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Title: The Effects of Morphine and Naloxone on Serum Levels of Luteinizing Hormone in Intact and Ovariectomized Pony Mares during the Breeding and Nonbreeding Seasons

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D. W. Holtan

Intact and ovariectomized (OVX) pony mares were randomly assigned to treatments of morphine (M), naloxone (N), or a saline control (C) to determine if endogenous opioid peptides (EOP) play a role in equine seasonal breeding. Morphine was expected to decrease luteinizing hormone (LH), whereas N (an opiate antagonist) should increase it. These opiate analogs were given to normally cycling mares on the second day of estrus, and to winter anestrous mares having no follicular activity (follicles less than 5 mm diameter). All treatment groups (n=3) received a single injection (1 mg/kg body weight, iv) on zero hour, except the winter N treatments which had three hourly doses (1 mg/kg body weight, iv). Serial blood samples taken on treatment day showed no difference ($P>.05$) in LH patterns in the winter (M, 1.4±.1; N, 1.7±.1; C, 1.7±.1 ng/ml; $\bar{x}$±SE). In the summer, LH patterns (M, 9.8±.2; N, 20.1±.9; C, 16.5±.5) were different ($P<.01$) between M and N treatments, but not when compared to controls. However, this difference seemed to be due to the 24 h

sample of one N treated pony, which had high LH levels. Days in estrus were unchanged in all cycling ponies (M, 7.0±0.0; N, 8.3±2.3; C, 7.3±.9 d). Three ponies that received daily injections of M throughout estrus also had similar lengths of estrus (9.0±1.0). Two OVX ponies were treated three times each on a switchback type of experiment in the summer and the winter. Again, there was no difference in LH except that due to season (summer, 33.6±1.0; winter, 1.4±.1 ng/ml; $P<.05$). These opiate analogs at this treatment dose indicated that EOP may not play a major role in equine reproduction as related to LH.

The Effects of Morphine and Naloxone on Serum Levels of Luteinizing Hormone in Intact and Ovariectomized Pony Mares during the Breeding and Nonbreeding Seasons

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Jane F. Dietz

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Head of Department of Animal Science

Redacted for Privacy

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CHAPTER 1

INTRODUCTION

The artificial control of the mare's breeding season (a seasonal breeder) is desired by many people who breed and(or) show horses. There are a number of reasons to try to control the mare's reproductive cycle at different times of the year. The major reason is the universal birthdate of January first imposed on most horse breeds. This standard birthday makes most horsebreeders desire their foals to be born early in the year. Due to the eleven month gestation of mares, these early foals can be difficult to obtain since the physiological breeding season begins in April and May (Ginther, 1974; Wesson and Ginther, 1981). The natural breeding season of the mare must be advanced to approximately February fifteenth to get January foals. Before this goal can be practically achieved, more information on the hormonal control of equine seasonal breeding is necessary. An examination of the seasonal events in horses, and a comparison with seasonal rodents and nonseasonal animals may help elucidate the endocrine events of the mare.

The mare is seasonally polyestrous, having a period of ovarian quiescence in the fall and winter and a period of ovarian activity in the spring and summer (Ginther, 1979). Turner et al. (1979) found that the size and number of follicles, and the concentration

of luteinizing hormone (LH) were lowest in the winter anovulatory season. In a survey of ponies at slaughter, Wesson and Ginther (1981) determined that the percentage of ponies ovulating was lowest (10 to 16%) in January through April. It increased to one hundred percent throughout the months of June to August. The ovaries become active in the spring (Turner et al., 1979) producing steroids used in the feedback systems in the ovulatory season (Freedman et al., 1979) and usually releasing mature eggs approximately every 21 d (Hughes et al., 1975; Ginther, 1979). The yearly pattern of the ovulatory hormone LH best reflects the changes occurring in the mare's reproductive status. At the onset of the anestrous or non breeding season, Snyder et al. (1979) concluded from a pony study that the lack of an adequate LH surge causes the failure of ovulation. Then LH remains low for the winter at a basal level of about 1 to 2 ng/ml serum (Garcia and Ginther, 1976).

In the winter, the ovaries have no influence on serum LH concentrations (Freedman et al., 1979; Garcia et al., 1979). However, in the ovulatory or breeding season, Freedman et al. (1979) noted that the ovarian steroids begin to exert positive or negative feedback effects on LH secretion, depending on the stage of the estrous cycle. In diestrus, estrogens have a negative effect on LH release, whereas during estrus they have a positive effect. Before and during estrus, LH slowly increases in an episodic or pulsatile manner (Evans et al., 1979). The response of the ovary is to produce more estrogens which bring the mare into standing heat or estrus. These high serum levels of estrogen are also

responsible for the LH surge that causes ovulation. Stabenfeldt et al. (1975) noted that most other animals have a preovulatory LH surge 12 to 24 h before ovulation. However, the mare is unusual in that her surge takes 2 to 4 d to increase, reaching a maximum up to 48 h after ovulation. In a two year study of the estrous cycle, Hughes et al. (1975) found that LH peaks on the average every 20.6 d with a range of 13 to 34 d. This peak usually indicates that the mare has ovulated recently. They also observed that the average length of estrus is 5.7 d with a range of 1 to 24 d. Peak levels of LH vary depending on the individual mare. Luteinizing hormone profiles seem to be the same in both ponies and light riding horses, with LH the highest at or after ovulation (Miller et al., 1980).

Photoperiod can be directly linked to the increases and decreases in serum LH seen in the spring and fall, respectively. Experiments using ovariectomized (OVX) mares and(or) those employing artificial lighting (in intact and OVX mares) best reflect the role of photoperiod in the mare. The OVX mare has no ovarian steroids to complicate the effects of long and short hours of light. However, both Freedman et al. (1979) and Garcia and Ginther (1976) have shown that LH fluctuates from low, constant amounts in the winter, to high, constant amounts in the summer. There seems to be both a central nervous system (CNS) and a pituitary component responsible for these basal circannual LH rhythyms, which is entrained to photoperiod, and independant of the ovaries (Garcia et al., 1979). Intact mares show the same seasonal fluctuations in LH. However,

these changes are complicated by ovarian involvement. As mentioned above, the ovarian steroids influence the 21 d LH cycle of the mare that occurs in the breeding season. The use of artificial lighting also shows the involvement of photoperiod in mare seasonality. Anestrous (nonbreeding) mares respond to 16 h of light per day by resuming the estrous cycle 2 to 3 mo earlier in the year (Sharp and Ginther, 1975; Freedman et al., 1979). Conversely, Kooistra and Ginther (1975) housed cycling mares in 9 h of light per day and they reverted to the anestrous state. Luteinizing hormone levels change in OVX mares when they are exposed to different lighting regimens. Long hours of light (16 h light) in the winter cause an increase in LH levels, comparable to those found in the summer. Nine hours of light in the summer reduces LH back down to winter concentrations. However, lighting as a common method of inducing an early breeding season can become costly without adequate facilities, and therefore impractical to the average horseperson.

In seasonal breeders such as the horse, mink, and golden hamster, the hormonal factor(s) suppressing LH or it's control, luteinizing hormone releasing hormone (LHRH), each winter is(are) unknown. Ginther (1979) states that the mare may have an increase in some type of "antigonadal factor" in the winter that inhibits LH secretion and therefore, the estrous cycle. This "factor" is thought to decrease in the spring as the photoperiod increases. But, no such compound has yet been identified. The decrease in photoperiod is perceived by the eye which relays the information to

the pineal gland via the superior cervical ganglia. The removal of these ganglia (ganglionectomy) is the physiological equivalent to a pinealectomy (PIN-X), both causing the delay or suppression of anestrous in the hamster (Rusak, 1980). Sharp et al. (1979) ganglionectomized mares during the anestrous season, and found no effect the first year. The onset of the breeding season (first ovulation) was delayed the second spring after surgery. The same result occurs with ponies PIN-X in the winter, but not the summer months (Grubaugh et al., 1982). Thus, the mare responds slowly, but must receive the changes in photoperiod at critical seasons if she is to cycle normally. Mink also need to perceive an increase in hours of light per day before their reproductive season can begin (Travis and Pilbeam, 1980). Experiments using golden hamsters have implicated the pineal gland as a modifier of the reproductive cycle. Pinealectomy (Rusak, 1980) prevents the gonadal regression caused by short photoperiod in the male hamster.

Since short photoperiod inhibits the gonads, and PIN-X blocks this inhibition, there must be some compound in the pineal responsible for these changes. The pineal product believed to be involved with seasonal breeding is the indole, melatonin (MEL), which acts in an inhibitory manner on the gonads (Bittman and Zucker, 1981). Pinealectomized hamsters given MEL will revert to the nonbreeding state (Rusak, 1980). Rollag et al. (1980) gave MEL (1.6 to 12.8 ug) to cycling female hamsters. This treatment caused fifty percent of those hamsters to become acyclic. Melatonin inhibits the gonads, LH, follicle stimulating hormone (FSH), and prolactin in PIN-X as

well as intact male and female hamsters (Rusak, 1980), but not in animals which had lesions in the suprachiasmatic nucleus (SCN). This suggests that MEL cannot regress the gonads unless an area in the hypothalamus (the SCN) carrying LHRH containing neurons is intact. It is unknown whether MEL acts directly on the LHRH containing neuron, or through its neurotransmitter, norepinephrine (NE). However, it was found that in short photoperiod or acyclic hamsters NE turnover is low at a time when LH and FSH activity is suppressed (Steger et al., 1982). Norepinephrine (and LH, FSH, dopamine, and prolactin) must increase before testicular recrudescence occurs in the hamster.

The physiological control of the reproductive cycle in non-seasonal animals such as the rat, mouse, nonhuman primate, and woman (Ropert et al., 1981) has been elucidated as involving CNS and(or) hypothalamic neurotransmitters, and possibly endogenous opioid peptides (EOP, for a review, see Barraclough and Wise, 1982). Experimental administration of beta endorphin (END, an endogenous opiate) and opiate analogs affect LH patterns in these animals. Opiate agonists and antagonists act competitively, one inhibiting the action of the other. This competition suggests that opiates act at a stereospecific (only the levorotatory isomers have biological activity) receptor site (Snyder, 1977), which Pasternak (1980) has localized on certain nerve terminals. These receptors have been found in the hypothalamus which implies a physiological role for EOP at such sites (Drouva et al., 1980). Bloom (1981) used fluorescent antibodies to enkephalin (an endogenous opiate) and

found that the EOP are located in nerve cells at the base of the hypothalamus and in anterior pituitary cells. The END containing cells in the hypothalamus are concentrated in the arcuate nucleus (Bloom, 1981) and other specific sites known to contain LHRH (Barraclough and Wise, 1982).

Morphine (Bruni et al., 1977; Ieiri et al., 1980a; Van Vugt and Meites, 1980), beta endorphin (Kinoshita et al., 1980), and other opiate agonists (Meltzer et al., 1978; Stubbs et al., 1978) decrease serum LH in the rat within 15 to 30 min after injection by several different routes. This decrease in LH can be extensive enough to block ovulation in rats (Barraclough and Sawyer, 1955) and also in women who are morphine addicts (Barraclough and Wise, 1982). Ieiri et al. (1980a) suggest that endogenous opiates may affect the regulation of the proestrus LH surge in the rat. The opiate antagonists (naloxone, naltrexone, etc.) can block the opiate induced LH decrease in female rats (Ieiri et al., 1980a) or in both sexes when injected concurrently with or after the morphine injection (Grandison et al., 1980; Kinoshita et al., 1980). Alone, these antagonists lead to an increase in serum LH in prepubertal (Blank et al., 1979) and mature rats (Bruni et al., 1977; Van Vugt and Meites, 1980), and in the woman (Quigley and Yen, 1980). Experiments using rat pituitary cell cultures treated with opiates have shown no direct effect on LH secretion (Grandison et al., 1980). Thus the hypothalamus may be the site of action of opiates. Cicero et al. (1979) and Cicero (1980) showed that EOP are involved with testosterone negative feedback on the hypothalamus and LH

release in male rats. Because EOP act on the hypothalamus, they must either act directly on neurons containing LHRH and its synthesis or indirectly through the neurotransmitters that control LHRH release.

The hypothalamic cells containing LHRH release their product after neural stimulation (Norris, 1980). The neurotransmitters that may be affecting this release are either dopamine (Rotsztejn et al., 1978; Takahara et al., 1978; Delitala et al., 1981), norepinephrine (Kalra and Simpkins, 1981; Kalra, 1981), serotonin (Ieiri et al., 1980b), or a combination of these (Lohse and Wuttke, 1981; Barraclough and Wise, 1982). Experiments involving one or the other neurotransmitters have been inconclusive as all three can respond to the same stimuli, especially since dopamine and NE are in the same synthetic pathway. So far, none of the neurotransmitters have been completely ruled out as the LHRH releasing agent, but in a review of the subject (Barraclough and Wise, 1982), NE is generally accepted as the in vivo neurotransmitter responsible for LHRH release. Norepinephrine containing fibers stretch from the higher brains centers to the hypothalamus, where they are in close proximity to the LHRH containing cells. The release of NE from it's nerve ending, and the subsequent binding to alpha adrenergic receptors on the LHRH containing neuron may cause the secretion of LHRH. The LHRH then travels through the hypothalamic-hypophyseal blood portal system to the anterior pituitary where it releases LH from the gonadotroph cells (Norris, 1980).

As mentioned above, opiates cause the suppression of LH

secretion. It is believed that this process occurs in the hypothalamus by the suppression of NE release (turnover). Frederickson and Norris (1976) traced END containing neurons and found that they follow the same neural tracts as NE containing fibers from the brain to the hypothalamus. Although receptors for END have been located on neuronal membranes, these specific neurons have not yet been identified. Therefore, it is unknown whether they reside on neurons containing NE. Endogenous opioid peptides must bind to their membrane bound receptor to work (Frederickson and Norris, 1976; Snyder, 1977), indicating that the NE containing neuron carries them. In other words, END may be viewed as an inhibitory neurotransmitter. For example, it inhibits neuronal firing in systems such as the guinea pig ilium (Williams and North, 1979). Endorphin may act via a receptor to inhibit the release of NE to the LHRH containing neuron. The end result is a decrease in serum LH. One example of a physiological reason for this inhibition is involved with the negative feedback systems of steroids on the hypothalamus (and anterior pituitary) to keep LH at a low, basal level. Studies using fluorescent markers on estradiol (and testosterone in males) show that these steroids congregate around the neurotransmitters mentioned above (Barraclough and Wise, 1982). It is thought that the steroids inhibit neurotransmission. It is currently thought that EOP are involved with this feedback system of testosterone in male rats (Cicero et al., 1979; Cicero, 1980) and also estrogen's positive or negative feedback roles in females rats (Sylvester et al., 1982; Van Vugt et al., 1982). The steroids

may cause an END release which would inhibit the firing of the nerves containing NE. Without NE stimulation, LHRH is not released, and consequently no LH is released into the bloodstream. Ferin et al. (1982) showed with rhesus monkeys that opiates affect pulsatile LH release (frequency and amplitude of peaks), but have no control on estradiol's positive feedback as in the rat.

As noted above, EOP seem to play a role in the control of LHRH by NE in nonseasonal breeding animals. In the seasonal breeder, such as the golden hamster, Wilkinson et al. (1983) measured the opiate, beta adrenergic, and benzodiazepine (measure of gamma amino butyric acid) binding sites under the influence of long and short photoperiod. Opiate, but not beta adrenergic or benzodiazepine, binding sites increased in the whole brain during short as compared to long photoperiod. No receptor type seemed to change in the hypothalamus. Specific hypothalamic nuclei may have to be examined before a change is noted. Norepinephrine beta adrenergic receptors did not change in the hamster due to season. It is thought that NE binds to alpha, not beta, receptors on the LHRH containing neuron in male and female rats (Barraclough and Wide, 1982). If opiates are involved with seasonal breeding, the high levels in the winter in the hamster may exert a suppressive effect on the gonads. If they are involved in equine seasonality, they may be the "antigonadal factor" alluded to by Ginther (1979) that inhibits LHRH and(or) LH release. With this theory in mind, opiate analogs may also affect the mare's reproductive status. Naloxone or other opiate antagonists may compete with EOP and therefore allow increased

LH secretion in the winter, perhaps to the point that a mare may cycle earlier in the year. This advancement of the breeding season may then allow horse breeders to get their foals early in the year (January and February). Opiate agonists may also be of aid in the control of the mare's estrous cycle. By depressing LH, morphine may suppress or even block ovulation, or it may reduce the outward signs of behavioral estrus. On the other hand, naloxone may be a means of shortening estrus. This antagonist might allow early, controllable ovulations in the mare by allowing a premature release of LH. Therefore, the following experiments used the opiate analogs morphine and naloxone to try to manipulate the estrous and anestrous periods of the mare.

Running Head: Morphine and Naloxone in Pony Seasonality

CHAPTER 2

THE EFFECTS OF MORPHINE AND NALOXONE ON SERUM LEVELS OF LUTEINIZING HORMONE IN INTACT AND OVARIECTOMIZED PONY MARES DURING THE BREEDING AND NONBREEDING SEASONS[1]

Jane F. Dietz and D.W. Holtan[2]
Department of Animal Science
Oregon State University, Corvallis 97331

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[2]Reprint requests from D.W. Holtan, Department of Animal Science, Oregon State University, Corvallis 97331.

Summary

Intact and ovariectomized (OVX) pony mares were randomly assigned to treatments of morphine (M), naloxone (N), or a saline control (C) to determine if endogenous opioid peptides (EOP) play a role in equine seasonal breeding. Morphine was expected to decrease luteinizing hormone (LH), whereas N (an opiate antagonist) should increase it. These opiate analogs were given to normally cycling mares on the second day of estrus, and to winter anestrous mares having no follicular activity (follicles less than 5 mm diameter). All treatment groups (n=3) received a single injection (1 mg/kg body weight, iv) on zero hour, except the winter N treatments which had three hourly doses (1 mg/kg body weight, iv). Serial blood samples taken on treatment day showed no difference ($P>.05$) in LH patterns in the winter (M, 1.4±.1; N, 1.7±.1; C, 1.7±.1 ng/ml; $\bar{x}$±SE). In the summer, LH patterns (M, 9.8±.2; N, 20.1±.9; C, 16.5±.5) were different ($P<.01$) between M and N treatments, but not when compared to controls. However, this difference seemed to be due to the 24 h sample of one N treated pony, which had high LH levels. Days in estrus were unchanged in all cycling ponies (M, 7.0±0.0; N, 8.3±2.3; C, 7.3±.9 d). Three ponies that received daily injections of M throughout estrus also had similar lengths of estrus (9.0±1.0; $P>.05$). Two OVX ponies were treated three times each on a switchback type of experiment in the summer and the winter. Again, there was no difference in LH except that due to season (summer, 33.6±1.0; winter, 1.4±.1 ng/ml; $P<.05$). These opiate analogs at this treatment dose indicated

that EOP may not play a major role in equine reproduction as related to LH.

(Key Words: Morphine, Naloxone, Equine, Seasonal Breeders, Luteinizing Hormone.)

Introduction

The onset of the physiological breeding season of the mare (a seasonally polyestrous animal) begins in April and May (Ginther, 1974; Wesson and Ginther, 1981). Long photoperiod stimulates ovarian activity and luteinizing hormone (LH) secretion. A surge in LH lasting 2 to 4 d causes ovulation. These surges (and ovulations) occur in a cyclic pattern, every 20.6 d, throughout the summer months (Hughes et al., 1975). Snyder et al. (1979) concluded that in the fall the lack of an adequate LH surge suppresses ovulation at the onset of the anovulatory season. Photoperiod seems to be involved with the failure of this LH pulse, and may be responsible for LH remaining low (approximately 1 to 2 ng/ml serum) the rest of the winter (Garcia and Ginther, 1976).

In other seasonal breeders such as the mink (Travis and Pilbeam, 1980) and the hamster (Bittman and Zucker, 1981; Steger et al., 1982) short photoperiod (less than 12.5 h of light per day) causes gonadal regression and a suppression of LH secretion. Widmaier and Campbell (1981) found that the winter photoperiod is also responsible for the loss of circulating prolactin in female anestrous hamsters. Prolactin increases the number of LH receptors, plus it enhances LH binding to these receptors. The hypothesized antigonadal factor in the brain of seasonal animals that responds to decreasing photo-

period by lowering LH levels is not well understood. In hamsters, the pineal indole melatonin (MEL) decreases sexual function (Bittman and Zucker, 1981), similar to a short daily photoperiod. Melatonin acts on the hypothalamus (Rusak, 1980), at a site innervated by neurons containing luteinizing hormone releasing hormone (LHRH) as in the suprachiasmatic nucleus (SCN). It is unknown whether MEL exerts a direct or indirect inhibitory effect on these neurons.

In nonseasonal animals, the control of the LHRH containing neuron has been studied extensively (for a review, see Barraclough and Wise, 1982). The catecholamine norepinephrine (NE) may be responsible for LHRH stimulation (Clifton and Sawyer, 1980; Kalra and Simplins, 1981; Kalra, 1981; Barraclough and Wise, 1982). Other neurotransmitters may also be involved. Opiates decrease LH secretion at the level of the hypothalamus (Cicero, 1980; Van Vugt and Meites, 1980). This decrease is blocked by clonidine, a NE agonist (Kalra, 1981) which suggests the endogenous opioid peptides (EOP) may play a role in NE-LHRH inhibition. Opiate binding sites increase in the whole brain of hamsters under short photoperiod (Wilkinson et al., 1983), suggesting opiate involvement in seasonally breeding animals. With these concepts in mind, the following experiments used morphine (M, an opiate agonist) to mimic EOP suppression of LH. Naloxone (N, an opiate antagonist) was expected to block EOP receptor binding and therefore, increase LH. These two opiate analogs were used to study, and perhaps control, the physiological events involved with equine seasonal breeding.

Materials and Methods

Animals: Pony mares of the Shetland type were housed in small paddocks and were fed grass hay and oats throughout the year. Ponies were teased as described by Ginther (1974), with a vigorous pony stallion for behavioral estrus at least every two days. This schedule increased to daily teasing when the mares were in estrus, or during treatment periods. They were palpated per rectum to check ovarian activity immediately after the teasing was completed. Ponies assigned to winter anestrous treatments (February, 1982) displayed no signs of behavioral estrus and had no follicle larger than 5 mm diameter for at least one week pretreatment. During the summer breeding season (1981, 1982) ponies must have had at least one complete estrous cycle prior to treatment. Two mares in anestrus were ovariectomized (OVX) three months before the winter treatments began. They were used both in the winter and summer months as described below.

Treatments: Morphine[3], naloxone[4], or a .9% saline control (C) were used at a dose of 1 mg/kg body weight, iv, for all injections. Three ponies per treatment group were randomly chosen, and treatments began at 0900 h for each experiment, all ponies receiving one dose. Exceptions are all winter N treatments (intact and OVX mares), in which the mares were given three injections (0900, 1000, 1100 h)

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[3]Morphine sulphate, Eli Lilly, Indianapolis, Indiana.

[4]Naloxone hydrochloride dissolved in .9% saline, generously donated by Endo Laboratories, Garden City, New York.

during one treatment day. All ponies used in the breeding season portion of these experiments were treated on the second day of estrus, including three mares that received daily doses of M until they ovulated. The two OVX mares were used both winter and summer (1982) on a switchback type of experiment. They were treated three times, 7 d apart; each week the treatment was switched. In the winter, one pony received C the first week, N the second, and C again the third week. The second pony had reciprocal treatments (i.e., NCN). The same schedule was used in the summer, except M was substituted for N.

Blood Collection: On treatment day, blood samples were collected every 15 min for 3 h, then at 4 and 8 h (figure 1). The cycling mares were bled every 15 min for the first hour only, with an additional blood sample withdrawn at 12 h (figure 2). At least three pretreatment samples were collected on treatment day. Daily blood samples were obtained at 0900 h throughout estrus. Sampling continued for 2 d after ovulation. Anestrous and OVX mares were bled daily (0900 h) one week before and after treatment day. Ponies were bled by venipuncture, blood was clotted, centrifuged, and serum was decanted and frozen until assayed for LH by radioimmunoassay (RIA).

Radioimmunoassay: Luteinizing hormone was analysed by RIA as described by Niswender et al. (1969) and adapted for the equine by Nett et al. (1975). In our laboratory, first antibody (N-15 anti-ovine LH, supplied by G.D. Niswender) was used at a final dilution of 1/100,000. Goat anti-rabbit gamma globulin (supplied by J.J. Reeves) was used at a 1/125 final dilution for the second antibody.

Ten micrograms of ovine LH (NIH-LH-S18) were iodinated as needed for radioligand (25,000 cpm/tube). Purified (E98A) equine LH (Licht et al., 1979) was used to generate a standard curve (0 to 10 ng/tube) which was run in each assay in triplicate. Sensitivity of the assay was .05 ng/tube. Inter- and intraassay coefficients of variation were 14.1 and 5.8%, respectively. Recovery of purified hormone added to serum was linear over the range of 1.0 to 50.0 ng, and was parallel to the standard curve from 91.8 to 15.6% inhibition. There were no serum effects as 10, 50, 100, and 200 µl aliquots had equivalent values.

Statistics: Split-plot analysis of variance of treatment over time was used to analyse serum LH. The cycling mares were examined only until the 24 h posttreatment sample to avoid complication due to different lengths of estrus in mares. Length of estrus was analysed separately by one-way analysis of variance (Steel and Torrie, 1960).

Results and Discussion

Behavioral Effects of Opiate Analogs: In the anovulatory season, the initial protocol was to give three hourly injections of M and N. However, the one pony that received all three M doses had very erratic behavior from the adverse effects of morphine. Hay was picked up as if to be eaten, but the chewing reflex was absent. Locomotor activity was continuous, even when the mare was restrained; this was accompanied by slight muscle tremors. Immediately after the first injection, the eyes dialated. Throughout the entire

time period, this mare never urinated or defecated. Both pulse and respiration increased after each injection and reached a maximum of 128 per minute after the third injection. The mare's temperature increased slowly at first, rising considerably (from 99.6 pre-treatment to 104.4°F) after the last injection. This increase was accompanied by a lack of perspiration. These affects are in contrast to most other animal species which respond in an opposite manner. They usually show depressed vital signs (such as pulse, respiration, and temperature), constricted eyes, and reduced locomotor activity.

Three hours after the third M injection, N was administered as an antidote (.44 mg/kg body weight) to counteract the general hyperexcitability of this mare. The N injection caused the mare to immediately stop picking up hay. Hay already present in the mouth was chewed. Reduced locomotor activity was apparent within a few minutes, and the eye dialation disappeared by 10 min post N injection. Pulse and respiration decreased dramatically, dropping below 100 per minute after 30 and 45 min, respectively. The mare started sweating profusely, concurrent with the drop in temperature. Temperature returned to normal (101.2°F) one hour after N was injected.

The ponies which received one M dose displayed mild behavioral changes within 15 min posttreatment. These signs included an increase in locomotor activity and elevated pulse, respiration, and temperature. None of these effects were long lasting; nor were they as extreme as that seen with three injections. Naloxone

also changed mare behavior. Some had a temporary appetite loss, as do rodents (Brown and Holtzman, 1979) and sheep (Baile et al., 1980). Other mares had a short term, mild diarrhea.

Anestrous Mares: There was no difference ($P>.05$) in LH secretion due to treatment (M, 1.4±.1; N, 1.7±.1; C, 1.7±.1 ng/ml; $\bar{x}$±SE). Figure 1 shows serum LH levels of the anestrous mares; each line represents the average of three ponies per treatment group. As is clearly shown, N did not cause LH to surge in the mare within 15 to 30 min after administration as occurs in the rat (Bruni et al., 1977; Blank et al., 1979) or the woman (Quigley and Yen, 1980; Ropert et al., 1981). The mares received a dose of N comparable to that used in the rat on a per weight basis. Meites et al. (1979) used .2, 2.0, or 5.0 mg/kg body weight, whereas both Cicero et al. (1979) and Kinoshita et al. (1980) used 1.0 mg/kg. Monkeys react to a lower dose (Gold et al., 1979). Since ponies are large animals and the N was in a limited quantity, doses higher than 1.0 mg/kg were not feasible. Higher systemic levels of N or a different route of administration (such as intraventricular injection or subcutaneous implants) may have induced LH to increase.

As previously stated, LH is at a low, basal level in the winter. This reduced LH secretion may help explain why M had no effect on the anestrous mares; it was already suppressed by some type of endogenous factor. Even the pony that received three M doses had no change in LH patterns (data not shown). The amount of M used in these mares was lower than doses used in the rat. Ieiri et al. (1980a) used 2.0 or 5.0 mg/kg, whereas Meites et al. (1979)

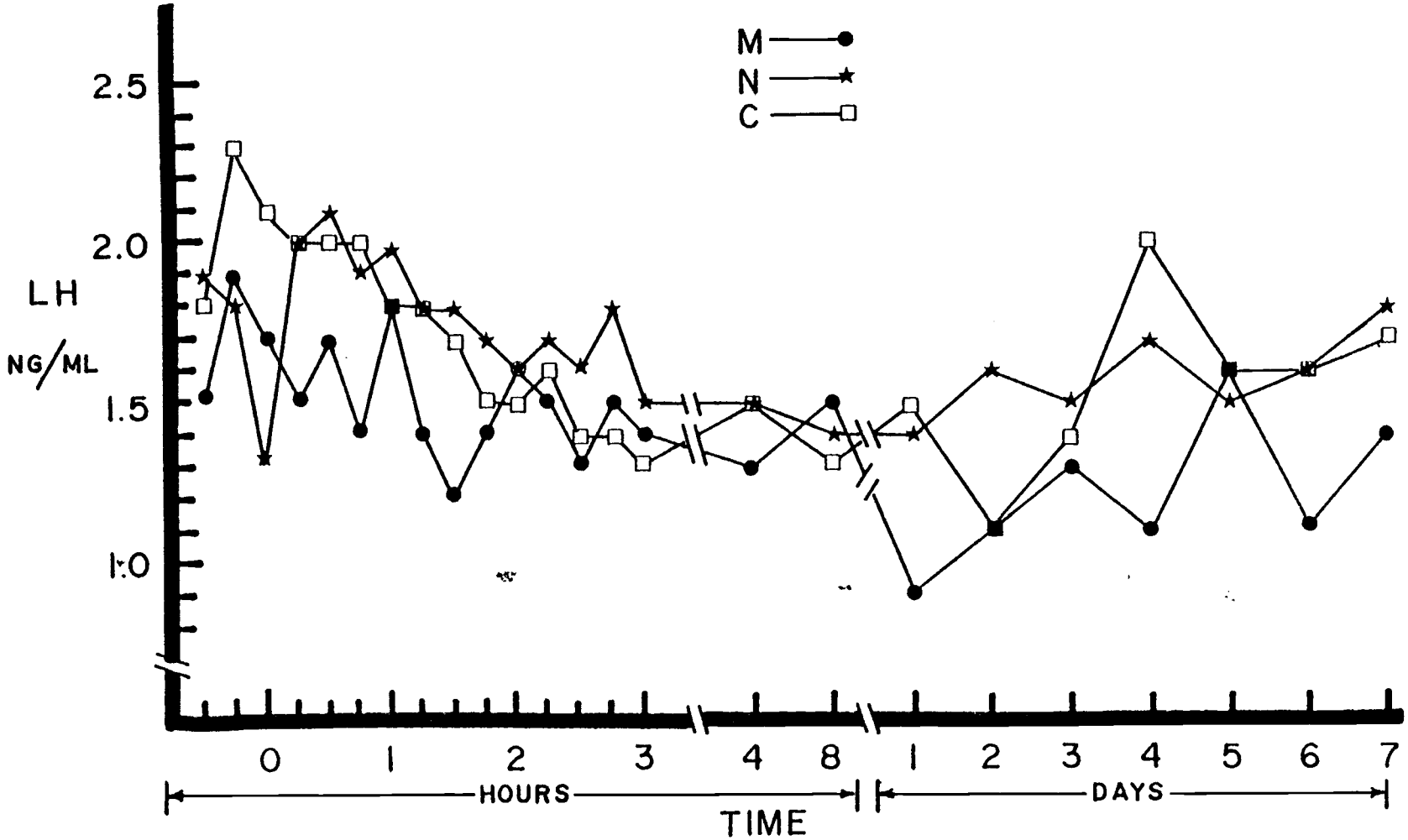

Figure 1: The effects of morphine (M), naloxone (N), or saline (C) on serum LH in intact anestrous mares. Pooled standard error of the mean equals .97. Treatment at zero hour.

used 2.0 or 10.0 mg/kg. Rats are catatonic at the 10 mg/kg dose. As stated previously, ponies cannot tolerate high systemic doses.

All anestrous ponies but one had some degree of ovarian enlargement. However, there were no follicles larger than 5 mm in diameter before or after treatment, except one had a follicle grow to 15 mm. Most mares showed no true signs of behavioral estrus, although some unseasonable estrus was noted. These signs were sporatic, weak, and irregular, a common occurrence during the nonbreeding season (Ginther, 1974).

Estrous Mares: There was no difference ($P>.05$) in LH profiles due to M or N treatments when compared to C. Mean concentration on treatment day were as follows: M, 9.8±.1; N, 20.1±.9, C, 16.5±.5 ng/ml; $\bar{x}$±SE. However, LH was different ($P<.01$) when M was compared to N (figure 2). This difference seems to be due to one pony in the N treatment group and her 24 h blood sample. This pony ovulated on the fourth day of estrus, or one day after the 24 h sample was withdrawn. The high concentration of LH at this time may be due to the LH surge that accompanies ovulation. The M treated ponies also started treatment with lower LH concentrations. Luteinizing hormone was expected to increase within 15 to 30 min post injection of N, as in other animals. Regression analysis of treatment day showed no changes ($P>.05$) in LH secretion patterns over time between ponies or treatments.

The length of estrus was unchanged ($P>.05$) due to M or N (N, 7.0±0.0; N, 8.3±2.3; C, 7.3±.9 d; $\bar{x}$±SE). Ovulation was not advanced by one N injection, nor was it delayed by one M injection,

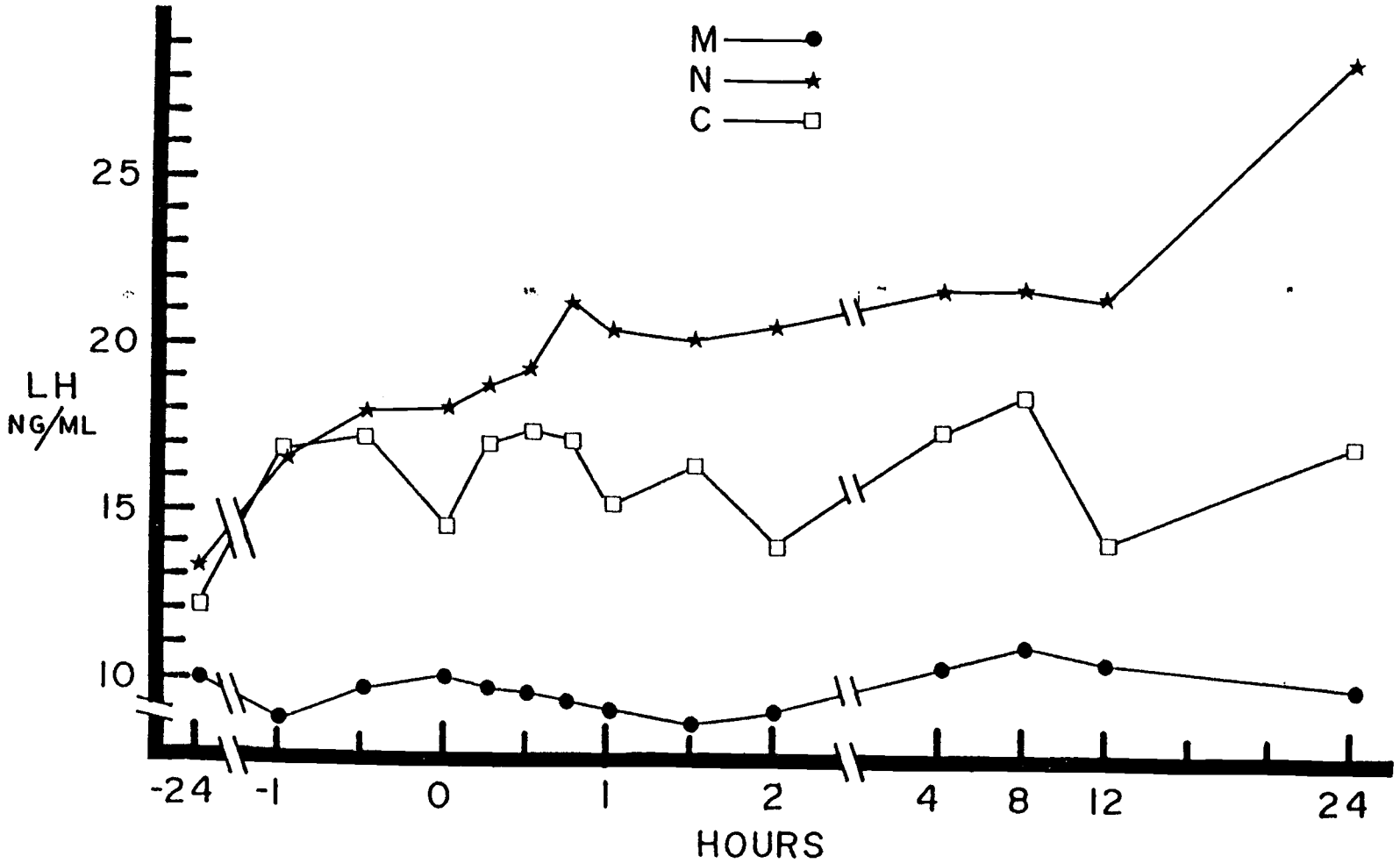

Figure 2: The effects of morphine (M), naloxone (N), or saline (C) on serum LH in intact cycling mares. Pooled standard error of the mean equals 5.06. Treatment at zero hour.

including the three ponies that received daily morphine (DM) treatments during estrus (figure 3). Estrus was not different ($P>.05$) in these DM treated ponies (DM, 9.0±1.0 d). The number of days these ponies were in estrus were comparable to other reports (Hughes et al., 1975; Ginther, 1979).

Behavioral estrus was not affected by a single injection of either M or N. However, two of the DM treated mares seemed to have slight behavior modifications. After the second day of M treatment, these two ponies had reduced signs of standing heat. The number of negative signs increased at this time, including kicking, biting, or ears laid back against the head when approached by a stallion. This behavior subsided within a few days and eventually the mares ovulated. Follicular dynamics (growth and development of follicles) were as expected for the breeding season (i.e., they were larger and more numerous than those of the nonbreeding season).

Ovariectomized Mares: The OVX mares (n=3) LH levels did not change ($P>.05$) over time from treatment within each season (figure 4). Morphine did not decrease high summer levels of LH (33.6±1.0 ng/ml; $\bar{x}$±SE), and N did not increase low winter levels (1.4±.1 ng/ml). Luteinizing hormone did change ($P<.05$) from winter to summer. This fluctuation in LH concentration season to season substantiates other reports of a nonovarian control of LH secretion in the mare (Garcia and Ginther, 1976; Freedman et al., 1979). This rhythym maybe entrained to an environmental factor such as photoperiod. The opiate analogs seemed to have no effect on the seasonal changes

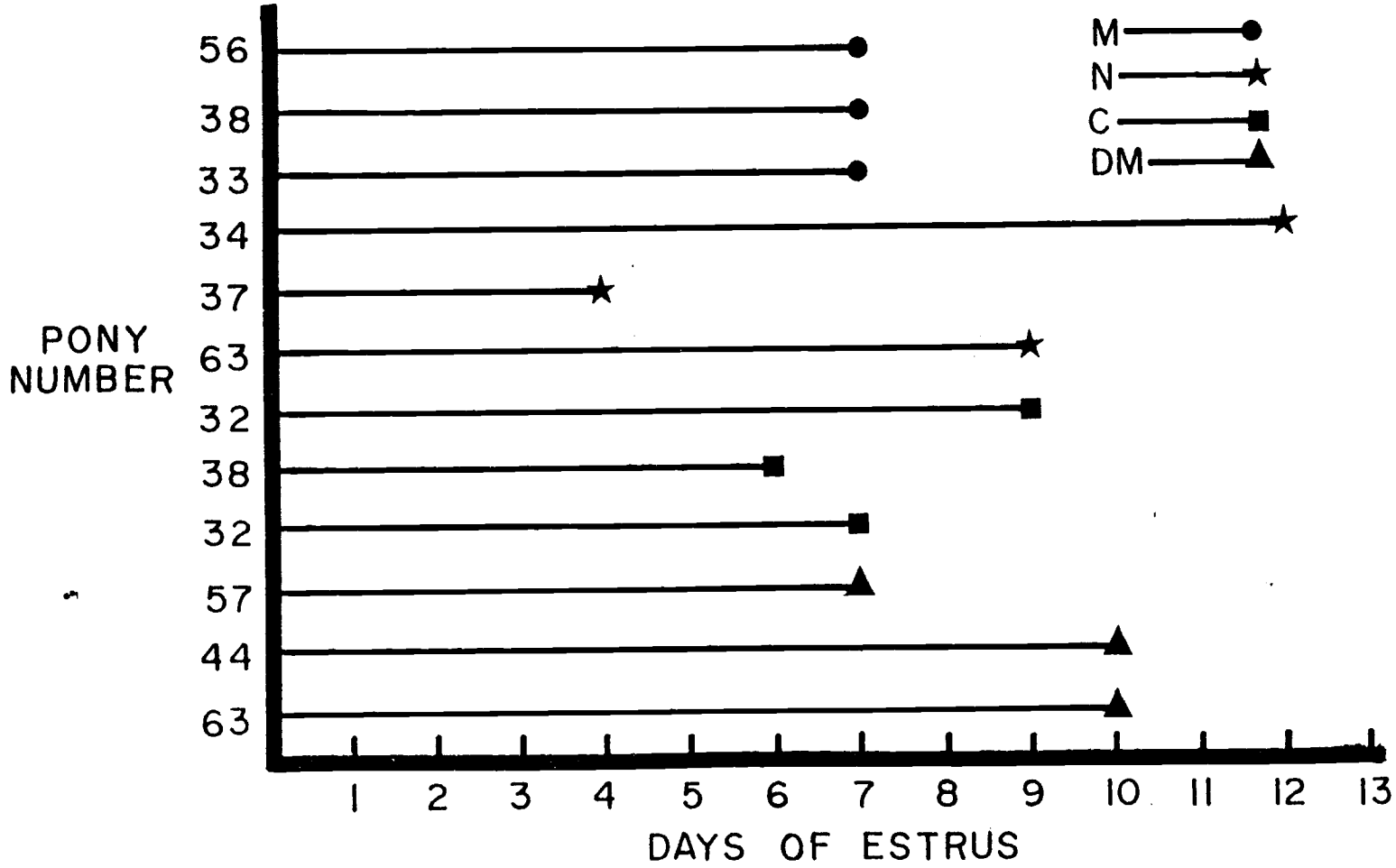

Figure 3: The effects of morphine (M), naloxone (N), or saline (C) on length of estrus in cycling mares. Single or daily (DM) treatment began on day 2 estrus.

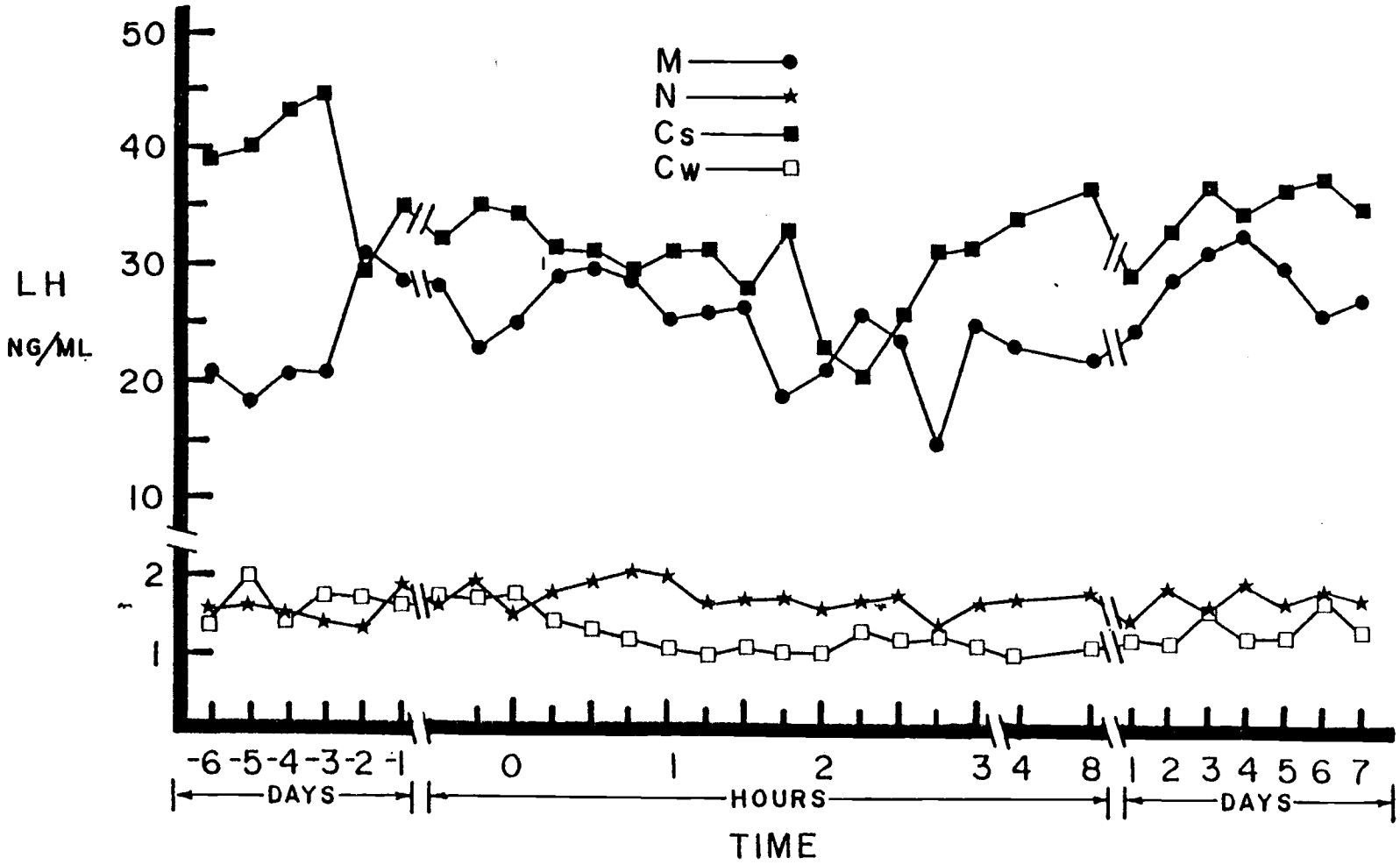

Figure 4: The effects of morphine (M) or saline (Cs) in the summer, and naloxone (N) or saline (Cw) in the winter on serum LH in ovariectomized mares. Pooled standard error of the mean for winter equals .1 and for summer equals 1.0.

in LH in the mare. However, in OVX rats (Sylvester et al., 1982) an estrogen or estrogen/progesterone pretreatment before opiate exposure changes their LH response due to the negative feedback systems of steroids. A monkey with low systemic estrogen levels (OVX or early follicular phase) has no END in the hypophyseal portal blood (Wehrenberg et al., 1982). The OVX mare may need a steroid pretreatment to allow the EOP system to work.

Conclusions: The above data seem to show no opiate involvement in equine seasonal breeding. Other species show effects within 30 min after treatment. But the mare may need another protocol. Intraventricular injections may be a means to use less opiate for an effective experiment. Obviously, due to technical problems, this method will not immediately solve the horseowner's problem of controlling the mare's estrous cycle. Information about opiate involvement in the control of reproduction in the equine will be valuable. If subcutaneous implants or high continuous doses of N increase LH, one or the other could become a practical way to routinely administer these products. Naloxone implanted in the anestrous season for a period of weeks or months might increase LH, advancing the onset of the ovulatory season.

Treatments involving more than one compound (such as a pretreatment of estrogen, estrogen/progesterone, or a melatonin inhibitor before N, given to anestrous mares) may change LH secretion. As explained above, OVX animals may require steroidal stimulation for opiate action. In the acyclic mare, LHRH causes an increase in LH, whereas estrogen decreases it. If estrogen is

injected before the addition of LHRH, then LH increases more than it would with LHRH alone (Vivrette and Irving, 1979). This experiment shows the importance of the timing of hormonal treatments. Length of time a treatment continues is also important. Increased photoperiod in the winter must begin in November to allow enough time for the physiological change from the nonbreeding to breeding seasons to occur in February. Perhaps N needs a similar time lapse before the mare will respond. The field of equine reproduction has important questions that need answering. Manipulation of the mare's reproductive cycles and seasons may be possible once these questions become common knowledge.

CHAPTER 3

CONCLUSION

The preceeding experiments suggest that the EOP have little or no involvement in equine reproduction. Other physiological factors may suppress opiate action on reproduction at certain times of the season or estrous cycle. In the mare's anestrous season, LHRH may be blocked by MEL in such a fashion that N cannot counteract this suppression. Melatonin may act on the LHRH containing neuron, whereas N may act on NE. These locations of action would explain naloxone's ineffectiveness. During the ovulatory season, the steroids estrogen and progesterone, along with their feedback systems, play a major role in LH control. In primates (Ropert et al., 1981), EOP effect LH secretion only when the steroids are at high, systemic levels, as in the diestrus phase in the monkey and woman (Quigley and Yen, 1980; Wehrenberg et al., 1982). Perhaps the equine has similar times of opiate activity and quiescence.

Endogenous opioid peptide involvement in mare seasonal breeding was not elucidated in our experiments. A change in protocol may show other results. Hormones fluctuate on a daily and(or) weekly pattern, as does LH throughout the estrous cycle. Ponies were all treated at the same time of day (0900 h). It is unknown whether or not EOP have a diurnal rhythym, and if so, when they reach their maximum. Melatonin concentration changes during the day in the hamster (Rollag et al., 1980) and mare (Kilmer et al.,

1982) reaching a peak at night. These high levels of MEL may block the opiate response to mares. A different time of day for treatment may have allowed the pony mares to respond.

Another factor to consider is the stage of the estrous cycle. During the ovulatory or breeding season the ponies were treated on the second day of estrus, which corresponds to the early follicular stage of the cycle. At this time LH has not yet started it's ovulatory surge. Progesterone is low and decreasing while estrogen is low but beginning to increase. These steroids are also low in the OVX mare. It is possible that opiates need a steroidal influence before they will act, including in the OVX mare. In primates, M and N are effective at times of the cycle when the steroids are at high systemic levels. In nonhuman primates (Wehrenberg et al., 1982) EOP disappear from the portal blood supply during times of low estrogen. This disappearance occurs in both the early follicular phase of the cycle and in OVX monkeys (Ferin et al., 1982; Wehrenberg et al., 1982). Morphine will decrease LH only when estrogen (and EOP) levels are high. In fact, the OVX monkey needs a steroid pretreatment before an opiate effect is observed. Women react to opiates at high steroid periods of the menstrual cycle. Both Quigley and Yen (1980) and Ropert et al. (1981) state that N infusion (1.6 mg/h) results in an LH response during the late follicular and mid luteal stages, but not the early follicular stage of the menstrual cycle.

In the rat, M is most effective at lowering LH during the proestrus stage of the cycle. Massive doses of M (20 to 50 mg)

injected at proestrus will block the LH induced ovulation (Barraclough and Sawyer, 1955). Lower doses of M will decrease LH secretion at this time, but do not stop ovulation (Ieiri et al., 1980a). As stated above, the protocol we followed was during the early follicular stage. Naloxone and M do not seem to affect the mare (or other species) at this time. However, due to the tremendous variation in estrus lengths in ponies, a treatment closer to expected ovulation date was not possible. For instance, if the fifth day of estrus had been chosen for treatment day, some ponies would already have ovulated, some would ovulate during treatment, and the rest would wait 2 to 4 d posttreatment. This individual variation would invalidate any treatment result (i.e., was LH increasing due to ovulation, or N?). A treatment during diestrus (mid luteal stage) may be effective at modifying LH secretion, and should be undertaken.

The route of opiate administration may play a role in treatment efficacy. These ponies were treated intravenously. The blood-brain barrier must have been crossed as shown by the behavorial effects. Naloxone was effective in blocking M action, as was apparent in the mare that received N after three M injections. This antagonism suggests that the opiates acted at a specific receptor site. However, these intravenous injections have no apparent effect on LH secretion. Thus, a different route of administration may be necessary. Intraventricular injection of opiates might work. In general, doses of opiate agonists 100 to 1,000 fold smaller than iv preparations have the same effectiveness in

decreasing LH if injected into the rat ventricle (Meltzer et al., 1978; Kinoshita et al., 1980; Goldberg et al., 1982; Motta and Martin, 1982). Since cannulation of ponies is costly and difficult, this method is only appropriate for research purposes. In vitro experiments utilizing hypothalami and pituitaries would allow specific structures to be examined, and treated with opiates. This method would elucidate whether or not opiates are involved in the above brain areas.

The most practical method of opiate administration may be subcutaneous implants. They are easy to insert and would release a continuous amount of product. If N or N plus steroids were implanted early in the winter and maintained throughout the anestrous season, then LHRH may be released. Luteinizing hormone may then increase and the breeding season may then be advanced. These treatments may take several months to act. For example, a 16 h lighting regimen takes 2 to 3 mo to bring an anestrous mare into heat. Ganglionectomy (Sharp et al., 1979) and pinealectomy (Grubaugh et al., 1982) take at least one year to change the mare's reproductive status.

The above suggestions may help determine if EOP are involved in equine seasonal reproduction. However, opiates may not be a part of the mare's physiological control of these systems. There are many other compounds in the brain (peptides, proteins, indoles, etc.) which may influence the mare's seasonal breeding. Indoles such as MEL effect the reproductive cycle in many species. However, the location of MEL action on the hypothalamus is unknown. It may

suppress LHRH or NE directly without any aid from EOP systems. Other compounds besides MEL may inhibit reproduction at the level of the pituitary. Endogenous opiates may control reproduction through other hormones such as follicle stimulating hormone, prolactin, or growth hormone. These pituitary hormones were not examined in the above experiments. There are many aspects to be examined before the hormonal events of the mare can be elucidated, including whether or not opiates are involved.

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