EFFECTS OF GONADOTROPIN RELEASING HORMONE ON FOLLICULAR DEVELOPMENT AND SERUM GONADOTROPINS IN SEASONALLY-ANOVULATORY MARES

By

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ABSTRACT

EFFECTS OF GONADOTROPIN-RELEASING HORMONE AND GONADAL STEROIDS IN THE SEASONALLY-ANOVULATORY MARE

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Two experiments were conducted with seasonally-anovulatory pony mares to study effects of gonadotropin-releasing hormone (GnRH), estradiol-17β ($E_2$17β) and progesterone (P_4) on follicular development and gonadotropin concentrations.

In both experiments, all mares which were injected with exogenous hormones received injections every eight hours for 14 days. In Experiment 1, GnRH injections in lighted mares (16 hrs. light:8 hrs. dark), caused a significant increase in ovarian follicle size by the last day of treatment and ovulation in two of three mares injected. $E_2$17β and P_4 injections had no effect on follicular development and ovulation. In Experiment 2, both GnRH and GnRH + $E_2$17β stimulated follicular growth in lighted-mares but did not advance the time of first ovulation in comparison to lighted-controls. This was due to the lateness in the anovulatory season when the experiment

was conducted. LH but not FSH was elevated during the course of GnRH or GnRH + $E_2$17β treatment. The greatest elevation in LH seemed to occur in GnRH + $E_2$17β treated mares.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

Figure

CHAPTER I

INTRODUCTION

A standard January 1st birthdate for foals of major breed associations has been used in an attempt to allow more uniform age determination of show and race horses. Therefore, breeders are presently attempting to have foals born as close to this date as possible to insure growthier, more mature foals. However, such breeding programs dictate that mares must be bred during the anovulatory phase of the equine reproductive cycle nearly three months before the normal ovulatory season. Many mares may exhibit signs of estrus during the seasonal-anovulatory period, however, only 18-25% are capable of ovulation and conception at this time (Day, 1939; Quinlan et al., 1951; Osborn, 1966; Van Niekerk, 1967a; Ginther, 1974; and Greenhoff and Kenney, 1975). Induction of ovulation during the seasonal-anovalatory period would be desirable to increase the percentage of mares capable of conceiving at this time.

Among the farm species, both the ewe and the mare are seasonally-polyestrous. The ewe enters the seasonal-ovulatory period in response to decreasing photoperiod; whereas, the mare enters the seasonal-ovulatory period in response to increasing photoperiod. Many researchers have

shown that reproductive function in both species can be manipulated by artifically altering the photoperiod (Burkhardt, 1947; Yates, 1949; Niskihawa, 1959; Loy, 1968; Oxender et al., 1977; and Ovtavant, 1977). For example, 16 hours of light:8 hours of darkness appear to be capable of inducing ovulation in the seasonally-anovulatory mare (Kooistra and Ginther, 1975). Researchers have found that artifically increasing the photoperiod will cause seasonally-anovulatory mare to begin ovulating within 59 to 120 days, depending upon the amount of ovarian activity present at the time the phototreatment was increased (Day, 1940; Burkhardt, 1947; Loy, 1968; Niskihawa, 1959; Kooistra and Ginther, 1975; and Oxender et al., 1977). Since up to three months of increased photoperiod may be required to induce ovulation in seasonally-anovulatory mares, a method of shortening this interval would be desirable. Potential to reduce the length of the photoperiod treatment may rest in the combination of hormone administration and increased photoperiod.

A new synthetic peptide containing 10 amino acids identified as gonadotrophin releasing hormone (GnRH) has been used to induce ovulation in various species. Mares treated with GnRH have shown two to three fold increases in serum LH concentrations (Ginter and Wentworth, 1974; Garcia and Ginther, 1975; Irvine et al., 1975; Evans and Irvine, 1976; and Oxender et al., 1977). A summary of

the data indicates that exogenous GnRH administration significantly increased LH concentrations for 2 to 8 hours in both seasonally-anovulatory mares and seasonally-ovulatory mares with a decline to baseline within 24 hours following treatment. Evans and Irvine (1976) also reported that a single injection of GnRH resulted in a 3.7 fold greater increase in blood levels of FSH than in LH in seasonally-anovulatory mares. In the ovulatory season, the transient LH surge following a single injection of GnRH did not reduce the interval from estrus to ovulation in mares (Garcia and Ginther, 1975; and Ginther and Wentworth, 1975). It should be noted that during the equine estrous cycle, endogenous LH concentrations remain elevated for 2-4 days during the periovulatory period (Whitmore et al., 1973; Noden et al., 1974; and Pattison et al., 1974). Thus, Ginther and Wentworth (1974) suggested that repeated injections of GnRH may be necessary to duplicate the normal LH response of the mare in stimulating follicular development and ovulation.

Estradiol concentrations in peripheral blood reach maximal levels prior to ovulation (Noden et al., 1975) and may influence pituitary LH and FSH release. In seasonally-anovulatory mares, the estradiol concentrations have been shown to be static and approximately 2.5 pg/ml; whereas prior to ovulation estradiol concentrations would be about five to ten pg/ml (Noden et al., 1975). In the ewe (Reeves

et al., 1970; and Reeves et al., 1974), woman (Keye and Jaffe, 1973), and mare (Garcia and Ginther, 1975) pretreatment with estradiol-17β enhanced the LH and FSH response to a does of GnRH. Data reported by Pattison et al., (1974) in the mare have shown an increase in estradiol concentrations corresponding with increasing LH levels and the onset of estrus. This evidence suggests that estradiol may play a significant role in the induction of estrus and ovulation.

During the seasonally-anovulatory period, peripheral progesterone concentrations in the mare are usually below 1 ng/ml (Oxender et al., 1977). However in the cycling mare, progesterone concentrations have been shown to be below 1 ng/ml during estrus and rise two days after ovulation to peak levels of 10-12 ng/ml by approximately day 10 post-ovulation (Short, 1959; Van Hensburg and Van Niekerk, 1968; Sharp, 1972; Stabenfeldt et al., 1972; Sharp and Black, 1973; Squires et al., 1974; and Noden et al., 1975. Van Niekerk et al. (1973) have suggested that exogenous progesterone administration resulted in an accumulation of FSH and LH in the anterior pituitary which was released upon withdrawal of the exogenous progesterone. Subsequently, Garcia and Ginther (1978) demonstrated that exogenous progesterone significantly depressed blood levels of LH in ovariectomized mares. Theoretically, the release of FSH and LH following withdrawal of progesterone would

result in follicular development and ovulation. Van Niekerk (1973) demonstrated that exogenous progesterone administration resulted in estrus and ovulation within 3-8 days following withdrawal in mares with active but noncycling ovaries. However, mares with inactive ovaries (few palpable follicles) did not respond to this treatment. Progesterone treatment in conjunction with increased photoperiod in the seasonally-anovulatory mares apparently has not been studied.

This research was conducted to study the effects of several exogenously administered hormones on the stimulation of follicular development and induction of ovulation in seasonally-anovulatory mares exposed to an increased photoperiod (16 hours of light:8 hours of dark).

CHAPTER II

LITERATURE REVIEW

Equine Reproductive Seasonality

Seasonal Variation

In mammalian species, the ewe and the mare are known to be seasonally polyestrous, responding to decreasing photoperiod and increasing photoperiod respectively. In the horse mare, the seasonal-ovulatory period is from approximately late March to October in the northern hemisphere. However, the pony mare has a much shorter seasonal-ovulatory period from late May to September (Ginther, 1979). The length of photoperiod appears to be the major factor in regulating seasonal reproductive patterns in the mare, and artificially increased photoperiod has been documented to have the greatest influence on stimulating an early onset of the breeding season (Burkhardt, 1947; Sharp and Ginther, 1974; Oxender et al., 1977; and Ginther, 1979). However, environmental temperature, humidity, and nutrition have been reported to have an effect on the mare's reproductive pattern (Quinlan et al., 1951; Van Niekerk et al., 1967a; Greenhoff and Kenney, 1975; and Sharp and Ginther, 1975).

The longest day of the year (June 21, 16 hours of light:8 hours of dark) is the summer soltice and the winter soltice occurs on the shortest day of the year (December 21, 8 hours of light: 16 hours of dark). The greatest number of mares are ovulating and the highest conception rate occurs around the summer soltice and decreases thereafter (Ginther, 1974; Kenney et al., 1975; and Ginther, 1979). In comparison, only 18-25% of mares are ovulating in February and March in the northern hemisphere (Day, 1939; Quinlan et al., 1951; Osborne, 1966; and Ginther, 1974).

Mares may exhibit estrus every month of the year, but much greater regularity in the length of estrus occurs during the months of May through October (Ginther, 1974; Kennedy et al., 1975; and Ginther, 1979). Most mares enter a seasonal-anovulatory state corresponding with decreasing photoperiod (Ginther, 1974; Kennedy et al., 1975; and Ginther, 1979). Since mares may exhibit signs of estrus during the seasonal-anovulatory period, ovulation is a better indicator of seasonality than estrus (Ginther, 1979).

The estrus associated with the first ovulation of the ovulatory season was greatly prolonged in mares in which the first ovulatory estrus began in March or April (Ginther, 1974; and Ginther, 1979). As the ovulatory season approaches the summer soltice, the duration of estrus becomes shorter (Ginther, 1979). However, the length of estrus and diestrus changes as the ovulatory season

progresses (Ginther, 1974). Estrus becomes progressively shorter from April to June, then increased during August to October. Whereas, the length of diestrus increases as the ovulatory season progresses corresponding with the estrus length decreasing (Ginther, 1974).

In summary, the general overview of equine reproductive seasonality is the following: 1) biologic variation between mares; 2) behavioral estrus is a poor indicator of ovarian function since estrus without ovulation often occurs during the anovulatory season; 3) length of behavioral estrus becomes more regular during the ovulatory season and becomes a much more reliable indicator of ovarian function although ovulation unaccompanied by estrus can occur; 4) the first estrus of the ovulatory season is more prolonged than subsequent estrus periods occuring later in the ovulatory season; and 5) the length of estrus and diestrus change as the ovulatory season progresses.

Estrus and Ovulation

During the ovulatory season, the estrous cycle ranges from 18-23 days in length with diestrus ranging from 14-16 days and estrus from 4-9 days (Quinlan et al., 1951; Van Niekerk, 1967b; Ginther et al., 1972; and Ginther, 1974). Most researchers have indicated ovulation 24 to 48 hours before the end of estrus (Day, 1940; Ginther et al., 1972; Whitmore et al., 1973; and Ginther, 1974). The size

of the follicle just prior to ovulation has been reported to be 25 to 65 mm in diameter in horse mares and 25 to 45 mm in pony mares (Day, 1940; and Ginther et al., 1972).

Reproductive Hormonal Events During the Estrous Cycle

Gonadotrophins

The two major hormones responsible for affecting ovarian follicular development in mammals are Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). In general, FSH is thought to promote the growth and development of ovarian follicles. More specifically, FSH is thought to stimulate the development of follicles from the antral to the preovulatory surge, and will produce biochemical changes such as increased oxygen uptake and protein synthesis in the theca cells (Austin and Short, 1972). Evans and Irvine (1975 and 1976) suggest that developing follicles are "primed" to maturation by two or more surges of FSH at approximately ten day intervals. More recent research by Miller et al. (1977) reported a statistical significance in FSH surges between days 9 and 11 prior to ovulation in pony mares. However, only one mare exhibited the early and middiestrus surges of FSH similar to that reported by Evans and Irvine (1975). The remaining four mares had a significant increase in FSH concentrations only between day 9 and 11 prior to ovulation followed by a rapid decline by day 16 (Evans and Irvine, 1976; and Miller

et al., 1977). More conclusive research will be required before any accounting for these differences can be made. The role of these two hormones in development of follicles in the equine has not been completely elucidated.

Researchers have utilized antiserum against equine pituitary extracts to study the effects on estrus, follicular development and ovulation in pony mares (Pineda and Ginther, 1972; Pineda et al., 1972; and Pineda et al., 1973). They concluded that antiserum against the equine pituitary fraction inhibited the activity of the endogenous gonadotropins. When given on days 2 through 6 of estrus, the antiserum blocked ovulation and resulted in degenerative changes in the largest follicle (Pineda and Ginther, 1972). The same treatment given on days 3 through 7 of diestrus caused regression of the corpus luteum but increased the diameter of the largest follicle (Pineda et al., 1972). In a subsequent experiment, a similar treatment of antiserum was given on days 1 to 4, 4 to 7, and 7 to 10 of diestrus (Pineda et al., 1973). This study was conducted to study the effects of antiserum (same dosage for all days studied) on follicles during different days of diestrus to provide insight into the effect of endogenous gonadotropins on follicular development, maintenance, and atresia during diestrus. It should be remembered that the effect on circulating levels of gonadotropins and on atretic changes of follicles was not directly studied. From this study and

earlier studies utilizing equine pituitary antiserum it was concluded that: 1) early in diestrus, development of follicles in all categories may be dependent on endogenous pituitary gonadotropins; 2) during middiestrus, the gonadotropins may induce atresia of large follicles; 3) toward the end of diestrus, gonadotropins, may promote development of large follicles; and 4) during estrus, development of the largest follicle is also dependent on the gonadotropins.

LH induces ovulation in certain ovarian follicles. LH also stimulates steriod synthesis in the theca interna, granulosa, interstitial, and luteal cells (Austin and Short, 1972). The major site of action of LH in steriod biosynthesis appears to be at the level of cholesterol conversion to pregnenolone (Austin and Short, 1972). Basal secretion of LH seems to be sufficient for steriod synthesis; however, a surge of LH (ovulatory surge) is needed to cause ovarian follicles to ovulate. In the mare, the ovulatory surge of LH is approximately four to five days in duration (Ginther, 1979). This is in sharp contrast to most mammalian species in which to ovulatory surge of LH is only 12 to 24 hours in duration (Austin and Short, 1972).

Serum LH has been quantified in the peripheral blood of mares by several laboratories (Whitmore et al., 1973; Noden et al., 1975; Evans and Irvine, 1976; and Garcia and Ginther, 1976). Depending on the assay procedures

utilized by the above authors, the magnitude of LH concentrations differed. The general agreement is that LH concentrations are low during mild middiestrus with a marked increase a few days prior to the onset of estrus reaching maximum levels 1 to 2 days post ovulation and then gradually declining during the next four to six days (Whitmore et al., 1973; Pattison et al., 1974; Noden et al., 1975; Evans and Irvine, 1976; and Garcia and Ginther, 1976). The total biological role of LH is not yet known. However, it has been suggested by Evans and Irvine (1975) that LH may play an important role in the maturation of the ovulatory follicle as well as have a lutotropic effect in the mare.

Gonadal Steriods

Estrogen secreted by ovarian follicles is thought to be the major hormone responsible for the occurrence of sexual receptivity (estrus) in estrual animals. Estradiol-17β is the major circulating estrogen in the mare. The circulating concentrations of estradiol-17β are more difficult to determine due to low concentrations (pg/ml) and the presence of several other estrogenic compounds in plasma. These estrogenic compounds may cross-react with estradiol-17β antisera and complicate quantification procedures and interpretation of the results. Reported levels of plasma or serum levels of estradiol-17β vary greatly (Pattison et al., 1974; Noden et al., 1975; Plotka

et al., 1975; and Oxender et al., 1977). Depending on the seasonal state, the serum estradiol concentrations reported by Pattison et al. (1974) for estrous and diestrous mares were 141 pg/ml and 20 pg/ml, respectively. The pattern of estradiol secretion reported by Noden et al. (1975) describes estradiol concentrations as being minimal during diestrus; but at the beginning of estrus, estradiol concentrations begin to increase reaching a maximum on approximately the day of ovulation. In seasonally-anovulatory mares estradiol concentrations have been reported to be approximately 2.5 pg/ml (Oxender et al., 1977).

Progesterone is secreted by the ovarian corpus luteum and is the major ovarian hormone secreted during diestrus. Numerous studies have reported progesterone concentrations during the estrous cycle. The earlier work was done with competitive protein binding assay techniques (Smith et al., 1970; Plotka et al., 1972; Stabenfeldt et al., 1972; Sharp and Black, 1973; Allen and Hadley, 1974; and Van Niekerk et al., 1975). Later studies utilized radioimmunoassay techniques (Squires et al., 1974; Noden et al., 1975; and Oxender et al., 1977). Although there is variation among reports, most researchers have reported that levels of progesterone are less than 1 ng/ml of serum during the estrous and the seasonal-anovulatory period. Progesterone concentrations increase within 24-36 hours

following ovulation with a peak concentration occurring at approximately middiestrus (Van Niekerk et al., 1975). Van Niekerk et al. (1973) also reported that between 10 and 14 days after ovulation the progesterone level decreased steadily with a rapid drop between days 14 and 16 (approximately 4 to 5 days prior to ovulation). Progesterone concentrations remains elevated until luteolytic surge of prostaglandin F2α occurs at approximately day 13 to 14 post-estrus (Douglas and Ginther, 1976). Following luteolysis, concentrations of progesterone decrease rapidly until the low estrual levels (less than 1 ng/ml) are reached.

Reproductive Hormonal Events During the Seasonal Anovulatory Period

Very little is known about the hormonal events during the seasonal-anovulatory period in the equine. The predominant amount of research that has been done has been concentrated on the study of gonadotropins.

Garcia and Ginther (1976) studied ovariectomized and intact pony mares for a twelve month period. In both ovariectomized and ovarian-intact mares, LH levels were low during winter and early spring and high during late spring. These data suggest that the depressed levels of LH during the anovulatory season (winter) may be independent of ovarian functions.

During the transition from the anovulatory to the ovulatory season, FSH and LH secretion appear to occur in a

reciprocal fashion in mares (Freedman et al., 1977a). Mean FSH concentrations fluctuated prior to day 20 before the first ovulation of the ovulatory season. FSH steadily declined from day 20 to day 1 (first ovulation of the ovulatory season). Mean LH concentrations remained low and static prior to day 8 and then rose to a maximum at day 1. Mean diameter of the largest palpable follicle increased gradually from day 16 and then increased rapidly between day 8 and day 1.

Even less is known about secretion of gonadal steriods during the anovulatory season. Hillman and Loy (1975) reported that urinary estrogens were below detectable levels in the mares with ovarian and behavioral inactivity during the fall and winter. Oxender et al., (1977) found low levels of estradiol (less than 3 pg/ml) and progesterone (less than 1 ng/ml) during the anovulatory season. This level of estradiol would be similar to that found during diestrus and the level of progesterone would be similar to that found during estrus.

Effects of Increased Photoperiod During the Seasonal-Anovulatory Period

The mechanism of action of increased or decreased photoperiod on reproductive function is not yet clearly understood in the horse. However, research conducted utilizing hamsters, ferrets, and rats has suggested that there is a relationship between melatonin levels in the

pineal gland and photoperiod (Reiter, 1973; Reiter, 1974; Wallen and Yochim, 1974; Herbert et al., 1975; Morin, 1975; Reiter et al., 1975; Tamarkin et al., 1976; and Turek, 1977).

In general, decreased photoperiod has been demonstrated to increase melatonin synthesis by the pineal gland. In the male hamster, melatonin has been demonstrated to have antigonadal action on the testes when it was exogenously administered (Turek, 1977). This may suggest that melatonin alters the gonadal activity by altering hypothalamic-hypophyseal activity but this still is not clearly understood (Turek, 1977; and Reiter, 1978).

In the female, whether hamster, ferret, or rat the pineal has been indicated to be essential for the animal to exhibit a reproductive response to increased or decreased artificial photoperiod (Wallen and Yochim, 1974; Hebert et al., 1975; and Reiter et al., 1975). Hebert et al. (1975) demonstrated that administration of melatonin effects the breeding season of ferrets so that a higher dose (1 mg) will drive females out of estrus and lower doses (.5 mg) will delay the onset of estrus in intact animals. Reiter et al. (1975) reported that melatonin acts like pinealectomy in preventing gonadal atrophy in light deprived female hamsters. These same authors concluded that melatonin actually prevents the pineal gland from interfering with normal pituitary-ovarian relationships in female hamsters.

Some recent work has been conducted to establish the seasonal changes in pineal melatonin-forming enzyme in pony mares (Wesson et al., 1977). Their preliminary data suggests an inverse relationship between increased pineal hydroxyindole-o-methyl transferase (HIOMT) and the percent of ovulatory mares. This increase of HIOMT with a decrease in percentage of ovulating mares and may suggest an antigonadal action of the pineal in the mare. However, more research is needed to determine the mechanism of action of increased or decreased photoperiod on reproductive function in the mare. Studies directed towards determining what relationship melatonin and the pineal gland have on the equine ovarian cycle are indicated. More recent studies have demonstrated that peptide products of the mammalian pineal gland also have anti-gonadotrophic activity. However, apparently studies concerning such compounds have not been reported in the mare.

The use of increased artificial photoperiod has been documented by several researchers to induce estrus and ovulation in the seasonally-anovulatory mare (Burkhardt, 1947; Nishikawa, 1959; Loy, 1968; Kenney et al., 1975; Sharp and Ginther, 1975; Kooistra and Ginther, 1975; and Oxender et al., 1977). The light to dark ratio that appears to be optimal in inducing ovulation is approximately 16 hours of light and 8 hours of dark (Kooistra and Ginther, 1975). Varying the amount of light by ± 2 hrs was less

effective than 16 hrs light. Increased photoperiod has been shown to have a major effect on inducing ovulation. However, it should be recognized that other factors such as temperature, humidity, and nutrition may be involved but photoperiod appears to be paramount (Day, 1939; Quinlan et al., 1951; Van Niekerk and Van Heerden, 1972; and Sharp and Ginther, 1975). Apparently, no studies have been conducted to study effects of different types of lighting on inducing ovulation in seasonally-anovulatory mares. However, the 200-250 watt incandescent light (providing at least 2 foot candles for 16 hours per day) is sufficient to induce ovulation in the mare (Loy, 1968; Cooper and Wert, 1975; and Oxender et al., 1977). The exposure of seasonally-anovulatory mares to increased photoperiod will induce ovulation within 59 to 120 days after initiation of lights (Burkhardt, 1947; Nishikawa, 1959; Loy, 1968; Kooistra and Ginther, 1975; Sharp and Ginther; and Oxender et al., 1977). Several studies have demonstrated that the interval to ovulation is significantly shorter for mares which had ovaries with some follicular development than for those mares in which papable follicular development was absent at the onset of increased photoperiod (Loy, 1968; Kooistra and Ginther, 1975; and Oxender et al., 1977).

Loss of winter hair coat also occurs with exposure to increased photoperiod (Burkhardt, 1947; Koositra and Ginther, 1975; Sharp and Ginther, 1975; and Oxender et al.,

1977). However, the correlation between loss of hair coat and increased photoperiod is not yet understood.

Effect of Exogenous Hormones During the Seasonal-Anovulatory Period

Progesterone

Attempts have been made to use progesterone to induce ovulation in seasonally-anovulatory mares (Van Niekerk et al., 1973; and Kenney et al., 1975). Van Niekerk et al. (1973) studied the effects of daily administration of 100 mg of progesterone in oil for approximately 7 days during the transition from the anovulatory to the ovulatory season. A behavioral characteristic of this phase of the seasonal reproductive cycle is extended estrous periods. Within two days following initiation of progesterone treatment, behavioral estrus was blocked; however, a return to estrus occurred within three days following progesterone withdrawal. The post-treatment estrus was accompanied by ovulation. Mares not in estrus but having ovarian follicular development responded in a similar fashion. Mares with only slight ovarian activity also responded but had a longer interval from end of treatment to onset of estrus. Progesterone treatment had no effect in mares with small ovaries without follicular development. Conception rate was greater than 60% in those mares responding to treatment.

In another study (Kenney et al., 1975) eight of nine mares came into prompt, positive behavioral estrus following

daily treatment with progesterone. Prior to treatment, eight of the mares were showing signs of behavioral anestrus and one mare had irregular cycles. Four of the mares became pregnant during the post-treatment estrus.

Estradiol-17β

In most species, estradiol-17β is the most potent naturally occurring estrogen and the main estrogen secreted by the ovary (Austin and Short, 1972). Estrogen has been shown to induce both FSH and LH release from the pituitary at the same time and thus may control release of both hormones by the same mechanism of activating a single releasing hormone (Reeves et al., 1974). Several authors have suggested that the role of estrogen is a positive feedback effect through the hypothalamus and/or pituitary in stimulating LH secretion. Such an effect has been reported in the ewe, monkey, cow, and mare (Goding et al., 1969; Pelletier and Signoret, 1969; Yamaji et al., 1971, Henricks et al., Wetteman et al., 1972; and Garcia and Ginther, 1976). It has been well established by several authors that a single injection of estrogen can induce release of LH from the anterior pituitary gland in the anestrous ewe (Goding et al., 1969; Radford et al., 1970; and Beck and Reeves, 1973). Garcia and Ginther (1975) reported that the mare was also similar to other species in which estrogen had a facilatory effect on LH response to GnRH treatment.

Treatment with estrogen has not been specifically studied as a method of inducing ovulation in the seasonally-anovulatory mare. However, Garcia and Ginther (1975) presented data indicating that estradiol treatment alone increased the plasma LH concentration during the ovulatory season in ovariectomized mares.

Equine Pituitary Extracts

In pony mares, gonadotropic hormones such as HCG, PMSG, and horse pituitary extracts injected in various concentrations and treatment regimes did not elicit a response during the anovulatory season (Day, 1940). However, Douglas and Ginther (1974) and Lapin and Ginther (1977) demonstrated in seasonally-anovulatory mares that single and multiple ovulations occurred following administration of crude and purified equine pituitary extracts (EPE) when given daily for a fourteen day period. Douglas and Ginther (1974) reported that administration of crude EPE or a relatively pure EPE was sufficient to induce ovulation in 20 out of 23 mares. They also showed that 20 mares (87%) ovulated with 11 mares (58%) having two or more ovulations. However, these mares failed to return to estrus or reovulate at the next expected ovulatory period. Lapin and Ginther (1977) used crude EPE to induce ovulation in seasonally-anovulatory mares and determined whether or not the ova are fertilizable. Mares were treated with EPE and EPE + HCG. These two groups had a significantly greater number of

ovulations per mare and per group as compared to saline treated controls. Mares receiving HCG also had significantly shorter intervals from day 13 to day of first ovulation. Six fertilized ova and two fresh non-fertilized ova were recovered, indicating that at least some of the induced ovarian follicles yielded normal ova. This confirms the previous work of Douglas and Ginther (1974) that equine pituitary extracts can induce single and multiple ovulations in seasonally-anovulatory mares. The use of EPE combined with increased photoperiod may offer potential for shortening the interium required to induce ovulation in seasonally-anovulatory mares with a continuation of ovulatory cycles.

Gonadotropin Releasing Hormone

Gonadotropin releasing hormone (GnRH) is a peptide containing 10 amino acids which is produced by the hypothalamus and is transported to the adenohypophysis through a system of portal vessels. This hypothalamic substance (GnRH) which affects secretion of LH and FSH has been recently isolated and synthesized. Synthetic and natural GnRH have been shown to cause a rise in plasma concentrations and pituitary release of both LH and FSH in several species (Schally et al., 1972; and Schally et al., 1976).

A single injection of GnRH was effective in stimulating LH release and ovulation in heifers (Kaltenbach

et al., 1974); ewes (Rippel et al., 1974b); and gilts (Baker et al., 1973). GnRH has been demonstrated to cause an LH surge in female rats exposed to increased photoperiod (Steger et al., 1976).

GnRH has been investigated by several researchers for its ability to induce ovulation in seasonally-anovulatory and ovulatory mares (Ginther and Wentworth, 1974; Garcia and Ginther, 1975; Heinze and Klug, 1975; Irvine et al., 1975; Evans and Irvine, 1976 and 1977; and Oxender et al., 1976). Researchers have indicated that more than a single injection is needed to maintain high serum LH levels (Ginther and Wentworth, 1974; Garcia and Ginther, 1975; Irvine et al., 1975; and Oxender et al., 1977b). Ginther and Wentworth (1974) demonstrated that a single intravenous injection of GnRH in seasonally anovulatory mares caused an immediate increase in plasma LH concentrations that gradually decrease within 2.5 hours to pretreatment levels. Administration of GnRH subcutaneously on day 2 of estrus caused a more prolonged rise in LH values which reached a maximum one hour post treatment and then decreased to pretreatment levels by 8 hours. In these experiments, there was no effect of the GnRH treatment on follicular development, time of ovulation, or length of estrus.

Evans and Irvine (1976) further characterized the effects of GnRH in the seasonally-anovulatory mare by measuring both FSH and LH plasma levels following a single

injection of GnRH. These researchers demonstrated that a single injection of GnRH will cause an increase in serum FSH at 0.5 hours after the injection that is 3.7 times the mean pretreatment concentration. This concentration of FSH is comparable to the peak which occurs during the estrous cycle. The induced increase in LH was much less than that of the cycling peak. This is of interest because it had been suggested that GnRH exerted primarily an LH releasing effect in the human, rat, sheep, and cow (Schalley et al., 1971; Kastin et al., 1972; Reeves et al., 1972; and Ahbar et al., 1974).

Ginther and Wentworth (1974) have shown that estradiol pretreatment had a facilatory effect on LH secretion following GnRH treatment. However, Booth et al. (1977) demonstrated that estradiol-17β pretreatment did not enhance LH release following GnRH administration in seasonally ovulatory mares.

A refractory period in LH release following repetitive injections of GnRH has been demonstrated in the ewe and the steer (Rippel et al., 1974a; and Tannen and Convey, 1977). Rippel et al. (1974a) concluded from their data that both the anestrous and ovariectomized ewe had a refractory period in the mechanism controlling GnRH induced LH release. Oxender et al. (1977b) have reported that daily repeated injections of GnRH in seasonally-ovulatory mares did not invoke a refractory response.

Rippel et al. (1974a) determined that successive injections of GnRH injected at 96 hours intervals resulted in LH release similar to the initial dose; however a decline in LH release occurred at 72 hour injection intervals. Serum LH levels in mares following daily injection of GnRH increased greater than three fold in 60 minutes after each consecutive GnRH injection. Magnitude and duration of serum LH release were similar after each of the daily treatments. It appears that pituitary concentrations of LH and FSH were not depleted which suggests pituitary secretion was not necessarily related to hormone content of the gland.

CHAPTER III

MATERIALS AND METHODS

Two experiments were conducted during the seasonal-anovulatory period utilizing 31 pony mares of mixed breeding and ranging in ages from 3 to 20 years. Experiment 1 utilized 18 mares and was conducted during the period of January 7 to June 1, 1977. Experiment 2 utilized 13 pony mares and was conducted during the period of March 18 to June 1, 1977.

In both experiments, all treatment groups, with the exception of ambient light controls, were maintained under a constant increased artificial photoperiod so that the mares were exposed to 16 hours of light and 8 hours of darkness. The 16 hour photoperiod was composed of 8 hours of ambient light plus 8 hours of incandescent light (at least 2 foot candles). Mares exposed to increased photoperiod were housed in a three sided barn in group pens for approximately 16 hours a day and outdoors for approximately 8 hours a day. Ambient controls were maintained under ambient conditions outdoors. All mares were fed hay without grain supplementation. Mares were randomly assigned to treatments and maintained under their respective experimental conditions for a period of 5 days prior to

initiation of the experiment to allow for acclimation to experimental conditions. For example, mares assigned to groups receiving increased photoperiod, were placed under lights and handled as they would be during the experiment. The first day following the five day adjustment period was considered as day 1 of the experimental period. Injections of hormones were initiated on day one and continued at eight hour intervals (0800, 1600, 2400 hours) through day 14. The first injection was given at 0800 hours on day 1 and the last injection was given at 2400 hours on day 14. All injections were given subcutaneously (SC) and all mares were teased daily with an intact pony stallion during day 1 thru 14 and at least every third day thereafter to detect occurrence of estrus. Criteria used to determine if a mare was in estrus were those published by Ginther et al. (1972). If estrus behavior was observed, mares were teased daily until estrus behavior no longer occurred. Ovarian palpation, per rectum, was performed in all mares at least every third day. Ovarian follicles less than 5 mm in diameter were recorded as 0 mm. If a follicle of greater than 20 mm in diameter was detected, palpation was performed daily until ovulation or follicular atresia occured. All blood samples were collected via jugular venapuncture prior to 0800 injection every day for the first 14 days and then every third day thereafter in both experimental groups.

Preparation of Treatments

The gonadotrophin releasing hormone (GnRH) used was an analogue (D-Leu-6) supplied by Abbott Laboratories. The dessicated powder was dissolved in saline to provide 200 mg of GnRH per 3 ml saline.

The Estradiol 17-β and progesterone used was a dessicated powder. Peanut oil was utilized as a diluent to obtain the final concentration of 40 mg of progesterone per 3 ml peanut oil and 400 ug of estradiol-17β per 3 ml of peanut oil for the treaments.

Blood Collection and Sample Storage

Every day at 0800 hours (prior to injection) for the first six weeks of the experiment period blood samples (20 ml) were collected. Blood samples were collected by jugular venapuncture utilizing a 20 ml vacutainer tube with a 20 guage 1½" vacutainer needle. The samples were placed in a refrigerator and allowed to stand to allow for sufficient plasma separation. Samples were centrifuged, the plasma poured off, placed in vials, and stored at -20°C for quantification of hormones.

Hormone Quantification

Serum concentrations of progesterone (Experiment 1 and 2), FSH (Experiment 2), and LH (Experiment 2) were determined by radioimmunoassay procedures.

Progesterone

The assay technique for progesterone was a modified form of the assay technique reported by Squires et al. (1974). However, bound and free fractions were separated by using polyethylene glycol (PEG) instead of charcoal dextian. PEG was added to all tubes while in an ice-water bath. Tubes were vortexed for 15 seconds and allowed to incubate for 30 minutes. Then samples were centrifuged for 20 minutes at 3000 rpms in a 4°C centrifuge. The supernatant was poured off into a vial with 5 ml of scintillation cocktail and counted.

Follicle Stimulating Hormone (FSH)

Equine plasma FSH concentrations were assayed using a double antibody technique developed by Evans and Irvine (1976). This assay utilized an antibody prepared against human FSH (hFSH) in combination with labeled hFSH. The standard equine FSH (nute ℓFSH; 90 IU/mg NIH-FSH-S1) was supplied by Dr. L. Nuti, University of Wisconsin. After 72 hour of preincubation of h-FSH antibody and Nuti equine FSH standards, ^{125}I-hFSH (10,000 cpm) was added and incubation continued for 60 hours. The antibody-hormone complex was separated from the free hormone by a 24 hour immunoprecipitation step using antirabbit gamma globulin prepared in sheep. Following the addition of 2 ml .01 m PO_4 - 0.15 m NaCL, (Ph 7.4) buffer to each of the assay tubes, separation was completed by centrifugation for 30 min at 1000 x g and

aspiration of the supernatant. The radio activity in the precipitate was counted in an autogamma spectrometer set to substract counts due to nonspecific binding.

Luteinizing Hormone (LH)

Equine plasma LH concentrations were assayed using a double antibody technique developed by Whitmore et al. (1974). The procedure was the same as that described by these authors.

Statistical Analysis

One way analysis of variance was used to study differences among groups for interval from day 1 to first ovulation (Experiments 1 and 2), first estrus (Experiment 1), and first loss of hair coat in tufts (Experiment 1). Comparisons among groups were made with orthogonal contrasts where indicated. Paired t tests were used to detect differences in the size of the largest palpable ovarian follicle between day 1 and day 14 (Experiments 1 and 2). FSH and LH concentrations were analyzed by split-slot analysis of variance using animal within treatment as the error term for day (Experiment 2). If significant main effects were present, orthogonal contrasts were used to detect differences among groups. LH and FSH were further analyzed by comparing concentrations after day 1 with the pretreatment sample (day 0) by Dunnet's t test.

Experimental Design

Experiment 1. This experiment was initiated during the nadir of the seasonal-anovulatory period (January 7). A total of eighteen pony mares were used (Table 1). Mares were randomly assigned to one of five treatment groups: (1) ambient controls, (2) light controls, (3) GnRH, (4) Progesterone, and (5) estadiol-17β . Group one was maintained under ambient conditions. Groups 2, 3, 4 and 5 were exposed to 16 hours of light:8 hours of darkness as described earlier. The GnRH treated mares were injected SC with 200 ug D-LEU-G-GnRH in 3 ml of saline per injection. Progesterone treated mares were injected with 40 mg progesterone in 3 ml of peanut oil per injection. Estradiol-17β treated mares were injected with 400 ug estradiol-17β in 3 ml of peanut oil per injection. Ambient control and light control mares were not injected.

The end points studied in this first experiment were: (1) the growth of the largest palpable ovarian follicle between day 1 and day 14; (2) interval from day 1 to ovulation; (3) interval from day 1 to first estrus; and (4) interval from day 1 to loss of winter hair coat in tufts.

Experiment 2. This experiment was initiated during the transitional period between the anovulatory season and the ovulatory season (March 18). A total of thirteen mares was assigned to one of three groups: (1) light controls, (2) GnRH, or (3) GnRH plus estradiol-17β . All three groups

were exposed to increased photoperiod as described in the aforementioned protocol. The doses, injection schedules, and vehicles in which the GnRH and Estradiol-17β were injected are the same as in Experiment 1. Light controls were not injected.

The end points studied in Experiment 2 were: (1) the growth of the largest palpable ovarian follicle between day 1 and day 14; (2) interval from day 1 to first ovulation; and (3) peripheral plasma concentrations of FSH and LH.

TABLE 1

EXPERIMENT 1 (JANUARY 7 - JUNE 1, 1977)

Groups	No. of Mares	Dosages[c]
Ambient Controls[a]	4	-
Light Controls[b]	3	-
GnRH[b]	3	200 ug/injection
P_4[b]	4	40 mg/injection
$E_2 17\beta$	4	400 ug/injection

[a]Maintained under ambient conditions.

[b]Exposed to increased photoperiod of 16 hours of light:8 hours of darkness.

[c]Injections were given every eight hours (0800, 1600, 2400) for 14 days.

TABLE 2

EXPERIMENT 2 (MARCH 18 TO JUNE 1, 1977)

Groups	No. of Mares	Dosages
Light Controls[a]	4	-
GnRH[a]	4	200 ug/injection
GnRH + $E_2 17\beta$[a]	5	200 ug/injection + 400 ug/injection

[a]Exposed to increased photoperiod of 16 hours of light:8 hours of dark.

CHAPTER IV

RESULTS

Experiment 1

Results for follicular estrous and ovulation endpoints are shown in Table 3. A significant increase in follicular size occurred only in GnRH-treated mares. By the last day of injection (day 14) all three GnRH-treated mares had ovulatory size follicles. It is interesting to note these follicles did not ovulate but regressed and a subsequent set of follicles developed and ovulated approximately 21 days after the last GnRH injection. Although injections on P_4, $E_2 17\beta$ and GnRH caused an earlier ($P<.05$) occurrence of estrus as compared to ambient and light controls, only GnRH injections significantly advanced the day of ovulation. Ovulation occurred 40 days earlier ($P<.05$) in light controls than in ambient controls.

Experiment 2

Data for follicular and ovulation endpoints are shown in Table 4. GnRH alone or in combination with $E_2 17\beta$-stimulated ($P<.05$) ovarian follicular development. The size of the largest palpable ovarian follicle on the last day of injections was 3-fold larger in GnRH treated and 6-fold larger in GnRH + $E_2 17\beta$ treated mares as compared to

TABLE 3

EXPERIMENT 1

EFFECTS OF INCREASED PHOTOPERIOD, GONADOTROPIN RELEASING HORMONE (GnRH), PROGESTERONE (P_4) OR ESTRADIOL-17β ($E_2$17β) AND IN SEASONALLY-ANOVULATORY PONY MARES

Groups	No. of Mares	Mean Follicular Diameter (mm ± SE)		Interval (days ± SE) from First Injection - to	
		First Injection	Last Injection	Estrus	Ovulation
Ambient Control	4	1.3 ± 0.8	2.5 ± 1.1	32.0[a] ± 3.1	116.7[a] ± 1.6
Light Control	3	0	0	45.6[a] ± 0.6	77.0[b] ± 0.9
GhRH	3	3.3 ± 1.4	33.3* ± 2.0	8.5[b] ± 1.0	34.0[c] ± 3.4
P_4	4	0	0	81.6[c] ± 4.2	82.0[ab] ± 2.0
$E_2$17β	4	0	0	11.7[b] ± 0.6	99.3[ab] ± 4.0

*Greater than diameter at first injection ($P<0.05$).

[a,b,c]Means within a column with different superscripts are different ($P<0.05$).

TABLE 4

EXPERIMENT 2

EFFECTS OF INCREASED PHOTOPERIOD, GONADOTROPIN RELEASING HORMONE (GnRH) GONADOTROPIN RELEASING HORMONE + ESTRADIOL-17β (GnRH + $E_2$17β) IN SEASONALLY-ANOVULATORY PONY MARES

Groups	No. of Mares	Mean Follicular Diameter (mm ± SE)		Interval (days ± SE) from First Injection to Ovulation
		First Injection	Last Injection	
Light Control	4	8.8 ± 1.3	5.0 ± 1.0	45.2 ± 1.8
GnRH	4	7.5 ± 1.1	20.0* ± 2.1	54.3 ± 3.1
GnRH + $E_2$17β	4	2.5 ± 1.1	13.0* ± 0.7	58.0 ± 1.6

*Greater than diameter at first injection ($P<0.05$).

follicle size on the first day of injections. Follicle size in control mares remained unchanged. Although the interval from first injection to ovulation in both GnRH and GnRH + $E_2$17β averaged 56.2 days and was similar to the 34 day interval in GnRH treated mares in Experiment 1, this interval was not reduced ($P>.05$) when compared to light controls (45.2 days). This is due to the period of the year in which Experiment 2 was conducted (March-April), which corresponds with the onset of the physiological breeding season (Ginther, 1979). This is further reflected when one compares the interval to ovulation in light control mares in Experiment 1 (77.0 days) to light control mares in Experiment 2 (45.2 days).

FSH and LH concentrations (ng/ml) are shown in Figures 1 and 2. In general, FSH concentrations increased ($P<.05$) in light controls during the treatment period and decreased ($P<.05$) in GnRH and GnRH + $E_2$17β groups. In controls FSH was elevated ($P<.05$) on days 7, 10, 12, 15, 16 and 17 (day 0 = first day of injection) for GnRH-treated groups. In GnRH injected mares, FSH increased ($P<.05$) on day 2 and decreased to pre-injection concentrations by day 7, remaining unchanged throughout the sampling period. In GnRH + $E_2$17β treated mares, FSH was 3-fold higher at the pre-injection sample in comparison to controls and by day 7 decreased significantly. Concentrations appeared to increase (not different than pre-injection concentration) until day 14 at which time FSH was lower ($P<.04$) than

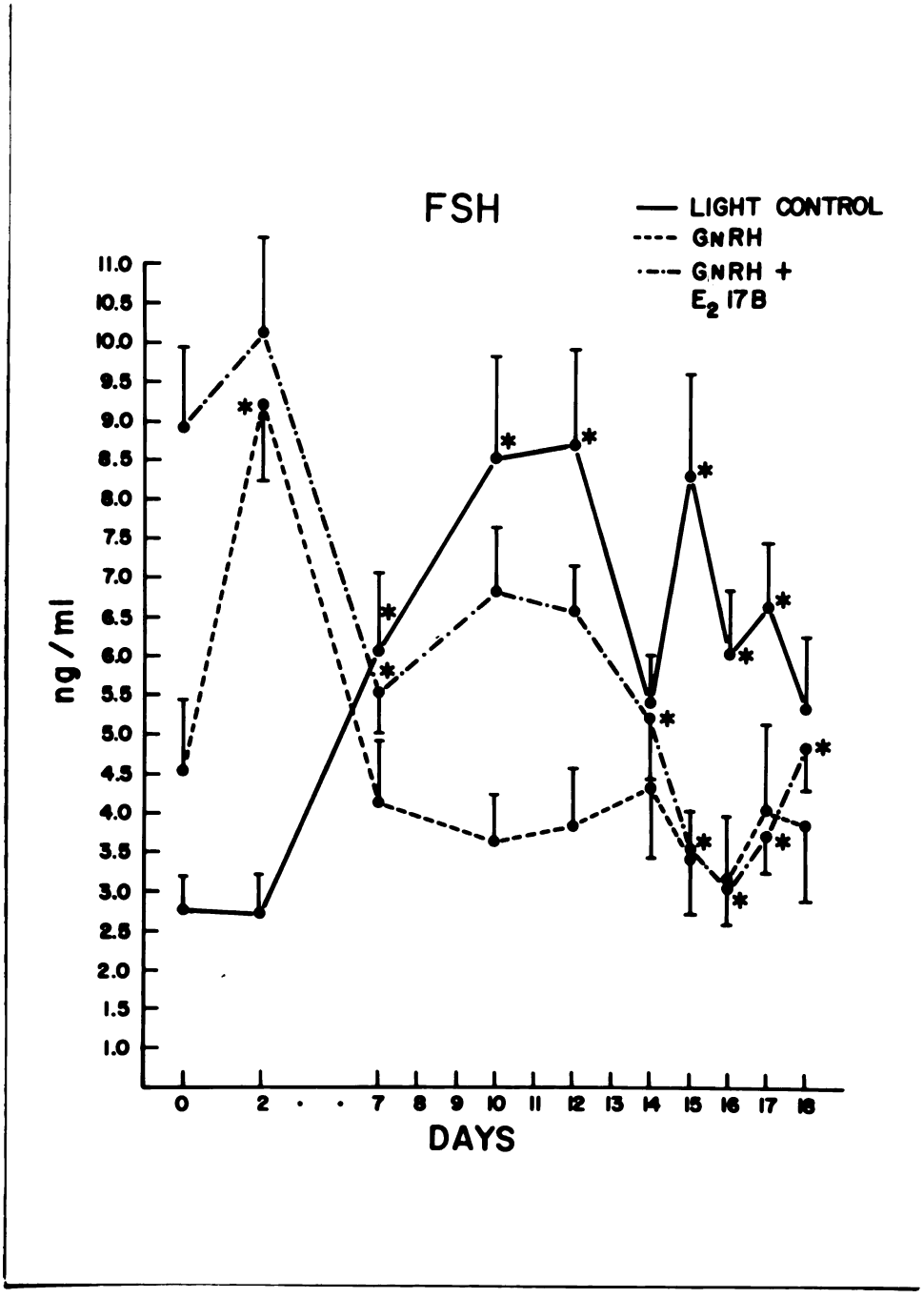

Figure 1. Plasma concentrations (ng/ml) of FSH (Experiment 2). Asterisks denote differences ($P<.05$) from pre-treatment concentrations within a treatment group.

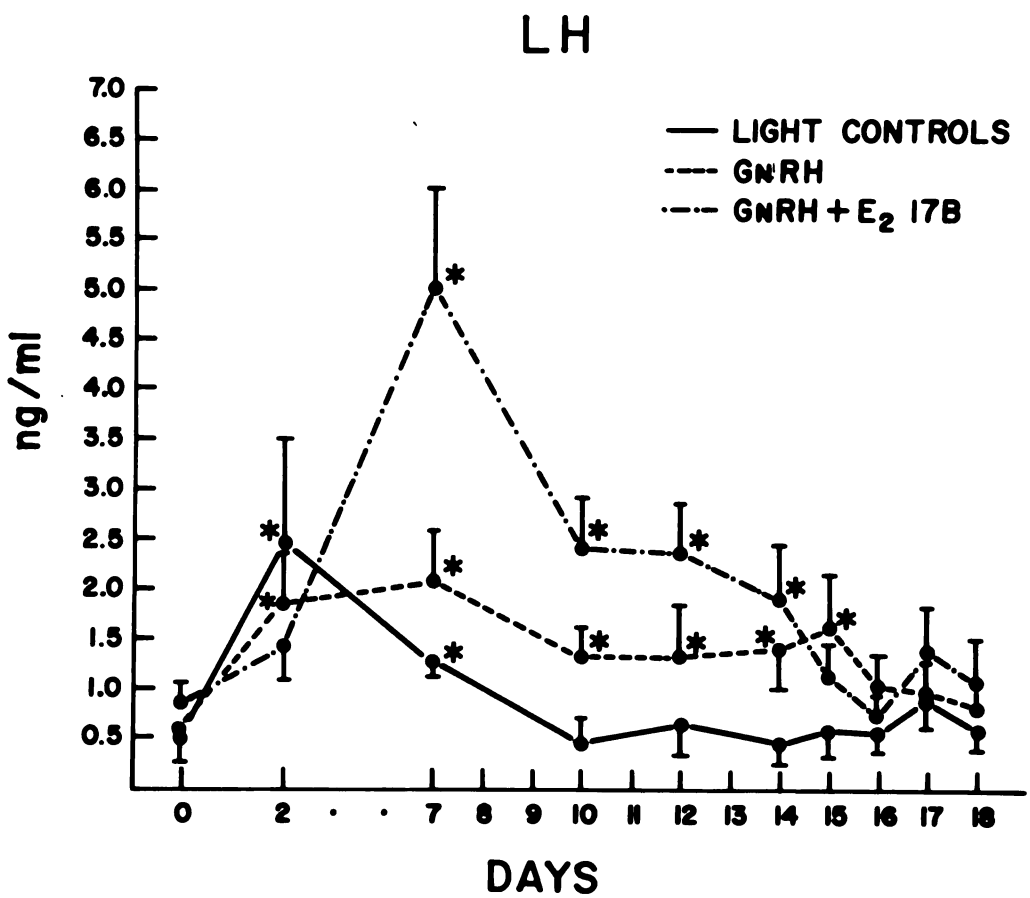

Figure 2. Plasma concentrations (ng/ml) of LH (Experiment 2). Asterisks denote differences ($P<.05$) from pre-treatment concentrations within a treatment group.

pre-injection concentrations and remained depressed ($P<.05$) throughout the sampling period.

A significant increase in LH concentrations occurred in all three groups in the treatment period. In controls LH was elevated on days 2 and 7 only. In GnRH injected mares, LH was elevated ($P<.05$) from days 2 through 15. The first day on which LH concentrations were significantly greater than pre-injection concentrations was day 7 in GnRH + $E_2$17β-treated mares. This was the highest increase seen in any of the groups and was fivefold greater than pre-injection concentrations. This increase contrasts with approximately a twofold increase in controls and GnRH-treated mares. In GnRH + $E_2$17β mares LH was also greater on days 10 through 14 but dropped to pre-injection concentrations on day 15 through 18.

CHAPTER V

DISCUSSION

The shortening of the anovulatory period by exposing seasonally-anovulatory mares to 16 hours of photoperiod (light controls) in Experiment 1 confirms numerous reports of this nature, the first of which was published in 1946 (Burkhardt). Furthermore, the interval from onset of lights to ovulation in Experiment 1 (approximately 80 days) was similar to that reported by Kooistra and Ginther (1975).

Daily injections of P_4 or $E_2 17\beta$ failed to advance ovulation in seasonally-anovulatory mares in the present study. Garcia and Ginther (1978) gave estradiol to ovariectomized mares during the anovulatory season and increased LH plasma concentrations whereas P_4 and a combination of P_4 and $E_2 17\beta$ had no effect. Apparently other studies with exogenous estradiol have not been reported. Other studies utilizing exogenous progesterone have been reported and the data indicate that exogenous progesterone is effective in advancing ovulation in anovulatory mares only when given during the transition from the anovulatory to the ovulatory season (Ginther, 1979).

The increase in follicular development seen in both Experiments 1 and 2 when GnRH alone or in combination with

$E_2$17β was injected for 14 days is supported by the findings of Evans and Irvine (1976 and 1977). These workers studied non-lighted anovulatory mares and used a GnRH injection regimen designed to duplicate the changes in FSH and LH which occur during the estrous cycle. Progesterone was also given to mimic diestrous concentrations. The addition of progesterone to the GnRH regimen seemed to improve follicular response to GnRH since follicles developed in mares not given P_4 but the follicles did not attain ovulatory size. Three of four mares given GnRH + P_4 ovulated but a corpus luteum did not develop. In Experiment 1 in the present study, ovulation occurred in two of three mares an average of 12.5 days after the first GnRH injection. However, ovulation did not occur in the third mare until day 75.

The pattern of FSH secretion in control mares in Experiment 2 was similar to that previously reported in seasonally-anovulatory mares. FSH concentrations seemed to be depressed in GnRH and GnRH + $E_2$17β-treated mares during the treatment period when the most rapid follicular growth was occurring. This is likely due to increased secretion of inhibin from growing ovarian follicles (Miller and Ginther, 1978).

The average LH plasma concentration seemed greatest in GnRH + $E_2$17β-treated mares. This would be expected since other reports have demonstrated estradiol enhances the stimulatory effects of GnRH on LH release (Vivrette and

Irvine, 1978). In general, LH concentrations were significantly elevated in both GnRH-treated groups throughout the treatment period. The reason a significant rise in LH occurred in control mares is unclear at present.

This study demonstrated that injections of GnRH at 8 hour intervals for 14 days stimulated ovarian follicular development in seasonally-anovulatory pony mares maintained under 16 hours of light:8 hours of darkness. Furthermore, these data offer promise of more efficient management of the broodmare when longer acting GnRH analogues or GnRH implants become available.

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