35mm, and then four times daily at 0600h, 1200h, 1800h, and 2400h until ovulation occurred. Bars with different superscripts are different (P<.05).

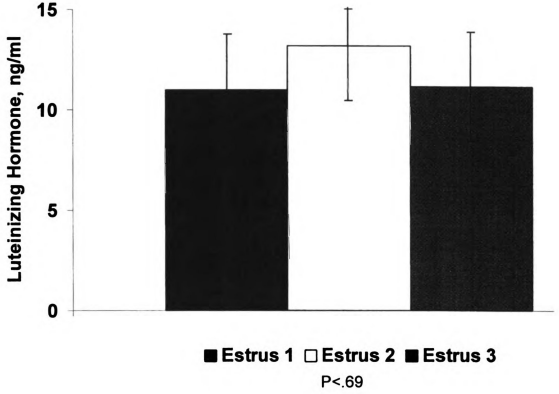
15
10
5
0
Luteinizing Hormone, ng/ml
■ Estrus 1 □ Estrus 2 ■ Estrus 3
P<.69

Figure 10

Figure 11: The effect of frequency of examination by rectal palpation and ultrasonography on serum estradiol 17-β concentration (pg/ml) in nulliparous mares during three successive estrus periods.

Mares were examined once daily at 1800h during Phase 1 of the estrus period, when the largest follicle was < 35 mm in diameter. Mares were examined four times daily at 0600h, 1200h, 1800h, and 2400h during Phase 2 of estrus, from the time the largest follicle was > 35mm in diameter until ovulation. Bars with different superscripts are different (P<.05).

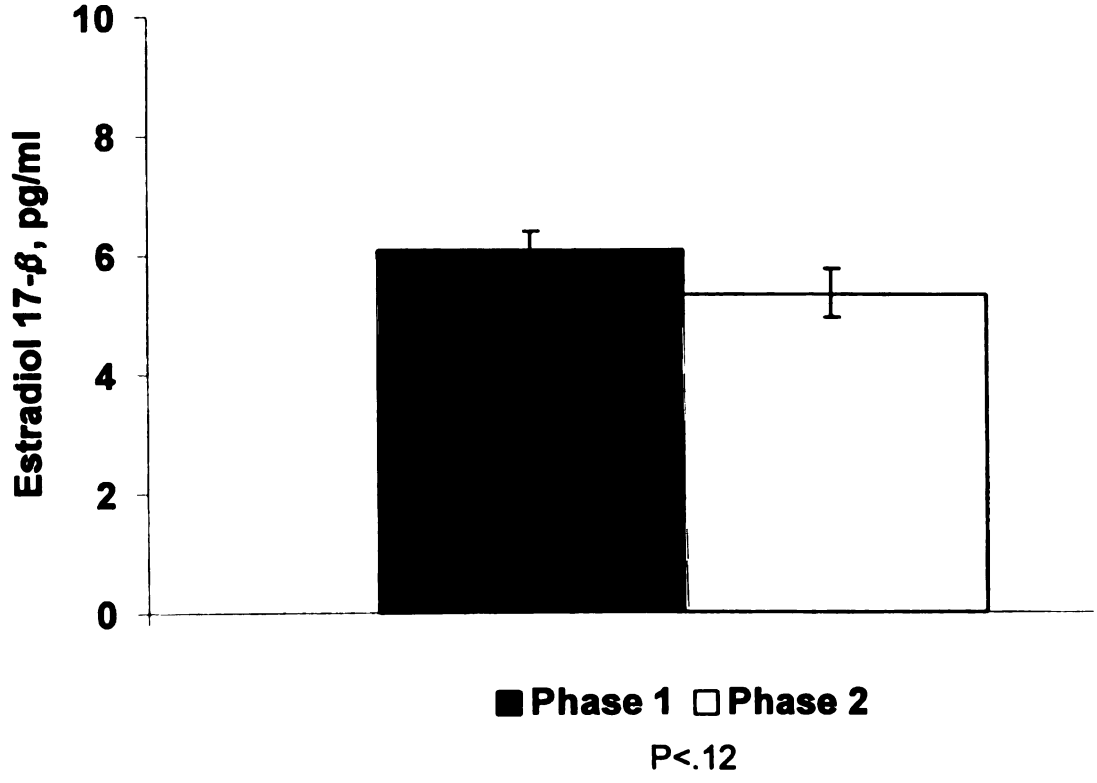

Figure 11

Figure 12: Serum estradiol 17-β concentration (pg/ml) in nulliparous mares that were examined by rectal palpation and ultrasonography over three consecutive estrus periods.

Mares were examined by palpation and ultrasonography once daily at 1800h until the largest follicle was > 35mm, and then four times daily at 0600h, 1200h, 1800h, and 2400h until ovulation occurred. Bars with different superscripts are different (P<.05).

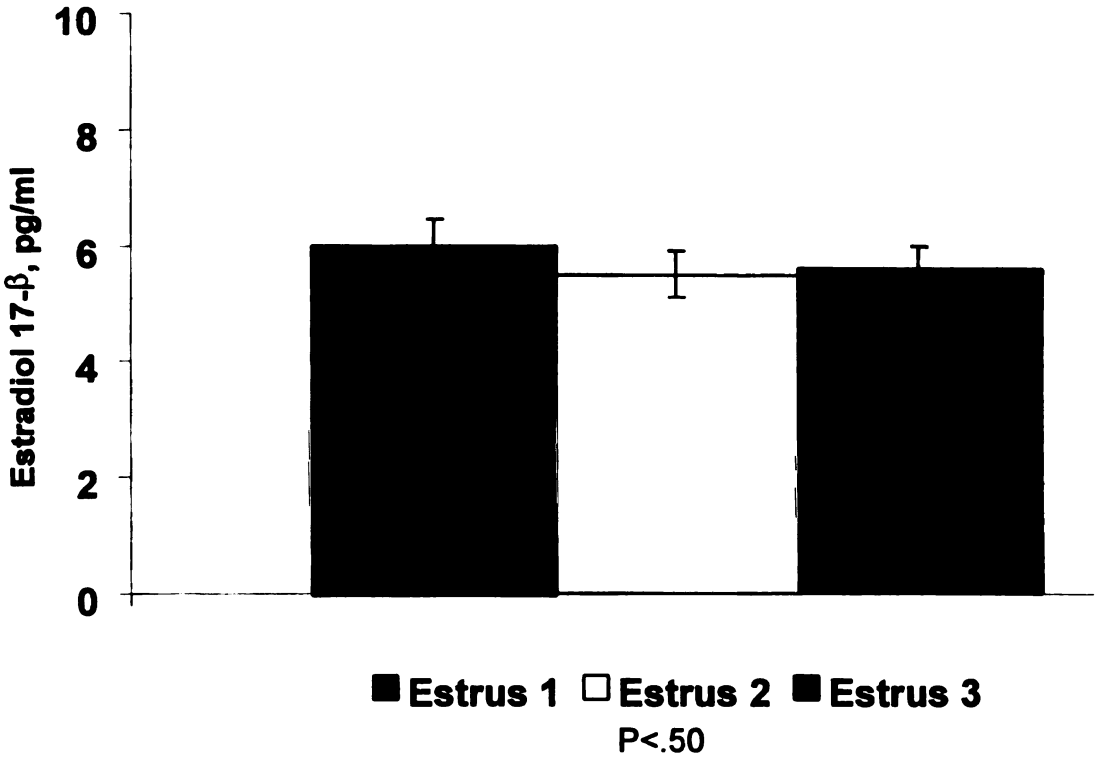

Figure 12

Discussion

Average duration of estrus in mares is reported as 4.5 to 8.9 days (Ginther 1993), so increased frequency of palpation did not affect expression or duration of estrus. Ovulation was also detected by ultrasonography in all mares in all cycles. This result is different than that found by Asa, et.al., (Asa 1982) who reported that increased cortisol blocked ovulation. However, in that experiment exogenous dexamethasone may have increased cortisol more than would be caused by frequent rectal palpation.

Although all mares exhibited behavioral estrus and ovulated, frequent palpation did increase concentrations of cortisol, and attenuated the expected late estrus increase in luteinizing hormone, and estradiol 17-β concentrations in mares.

Cortisol concentrations in blood at estrus are reported to be less than at diestrus (Ginther 1993), and concentrations are similar on different days within the estrus period. Whether variation in cortisol is regulatory to the estrous cycle is not known. But, cortisol at a nadir during the preovulatory period is consistent with follicular growth, pre-ovulatory surges of LH and ovulation. Exogenous glucocorticoids increased cortisol concentrations in blood, decreased luteinizing hormone and estradiol 17-β concentrations and blocked ovulation in mares (Asa 1982).

The increase in cortisol in frequently palpated mares reported here was expected because increased cortisol is a common physiological response to

changes in external environment. Although it is possible that the increase in cortisol is related to disturbing the sleep pattern of the mares, it is unlikely because all mares were disturbed at 2400 for blood sampling. Increased cortisol was not observed at 2400h except during Phase 2 of estrus, so it is unlikely that increased cortisol is due to sleep disturbance. This disruption occurred during all three successive periods of estrus, and within estrus during the early and late phases of estrus, so the increased cortisol concentration is attributable to the increased frequency of palpation.

Although the attenuation of LH concentrations reported here did not affect ovulation in this group of mares, the effect on luteinization of granulosal cells and subsequent progesterone production is unknown. If decreased LH at ovulation does not support luteinization fully, progesterone secretion would not develop fully. Luteal insufficiency will decrease embryo development, will decrease establishment of pregnancy and thus will limit fertility. Reducing palpation intensity could reduce incidence of luteal insufficiency. This result differs from data in cattle that increased endogenous cortisol following transportation or exogenous cortisol in vivo or in vitro, decreased LH (Stoebel 1982; Padmanabhan 1983; Nanda 1989). It is possible that the increase in cortisol in this experiment, although measurable, was not sufficient to reduce secretion of luteinizing hormone enough to block ovulation. This does not mean the decreased luteinizing hormone has no consequences. But, those effects were not elucidated in this study.

As with luteinizing hormone, it was expected that estradiol 17-β would be higher in phase 2 than phase 1 within the same period of estrus. Growth of the ovulatory follicle will continue until ovulation so there is a positive correlation between follicular size and estradiol production. Increased estradiol secretion is needed to stimulate the preovulatory surge of LH necessary for ovulation. Due to variability of length of estrus in days, comparison by phase of estrus as determined by follicle size provides a more accurate assessment of how mares responded to intensive palpation. Estradiol peaks 24 to 36 hours prior to the end of behavioral estrus, at the time of ovulation, so no difference in estradiol concentrations over the last 3 days of estrus could be due to the decrease in estradiol after ovulation. Variation among mares in time from ovulation to the cessation of behavioral estrus will increase variation in estradiol in Phase 2 and may mask differences in estradiol at the end of the estrus period. The attenuated increase in estradiol in Phase 2 of the estrous cycle of frequently palpated mares, could decrease myometrial activity, which is vital for embryonic mobility and maternal recognition of pregnancy. Estradiol is also necessary to increase endometral edema in preparation for an embryo.

Estradiol is not only important in behavioral estrus, but stimulates the increase in LH needed for ovulation and plays a vital role in preparing the endometrium for an embryo. Luteinizing hormone stimulates ovulation and has effects in formation of corpora lutea after ovulation occurs. In this study, frequent palpation did not decrease LH and estradiol sufficiently in this study to inhibit

ovulation or behavioral estrus. But, if decreased LH causes decreased progesterone during diestrus, there may be subsequent ramifications affecting reproductive success. Maternal recognition of pregnancy , maintenance of pregnancy, luteal development, luteal function, and the conceptus' ability to produce estrone sulfate and equine chorionic gonadotropin could potentially be affected by these changes in estradiol and luteinizing hormone. Further studies are necessary to elucidate what effects decreased estradiol and LH may have on maternal recognition of pregnancy and other aspects of reproductive physiology that could affect reproductive success in mares.

THE EFFECT OF DIFFERENT REPRODUCTIVE MANAGEMENT SYSTEMS ON ESTRADIOL 17-β, LUTEINIZING HORMONE, CORTISOL, AND β-ENDORPHIN IN MARES

Introduction

Foaling rates in domestic mares are lower (Ginther 1993) than foaling rates of feral mares (Daels 1995). A 10% increase in reproductive efficiency in domestic mares has been reported since ovarian palpation and ultrasonography have become common place (Ginther 1993); however, foaling rates in domestic mares are still 10 to15% lower than in feral mares. Decreased foaling rates are costly for breeders, as foals are the primary source of income for breeders. In fact, except for foals, there is no other marketable product from horses such as milk or meat.

Results of a previous study in our laboratory are that increased frequency of ovarian palpation and ultrasonography increased serum cortisol concentrations in mares (Brady 1997), and attenuated the late estrus increase in estradiol 17-β and luteinizing hormone concentrations. Exogenous cortisol inhibits follicular growth, ovulation, and estradiol secretion in mares (Asa 1982). Secretion of β-endorphin inhibits release of luteinizing hormone (Conove 1993) in sheep. Decreased concentrations of luteinizing hormone and estradiol could lead to a variety of reproductive problems. Decreased estradiol can result in failure to exhibit estrus, or failure of the endometrium to become prepared for an embryo. Decreased luteinizing hormone can result in failure to ovulate or

incomplete luteinization of the ovulated follicle, leading to decreased progesterone production.

Domestic mares are reproductively managed under a wide variety of management systems. These systems range from no human involvement to extremely intensive human involvement. In some minimal intensity management systems, mares will be housed with a stallion throughout the breeding season, and may see humans only rarely throughout the season. In contrast, some management systems involve much human involvement, and human intervention at every phase of the reproductive process. We hypothesized that reproductive management systems involving increased human intervention in the reproductive management of mares would increase plasma cortisol and β-endorphin, with a subsequent decrease in plasma estradiol and luteinizing hormone, compared to management systems with less human involvement.

Materials and Methods

Twenty-four Arabian mares at the Michigan State University Horse Teaching and Research Center were used for this study during the breeding season from May 1 to August 30,1997. All mares were housed on pasture for the duration of the study. Mares were blocked by age (young - <9 years (n=12), old - ≥ 9 years (n=12)) , and lactation (lactating (n=12) or non-lactating (n=12)) , and assigned to one of three reproductive management systems, with differing levels of human intervention (low intensity(n=8), medium intensity (n=8) and high

intensity (n=8)). Duration of treatment was one period of estrus. All lactating mares were in the first 30 days of lactation.

Low intensity mares (n=8) were managed with minimal intervention by technology and people. Mares were confined to a 30 acre pasture at the MSU Horse Teaching and Research Center with a stallion experienced at pasture breeding. Mares and the stallion had a minimum of one week of acclimation to this physical and social situation before jugular blood was sampled. Mares were exposed to the pasture breeding scenario in three groups. The first group was young mares with foals (n=2 mares/foals), the second group was all non-lactating mares (n=4), and the third group was old lactating mares (n=2 mares/foals). So, for three segments of the experiment, stallion to mares ratio was 1:2, 1:4, and 1:2, respectively. These divisions were made to avoid having non-lactating mares and lactating mares grouped together. Also, the old lactating mares foaled later than the young lactating mares, and had the lactating mares all been exposed to the treatment together, there would have been a 90 day difference in stage of lactation. Mares were removed as a group after all mares in the group had been through one estrus. In a situation where the stallion ostracized one non-lactating mare from the herd, removing the stallion overnight, and then reintroducing him to the mares the next day eliminated the problem. The stallion was also removed from the non-lactating group when he was injured while mating. The mares remained in the pasture together, and he was reintroduced at the discretion of the farm veterinarian. In the subsequent group,

the stallion was removed from the pasture, the new mares were introduced, and he was re-introduced. There were no further problems with this treatment group.

Mares were observed at least thrice daily for signs of behavioral estrus. Mares were determined to be in estrus when they exhibited one or more of the following behaviors: approached the stallion, raised their tails, squatted, and urinated. The end of estrus was identified as the time when the mares kicked, or squealed during teasing or did not did not approach the stallion. Interaction between the mares and humans occurred only when blood was sampled. For sampling, two people entered the pasture with a halter and lead rope, blood tubes, and needles. Mares were caught and haltered in the pasture, one person held the mare, and the second person sampled blood by jugular venipuncture. Mares received a piece of apple after sampling as positive reinforcement for catching and sampling. This method enabled us to sample mares without removing them from the experimental environment.

The medium intensity management system tested was hand mating (n=8). Mares were teased for estrus daily at 1200h with a stallion to determine onset of estrus. The same stallion was used to tease hand-mated mares for the entire experiment. Mares were confined in 2 large pens, affording free access to the stallion, who was released into a center pen. One of three fertile stallions, different from the teaser, bred mares every other day from day 2 of estrus to the end of behavioral estrus. Each mare was bred to the same stallion throughout the estrus period. Mares were prepared for mating one at a time. Mares were led into palpation stocks, rear doors were closed, and lead ropes were secured

at the front of the palpation stocks. Mares were then prepared for breeding using minimum contamination technique, backed out of the palpation stocks, and led to the adjacent breeding area, where they were restrained for mating for 5 to 10 minutes with a halter and lead rope and a chain twitch. Mares were held facing a solid wall, and directed toward the wall if they moved during mating. Lactating mares were separated from their foals for pre-breeding preparation and mating, a total of 15 to 20 minutes.

The highest intensity management system tested was artificial insemination (n=8). Mares were pen teased daily at 1200h using the same method and the same stallion for teasing as the medium intensity mares. Mares were prepared for ultrasonography one at a time. Mares were led into palpation stocks, rear doors were closed, and lead ropes were secured at the front of the palpation stocks. Ovaries were palpated rectally and examined by ultrasonography with a 6.25 MHz probe (Pie Medical) every other day from day 2 of estrus until the largest follicle reached 35mm in size. The ovaries were then examined by palpation and ultrasonography daily until ovulation occurred. Mares were prepared for mating using minimum contamination technique, and were inseminated daily from the time the follicle reached a size greater 35mm until ovulation was detected by ultrasonography. Criteria for ovulation were absence of a dominant follicle and presence of an ovulation depression, corpus hemorrhagicum, or corpus luteum.

For mares in all treatment groups, blood was sampled by jugular venipuncture at 0600, 1200, and 1800 hours from the first day of behavioral

estrus to the first day of diestrus. Blood was collected in EDTA tubes, centrifuged, and plasma was harvested and stored at -20° C until assayed for estradiol, luteinizing hormone, and cortisol. To quantify β-endorphin, blood was sampled at 1800 h on days one and four of estrus. Blood to be assayed for β-endorphin was collected in EDTA tubes, treated immediately with Aprotinin (500 KIU/ml), and kept on ice until centrifuged (<15 minutes). Harvested plasma was acidfied with 1 M acetic acid and stored at -20°C until assayed.

All hormones of interest were quantified in plasma by radioimmunoassay: cortisol (Diagnostic Products Corporation), β-endorphin (Peninsula Laboratory), luteinizing hormone (Whitmore 1973) and estradiol (Diagnostic Products Corporation). All data were analyzed with SAS for Mixed Models, and Least Squared means are reported. Estradiol 17-β and luteinizing hormone data were analyzed with auto-regression to adjust for heterogeneous variance. Data from day 1 of estrus was used as a covariate for data from day 4 of estrus in β-endorphin analysis. Data were also analyzed by quadratic regression to identify correlative relationships between cortisol and estradiol 17-β, and cortisol and luteinizing hormone. Significance was defined as $P<.05$.

Results

Breeding system did not affect concentration of β-endorphin. Variation in β-endorphin among mares was large (27-88%) and is a major reason no effect of treatment was detected(Figure 13). There also was no difference in β-endorphin concentrations between lactating and non-lactating mares (Figure 14),

or between young and old mares (Figure 15). It was hypothesized that increased β-endorphin would decrease luteinizing hormone, possibly by decreasing LHRH action in the anterior pituitary. If β-endorphin is affected by management system, and mediates secretion of LHRH and LH, sampling blood immediately before and after breeding may increase detection of effects.

The trend ($P<.07$) for the highest cortisol concentration in the hand mated mares (Figure 16) may be due to management techniques, such as twitching and isolation from herd mates, typically used in hand mating. It was anticipated that pasture bred mares would have the lowest cortisol concentration. This suppostition was not supported by the results of this study. It was expected that the increased physiological and metabolic demands of lactation, and separation of mares and foals for mating, would increase cortisol concentration in lactating mares. However, lactating mares had lower cortisol than non-lactating mares (Figure 17). The diurnal pattern of cortisol secretion was also affected in this study (Table 5).

Plasma luteinizing hormone concentration was higher in hand mated than in artificially inseminated or pasture bred mares (Figure 18). The trend for higher cortisol concentration in hand mated mares than in artificially inseminated mares indicates that cortisol likely did not have a negative effect on luteinizing hormone secretion in hand mated mares. However, it is important to remember that increased concentration does not necessarily mean increased activity. The assay used to quantify cortisol in this experiment measured total cortisol, and did

not differentiate between free cortisol and cortisol bound to cortisol binding globulin. Luteinizing hormone was higher in non-lactating hand-mated mares, than non-lactating mares that were pasture bred or artificially inseminated (Figure 19). Younger mares (< 9 years) had higher luteinizing hormone concentrations than older mares (9 years) (Figure 20). Lactating mares had lower LH concentrations than non-lactating mares (Figure 21), which is consistent with findings in other species (Faltys 1985). When tested within and among groups, there was no correlation between cortisol and luteinizing hormone in this experiment.

Plasma estradiol 17-β concentrations were lower in pasture bred mares than in artificially inseminated mares (Figure 22). Pasture bred mares tended to have higher cortisol concentrations than artificially inseminated mares (Figure 16), so this supports the hypothesis that increased cortisol may decrease estradiol. There was no correlation between cortisol and estradiol in this experiment. Estradiol concentrations were higher in lactating mares than non-lactating mares (Figure 23), and higher in old mares than young mares (Figure 24). Analysis of the interaction of lactation and management system showed that estradiol was higher in lactating mares that were hand mated or artificially inseminated than in non-lactating mares that were hand mated or artificially inseminated (Figure 25).

Figure 13: The effect of pasture breeding, hand mating, and artificial insemination on plasma β-endorphin concentrations (ng/ml) in mares.

Pasture bred (PB) mares were on pasture with an experienced breeding stallion throughout estrus. Hand mated (HM) mares were mated every other day from the second day of behavioral estrus until the end of behavioral estrus. Artificially inseminated (AI) mares were examined with rectal palpation and ultrasonography every other day from the second day of behavioral estrus until ovulation, and inseminated daily from the time the largest follicle was 35mm in diameter until ovulation. Bars with different superscripts are different ($P<.05$). N=8 per group.

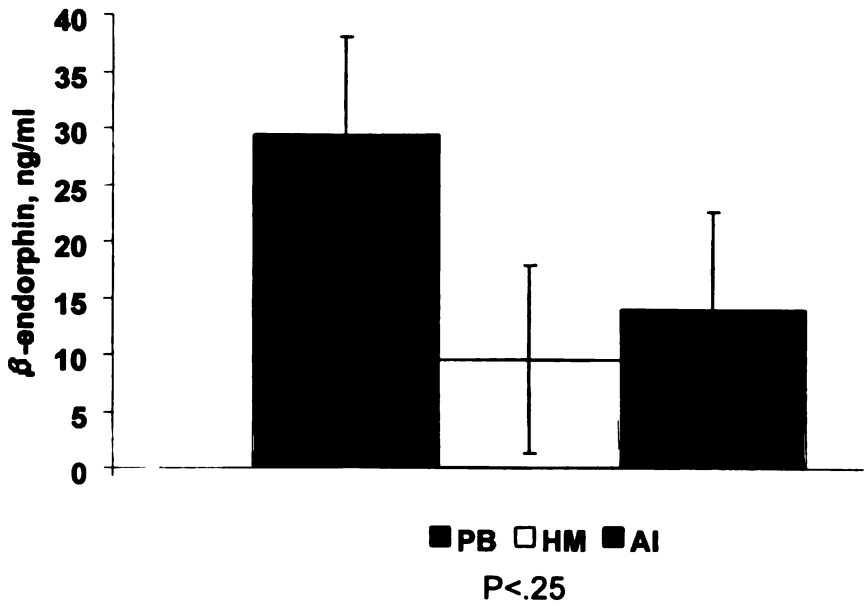

Figure 13

Figure 14: The effect of lactation state on plasma β-endorphin concentrations (ng/ml) in mares.

Bars with different superscripts are different (P<.05).N=12 per group.

ntrations

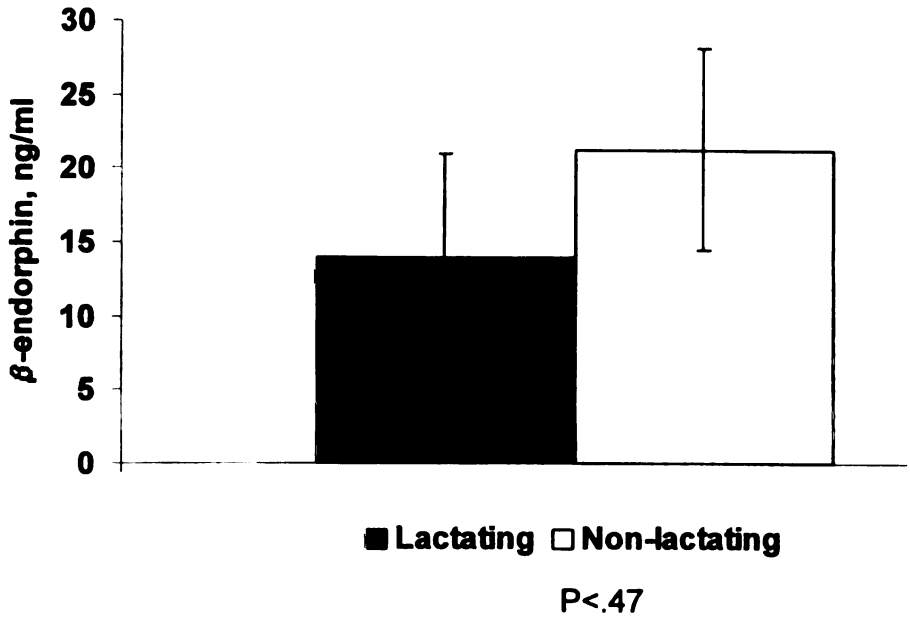

Figure 14

Figure 15: The effect of age on plasma β–endorphin concentrations (ng/ml) in mares.

Bars with different superscripts are different (P<.05). N=12 per group.

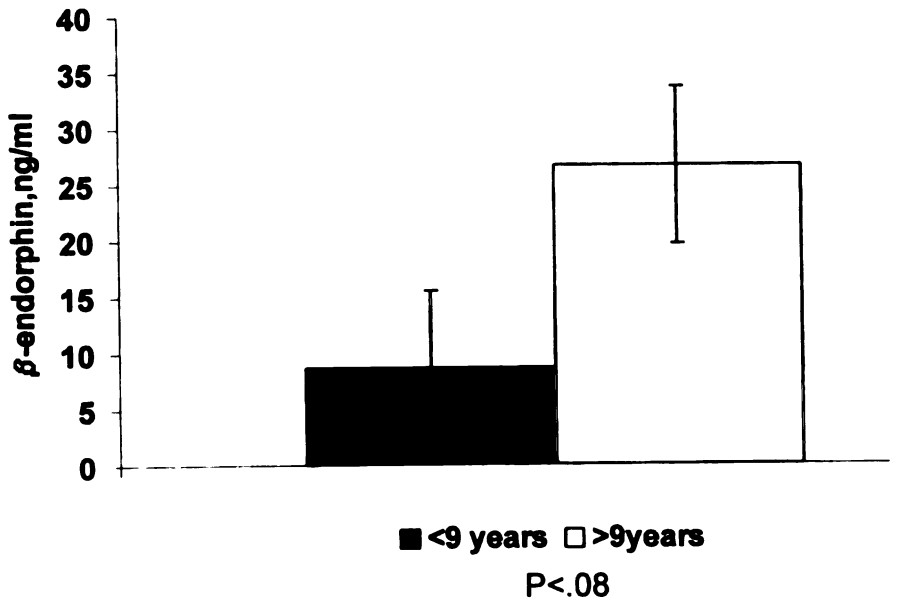

Figure 15

Figure 16: The effect of pasture breeding, hand mating, and artificial insemination on plasma cortisol concentrations (ng/ml) in mares.

Pasture bred (PB) mares were on pasture with an experienced breeding stallion throughout estrus. Hand mated (HM) mares were mated every other day from the second day of behavioral estrus until the end of behavioral estrus. Artificially inseminated (AI) mares were examined with rectal palpation and ultrasonography every other day from the second day of behavioral estrus until ovulation, and inseminated daily from the time the largest follicle was 35mm in diameter until ovulation. Bars with different superscripts are different ($P<.05$). N=8 per group.

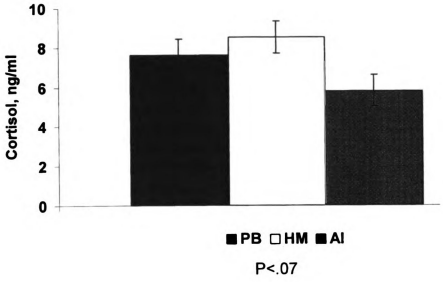

Figure 16

Figure 17: The effect of lactation state on plasma cortisol (ng/ml) concentrations in mares.

Bars with different superscripts are different (P<.01). N=12 per group.

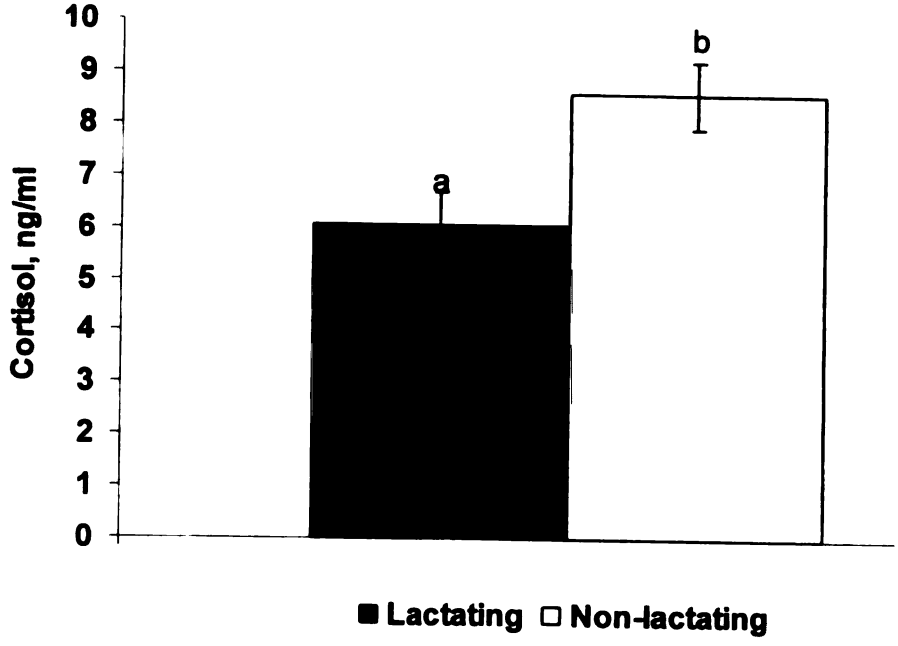

Figure 17

Figure 18: The effect of pasture breeding, hand mating, and artificial insemination on plasma luteinizing hormone concentrations (ng/ml) in mares.

Pasture bred (PB) mares were on pasture with an experienced breeding stallion throughout estrus. Hand mated (HM) mares were mated every other day from the second day of behavioral estrus until the end of behavioral estrus. Artificially inseminated (AI) mares were examined with rectal palpation and ultrasonography every other day from the second day of behavioral estrus until ovulation, and inseminated daily from the time the largest follicle was 35mm in diameter until ovulation. Bars with different superscripts are different ($P<.01$). N=8 per group.

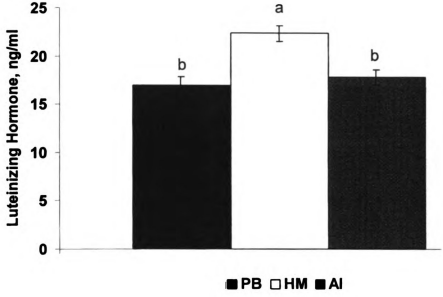

Figure 18

Figure 19: Interaction of lactation state and reproductive management system on plasma luteinizing hormone concentrations (ng/ml) in mares.

Pasture bred (PB) mares were on pasture with an experienced breeding stallion throughout estrus. Hand mated (HM) mares were mated every other day from the second day of behavioral estrus until the end of behavioral estrus. Artificially inseminated (AI) mares were examined with rectal palpation and ultrasonography every other day from the second day of behavioral estrus until ovulation, and inseminated daily from the time the largest follicle was 35mm in diameter until ovulation. Bars with different superscripts are different ($P<.05$). N=4 per group.

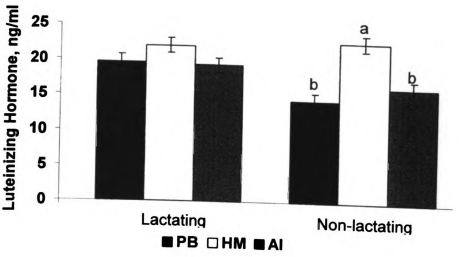
Luteinizing Hormone, ng/ml
25
20
15
10
5
0
a
b
b
Lactating
Non-lactating
PB HM AI

Figure 19

Figure 20: The effect of age on plasma luteinizing hormone concentrations (ng/ml) in mares.

Bars with different superscripts are different (P<.01). N=12 per group.

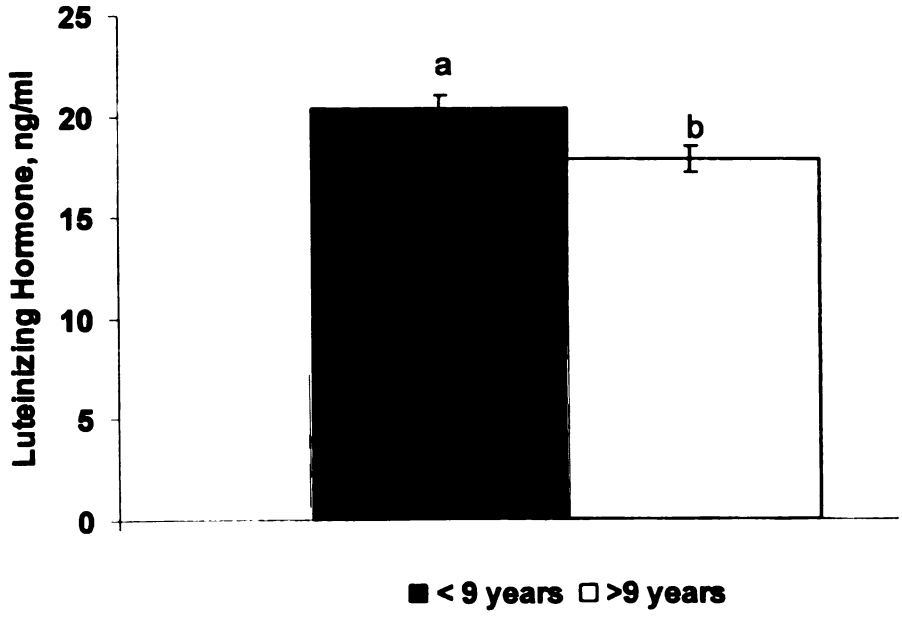

Figure 20

Figure 21: The effect of lactation state on plasma luteinizing hormone (ng/ml) concentrations in mares.

Bars with different superscripts are different (P<.01). N=12 per group.

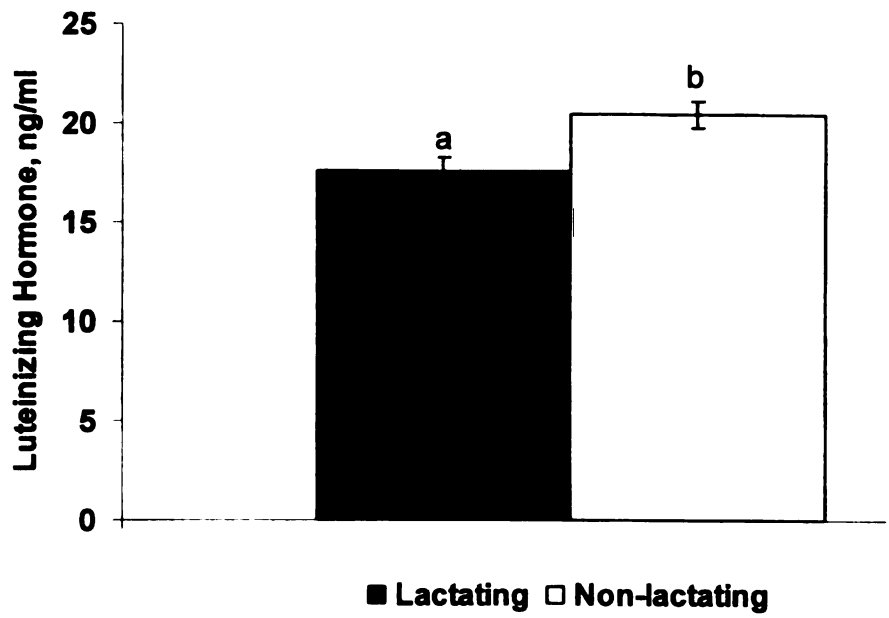
25
20
15
10
5
0
Luteinizing Hormone, ng/ml
a
b
Lactating
Non-lactating

Figure 21

Figure 22: The effect of pasture breeding, hand mating, and artificial insemination on plasma estradiol 17-β concentrations (pg/ml) in mares.

Pasture bred (PB) mares were on pasture with an experienced breeding stallion throughout estrus. Hand mated (HM) mares were mated every other day from the second day of behavioral estrus until the end of behavioral estrus. Artificially inseminated (AI) mares were examined with rectal palpation and ultrasonography every other day from the second day of behavioral estrus until ovulation, and inseminated daily from the time the largest follicle was 35mm in diameter until ovulation. Bars with different superscripts are different ($P<.05$). N=8 per group.

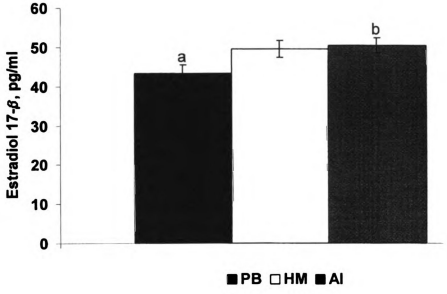

Figure 22

Figure 23: The effect of lactation state on plasma estradiol 17-β concentrations (pg/ml) in mares.

Bars with different superscripts are different (P<.0001). N=12 per group.

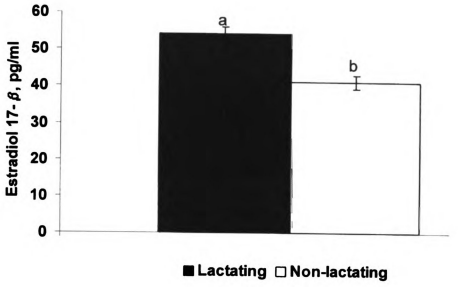
60
50
40
30
20
10
0
Estradiol 17- β, pg/ml
a
b
■ Lactating □ Non-lactating

Figure 23

Figure 24: The effect of age on plasma estradiol 17-β concentrations (ng/ml) in mares.

Bars with different superscripts are different (P<.01). N=12 per group.

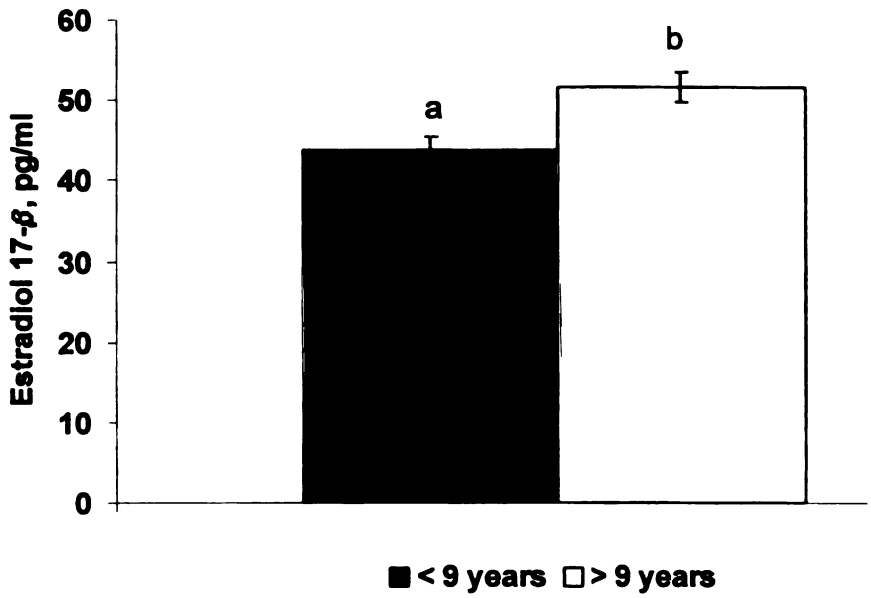

Figure 24

Figure 25: Interaction of lactation state and reproductive management system on plasma estradiol 17-β concentrations (pg/ml) in mares.

Pasture bred (PB) mares were on pasture with an experienced breeding stallion throughout estrus. Hand mated (HM) mares were mated every other day from the second day of behavioral estrus until the end of behavioral estrus. Artificially inseminated (AI) mares were examined with rectal palpation and ultrasonography every other day from the second day of behavioral estrus until ovulation, and inseminated daily from the time the largest follicle was 35mm in diameter until ovulation. Bars with different superscripts are different ($P<.01$). N=4 per group.

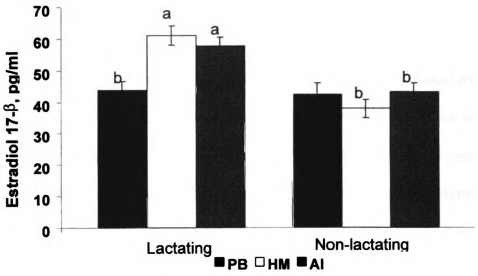
70
60
50
40
30
20
10
0
Estradiol 17-β, pg/ml
a
a
b
b
b
Lactating
Non-lactating
PB HM AI

Figure 25

Discussion

There was no difference in β-endorphin concentration between treatments (Figure 14). β- endorphin may a more reliable measure of acute than chronic stress, because of its short half -life. More frequent sampling of β-endorphin, or sampling closer to the actual time of mating, may determine if increased β-endorphin could cause the decreased concentration of luteinizing hormone in artificially inseminated or pasture bred mares.

Cortisol concentrations tended to be higher in hand mated mares than in pasture bred or artificially inseminated mares. Hand mated mares were restrained with a twitch, which is known to increase cortisol in horses (Colborn 1991). It was not expected that cortisol concentrations in hand mated mares would remain elevated, because the mares were twitched only every other day for less than 5 minutes. The hand mated mares were separated from their foals, but the same is true of artificially inseminated mares. All mares were unrestrained for teasing, and hand mated and artificially inseminated mares were teased together, so differences in teasing and separation from foals would not account for the difference in cortisol concentrations.

It was expected that the pasture bred mares, with minimal human intervention, would have the lowest cortisol concentrations. These mares, although acclimated to the situation, were perhaps still not adjusted to living in close proximity to a stallion. Or, maybe the expected result was wrong, and mares coexisting with a stallion have increased cortisol, compared to mares in all

female bands, regardless of how long they are together. There were no overt signs of distress after a brief (20-30 minute) adjustment period on the first day of acclimation. After an initial period of investigative behavior, the stallion and the mares grazed quietly and remained loosely grouped throughout the duration of the treatment period.

Cortisol was higher in non-lactating mares than in lactating mares. It was expected that the physiological stresses of lactation would increase cortisol in lactating mares, this expectation was not observed. This may be because of increased clearance in cortisol in lactating mares (through milk), changes in hepatic steroid metabolism related to lactation, or because of suckling induced reduction of cortisol binding globulin (CBG). Cortisol that is bound to CBG is biologically inactive, but the assay used for this experiment measured total cortisol, not free cortisol. If CBG in mares acts similarly to that in cattle, the result would be higher free cortisol in lactating than non-lactating mares (Faltys 1985). Therefore, more free cortisol would be available to affect gonadotropes and to reduce the LH secretion associated with suckling(Zalesky 1990; Gazal 1998). .

It has been reported that cortisol in mares peaks in the morning (0600h-0900h) and reaches a nadir in the evening (1800h-2100h) (Irvine 1994). This pattern differs from what was seen in the current experiment, where the nadir was at mid-day. Irvine, et.al. also reported that the circadian rhythm of cortisol secretion in horses is easily disturbed by changes in the environment, but can be entrained by external cues such as feeding and handling. The circadian rhythm

in mares in this study may have been shifted by the schedule to sample blood in which the mares were disturbed daily at 0600h, 1200h, and 1800h.

Although it has been reported in cattle that increased cortisol decreases LH, (Stoebel 1982; Padmanabhan 1983; Nanda 1989) that result was not detected in this study. It has been reported that progesterone concentration at 15 days post ovulation is higher in older mares than younger mares(Vanderwall 1993), so there is not a decrease in progesterone production associated with age in mares.

It is interesting to note that in all treatment groups, 30 day pregnancy rates were within the industry ranges (Ginther 1993) (Appendix II). With pregnancy data alone, however, there were insufficient animals in this study to infer anything about the success of the different management systems examined.

SUMMARY AND CONCLUSIONS

These experiments show that there are differences in cortisol, luteinizing hormone and estradiol 17-β in mares that are managed with differing levels of human intervention. In experiment 1 there was a clear increase in cortisol in frequently palpated mares, and attenuations in LH and estradiol concentrations. These results led to the second experiment. Do the different management systems and associated levels of management intensity have effects on cortisol, estradiol and luteinizing hormone that could effect reproductive success?

There were no differences in cortisol concentrations between the three levels of management intensity in the second experiment. Luteinizing hormone was increased in hand mated mares compared to pasture bred or artificially inseminated mares. Artificially inseminated mares had higher estradiol 17-β concentrations than did pasture bred or hand mated mares. There was no correlation between estradiol or LH and cortisol in the second experiment. This indicates that the changes in cortisol concentrations related to different levels of management intensity are not sufficient to change reproductive hormones. For many reasons it is not appropriate to compare data between experiments. But, it is interesting to note that cortisol concentrations for all mares in experiment 2 are lower than in mares of experiment 1 in which management was very intensive. In addition, in experiment 2 estradiol 17-β concentrations are much higher (and more similar to values reported in the literature) than in experiment 1. The two

experiments were conducted in different years, with different sampling protocols, and animals of different ages and physiological states, so direct comparisons are not appropriate, and no conclusions can be generated. But, it would be interesting to conduct a controlled study to quantify the effects of different levels of palpation intensity, and subsequent effects on estradiol and LH.

Continuing studies on the effects of management intensity on reproduction are needed to clarify the potential effects on fertility in mares. As the most intensively and invasively reproductively managed of livestock species, mares are more vulnerable to sub-fertility related to human intervention. Diagnostic and therapeutic intervention practices are used aggressively in mares, possibly because mares are frequently not culled for reproductive reasons. A thorough understanding of the effects of these practices on the reproductive biology of mares is needed to make sound management decisions regarding their use.

APPENDICES

APPENDIX I

Estrogen Assay

Diagnostic Products Corporation-estradiol double antibody
Journal of Reproduction and Fertility 89:643-653, 1990

I. Preparation of estradiol-17β stocks

A. Weigh 10 mg of estradiol and add to 1 L of 100% ETOH
Yield=10,000 ug/1,000,000 ul = 1 ug/100 ul = .01 ug/ul = 10 ng/ul
Stock A = 10,000 pg/ul

B. Take 10 ul of Stock A and add to 100 ml of 100% ETOH
Yield=100,000 pg/100,000 ul
Stock B = 1 pg/ul

C. Take 10 ml of Stock B and add to 90 ml of 100% ETOH
Yield=10,000 pg/100,000 ul
Stock C = 0.1 pg/ul

D. Take 1 ml of Stock B and add to 99 ml of 100% ETOH
Yield=1000 pg/100,000 ul
Stock D = 0.01 pg/ul

II. Preparation of estradiol-17β standards

A. Add appropriate volume of stock. Evaporate ETOH in a water bath at 56C.

B. Add 100 ul of PBS-gelatin into each tube and vortex for 1 minute at a speed of 3.5 on a multitube vortexer (VWR).
Note: standards are usually prepared in duplicate.

Standard pg/tube	ul of Stock D	ul of Stock C
0.10	10	--
0.25	25	--
0.50	50	--
1.00	100	--
2.00	200	--
5.00	--	50
10.00	--	100
20.00	--	200
40.00	--	400

Note: there will be no need to transfer the standards to any other tube for assay.

Estrogen Assay
(Sample extraction)

Step 1. Pipette 200 ul of plasma or serum sample into 16x100 mm borosilicate glass tubes (extraction tubes) labeled accordingly. This is usually done in duplicate.

Note: if more than one rack of tubes is involved, process the first rack to Step 4 before proceeding with the next rack. This will allow all the racks to go through the remaining steps simultaneously.

Step 2. Pipette 2 ml of "fresh" diethyl ether into extraction tubes under a ventilated hood, cover the rack of tubes with Parafilm and vortex for 1 minute at a speed of 3.5 on a multitube vortexer (VWR).

Step 3. Let the tubes set for approximately 1 minute after vortexing and then place the rack of tubes into a container partially filled with dry ice and methanol. Set to freeze for approximately 1 minute.

Step 4. Decant the organic phase into a second set of assay tubes labeled correspondingly to each extraction tube.

Step 5. Place the rack of assay tubes into a water bath partially filled with hot water (approximately 56C) to evaporate off the ether.

Step 6. Add 100 ul of PBS with .1% gelatin (PBSG; assay buffer) into each tube and vortex the rack of tubes for 1 minute at a speed of 3.5 on the multitube vortexer.

Estrogen Assay
(Modified DPC procedure)

Step 1. Gather up the standard curve and sample tubes and add at the front of the assay Total count tubes (TCT), nonspecific binding tubes (NSB) and maximum binding or zero standard tubes (MB). Add 100 ul of PBSG to the NSB and MB tubes.

Step 2. Add 30 ul of estradiol antiserum (Stock #E2D1 for 100 tubes or #5E2D1 for 500 tubes) to all tubes except the NSB and TCT tubes, vortex and let incubate for 2 hours at room temperature.

Step 3. Add 75 ul (or approximately 25,000 cpm) of ^{125}I-estradiol (Stock #E2D2) to all tubes, vortex and let incubate for 1 hour at room temperature.

Step 4. Add 1 ml of cold precipitating solution (Stock #N6 for 100 tubes or 5N6 for 500 tubes) to all tubes except TCT tubes, vortex and let incubate for 10 minutes at room temperature.

Step 5. Centrifuge all tubes except TCT tubes for 30 minutes at 1,500xg (approximately 3,000 rpms).

Step 6. Decant all tubes except TCT and let drain up-side-down for approximately 30 minutes before gamma counting.

Note: it is not necessary to purchase the whole kit, only those items (see Stock numbers above or in catalog) you will need can be gotten since you are preparing your own standards. Apart from preparing your own standards and using 100 ul of standard and sample, Steps 2 and 3 have been modified from the company procedure.

APPENDIX II

Table 1: Effects of palpation frequency[1], successive estrous cycle, and time of day on serum cortisol (ng/ml)[2] in nulliparous mares

Variable	N	LS Mean	Standard Error	P Value
Phase				.0001
1	18	47.02	1.27	
2	18	53.77	1.02	
Period of estrus				.0001
1	6	55.37[a]	1.42	
2	6	50.15[b]	1.33	
3	6	45.68[c]	1.31	
Time of day				.0013
0600	18	53.27	1.53	
1200	18	54.05	1.48	
1800	18	46.12	1.97	
2400	18	48.14	1.52	

[1] Phase 1-palpated once daily from first detection of estrus until the largest follicle ≥35mm in diameter ; Phase 2- Palpated four times daily from largest follicle >35mm in diameter until ovulation

[2] Values within variable with different superscripts are different (P<.05)

Table 2: Effects of the interaction of palpation frequency[1] and estrus period[2], and the interaction of palpation frequency and time of day on serum cortisol (ng/ml)[3] in nulliparous mares

Interaction	N	LS Mean	Standard Error	P Value
Estrus period x Phase				.02
1x1	6	61.89[a]	1.84	
1x2	6	48.86[b]	2.24	
2x1	6	51.89	1.73	
2x2	6	48.39	2.07	
3x1	6	47.54	1.75	
3x2	6	43.81	2.0	
Time of day x Phase				.008
0600x1	18	54.74	2.18	
1200x1	18	55.67	2.10	
1800x1	18	48.65	1.66	
2400x1	18	56.04[a]	2.18	
0600x2	18	51.81	2.15	
1200x2	18	52.42	2.04	
1800x2	18	43.60	3.58	
2400x2	18	40.25[b]	2.10	

[1] Phase 1-palpated once daily from first detection of estrus until the largest follicle ≥35mm in diameter ; Phase 2-palpated four times daily from largest follicle >35mm in diameter until ovulation

[2] Three consecutive estrus periods

[3]Values within variable with different superscripts are different (P<.05)

Table 3: Effects of palpation frequency[1], successive estrous cycle, and time of day on serum luteinizing hormone (ng/ml)[2] in nulliparous mares

Variable	N	LS Mean	Standard Error	P Value
Phase				.10
1	18	12.07	2.18	
2	18	11.51	2.19	
Period of estrus				.69
1	6	11.01	2.75	
2	6	13.19	2.72	
3	6	11.15	2.73	
Day of estrus				.0001
1	18	6.52^{a}	2.11	
2	18	8.84^{abc}	2.13	
3	18	11.28^{bc}	2.32	
4	18	13.43^{b}	2.53	
5		15.01^{b}	2.71	
6		15.65^{b}	2.75	
Time of day				.003
0600	18	10.37^{a}	2.22	
1200	18	10.87^{a}	2.22	
1800	18	12.58^{b}	2.22	
2400	18	13.33^{b}	2.22	

[1] Phase 1-palpated once daily from first detection of estrus until the largest follicle ≥35mm in diameter ; Phase 2- Palpated four times daily from largest follicle >35mm in diameter until ovulation

[2] Values within variable with different superscripts are different (P<.05)

Table 4: Effects of the interaction of palpation frequency[1] and estrus period[2] on serum luteinizing hormone (ng/ml)[3] in nulliparous mares

Variable	N	LS Mean	Standard Error	P Value
Estrus period x Phase				.002
1x1	18	10.78	2.77	
2x1	18	14.06	2.73	
3x1	18	11.36	2.74	
1x2	18	11.24	2.76	
2x2	18	12.33[a]	2.75	
3x2	18	10.96[b]	2.75	

[1] Phase 1-palpated once daily from first detection of estrus until the largest follicle ≥35mm in diameter ; Phase 2- Palpated four times daily from largest follicle >35mm in diameter until ovulation

[2] Three successive estrus periods

[3] Values within variable with different superscripts are different (P<.05)

Table 5: Effects of palpation frequency[1], successive estrous cycle, day of estrus[2] and time of day on serum estradiol 17-β (ng/ml)[3] in nulliparous mares

Variable	N	LS Mean	Standard Error	P Value
Phase				.12
1	18	6.06	.35	
2	18	5.35	.41	
Period of estrus				.50
1	6	6.03	.41	
2	6	5.49	.40	
3	6	5.61	.38	
Day of estrus				.0001
1	18	3.95[a]	.31	
2	18	5.79	.98	
3	18	6.55	1.03	
4	18	5.69[b]	.45	
5	15	6.50[b]	.64	
6	15	5.76[b]	.59	
Time of day				.03
0600	18	5.80	.41	
1200	18	4.89[a]	.45	
1800	18	6.32[b]	.39	
2400	18	5.81	.39	

[1] Phase 1-palpated once daily from first detection of estrus until the largest follicle ≥35mm in diameter ; Phase 2-palpated four times daily from largest follicle >35mm in diameter until ovulation

[2] Day 1 is the last day of behavioral estrus

[3]Values within variable with different superscripts are different (P<.05)

Table 6: Effects of pasture breeding[1], hand mating[2], and artificial insemination[3] on plasma β-endorphin (ng/ml)[4] in mares

Variable	N	LS Mean	Standard Error	P Value
Management System				.25
Pasture Breeding	8	29.39	8.60	
Hand Mating	8	9.55	8.24	
Artificial Insemination	8	13.94	8.65	
Age (years)				
<9	12	8.62	6.83	.08
≥9	12	26.64	7.03	
Lactation				.47
Lactating	12	13.99	7.01	
Non-Lactating	12	21.27	6.86	

[1] Mares on pasture with breeding stallion for duration of estrus period
[2] Mares hand mated every other day from onset of estrus to end of estrus
[3] Mares rectally palpated and artificially inseminated
[4]Values with different superscripts are different (P<.05)

Table 7: Effects of pasture breeding[1], hand mating[2] and artificial insemination[3] on plasma cortisol (ng/ml)[4] in mares

Variable	N	LS Mean	Standard Error	P Value
Management System				.07
Pasture Breeding	8	7.60	.83	
Hand Mating	8	8.52		
Artificial Insemination	8	5.81	.78	
Lactation				.01
Lactating	12	6.05	.65	
Non-Lactating	12	8.58	.65	
Time				.0001
0600	24	7.35[a]	.49	
1200	24	6.49[b]	.49	
1800	24	8.09[c]	.49	

[1] Mares on pasture with breeding stallion for duration of estrus period
[2] Mares hand mated every other day from onset of estrus to end of estrus

[3] Mares rectally palpated and artificially inseminated
[4]Values with different superscripts are different (P<.05)

Table 8: Effects of the interaction of lactation state and day[1], and the interaction of management system[2] and time on plasma cortisol (ng/ml)[3] in mares

Variable	N	LS Mean	Standard Error	P Value
Lactation x Day				.03
Lactating x1	12	5.91	.80	
Lactating x2	12	5.60	.73	
Lactating x3	12	6.59	.74	
Lactating x4	12	6.01	.76	
Lactating x5	10	5.96	.79	
Lactating x6	10	6.22	.82	
Non-lactating x1	12	6.92[a]	.80	
Non-lactating x2	12	6.72	.74	
Non-lactating x3	12	8.40	.74	
Non-lactating x4	12	9.41[b]	.75	
Non-lactating x5	10	9.17	.75	
Non-lactating x6	10	8.22[b]	.78	
Management System x Time				.02
Pasture bred x0600	8	7.28	.89	
Pasture bred x1200	8	7.55	.88	
Pasture bred x1800	8	7.99	.89	
Hand mated x0600	8	8.61	.84	
Hand mated x1200	8	7.12	.85	
Hand mated x1800	8	9.84	.84	
Artificially inseminated x0600	8	6.17	.81	
Artificially inseminated x1200	8	4.82[a]	.82	
Artificially inseminated x1800	8	6.43[b]	.81	

[1] Day 1 is last day of behavioral estrus
[2] Pasture bred-mares on pasture with breeding stallion for duration of estrus period; Hand mated-mares hand mated every other day from onset of estrus to end of estrus; Artificially inseminated-mares rectally palpated and artificially inseminated
[3]Values with different superscripts are different (P<.05).

Table 9: Effects of pasture breeding[1] ,hand mating[2] and artificial insemination[3] on plasma luteinizing hormone (ng/ml)[4] in mares

Variable	N	LS Mean	Standard Error	P Value
Management System				.0005
Pasture Breeding	8	17.00[b]	.82	
Hand Mating	8	22.34[a]	.79	
Artificial Insemination	8	17.80[b]	.76	
Age				.01
< 9 years	12	20.29[a]	.66	
≥ 9 years	12	17.79[b]	.66	
Lactation				.005
Lactating	12	17.58[a]	.65	
Non-Lactating	12	20.51[b]	.66	

[1] Mares on pasture with a stallion for duration of estrus period
[2] Mares hand mated every other day from onset of estrus to end of estrus
[3] Mares rectally palpated and artificially inseminated
[4]Values with different superscripts are different (P<.05)

Table 10: Effects of interaction of age and management system[1], and lactation and management system, on plasma luteinizing hormone (ng/ml)[2] in mares

Variable	N	LS Mean	Standard Error	P Value
Age x Management System				.06
< 9 years xPasture bred	4	19.59	1.17	
<9 years x Hand mated	4	22.01	1.05	
<9 years x Artificially inseminated	4	19.30	1.05	
≥9 years x Pasture bred	4	14.42	1.11	
≥9 years x Hand mated	4	22.65	1.16	
≥9 years x Artificially inseminated	4	16.31	1.05	
Lactation x Management System				.02
Lactating x Pasture bred	4	17.24	1.07	
Lactating x Hand mated	4	19.02	1.56	
Lactating x Artificially inseminated	4	16.48	1.04	
Non-lactating x Pasture bred	4	16.76[b]	1.23	
Non-lactating x Hand mated	4	25.65[a]	1.06	
Non-lactating x Artificially inseminated	4	19.13[b]	1.05	

[1] Pasture bred-mares on pasture with a stallion for duration of estrus period;hand mated-mares hand mated every other day from onset of estrus to end of estrus; artificially inseminated-mares rectally palpated and artificially inseminated
[2]Values with different superscripts are different (P<.05).

Table 11: Effects of pasture breeding[1] , hand mating[2] and artificial insemination[3] on plasma estradiol 17-β (pg/ml)[4] in mares

Variable	N	LS Mean	Standard Error	P Value
Treatment				.05
Pasture Bred	8	43.15[b]	2.24	
Hand Mated	8	49.42	2.11	
Artificially Inseminated	8	50.50[a]	1.97	
Age				.003
< 9 years	12	43.72[a]	1.73	
≥ 9 years	12	51.66[b]	1.75	
Lactation				.0001
Lactating	12	54.23[a]	1.77	
Non-Lactating	12	41.15[b]	1.84	

[1] Mares on pasture with breeding stallion for duration of estrus period
[2] Mares hand mated every other day from onset of estrus to end of estrus
[3] Mares rectally palpated and artificially inseminated
[4]Values with different superscripts are different (P<.05)

Table 12: Effects of interactions of age and lactation, and lactation and management system[1], on plasma estradiol 17-β (pg/ml)[2] in mares

Variable	N	LS Mean	Standard Error	P Value
Age x Lactation				.10
< 9 years x Lactating	6	48.23	2.28	
< 9 years x Non-lactating	6	39.20	2.58	
≥ 9 years x Lactating	6	60.22	2.56	
≥9 years x Non-lactating	6	43.09	2.44	
Lactation x Treatment				.007
Lactating x Pasture bred	4	43.82[b]	2.85	
Lactating x Hand mated	4	61.02[a]	3.13	
Lactating x Artificially inseminated	4	57.86[a]	2.77	
Non-lactating x Pasture bred	4	42.48	3.46	
Non-lactating x Hand mated	4	37.83[b]	2.84	
Non-lactating x Artificially inseminated	4	43.14[b]	2.80	

[1] Pasture bred-mares on pasture with breeding stallion for duration of estrus period; Hand mated-mares hand mated every other day from onset of estrus to end of estrus; Artificially inseminated-mares rectally palpated and artificially inseminated
[2]Values with different superscripts are different (P<.05)

APPENDIX III

Table 13: Pregnancy rates at 30 days post-treatment in pasture bred[1] , hand mated[2] and artificially inseminated[3] mares

Management System	Number Pregnant	Number Bred	Percent Pregnant
Pasture Bred	3	5[4]	60
Hand Mated	6	8	75
Artificially Inseminated	5	6[5]	84

[1] Mares on pasture with a stallion for duration of estrus period
[2] Mares hand mated every other day from onset of estrus to end of estrus
[3] Mares rectally palpated and artificially inseminated
[4] Three mares treated with prostaglandin f2α 9 days after treatment period
[5] Two mares inseminated with vehicle

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LITERATURE CITED

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