EFFECT OF NALOXONE ON SERUM LUTEINIZING HORMONE CONCENTRATIONS DURING THE EARLY POSTPARTUM PERIOD AND THE ESTROUS CYCLE IN PRIMIPAROUS AND MULTIPAROUS HOLSTEIN COWS

by

AMIN AHMADZADEH

Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Dairy Science

APPROVED:

M. A. Barnes

M. A. Barnes, Chairman

D. Michael Denbow R. E. Pearson

D. M. Denbow R. E. Pearson

W. E. Vinson

W. E. Vinson

November, 1994

Blacksburg, Virginia

EFFECT OF NALOXONE ON SERUM LUTEINIZING HORMONE CONCENTRATIONS DURING THE EARLY POSTPARTUM PERIOD AND THE ESTROUS CYCLE IN PRIMIPAROUS AND MULTIPAROUS HOLSTEIN COWS

by

AMIN AHMADZADEH

M. A. Barnes, Chairman

Dairy Science

(ABSTRACT)

Four experiments were conducted to investigate the effect of naloxone, an opioid receptor antagonist, on pituitary LH secretion in Holstein cows during two periods after parturition and two phases of the estrous cycle. In experiment 1, 24 cows (12 primiparous; 12 multiparous) received either saline (n=12) or 1 mg/kg naloxone (n=12) i.v. at 14 ± 1 days postpartum. Blood samples were collected at 15-minute intervals for 2 hours before and 2.5 hours after naloxone or saline. Serum LH concentrations increased ($P < .05$) in response to naloxone injection in both primi- and multiparous cows. Saline injection did not affect LH concentrations. In experiment 2, 27 cows (13 primiparous; 14 multiparous) received either saline (n=14) or 1 mg/kg naloxone (n=13) i.v. at 28 ± 1 days postpartum. Blood samples were collected as in the previous experiment. Naloxone did not affect serum LH concentrations in either primi- or multiparous cows at 28 days postpartum. In experiment 3, estrous cycles were synchronized via prostaglandin administration (25 mg) in 22 cows (10 primiparous; 12 multiparous). Cows received either saline (n=11) or 1 mg/kg naloxone (n=11) i.v. during the luteal phase of the estrous cycle. Blood samples were collected as in the

previous experiments. Luteinizing hormone concentrations were not affected by naloxone in either primi- or multiparous cows during the luteal phase of the estrous cycle. In experiment 4, the same cows used in experiment 3 received a second dose of prostaglandin (25 mg). Thirty-six hours later, during the follicular phase of the estrous cycle, the cows received either saline (n=9) or 1 mg/kg naloxone (n=11) i.v. Naloxone increased ($P < .05$) serum LH concentrations in both primi- and multiparous cows in the follicular phase. These results suggest that LH release in the early postpartum dairy cow is regulated, at least in part, by endogenous opioid peptides, and the ability of naloxone to affect LH secretion may change as days postpartum increases, perhaps due to changes in degree of inhibition by endogenous opioid peptides, and(or) changes in serum progesterone concentration due to onset of ovarian activity during postpartum period. It appears that the modulation of LH secretion may be mediated via opioids during the follicular phase of the estrous cycle. However, an opioid-mediated mechanism for modulation of LH secretion was absent or overridden by progesterone feedback during the luteal phase of the estrous cycle.

DEDICATION

I dedicate this thesis to my parents, Hashem and Mansoureh Ahmadzadeh for their love and support.

Acknowledgments

I would like to express my sincere gratitude and appreciation to the people who assisted, advised, and encouraged me during my masters program at Virginia Tech. I would especially like to thank my major advisor, Dr. M. A. Barnes for his support and encouragement through every step. Thank you for your guidance, advice, and friendship. I would also like to thank the other members of my committee: Dr. D. M. Denbow who gave me a totally new view of neuro-endocrinology in his physiology and neurochemistry classes and Dr. R. E. Pearson who assisted me tremendously with the statistical analysis and other means regarding my research. Dr. W. E. Vinson also deserve thanks for his insight and constructive criticism of this research. I wish to express my appreciation to Dr. R. M. Akers for radioiodination of the hormone and his input in regard to radioimmunoassay and to Dr. M. L. McGilliard for his suggestions and intuition.

Thanks to John Gibbons for teaching me about ultrasonography, Billy Walker for his help at various times and for making everything fun and enjoyable, Sher Nadir my officemate who unselfishly contributed to all aspects of my graduate study, and to Greg Bethard for help bleeding the cows while I had to attend class. Jim Rohl deserves special thanks for all his help with this research during the hot summer of 1993 and the help with writing my thesis. Thank you for being a friend and a wonderful listener in all situations.

I am unable to give enough appreciation to my parents Hashem and Mansoureh. They deserve way more than that. Thank you father, for your support, encouragement and all the good discussions about the dairy industry. Thanks Mom, for your patience and words of wisdom. You are a fine human being. My sincere gratitude goes to my brother Ali and my sister Sudi here in the U.S., and my other sister Elham back home,

who helps my mom and dad and thus enabled me to come to the U.S. to study. I love you all from the bottom of my heart.

Finally I'd like to recognize my other friends, Alan Pasquino for endless assistance in computing, my good buddy Dr. Dan Weigel who was always there to help me in SAS and who coached me in softball, Patricia Boyle for her help in various laboratory procedures, and last but not the least, the Virginia Tech Dairy Farm crew who made this research possible. Thank you all.

TABLE OF CONTENTS

TITLE i

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

LIST OF TABLES x

LIST OF FIGURES xii

INTRODUCTION 1

REVIEW OF LITERATURE 3
- Opioid Receptors and their Distribution 3
- Opioid peptides, their Family, and their Distribution 5
  - Enkaphalin Family 6
  - β-Endorphin Family 6
  - Dynorphin Family 7
- Central Nervous System Location of EOP Perikarya 7
- Physiological Role of Endogenous Opioids 8
  - Endogenous Opioid Control of Gonadotropin Secretion 10
- Exogenous Opioid and their Influence upon Gonadotropin Secretion 10
  - Opioid Antagonists 12
- How Opioid Peptides Affect Gonadotropin Secretion 13
- Physiological Significance of EOP 15
  - Opioids and gonadal steroids feedback mechanism 16
  - Postpartum period and EOP 19
  - Energy balance and EOP 20
- Function of the Hypothalamus and Pituitary During the Postpartum Period . 21

EXPERIMENTAL OBJECTIVES AND RATIONALES 24

MATERIALS AND METHODS 26

Experiment 1 . . . 26
Experiment 2 . . . 27
Experiment 3 . . . 28
Experiment 4 . . . 29
Experimental Procedures . . . 29
Treatment . . . 29
Blood Collection Procedure . . . 29
Feed Intake and Energy Balance Estimation . . . 31
Hormone Determinations . . . 32
Statistical Analysis . . . 33

EXPERIMENT 1

THE EFFECT OF NALOXONE ON LUTEINIZING HORMONE SECRETION ON DAY 15 POSTPARTUM IN HOLSTEIN COWS . . . 37
Results . . . 37
Discussion . . . 38

EXPERIMENT 2

THE EFFECT OF NALOXONE ON LUTEINIZING HORMONE SECRETION ON DAY 28 POSTPARTUM IN HOLSTEIN COWS . . . 53
Results . . . 53
Discussion . . . 55

EXPERIMENT 3

THE EFFECT OF NALOXONE ON LUTEINIZING HORMONE SECRETION IN THE LUTEAL PHASE OF THE ESTROUS CYCLE IN HOLSTEIN COWS . . . 66
Results . . . 66
Discussion . . . 67

EXPERIMENT 4

THE EFFECT OF NALOXONE ON LUTEINIZING HORMONE SECRETION IN THE FOLLICULAR PHASE OF THE ESTROUS CYCLE IN HOLSTEIN COWS . . . 77

Results 77
Discussion 78

SUMMARY AND CONCLUSIONS 89

LITERATURE CITED 93

APPENDICES 105

Appendix A. Analysis of variance tables for experiment 1 106

Appendix B. Analysis of variance tables for experiment 2 109

Appendix C. Analysis of variance tables for experiment 3 113

Appendix D. Analysis of variance tables for experiment 4 117

VITA 119

LIST OF TABLES

TABLE

1 Ingredient and chemical composition of the total mixed ration (TMR) 36

EXPERIMENT 1

2 Mean serum luteinizing hormone concentrations in Holstein cows treated with either saline or opioid antagonist (naloxone) on day 15 postpartum 45

3 Mean serum luteinizing hormone concentrations in primiparous and multiparous Holstein cows before and after gonadotropin releasing hormone (GnRH) administration on day 15 postpartum 46

4 Mean net energy balance and serum non-esterified fatty acids (NEFA) concentrations in primiparous and multiparous Holstein cows at 15 days postpartum 47

5 Correlation coefficient for the variables net energy balance (NEB), serum non-esterified fatty acids (NEFA) concentration (μEq/L), milk yield, basal serum luteinizing hormone concentrations (ng/ml), and percent increase luteinizing hormone concentration in response to naloxone at 15 days postpartum . . . 48

EXPERIMENT 2

6 Mean serum luteinizing hormone concentrations in Holstein cows treated with either saline or opioid antagonist (naloxone) on day 28 postpartum 59

7 Mean serum luteinizing hormone concentrations in primiparous and multiparous Holstein cows before and after gonadotropin releasing hormone (GnRH) administration on day 28 postpartum 60

8 Mean serum non-esterified fatty acids (NEFA) concentration and net energy balance in Holstein cows at 15 and 28 days postpartum 61

EXPERIMENT 3

TABLE

9 Mean serum progesterone concentrations in Holstein cows in the luteal and follicular phase of the estrous cycle 71

10 Mean serum luteinizing hormone concentrations in Holstein cows treated with either saline or opioid antagonist (naloxone) in the luteal phase of the estrous cycle 72

11 Mean serum luteinizing hormone concentrations in Holstein cows before and after gonadotropin releasing hormone (GnRH) administration in the luteal phase of the estrous cycle 73

12 Mean basal luteinizing hormone concentrations in Holstein cows in the luteal and follicular phase of the estrous cycle 74

EXPERIMENT 4

13 Mean serum luteinizing hormone concentrations in Holstein cows treated with either saline or opioid antagonist (naloxone) in the follicular phase of the estrous cycle 83

14 Mean serum luteinizing hormone concentrations in Holstein cows before and after gonadotropin releasing hormone (GnRH) administration in the follicular phase of the estrous cycle 84

LIST OF FIGURES

FIGURE

EXPERIMENT 1

1 Mean serum luteinizing hormone concentrations in saline- (n=12) and naloxone-treated (n=12) Holstein cows at day 15 postpartum 49

2 Mean serum luteinizing hormone concentrations in 3 Holstein cows (#859, #896, #646) which did not respond to naloxone on day 15 postpartum 50

3 Mean serum luteinizing hormone concentration before and after gonadotropin releasing hormone (GnRH) injection in saline- and naloxone-treated primiparous Holstein cows on day 15 postpartum 51

4 Mean serum luteinizing hormone concentration before and after gonadotropin releasing hormone (GnRH) injection in saline- and naloxone-treated multiparous Holstein cows on day 15 postpartum 52

EXPERIMENT 2

5 Mean serum luteinizing hormone concentrations in saline- (n=7) and naloxone- (n=8) treated Holstein cows with serum progesterone concentrations less than 1 ng/ml on day 28 postpartum 62

6 Mean serum luteinizing hormone concentrations in saline- (n=7) and naloxone- (n=5) treated Holstein cows with serum progesterone concentrations greater than 1 ng/ml on day 28 postpartum 63

7 Mean serum luteinizing hormone concentrations in saline- (n=14) and naloxone- (n=13) treated Holstein cows on day 28 postpartum 64

8 Mean serum luteinizing hormone concentration before and after gonadotropin releasing hormone (GnRH) injection in primi- and multiparous Holstein cows on day 28 postpartum 65

EXPERIMENT 3

FIGURE

9 Mean serum luteinizing hormone concentrations in saline- (n=11) and naloxone- (n=11) treated Holstein cows during the luteal phase of the estrous cycle . 75

10 Mean serum luteinizing hormone concentration before and after gonadotropin releasing hormone (GnRH) injection in saline- and naloxone-treated Holstein cows in the luteal phase of the estrous cycle 76

EXPERIMENT 4

11 Mean serum luteinizing hormone concentrations in saline- (n=6) and naloxone- (n=6) treated primiparous Holstein cows in the follicular phase of the estrous cycle 85

12 Mean serum luteinizing hormone concentrations in saline (n=3) and naloxone- (n=5) treated multiparous Holstein cows in the follicular phase of the estrous cycle 86

13 Mean serum luteinizing hormone concentrations before and after naloxone (1 mg/kg BW) administration in cow #867(▲) which did not exhibit an increase in serum LH concentrations but responded to GnRH injection 87

14 Mean serum luteinizing hormone concentration before and after gonadotropin releasing hormone (GnRH) injection (time=1400) in saline- and naloxone-treated Holstein cows in the follicular phase of the estrous cycle 88

Introduction

Following parturition in the cow, there is a transitional period during which the animal is not cyclic. During this period of involution reproductive organs recover from the effects of pregnancy. During pregnancy the hypothalamic-hypophysial axis is suppressed by high concentrations of progesterone and estradiol in the blood (Nett, 1987). The high concentration of these steroids appears to inhibit secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus, resulting in inadequate stimulation of pituitary gonadotrophs to maintain synthesis of luteinizing hormone (LH). This results in a depletion of LH in the anterior pituitary gland that must be restored after parturition so that normal estrous cycles can begin. Although the circulating concentration of LH is lower in postpartum cows than in cyclic cows, the circulating concentrations of FSH are not suppressed (Moss et al., 1985). This may be due to relative lack of follicular development during late gestation, and without negative feedback of folliculostatin, secretion of FSH remains normal (Miller et al., 1982). The mechanism which causes such effects on the hypothalamus-pituitary axis and prevents cows from initiating cyclic activity is not clearly understood. A better understanding of the early postpartum period and endocrine and neuroendocrine mechanisms which regulate it can help to lead to more effective treatments to shorten this transition period.

There are several components of the hypothalamic-hypophysial axis, which, if their function were suppressed, could lead to reduction in the secretion of LH, probably manifested by a decrease in frequency and(or) amplitude of LH pulse (Nett, 1987).

These factors include the amount of GnRH synthesized and stored in the hypothalamus, direct inhibition of the secretion of GnRH without a concomitant reduction in the content of GnRH in the hypothalamus, the amount of GnRH secreted into the hypophysial portal circulation, the number of receptors for GnRH in the anterior pituitary gland , and the amount of LH synthesized and stored in the anterior pituitary gland.

It is known that opiate alkaloids can impair reproductive function in the human, and the inhibition of sexual function by chronic opiate treatment has been practiced (Szekely, 1982). Male opiate addicts have depressed serum testosterone level, and female addicts were repeatedly reported to experience amenorrhea, infertility, and spontaneous abortion. There is no indication that opiates directly affect the sexual organs but their suppressive effects on gonadotropin secretion is well documented (Szekely, 1982). The fact that exogenous opioids can bind to receptors in the brain led to the discovery of endogenous compounds which act like exogenous opioids and utilize the same receptors. Since this discovery, there has been extensive research on the possible physiological role of endogenous opioid peptides (EOP) in humans and animals. Endogenous opioid peptide control mechanisms for LH secretion have been implicated in various reproductive states in a wide spectrum of mammals, including beef cattle (Whisnant et al., 1986a,b,c) and dairy cows (Nanda et al., 1989). It appears that in postpartum beef cows, EOP affect LH secretion by inhibiting the release of GnRH. Because of the extremely important role of LH in ovulation, development of follicles, and resumption of cyclicity during the early postpartum period, this possible role of EOP, from an economic point of view, may play an important role in dairy cattle reproduction and production. The objectives of this study were to investigate the roles of EOP on modulation of LH secretion in the early postpartum period and during different phases of the estrous cycle in Holstein cows.

Review of Literature

Since 1975 a large variety of different opioid peptides have been isolated and characterized their nomenclature becoming somewhat confusing. The generic term endorphin was originally proposed to describe all endogenous opioids. However, this term is similar to β-endorphin, one of the specific opioid peptides (Malven, 1993). The term opioid is sometimes used, but it must be prefaced with either endogenous or exogenous to distinguish drugs from endogenous compounds. The accepted generic term is endogenous opioid peptides (EOP), which is both descriptive and correct, and is reserved for compounds that possess bioactivity similar to opioid agonist drugs (Malven, 1993). Furthermore, an opiate is an exogenous compound derived from the juice of opium poppy, whereas an opioid is an endogenous compound with morphine-like activity. Thus, receptors which mediate the effects of both opioids and opiates are referred to as opioid receptors since their physiological ligands are opioids (Brooks et al., 1986a). Drugs such as naloxone, which antagonize the effect of both morphine and the endogenous opioids are generally referred to as opiate antagonists. However, naloxone mediates its effects via opioid receptors, and therefore for the purpose of this review will be referred to as an opioid antagonist.

Opioid Receptors and their Distribution.

The original discovery of opioid receptors involved the binding of radiolabeled naloxone to brain homogenate (Pert and Snyder, 1973). Naloxone is a drug known to antagonize most of opioid bioactivity, thus its competitive binding to the same moiety as opioid drugs was not unexpected. Based on comparison of activity in various bioassays and binding affinities for many synthetic opioid-related drugs, a number of subtypes of opioid receptors have been identified. The selectivity of each subtype for any given EOP or antagonist is never absolute, so that the selectivity of binding always depends on dosage (Goldstien and Naidu, 1989). For example, naloxone can antagonize most subtypes if the dosage is sufficiently high, however it has higher affinity for the *mu* receptor in a moderate dose.

Opioid receptors are widespread in neural tissue and not surprisingly, because there are a variety of ligands, there is now strong evidence to suggest that they are not all identical (Brooks et al., 1986a). The three primary receptor subtypes are known as *mu* (μ), *delta* (δ), and *kappa* (κ), with an additional one called *epsilon* (ϵ) (Malven, 1993). The regional distribution of opioid receptors closely parallels that of their ligands, except in the anterior pituitary which contains low receptor numbers (Hayens et al. 1989). Previous studies (Leshin et al. 1991;Trout and Malven, 1988) found opioid receptors in various regions of the bovine brain. For instance, very high receptor density has been demonstrated in the median eminence (ME) and preoptic area (POA) of the hypothalamus, a fact which is of particular interest because many of the releasing hormones controlling anterior pituitary function have nerve endings in these regions. In support of this idea, Leshin et al. (1991) showed that luteinizing hormone releasing hormone (LHRH) release from ME and POA was induced by naloxone.

Extrahypothalamic sites involved in the control of gonadotropin secretion have also been shown to have a high degree of opioid receptor binding. The periaqueductal central grey region of the brain has anatomical connections with the mediobasal hypothalamus (Hayens et al., 1989) and has been shown to exert an inhibitory influence upon gonadotropin secretion (Carrer and Taleisnik, 1972). This region of the brain has a high concentration of opioid receptor binding. Also, concentration of opioid receptors in different regions of the brain may vary depending upon the physiological state of the animal. For instance, Trout and Malven (1988) found a higher concentration of receptors in the POA of anestrous suckled cows than of cyclic suckled cows.

The tissue distribution of opioids and their subtype receptors is highly heterogenous in the central nervous system (CNS) (Mansour et al., 1987). They also occur in the gut and in the vas deferens of the male reproductive tract. In both of these muscular tissues, opioid antagonists inhibit contractile activity, and both have been used for opioid bioassays (Malven, 1993). As mentioned previously, because opioid antagonists have the ability to bind with opioid receptors, it seems logical that the function of these receptors is to mediate the effects of some endogenous substances with opioid-like activities.

Opioid peptides, their Family, and their Distribution

Development of knowledge about endogenous opioid peptides began with discovery of specific binding of opioid drugs to the homogenate of brain tissue. The fact that specific receptors might exist stemmed from the observation that electrically stimulated contractions of a segment of mouse vas deferens or guinea pig ileum could be prevented by the administration of an opiate analgesic such as morphine (Henderson et al., 1972). This discovery raised the question of the normal physiological role for such receptors in animals or humans that were not administered derivatives of the opium poppy. The obvious answer was the possible existence of endogenous compounds capable of interacting with these receptors. The first two of these compounds to be discovered by Hughes and co-workers (1975) were methionine-enkephalin and leucine-enkephalin. They were soon followed by discovery of β-endorphin and several years later by the discovery of dynorphin. Each of these neuropeptides is a member of a family of peptides, all members of which share a common precursor (Malven, 1993).

Endogenous opioid peptides are divided to three main groups, which are derived from a specific precursor. These are summarized as follows(for complete review see Malven, 1993):

Enkephalin Family of EOP. The first EOP's to be discovered were the two pentapeptides, methionine-enkephalin ([Met]-ENK) and leucine-enkephalin ([Leu]-ENK), which differ in only one amino acid, methionine and leucine (Hughes et al., 1975). A precursor was subsequently discovered and found to contain six replications of the [Met]-ENK sequence and one sequence of [Leu]-ENK. The precursor is called proenkephalin or more specifically proenkephalin A to distinguish it from proenkephalin B. Proenkephalin B is the name for the precursor of dynorphin-related peptides. These naturally occurring EOPs of the enkephalin family share higher affinities to the delta subtype of opioid receptors (Malven, 1993).

β-Endorphin Family of EOP. A protein called β-lipotropin based on its lipid-mobilizing bioactivity was discovered in pituitary extracts of domestic ungulates (Li and Chung, 1976). When pituitary extracts from camels were examined, they lacked β-lipotropin but contained part of its sequence in a smaller peptide, originally named C-fragment (Bradbury et al. 1976). This C-fragment was found to contain opioid bioactivity, and it was assigned a new name, β-endorphin and later found not to be unique to camels.

A large precursor for β-endorphin called pro-opiomelanocrotin (POMC) was subsequently identified and found to give rise to a variety of biologically active hormones and neuropeptides (Imura et al., 1983). The cleavage of POMC to constitute peptides appears to differ among tissues (Holt, 1986). The N-terminal fragment of POMC is cleaved from the N-terminus of ACTH, leaving a transient compound sometimes called pro-opiocortin, which is rapidly cleaved into ACTH and β-lipotropin (Malven, 1993). These initial steps probably occur in all tissues. Eighty percent of β-lipotropin is secreted in adenohypophysial tissue of the pars anterior, but approximately 20% of it is cleaved into β-endorphin. β-endorphin contains 31 amino acids and in terms of activity, favors the mu receptor subtype. β-endorphin family of EOP is unique because there are many other neuropeptides derived from POMC which don't have any known opioid activity.

Dynorphin Family of EOP. After discovery of [Met] and [Leu]-ENK, research demonstrated some other opioid bioactive peptides which had [Leu]-ENK at their N-terminus but were larger than the [leu]-ENK molecule. Because of their extreme potency in some bioassays, the name dynorphin was given to these peptides (Goldstein and Naidu, 1989). Later, 3 types of dynorphin were classified and called A, B, and β-neo-endorphin. The precursor for this family is prodynorphin, and is also called proenkephalin-B. Bioactive peptides of this family of EOP's share high affinities for binding to the kappa subtype of opioid receptors (Holt, 1986).

Central Nervous System Location of EOP Perikarya

Following the elucidation of the three EOP families, immunocytochemical techniques were used to locate neuronal perikarya specific to each family. These studies revealed highly heterogeneous distributions which differ among EOP families (Goldstien and Naidu, 1989). The enkephalin family is distributed more widely than other families. It can be found in different areas of the hypothalamus such as the arcuate nucleus (ARC), paraventricular (PVN), and lateral hypothalamic area (LHA) and is also found in the ventromedial nucleus, dorsal medial nucleus, and mammillary bodies. Enkephalin is also strongly expressed in the medulla of adrenal gland.

The β-endorphin family is distributed in perikarya of only two CNS locations which are the ARC of the hypothalamus and the nucleus of the solitary tract in the brain stem, and also pituitary (Hayens et al., 1989;Malven, 1993). β-endorphin-containing neurons that are located in the ARC of the mediobasal hypothalamus project into the ME, POA and amygdala (Haynes et al., 1989) region, which are known to influence gonadotropin secretion.

The distribution of the dynorphin family in the hypothalamus is similar to that of enkephalins. Additionally, dynorphins can be found in the super optic nucleus (SON) (Malven, 1993). Dynorphin related peptides are also present in a few other non-limbic regions. The prodynorphin gene appears to also be expressed in the LH-containing cells of the adenohypophysis, but the processing of the precursor does not proceed far enough to yield any small peptides with opioid bioactivity (Malven, 1993).

Physiological Role of Endogenous Opioids

Although characterization discoveries of opioid receptors and EOP began as an effort to understand and perhaps treat or prevent drug addiction (Malven, 1986), most studies have dealt with roles for EOP in opiate-free animals and humans. Information about these physiological roles for EOP depends very much on a single research approach, notably the administration of the opioid receptor antagonist naloxone to drug-free subjects. In this research model, the investigator is searching for any effect on hormone secretion and(or) a physiological parameter as a result of the administration of naloxone. For instance, if a hormone secretion changed as a result of opioid antagonism while the animal was not being treated with exogenous opioid drugs, one may conclude that an endogenous opioid modulation of that hormone had been competitively removed and the secretion of that hormone was being influenced by EOP's at the time of administration of the opioid receptor antagonist (Haynes et al., 1989). This EOP influence should resume as the effect of the exogenous antagonist wanes. It should be noted that the observed changes are usually very short-lived (less than 1 h) because naloxone -induced antagonism of opioid receptors is very short-lived (Malven, 1993). This type of evidence usually provides the initial suggestion that EOPs modulate some variable (i.e, hormone) implying that perhaps brain areas known to control the variable contain high concentrations of EOPs or their receptors.

In addition to those that are related to the EOP-induced analgesia several other types of behavior appear to be modulated by EOPs . Stressful stimuli provoke release of ACTH and PRL from the adenohypophysis, and EOPs may mediate some of these stimulatory effects because naloxone administration can decrease the amount of stress-induced release. In contrast, secretion of LH by the adenohypophysis may be decreased by EOPs in certain situations (Malven, 1993). Those experimental situations in which naloxone administration in females will increase LH, presumably by increasing LHRH release, involve stress-induced suppression, suckling-induced anestrus, and gonadal feedback involving progesterone. There is some evidence which indicates that the increase of LH after naloxone administration could be a direct effect on the adenohypophysis (Chao et al., 1986). Thus, EOPs that inhibit LHRH may be activated during these states to prevent secretion of LHRH and LH. Such a physiological role of EOPs would appear to suppress female reproduction when conditions are inappropriate for fertility (Chao et al., 1986).

Endogenous Opioid Control of Gonadotropin Secretion

Opioid peptides, and their analogues in general, are considered to have a suppressing effect on gonadotropin secretion, as is the case with exogenous opiates. Studies indicated that different types of opioid peptides and their analogues and also their route of administration resulted in different effects upon gonadotropin secretion (Brooks et al., 1986a). For example, [Met]enkephalin inhibited the release of LH in mature male rats when administered systemically (Bruni et al., 1977). However, β-endorphin elicited an increase in LH levels when given via the lateral cerebral ventricle of anaesthetized adult male rats (Takahara et al., 1978). Further investigation into what appears a confusing area is obviously required to clarify the situation, but it has been postulated that these conflicting results could be due to activation of different classes of opioid receptors (Kalra and Gallo, 1983). Overall, the literature favors an inhibitory role for opioid peptides and their analogues in the control of gonadotropin secretion.

Exogenous Opioid Peptides and their Influence upon Gonadotropin Secretion

The effects of exogenous opioids on gonadotropin secretion and other physiological behaviors were well documented long before the discovery of EOP (Szekely, 1982). The earliest convincing evidence that exogenous opioids could influence gonadotropin secretion was produced by Barraclough and Sawyer (1955) who showed that if morphine was given to rats on the afternoon of proestrus, ovulation was inhibited and this was reversed by naloxone (an opioid antagonist) (Packman and Rothchild, 1976). Evidence for a hypothalamic site of action was provided by Cicero et al. (1977) with the observation that morphine did not alter the pituitary content of LH or the response of the pituitary to GnRH. Morphine also affected tonic LH secretion in intact and gonadectomized animals since the drug suppressed LH secretion in the female monkey by reducing the number of LH pulses (Bruni et al., 1977;Ferin et al., 1982).

The use of opioid peptides and their analogues poses problems in the above type of study for the following reasons:

1) One factor to consider when measuring hormone secretion or any other physiological parameter after opioid or opiate agonist injection is whether or not the observed differences were indeed the result of specific activation of opioid receptors. In other words, their specificity for opioid receptors cannot always be guaranteed, especially when used in high doses (Brooks et al., 1986a). This reason justifies the use of an opioid antagonist with concurrent administration of an opioid agonist in order to demonstrate that the effects are specifically opioid receptor-mediated.

2) Modification of opioid peptides to increase their stability and (or) potency may alter their binding specificity to the various subclasses of their receptors. For instance FK33-824, a [Met]-enkaphalin analogue, binds to *mu* receptors rather than *delta* receptors, which are preferred by endogenous or synthetic [Met]-enkephalin (Kosterlitz et al., 1980).

3) Administration of an opioid agonist may influence hormone secretion, but this does not discern whether a physiological role for that agonist or the receptor which it is activating exist (Brooks et al., 1986a). For these reasons, experiments investigating the effects of opioid antagonists on circulating hormone levels are generally favored.

Opioid Antagonists

Some of the earliest evidence that endogenous opioid peptides could participate in the physiological control of gonadotropin secretion was provided by the use of naloxone, an opioid antagonist which acts through receptor competition. Naloxone is relatively unique because it appears to lack any agonist bioactivity even at high dosage (Malven, 1993). Therefore, it is very useful for *in vivo* studies in drug-free subjects. As was previously explained, any parameter that changes as a result of naloxone treatment was likely being modulated by some EOP at the time of naloxone administration.

Naloxone (N-allyl-noroxymorphone-hydrochloride) is generally considered to be a pure antagonist (Blumberg and Dayton, 1974) although it is recognized that at high doses it may show some lack of specificity with regard to opioid receptors (Chang, 1984). Following intravenous administration in man, naloxone has a maximal effect within 20 min, a half-life in plasma of 64 min and duration of action of one to four hours (Gold and Pottash, 1982). Peak brain concentration of naloxone is achieved within 15 min after administration and declines by 50 percent within one hour. This accounts for the rapid onset, but short duration of action by naloxone (Berkowitz, 1976). The action of naloxone in sheep and cattle is also of relatively short duration (Brooks, et al., 1986a).

Although naloxone is relatively specific to opioid receptors and favors the *u* type, it will bind to other receptor subtypes (Chang, 1984). As a result, the particular receptor varieties, and by inference the opioid involved, cannot easily be deduced from such studies. Also, when experimental antagonism of opioid receptors fails to produce a change, the negative results are not definitive (Malven, 1986). It is possible that the opiodergic effect on hormone secretion was so high that the dose of opioid antagonist was insufficient to establish competitive inhibition. Furthermore, it may have been that the route of administration did not result in antagonism of the relevant subtype of opioid receptor in the tissue site where the EOP was acting to modulate the measured hormone (Malven, 1993) or perhaps other factors (endocrine factors) modulate the secretion of the measured hormone and thus overcome the effect of antagonism of opioid receptors on that hormone.

How Opioid Peptides Affect Gonadotropin Secretion

Opioid or opiate inhibition of LH secretion can generally be overridden by injection of GnRH, known to act at the pituitary, indicating that the site of action of opioids is likely within the hypothalamus or through the neurons contained within it (Ferin et al., 1982;Grossman and Rees, 1983). In the human, detailed studies utilizing frequent blood sampling indicated that the increase in LH as a result of naloxone treatment resulted from an increased frequency and amplitude of LH episodes, which is directly related to pulsatile release of GnRH (Quigley and Yen, 1980). In addition naloxone-induced increases in LH have been shown to be blocked by an antagonist to GnRH (Blank and Roberts, 1982). Furthermore, morphine methyl-iodide and naloxone methyl-iodide, compounds which are unable to cross the blood brain barrier, were effective in modulating LH secretion (Brooks et al., 1986a). However, surgical ablation of the median eminence abolished this response (Panerai et al., 1983), lending support for a hypothalamic site of action for opioid control of gonadotropin secretion.

It is possible that endogenous opioids may exert their effect via interaction with other neurotransmitter compounds such as dopamine, serotonin, and(or) catecholamines. The evidence for dopamine involvement in the control of gonadotropin secretion is not conclusive. Most data indicate that opioid inhibition of gonadotropin secretion is unlikely to be mediated via dopamine systems. For instance, in ovariectomized rats pretreated with estradiol and progesterone, injection of a dopamine agonist or antagonist failed to affect basal LH in response to naloxone (Kalra and Simpkins, 1981).

Serotonin is generally considered to be an inhibitory neurotransmitter of gonadotropin secretion, as demonstrated by a reduction in LH secretion following its injection into the third ventricle in ovariectomized rats (Schneider and McCann, 1970). Several studies showed an increase in serotonin turnover in the hypothalamus after opioid treatment (Spampinato et al., 1979). This could be an indication that the inhibitory effect of opioid on gonadotropin secretion is exerted via serotonin. This became evident when Ieiri et al. (1980) showed that naloxone-stimulated LH release in prepubertal female rats was suppressed by prior injection of 5-hydroxytryptophan, which increases the hypothalamic serotonin content. This effect was reversed when hypothalamic serotonin was reduced by parachlorophenylalanine .

A large body of evidence demonstrates that noradrenergic neurons are involved in the control of gonadotropin secretion, generally having a stimulatory effect (McCann, 1983). Morphine, which suppresses LH secretion, reduced the release of noradrenalin from the brain nerve terminals in the rat (Diez-Guerra et al., 1986), and naloxone, which increases LH secretion, increased noradrenaline turnover within the hypothalamus (Rasmussen et al., 1988). This may indicate that opioids modulate gonadotropin secretion with catecholaminergic neurons as the intermediary neurotransmitter. Naloxone increased LH secretion in estrogen-progesterone injected ovariectomized rats. This effect was reversed when noradrenaline was suppressed using an α-adrenergic receptor blocker phenoxybenzamine (Kalra and Simpkine, 1981). Overall, studies involving opioids and their effects upon LH secretion suggest that the interaction between EOP and other neuro-transmitters should not be excluded.

Physiological Significance of EOP

An opioid control mechanism for LH secretion has been implicated during various reproductive states in a wide spectrum of mammals including rats (Bruni et al., 1977), monkeys (Ferin et al., 1982), chimpanzees (Gosselinet et al., 1983), sheep (Malven et al., 1984; Brooks et al., 1986b), and pigs (Barb et al., 1985). In beef heifers, stimulation of EOP receptors with bromazocine (an opioid agonist) decreased plasma LH concentration (Short et al., 1987). Peck et al. (1988) demonstrated a similar effect of morphine on LH secretion in the postpartum cow and steer.

The opioid receptor antagonist naloxone stimulated release of LH in postpartum rats (Sirinathsinghii & Martini, 1984), women (Ried et al., 1981), ewes (Greg et al., 1986), gilts (Barb et al., 1986a,b), and beef cows (Whisnant et al., 1986d; Greg et al., 1986). The intensity of EOP modulation of pulsatile gonadotropin secretion is profoundly influenced by the endocrine state of the animal (Barb et al., 1991). The ability of naloxone to disinhibit serum LH in male and female rodents has been reported to vary with age relative to puberty (Sylvester et al., 1985), time of the day (Blank & Mann, 1981), dietary intake (Dyer et al., 1985), stage of estrous cycle (Piva et al., 1985), and gonadal steroids (Gabriel et al., 1983).

Opioids and gonadal steroid feedback mechanism

According to Haynes et al. (1989) opioidergic control over LH secretion exists throughout the postnatal period but not during the powerful negative feedback of estradiol in sheep. These workers speculated that this negative feedback works through routes not involving opioids. This negative feedback is so strong that it masks any possibility of demonstrating opioid involvement by the use of antagonist drugs. Opioids have been shown to play a part in the modulation of LH secretion during the reproductive cycle of non-farm species. The degree of opioidergic effect, which can be demonstrated by administration of opioid antagonists, seems to be related to the particular steroid milieu prevalent at the time. In the human (Quigley and Yen 1980), naloxone was ineffective during the early follicular phase, when ovarian steroid concentrations in blood are low, but during the late follicular phase and luteal phase LH secretion was increased as a result of naloxone administration. The monkey responded similarly (Van Vuget et al, 1984).

Farm animals also show a opioidergic regulation of LH during the estrous cycle. However, there are differences as to which steroids appear to be important. Also, age relative to puberty and parity could play a role in opioidergic modulation of LH. Malven et al. (1984) reported that a single bolus injection of naloxone increased serum LH concentration only in ewes with elevated serum progesterone concentration. Also, naloxone infusion increased LH pulse frequency during the luteal phase in ewes (Currie and Rawlings, 1989). In contrast, Currie and Rawlings (1987) observed that naloxone infusion increased basal serum LH concentration and LH pulse amplitude but not pulse frequency during the luteal phase in the ewe. Brooks et al. (1986b) demonstrated that naloxone caused a significant increase in LH pulse frequency and amplitude during the follicular phase of the estrous cycle in sheep. Currie and Rawlings (1987) and Whisnant and Goodman (1988), using naloxone and WIN 44, 431-3 (long-acting EOP antagonist), respectively, found a significant increase in LH pulse amplitude during the follicular phase, but this was accompanied by a significant decrease in pulse frequency resulting in a non-significant increase in mean LH concentration. However, the sheep has a short follicular phase and this should be considered when attempting to investigate which specific steroids may play a role with opioid modulation of LH secretion. While plasma progesterone levels are low in the follicular phase of the cycle, it is possible that the residual effects of progesterone may persist during this period (Haynes et al., 1989). The effect of naloxone on pulse frequency and(or) pulse amplitude of LH is important because it may explain in part the site of action of opioid peptides at particular stages of the estrous cycle.

Similar to the ewe, EOP modulation of LH secretion seems to vary with the stage of estrous cycle in the pig and cow. In the sexually mature gilt, naloxone enhanced LH secretion during the luteal phase but not during the early or late follicular phase of the estrous cycle (Barb et al., 1985,1986a). In addition, naloxone failed to elicit an increase in LH secretion in ovariectomized gilts unless progesterone was administered (Nilaver et al., 1979).

Short et al. (1987) observed that the EOP antagonist (quadazocine) increased mean plasma LH concentration in 3-year-old heifers during the follicular phase of the estrous cycle by increasing pulse amplitude. In contrast, LH secretion was not altered by a similar dose of quadazocine during the luteal phase. The authors suggested that a higher dose of the antagonist may be required to evoke LH secretion during the luteal phase compared with the follicular phase of the bovine estrous cycle (Short et al., 1987). Mahmoud et al. (1989) suggested that EOP modulate LH secretion throughout the estrous cycle because naloxone increased LH secretion during both the luteal and follicular phase in yearling heifers. However, this effect, using a similar dose of naloxone, was not demonstrable in multiparous cows. The degree of opioid inhibition may be greater in cows undergoing cyclic activity compared to the postpartum anestrous, state and it is possible that the dose of the opioid antagonist was not sufficient to elicit a response (Chang et al., 1988). Thus, opioids are antagonized by this dosage (1 mg/kg BW) of naloxone in prepuberal and in postpubertal yearling heifers undergoing estrous cycles, but apparently not in the postpartum cycling cow. The cycling cow then differs in this respect from the other investigated species. Mahmoud et al. (1989) suggested that EOP modulation of LH secretion during the estrous cycle is either a parity- or an age-dependent mechanism.

Yang et al. (1988) suggested that the effects of estradiol and progesterone on the GnRH pulse generating system may be mediated via different neural systems, such that progesterone but not estradiol activates the opiodergic system. Nanda et al. (1991) argued that cows under the influence of the negative feedback effects of estrogen or progesterone failed to demonstrate an active EOP modulation of LH release. They suggested that lack of LH response to naloxone in their study may have been due to the fact that opioids are not the main or only active LH modulator at this phase. Also, in ovariectomized dairy cows, naloxone caused an immediate LH pulse above the preceding basal value 15 min after administration (Nanda et al., 1989). Overall, it seems that opioids may modulate LH secretion during different phases of the estrous cycle in farm animals but the degree of opioid inhibition may vary in different species and may differ under the influence of the predominant ovarian steroids.

Postpartum period and EOP

As previously mentioned several studies have demonstrated EOP involvement in controlling gonadotropin secretion during the postpartum period in domestic farm animals. Inability of postpartum animals to generate LH pulse frequencies, perhaps due to inhibition of GnRH release, led investigators to examine the inhibitory role of EOP on gonadotropins, mostly using opioid antagonists. In an experiment with autumn-lambing ewes, naloxone significantly increased LH concentrations when administered between d 10 to 26 postpartum. However, the response was not consistent and declined by d 26 (Gregg et al., 1986). Naloxone treatment increased LH secretion in lactating beef cows (Whisnant et al., 1986b,c,d). It appears that opioid concentration, and hence the opiodergic depression of LH, changes during lactation in ewes and the beef cow. Whisnant et al. (1986c) suggested that the ability of naloxone to increase serum LH concentration changes during the postpartum period because the dose of naloxone (200 mg) which evoked an increase in LH secretion at d 42 postpartum failed to influence LH concentration at d 14 or 28 postpartum. In support of this concept, Whisnant et al. (1986b) discovered that a larger dose of naloxone (400 and 800 mg) was necessary to increase LH secretion in beef cows on d 14 and 28 postpartum than on d 42. It should be noted that there are other possible explanations for the observed differences in these studies which do not directly involve EOP. Factors such as sensitivity of the hypothalamic-pituitary axis to energy balance, estradiol, and or stress are among those factors.

There are other factors that increase the length of the anestrous postpartum interval by suppressing LH concentration in peripheral blood. One of the most important factors is suckling. Recently, much speculation has focused on EOP as factors that might be involved in the inhibitory role of suckling stimulus on LH secretion.

In beef cows at d 35 to 40 postpartum, calf removal for 48 hours increased LH secretion and abolished the response to naloxone (Whisnant et al., 1986a). In contrast, Cross et al. (1987) demonstrated that in cows at d 24 postpartum, there was very little change in LH in response to weaning and both weaned and suckled cows responded to naloxone. In support of this, Rund et al. (1989) reported that naloxone increased LH secretion in intact or ovariectomized suckled and non-suckled postpartum beef cows. However, the magnitude of LH response was variable, with the greatest response occurring in the ovariectomized groups. It was concluded that there is both a suckling stimulus and ovarian component which contributes to EOP inhibition of LH secretion. However, because EOP modulation of LH secretion was apparent in both the absence of ovaries and suckling stimulus, it was hypothesized that the steroid milieu of pregnancy and its effect on EOP modulation of LH secretion persist during the early postpartum period.

Energy balance and EOP

An interaction between dietary energy level and EOP modulation of gonadotropin secretion in postpartum cows may exist because naloxone stimulated LH secretion in cows which were fed a high energy ration but not in cows on restricted energy diet (Holloway et al., 1987). Conner et al. (1990) reported that dietary energy intake prepartum affected preoptic area methionine-enkaphalin and GnRH content, while pituitary LH content was correlated with body condition at parturition.

The mechanism by which the net energy balance (NEB) of the cow can modulate the hypothalamic-hypophysial-gonadal axis is unknown. In dairy cattle, days postpartum to first ovulation was highly correlated with days postpartum to negative NEB (r=.76) (Canfield and Butler, 1990). Days postpartum to first ovulation was also highly correlated with the changes in LH pulse frequency (r=.91). Comparing individual series of plasma LH concentrations prior to and following NEB nadir, the LH frequency increased, the LH baseline increased, mean LH increased and LH pulse amplitude declined. These changes associated with negative NEB nadir appear to be removed with increasing days postpartum. It is suggested that NEB plays an integral role in the resumption of ovulatory ovarian cycles in postpartum dairy cows. This argues strongly that the changes in LH secretion are related to changing energy balance status and are not strictly a phenomenon of advancing days postpartum (Canfield and Butler 1990). These workers proposed that some factors representative of the animals metabolic state, either centrally or peripherally, could act as a signal of NEB status to the hypothalamus or higher neuroendocrine control centers. In this study the high within-animal correlations between non-esterified fatty acids (NEFA) and NEB suggest that this metabolite may provide one such potentially important signal. Additionally, NEFA can change rapidly in plasma (Bauman et al., 1988) and is able to penetrate the blood brain barrier (Mcllawin and Bachelard, 1985). However, the factor(s) by which NEB status is conveyed to the brain and how it may regulate the function of the hypothalamic-gonadal axis is unclear.

The action of ovarian steroids on the LH pulse generator appears to be a manifestation of EOP inhibition of GnRH secretion and this inhibition is lost with age and(or) parity in beef cows (Whisnant et al., 1986b,c). In addition to the suckling stimulus, other EOP mediated feed back mechanisms may be involved with modulating LH secretion in the postpartum cow (Barb et al. 1991).

Webb et al. (1977) and Williams et al. (1982) reported that LH response to GnRH increased until d 20 postpartum in beef cows. Moss et al. (1985) reported that in suckled beef cows, pituitary LH content increased in the early postpartum period and reached levels similar to those of luteal phase cows by d 30 postpartum. Therefore, the change in LH response to naloxone during the postpartum period may in part be related to increased pituitary responsiveness to GnRH.

It has been hypothesized that pulsatile release of LH may be suppressed in the early postpartum period due to decreased frequency of GnRH-pulses secreted by the hypothalamus (Walters et al., 1982). If this hypothesis is correct, prolongation of the postpartum period may be due to an alteration of the mechanism regulating GnRH release. Carruthers et al. (1980) observed no changes in hypothalamic content of GnRH during the postpartum period of suckling and non-suckling beef cattle. When GnRH content in the hypothalamus of postpartum cows was compared with content in normally cycling cows (Nett et al., 1987), content of GnRH in the POA of postpartum cows was over twice that observed in cycling cows, whereas there was three times as much GnRH in the hypothalamus of postpartum cows. This indicates that there are sufficient stores of GnRH to initiate cyclic activity. However, the release of GnRH may be suppressed, presumably by suckling-induced secretion of endogenous opiates (Gregg et al., 1986). Alternatively, the anterior pituitary may not be capable of responding to GnRH stimulus. This seems unlikely based on the number of receptors for GnRH in the anterior pituitary observed during the postpartum period (Nett et al., 1988). Pituitary content of LH remained low through at least d 15 postpartum but increased to levels observed during the estrous cycle by d 30 postpartum in beef cattle (Nett et al., 1987). The decreased pituitary content of LH early in the postpartum period may be a limiting factor in the initiation and maintenance of ovarian activity. However, that may not be the case in dairy cows because release of sufficient LH to induce ovulation can be stimulated by exogenous GnRH in dairy cows prior to d 14 postpartum (Fernandes et al., 1978; Carter et al., 1980). According to these studies, it seems there are adequate stores of LH and sufficient GnRH receptors in the anterior pituitary in postpartum dairy cows. Thus, if naloxone is not effective in increasing LH secretion during the early postpartum period (via the hypothalamus-pituitary axis), it is less likely due to insufficient LH content and GnRH receptors in the pituitary and perhaps other factor(s) modulate LH secretion and opioids are not the only modulator of LH.

If manipulation of LH secretion could be accomplished, it may be possible to shorten the postpartum anestrous period and control the process of ovulation which could be advantageous to commercial livestock farming. However, the mechanisms which control LH secretion during the early postpartum period and during the estrous cycle are not clearly understood and more fundamental research is required. The fact that EOP affect LH secretion in farm animals is important because it implies that they play a physiological role in regulation of LH secretion during the early postpartum period and estrous cycle and thus may be important regulators of reproductive efficiency in dairy cattle.

Experimental Objectives and Rationales

Objective 1

As discussed, LH plays an important role in regulating reproductive activity of dairy cattle and EOP may be involved in LH modulation. Circulating concentrations of LH are low during the early postpartum period in dairy cows (Edgerton and Hafs, 1973), apparently due to a decreased frequency of LH pulses. There is little information available concerning effects of EOP on pituitary LH secretion in dairy cattle. Thus, the first objective of this study was:

To investigate the role of EOP in modulation of LH secretion by characterizing serum LH in response to administration of an opioid antagonist (naloxone) in primiparous and multiparous dairy cows after parturition at days 15 and 28 postpartum.

Objective 2

The means by which the energy balance of the cow may modulate the hypothalamic-hypophysial-gonadal axis is unknown. Also, involvement of EOP modulation of LH secretion at the time of NEB nadir (d 14 to 17) in dairy cattle is not clear. Canfield and Butler (1990) suggested that the NEB of the cow affects days to first ovulation by suppressing LH pulse frequency. Thus, the second objective was:

To examine that EOP may be associated with the suppressing effect of NEB on LH secretion.

Objective 3

Compelling evidence indicates that EOP are involved in LH modulation during the estrous cycle in various species (Barb et al, 1991). These observations suggest that in certain situations (varies with the species) steroid modulation of the LH pulse generator may be mediated via opioid peptides in farm animal (Barb et al., 1991). However, involvement of EOP in mediating the feedback action of progesterone and estradiol on LH in dairy cattle is not clearly understood. Thus, the third objective was:

To determine the effect of naloxone on LH secretion in primiparous and multiparous dairy cattle during both the follicular (negative feedback of estradiol) and luteal phases (negative feed back of progesterone) of the estrous cycle.

To achieve these objectives, four experiments were conducted at the Virginia Tech Dairy Facilities, Blacksburg Virginia. The study was started in June of 1993 and finished in December of 1993.

Materials and Methods

Experiment 1

Animals. Twelve primiparous Holstein cows between 24 and 31 months of age (mean BW = 474 ± 46 kg) and 12 multiparous Holstein cows between 3 and 8 years of age (mean BW = 594 ± 54 kg) were acquired from the university herd. On the day of parturition all cows were weighed. At 5 d postpartum, all cows were housed in free stalls and fed total mixed ration via the pinpointer feeder in order to measure feed intake. One wk after parturition cows were weighed immediately after the afternoon milking and a 10 ml coccygeal blood sample was collected. This procedure was repeated weekly at the same time until the end of the fourth experiment and blood was subsequently analyzed for progesterone and NEFA content. In addition, milk yield data were obtained from the computerized milk meter and recorded on a daily basis. Daily feed consumption was measured starting 1 wk postpartum and continued until the end of the second experiment (d 28 postpartum). Ovarian activity of all cows was monitored weekly using a real time ultrasound scanner and rectal palpation beginning the second wk after parturition (one d prior to the first experiment). Furthermore, visual observation for estrus was conducted at least 3 times daily beginning on d 10 postpartum. Also, all cows were fitted with a K-mar^{R} heat detection device to aid in detection of estrus.

On the day prior to the experiments all cows were weighed, a blood sample was collected to be analyzed for progesterone concentration, and the ovaries were examined by ultrasound scanner. On d 15 (± 1) postpartum cows were randomly assigned to receive either saline (n=12) or 1 mg/kg naloxone (n=12). On the day of experiment all cows were fitted with jugular catheters at 0530. At 0930 serial blood collection at 15 min intervals was initiated. At 1130, naloxone (1 mg/kg BW) or saline (10 ml) was administered and serial blood sampling was continued until 1400. After the 1400 collection, 3.0 μg GnRH was administered i.v. and blood was collected for another hour at 15 min intervals. This low dose of GnRH was administered to test the pituitary competency to release of LH. At 1500 h, after the last blood sample was obtained, a 10 ml blood sample was collected from the tail vein for progesterone analysis. During blood sampling all cows were tied in individual stalls with access to the mixed ration ad libitum.

Experiment 2.

Fourteen primiparous Holstein cows (mean BW = 469 $\pm$ 41 kg) and 13 multiparous Holstein cows (mean BW = 580 $\pm$ 53 kg) were used for this experiment. Twenty-four of these cows were the same ones which were used in the first experiment. The 3 additional cows received a sham injection on d 15 postpartum and were housed and fed exactly as the other experimental cows.

On d 28 ($\pm$ 1) postpartum, cows were randomly assigned to receive either saline (n=15) or naloxone (1 mg/kg BW) (n=12). On the day prior to the experiment cows were weighed, a blood sample collected to be analyzed for progesterone, and the ovaries examined using an ultrasound scanner. This examination indicated the absence or presence of prominent ovarian structures. On the day of the experiment, all cows were fitted with jugular catheters as in experiment 1 and blood sampling was initiated as described previously. Once again, 2.5 h after saline or naloxone administration, 3.0 μg GnRH was administered i.v. and blood was collected for another hour. After the last blood sample was collected, a 10 ml blood sample was obtained for progesterone analysis.

Experiment 3.

As mentioned previously, ovarian activity of all cows was monitored using an ultrasound device and rectal palpation throughout the early postpartum period. Following the d-28 trial (experiment 2) and upon detection of a corpus luteum, 25 mg of prostaglandin ($PGF_{2\alpha}$) (Lutalyse, The Upjohn Co. Kalamazoo, MI) was administered i.m. to achieve luteolysis. The cows which exhibited estrus or developed a new corpus luteum after the injection of $PGF_{2\alpha}$ became the experimental subjects. Cows which did not exhibit estrus were examined by ultrasound 96 hours post $PGF_{2\alpha}$ injection to observe whether the corpus luteum had regressed. Two multiparous cows were eliminated from the study due to complications not related to the treatment. As a result, 12 primiparous and 10 multiparous cyclic Holstein cows, 53 $\pm$ 12 d postpartum, were used for this experiment. Cows were randomly assigned to receive either saline (n=11) or 1 mg/kg naloxone (n=11) to determine the effect of naloxone on LH secretion in the luteal phase of the estrous cycle. One day prior to the initial experiment all cows were weighed and a blood sample (10 ml) was obtained for progesterone analysis. The mean BW was 486 kg ($\pm$ 32) and 604 ($\pm$ 51) kg for primi- and multiparous cows, respectively. On d 14 post-$PGF_{2\alpha}$ injection or d 11 post-estrus, (mid luteal phase) the cows were fitted with jugular catheters and serial blood sampling was resumed using the same method described in experiment 1. At 1400 h, 3 μg GnRH was injected to test the competency of the pituitary for LH release.

Experiment 4.

Following experiment 3, at 2130 h a second dose of $PGF_{2\alpha}$ was administered to the same cows. Thirty-six h post-$PGF_{2\alpha}$ (early follicular phase), when the level of serum progesterone presumably has declined and estradiol concentration is elevated but has not yet peaked (relative to 15 hours prior to estrus; Nanda et al., 1991), serial blood collection was conducted. Once again, all cows were randomly assigned to receive either saline (n=11) or 1 mg/kg naloxone (n=11) i.v. On the day of the experiment, cows were placed in a stanchion and jugular catheters were inserted at 0530. Jugular blood samples were collected starting at 0930 at 15-min intervals for 2 h pre-naloxone or saline injection and 2.5 h post-naloxone or saline. At 1400, 3 μg GnRH was injected to test the competency of the pituitary for GnRH receptors. Estrous activity in response to the second dose of $PGF_{2\alpha}$ was monitored 3 times daily for 120 h post-injection. At that time the experiment was terminated.

Experimental procedures

Treatment and Blood Collection Procedures

All cows were randomly assigned to either a control or treatment group. Treatment group cows received naloxone hydrochloride (Sigma Chemical Corp., St.

Louis, MO)(1 mg/kg BW) and control group cows received 0.9% saline (10 ml) via a jugular catheter. Naloxone was dissolved in 10 ml sterile 0.9%-saline prior to administration.

On the day of all blood collection trials, using aseptic procedure, an indwelling jugular cannula was inserted in at 0530. Clotting of blood in cannulation tubing was prevented by infusion of a 3.5% citrate solution after cannulation and immediately after each blood sample was drawn. External tubing with a sample port was securely taped to the side of each cow's neck for easy blood withdrawal. Four hours post- cannulation, serial blood collection at 15 min intervals was initiated. After 2 h serial blood collection, at 1130 immediately after blood withdrawal, naloxone or saline was injected via jugular cannula ports and blood collection was continued for another 2.5 h at 15 min intervals. At 1400, 3.0 μg GnRH (cystorelin, Sanofi Animal Health, Inc. Overland Park, KS) was administered via the cannula port to all cows and blood samples were collected for another hour. The GnRH was administered in all experiments. In each sampling interval, the initial 2 ml of each sample was discarded, and a 10 ml sample then was transferred into collecting tubes. Blood samples were placed gently into the 13 ml collection tubes and were immediately placed in ice. Blood samples were then stored at 4°C for 24 h to allow time to clot. All samples were centrifuged for 35 min at 2,200 $\times$ g. Serum was harvested and stored at -20°C until assayed for LH content.

A weekly blood sample (10 ml) was collected via the tail vein by hypodermic syringe to be analyzed for progesterone and non-esterified fatty acid (NEFA) content. Serum samples were collected and placed into two aliquots. These serum samples were frozen at -20°C and -70°C until assayed for progesterone and NEFA content, respectively. Also, a 10 ml blood sample was obtained via the tail vein at the end of each experiment. These blood samples were transferred to the collection tube and placed in ice immediately after withdrawal. The blood samples were stored at 4°C for 24 h and then centrifuged. Serum was harvested and frozen as described previously.

Feed Intake and Energy Balance Estimation

All cows were fed a high energy total mixed ration via the pinpointer starting at parturition and continuing throughout the experiments feeder. Typical ingredients and chemical composition of the diet are presented in Table 1. A representative feed sample was taken from the ration weekly and nutrient content of the feed was determined by the Forage Test Laboratory at Virginia Tech. Milk fat test data were obtained from the Dairy Herd Improvement (DHI) Laboratory. Milk yield, BW, and milk fat were determined daily, weekly, and monthly, respectively. Although feed intake and milk yield were measured daily, only feed intake and milk yield for the 4 d prior to experiment 1 and 2 were used to calculate means for each experiment. Milk yield, milk fat content, mean daily feed intake, BW, and estimated net energy for lactation of the feed (obtained from 4 feed samples acquired while cows were fed via the pinpointer) were used to determine NEB of the cows during the trial. Net energy balance (Mcal) was determined via the following equations (NRC, 1989):

$NE_{consumed} = NE_{Feed} \times DMI$

$NE_{lactation} = .74\ [Milk_{kg} \times (0.4) + (milk\ fat_{kg} \times 15)]$

$NE_{required} = BW^{.75}(.08) \times X_i^{*} + NE_{lactation}$

$\text{Energy Balance (EB)} = NE_{consumed} - NE_{required}$

* Xi= 1.2 for 1st lactation, 1.1 for 2nd lactation , and 1 for others cows.

In addition, serum NEFA was measured weekly so that its relation to NEB status could be evaluated. Energy balance and irreversible loss rate of NEFA are highly correlated and serum NEFA concentrations can change rapidly during the first two months postpartum making them potential signals of change in NEB status (Canfield and Butler, 1990).

Hormone Determinations

Luteinizing Hormone. Concentration of LH was determined as described by Gutrie and Bolt (1983). Briefly, the purified LH (USDA-bLH-B-6) and serum samples in assay buffer (total volume=500 μl) were incubated with specific antiserum (USDA-309-684p, at a 1:35,000 dilution) for 24 h at room temperature. Next, ^{125}I radio-labeled LH (USDA-bLH-B-6) was added and incubated for an additional 48 h at room temperature. To obtain counts, a second antibody (anti-rabbit gamma globulin serum) at 1:8 dilution was added to all sample tubes and incubated for 48 h at 4^{o}C. The sample tubes then were centrifuged for 45 min at 4^{o}C. To separate bound from free LH, supernatant was poured off, sample tubes drained for 30 min, and then the pellets in the tubes were counted for 1 min in a gamma counter.

The specific antiserum bound 35% of radio-labeled LH in the absence of unlabeled hormone and the sensitivity of the assay was .06 ng/ml reference standard LH and defined as the concentration corresponding to 2 standard deviations less than mean zero dose tubes. All samples were assayed in duplicate, and intra- and interassay CV calculated from serum pools averaged 6.7% and 13.8%, respectively.

Non-esterified fatty acids. Non-esterified fatty acid serum concentration was determined using a commercially available enzymatic assay (Wako, Biochemical Diagnostic ; Brentwood , N.Y.) as modified by method of McCutcheon and Bauman (1986). This assay method utilizes enzymatic activation of NEFA to Acetyl Coenzyme

A. Acetyl Coenzyme A is then oxidized to produced hydrogen peroxide, which in the presence of peroxidase allows the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl-aniline with 4-aminoantipyrine) to form a purple color. The optical density of this color then can be measured with colorometric system with absorption maximum at 540 nm. Hence, the amount of NEFA in the sample can be determined from the optical density. Standard reference was provided as oleic acid. The assay system was modified to increase the number of analyzable samples per kit thus reducing the cost per tube. The volume of reference and unknown samples for the assay was reduced from 50 μl to 25 μl. The color reagents were further diluted by addition of double distilled deionized water (80% and 87% of total volume of color reagent A and B, respectively) to the reagents. Again, the volume of reagents which used in the assay were reduced to 40% of recommended volume. The intra and interassay CV were 4.3% and 4.9%, respectively.

Progesterone. Concentration of progesterone was quantified using solid-phase radioimmunoassay (Diagnostic product corp.;Los Angles, CA). The standard curve ranged from .1 to 20 ng/ml and the intra- and interassay CV were 6% and 5.3%, respectively.

Statistical Analysis

Serum LH data in all experiments were analyzed by least-squares analysis of variance by General Linear Model procedure using Statistical Analysis systems (SAS), SAS Institute, Cary, North Carolina. Blood samples were assigned to one of 4 periods.

Period 1 represented samples collected from 0930 h to 1130 h and was considered the basal period (9 samples); period 2 represented samples that were collected from 1145 to 1200 after naloxone or saline administration and were considered the treatment period (2 samples); period 3 represented samples that were collected from 1215 to 1400; and period 4 represented samples collected after GnRH injection (from 1400 to 1500; post-GnRH period). The experiment was designed to determine the effects of naloxone in 2 periods after administration.

The statistical model included treatment, parity, treatment × parity, cow within treatment × parity, period, period × treatment, period × parity, treatment × parity × period, and period × cow within treatment × parity. Treatment, parity and their interaction were tested using cow within treatment × parity as the error term. The rest of the effects were tested using period × cow within treatment × parity as the error term. When period × treatment effect were found to be significant ($P < .05$), non-orthogonal contrasts were used to compare least squares means for periods 1 vs 2, 1 vs 3, and 2 vs 3, within each treatment. All critical F values to determine significance of contrasts were adjusted using the improved Bonferroni F test (Games, 1977).

To analyze the effect of GnRH on serum LH concentration, the sampling interval was divided into 2 periods. The pre-GnRH period represented the 4 samples collected prior to GnRH administration and the post-GnRH represented 4 samples that were collected after GnRH treatment (period 4). Significant difference between the 2 periods was tested using the period × cow(treatment × parity) mean square as the error term in the same statistical model as above. When period × parity was significant ($P < .05$), non orthogonal contrasts were used to compare least squares means for pre- vs post-GnRH within each parity. Once again , these contrasts were evaluated by improved Bonferroni F test (Games, 1977).

The General Linear Model procedure was used to evaluate the effects of parity and days postpartum on NEB (mean NEB was calculated using NEB during 4 d prior to the experiment 1 and 2) and serum NEFA concentration. The statistical model included parity, cow within parity, day, day $\times$ parity. Effect of parity was tested using cow within parity as the error term. Simple correlation was used to compare mean NEB with corresponding serum NEFA concentrations, milk yield, basal LH concentration, and percent increase LH in response to naloxone.

Table 1. Ingredient and chemical composition of the total mixed ration (TMR)[a].

TMR Ingredient	Percent of TMR dry matter
Corn silage	28.6
Alfalfa silage	30.8
High-moisture corn	25.8
Blood meal	2.4
Soybean meal	7.3
Fat	2.7
mineral	2.4
Chemical composition	
DM	52.70
CP	17.38
ADF	20.05
NEL, Mcal/kg of DM	1.61

[a] Values based on mean of samples collected during the period that cows were fed via pinpointer feeder.

EXPERIMENT 1

The Effect of Naloxone on Luteinizing Hormone Secretion on Day 15 Postpartum in Holstein Cows

Results

Ultrasonography indicated absence of a corpus luteum in all cows on the day (d 14) prior to the experiment. Unexpectedly, one cow had high serum progesterone (2.6 ng/ml) on that day. No luteal structure was detected via ultrasound in this cow. All other cows had serum progesterone concentration below 1 ng/ml on the day of the experiment.

Serum LH concentrations increased by approximately 2-fold ($P < .05$) from period 1 to period 2 in the naloxone-treated group and decreased during period 3, but did not return to the basal level (Table 2). Serum LH concentrations did not change significantly between periods in saline-treated cows (Table 2). Serum LH concentrations in period 1 prior to the treatment were $.53 \pm .05$ and $.47 \pm .05$ ng/ml for saline- and naloxone-treated cows, respectively. There was no effect of parity on treatment response and the magnitude of LH response to naloxone was similar between primi- and multiparous cows. Two cows did not show an increase in serum LH secretion after naloxone administration, and the increase in LH secretion in response to naloxone was of a lower magnitude in another cow. However, data from these cows were included in the analysis of the effect of naloxone on LH secretion.

Effect of GnRH on LH secretion differed ($P < .01$) between primi- and multiparous cows. Gonadotropin releasing hormone injection increased ($P < .01$) mean serum LH concentrations during period 4 (post-GnRH) compared to the mean serum values during the pre-GnRH period only in primiparous cows (Table 3). No effect of GnRH was observed in the multiparous cows.

Serum NEFA concentrations and NEB did not differ between primi- and multiparous cows (Table 4). Net energy balance (NEB) of all cows on prior to the experiment ranged from -13.17 to .92 Mcal/d with a mean of -5.4 $\pm$.09 Mcal/d. Serum NEFA concentrations of all cows on the day prior to the experiment ranged from 110 to 490 μEq/L with a mean value of 290 $\pm$ 37 μEq/L. Across all animals, the NEB was negatively correlated with NEFA ($r= -.44$, $P < .05$) and milk yield ($r= -.43$, $P < .05$) (Table 5). A positive relationship was detected between NEFA and % LH ($r= .45$, $P < .15$), but NEFA was not correlated with milk yield, and basal LH concentration. Milk yield, basal LH concentration, and percent increase in LH response to naloxone (% LH) were not significantly correlated (Table 5).

Discussion

Results of this experiment indicate that naloxone (1 mg/kg BW) increased serum LH concentrations in postpartum dairy cows. Bolus injection of naloxone resulted in an increase in LH secretion within the first 45 min post injection. Serum concentration of LH declined to near basal level by 1 h after naloxone (Figure 1) because of the rapid onset, but short duration of action of naloxone. Because naloxone has little known action other than its antagonism of opioid receptors (Sawynok et al., 1979), any effect on hormone secretion after naloxone treatment presumably means that an endogenous opioid modulation of that hormone has been competitively removed (Haynes et al., 1989). As a result, the findings of this study appear to indicate a role for opioid peptides in the suppression of LH secretion in early postpartum dairy cows. Results of this study are in agreement with research in anestrous beef cows (Whisnant et al. 1986c,e), postpartum ewes (Malven and Hudgens, 1987), and suckled sows (Barb et al., 1986b). Also Nanda et al. (1989) reported an increase in LH secretion after naloxone administration in ovariectomized dairy cows. Conversely, results of this study are contradictory to the findings of Canfield and Butler (1991) who observed that infusion of naloxone (50 mg/h) did not change LH pulse frequency and pulse amplitude in early postpartum dairy cows.

The exact physiological mechanism which caused the increase in LH secretion in response to naloxone and the site of inhibitory action of opioids on LH secretion cannot be derived from this study. However, it may be speculated that the observed increase in LH secretion after opioid antagonist (naloxone) injection is due to removal of an inhibitory effect of EOP.

Opioid or opiate inhibition of LH secretion can generally be overridden by injection of GnRH (Ferin et al., 1982;Grossman and Rees, 1983) . These authors showed that opiates did not alter LH response to exogenous GnRH in the monkey and human. This appears to indicate that opiates exert their action at a site other than the pituitary, possibly the hypothalamus and(or) neurons impinging on the hypothalamus (Lakoski and Gabhart, 1981). Moreover, naloxone-induced increase in LH has been shown to be blocked by an antagonist to GnRH (Blank and Roberts, 1982). Surgical ablation of the ME abolished the suppressive effect of morphine on LH secretion lending support to the hypothalamic site of action for opioid control of LH secretion (Panerai et al., 1983). The above observations suggest that opioids modulate GnRH release through a direct action on the ME. However, many of the hypothalamic LHRH neurons in the bovine originate in the POA (Leshin et al., 1988). Endogenous opioid peptides are found throughout the hypothalamus and POA of cattle. The bovine POA also is a region where at least one type of opioid containing neurons (β-endorphin) was found in close association with LHRH perikarya and dendrites (Leshin et al., 1988). In addition the ME contains opioid neurons that terminate in close proximity to LHRH axons and terminals. Results reported by Leshin et al. (1991) indicated that the ME and POA were important sites for opioid modulation of LHRH secretion in cattle because 1) these 2 regions contained neurons that produce LHRH and opioid (at least β-endorphin), 2) opioid receptors were abundant in both regions, and 3) LHRH release from the POA and ME was induced by naloxone.

Results of the above studies, along with the findings of the present experiment suggest that EOP inhibition of LH secretion does exist in dairy cows. Furthermore, the inhibitory effects of EOP upon LH secretion in part may be mediated through specific opioid receptors which suppress GnRH secretion from the hypothalamus. In spite of these conclusions, direct EOP modulation of LH secretion from the pituitary remains a possibility. In the bovine, [Met]-enkephalin reduced the GnRH induced-release of LH from pituitary tissues (Chao et al., 1986). Similarly, in the ovine β-endorphin reduced the GnRH induced release of LH from pituitary cells (Matteri and Moberg 1985).

One of the cows (#646) in the present study, which did not show an increase in LH secretion after naloxone injection (Figure 2) had high serum progesterone concentration (2.6 ng/ml). This was not expected because no corpus luteum or luteal structure was detected via ultrasonography. Progesterone modulates LH secretion during the estrous cycle and is known to have a suppressive effect on LH secretion (Clarke et al., 1982;Goodman, 1978;Malven, 1993). This negative feedback effect of progesterone is prominent during the luteal phase of the estrous cycle when blood progesterone concentration is high. Thus, it is possible that the high serum progesterone level in this cow suppressed LH secretion and overrode the stimulatory effect of naloxone. Alternatively, a higher dose of naloxone might have been needed to enhance LH secretion when high concentrations of gonadal steroid were present. (the role of EOP, naloxone, and their interaction with gonadal steroids to modulate LH secretion will be discussed in experiment 3 and 4).

The LH data from another cow (#859), which did not show an increase in LH secretion after naloxone injection is represented in Figure 2. The blood profile of this cow indicated two consecutive increases in LH concentration (.95 and .9 ng/ml) prior to naloxone administration. These LH peaks were 95% higher than the preceding nadir in LH concentration, and it is possible that the dose of naloxone used may have been unable to further increase LH secretion. Byerley et al. (1992) demonstrated that LH concentrations failed to increase after naloxone only when an LH pulse was observed during the hour before the administration of naloxone. Additionally, when mean LH concentrations were elevated by LH pulse before naloxone, LH concentrations did not increase in response to naloxone until LH decreased to concentrations similar to preceding nadir. Byerley et al. (1992) suggested that a minimum time between LH pulses may be needed before responsiveness to opioid antagonism returns. The lack of response to naloxone in these two cows is less likely due to pituitary responsiveness to GnRH because both cows responded to exogenous GnRH with an increase in LH secretion. (Figure 2)

The blood profile of the third cow (#896) which did not show a profound increase in LH concentration after naloxone is also presented in Figure 2. The basal level of LH in this cow was .46 ng/ml and was similar to mean basal LH concentration of all cows. However, this cow had a milk yield of 49.8 kg/d at the time of the experiment. Mean milk yield for the experimental cows was 29.7 kg/d. Additionally, this cow was in a more severe negative NEB compared to other cows in the study (-13 vs -5.4 Mcal/d). Serum NEFA concentration in this cow was relatively high, which is an indication of high adipose mobilization for energy (Dunshea et al., 1989). Thus, it is likely that this cow was under severe metabolic stress. High blood cortisol concentration is related to stress and it has been shown that high serum level of cortisol inhibits LH secretion (Li and Wagner, 1983). In one study (Whisnant et al., 1986c) a cow which had elevated serum cortisol concentrations did not respond to naloxone. Also, Guillemin et al. (1977) demonstrated that stress resulted in the release of endogenous opioids, ACTH, and glucocorticoids. Hence, it is possible that in this cow the metabolic stress at the time of the experiment caused an increase in the blood cortisol and endogenous opioids, and therefore the dose of naloxone may have been insufficient to overcome the suppressing effect of high EOP in order to enhance LH release.

There is no clear explanation concerning the conflicting results found in the present study and the study by Canfield and Butler (1991). In the latter study, within-cow comparisons detected no significant changes in either LH pulse frequency or pulse amplitude following administration of naloxone (50 mg/h) in either non-lactating or lactating cows on d 7, 10 and 14 postpartum. One difference between the two studies is that in the Canfield and Butler (1991) study, naloxone was infused at the rate of 12.5 mg per 15 min and in our study naloxone was administered as a bolus injection of a relatively high dose. Also, progesterone concentrations in treated cows (unreported) in the Canfield and Butler (1991) study may have influenced LH response to naloxone.

The effect of parity on serum LH response to naloxone and its relationship to NEB and serum NEFA concentrations could not be investigated in this experiment. There were no significant differences in the NEB and serum NEFA concentrations between primiparous and multiparous cows at the time of experiment. Also, both primi- and multiparous cows responded to naloxone with an increase in LH secretion.

The role of NEB and serum NEFA in modulation of naloxone-induced release of LH is not clear from this study. In the present experiment NEB and serum NEFA concentrations had a weak correlation with basal LH concentration suggesting that these two factors may not be the main modulator of LH secretion in early postpartum dairy cattle. Canfield and Butler (1991) showed that in spite of large significant differences in NEB and plasma NEFA concentrations between postpartum lactating and non-lactating cows, LH pulse frequency was not different between the 2 groups on d 7 and 14 postpartum. However, postpartum interval to first ovulation was longer for the lactating cows. These investigations suggested that NEB and NEFA may affect the first postpartum ovulation by alteration of ovarian responsiveness to LH secretion.

A positive relationship between level of serum NEFA and the magnitude of LH response to naloxone was observed in the present study (Table 5). However, this correlation was insignificant. Moreover, there was considerable variation in NEB, serum NEFA concentration, and magnitude of LH response to naloxone. As a result, no conclusion concerning the role of energy balance and NEFA and their relationship to EOP modulation of LH could be drawn.

Unlike primiparous cows (Figure 3), multiparous cows did not respond significantly to exogenous GnRH. In saline-treated multiparous cows, GnRH injection increased LH concentration by more than two-fold (Figure 4), which appears to indicate that the pituitary was responsive to GnRH. A report by Nett et al. (1988) supports this idea based on the number of receptors for GnRH in the anterior pituitary observed during the early postpartum period in cattle. On the other hand, the naloxone-treated multiparous cows showed little increase in LH secretion after GnRH administration

(Figure 4), but this group did experience an increase in LH secretion in response to naloxone. In the case of the naloxone-treated group, perhaps the increase in LH secretion after naloxone injection reduced the LH stores in the pituitary to the extent that exogenous GnRH was not capable of evoking further LH secretion. The low magnitude of response to GnRH in the saline-treated group and lack of response to GnRH in the naloxone-treated group suggests that in the early postpartum period, pituitary content of LH is lower in multiparous cows than in primiparous cows and as a result multiparous cows did not experience a similar response to GnRH compared to primiparous cows. Alternatively, the dose of GnRH (3 μg) used in this study was lower on a bodyweight basis for multiparous cows than for primiparous cows. Thus, it is possible that the dose was insufficient to cause the anterior pituitary to secret LH in the multiparous cow on d 15 postpartum.

In summary, the results of this experiment provide evidence that an opiate antagonist increased LH secretion in both primi- and multiparous dairy cows on d 15 postpartum and this supports the theory that EOP play a part in the control of LH secretion during the early postpartum period. However, no role of either NEB or NEFA in the modulation of naloxone-induced LH release was detected in this study. These data do not provide information in regard to the site or mechanism of action of EOP in modulation of LH, but do support the concept that EOP are capable of suppressing pituitary LH release in early postpartum dairy cattle.

Table 2. Mean serum luteinizing hormone concentrations[1] in Holstein cows treated with either saline or opioid antagonist (naloxone, 1 mg/kg) on day 15 postpartum.

Treatment Group	No. cows	Period[2]	Serum luteinizing hormone concentration (ng/ml)
Saline	12	1	$.53 \pm .05$
		2	$.42 \pm .11$
		3	$.39 \pm .06$
Naloxone	12	1	$.47 \pm .05^{a}$
		2	$.89 \pm .11^{b}$
		3	$.57 \pm .06^{ab}$

[1]. Least squares means, ± SEM.
[2]. Period 1=Mean concentration of 9 blood samples collected before naloxone or saline administration from 0930 to 1130;period 2= Mean concentration of 2 blood samples collected after naloxone or saline administration from 1145 to 1200;period 3=Mean concentration of 8 blood samples collected from 1215 to 1400.
[a,b] Means with different superscripts in the same treatment group differ ($P < 0.05$).

Table 3. Mean serum luteinizing hormone concentrations[1] in primiparous and multiparous cows before and after gonadotropin releasing hormone (GnRH) administration on day 15 postpartum.

Parity	No. cows	Period[2]	Serum luteinizing hormone concentration (ng/ml)
Primiparous	12	Pre-GnRH	$.51 \pm .06^{a}$
		Post-GnRH	$1.68 \pm .06^{b}$
Multiparous	12	Pre-GnRH	$.41 \pm .06$
		Post-GnRH	$.60 \pm .06$

[1]. Least squares means, ± SEM.
[2]. Pre-GnRH = Mean concentration of 4 blood samples collected 1 h prior to GnRH administration;post-GnRH = Mean concentration of 4 blood samples collected 1 h after GnRH administration.
[a,b] Means with different superscripts in the same parity group differ ($P < 0.01$).

Table 4. Mean net energy balance[1] and serum non-esterified fatty acids (NEFA) concentrations[1] in primiparous and multiparous Holstein cows at 15 days postpartum.

Parity	No. cows	Serum NEFA concentration (μEQ/L)	Net energy balance (Mcal/d)
Primiparous	12	291 ± 37	-4.9 ± .9
Multiparous	12	304 ± 37	-5.9 ± .9

[1]. Least squares means ± SEM.

Table 5. Correlation coefficient for the variables net energy balance (NEB), serum non-esterified fatty acids (NEFA) concentration (μEq/L), milk yield, basal serum luteinizing hormone (LH) concentrations (ng/ml), and percent increase luteinizing hormone concentration in response to naloxone (% LH) at 15 days postpartum.

Variables	NEB	NEFA	Milk yield	Basal LH	% LH
NEB		-.44*	-.43*	.10	.01
NEFA			.25	-.12	.45†
Milk yield				-.10	-.13
Basal LH					-.22
% LH					

* $P < .05$
† $P < .15$

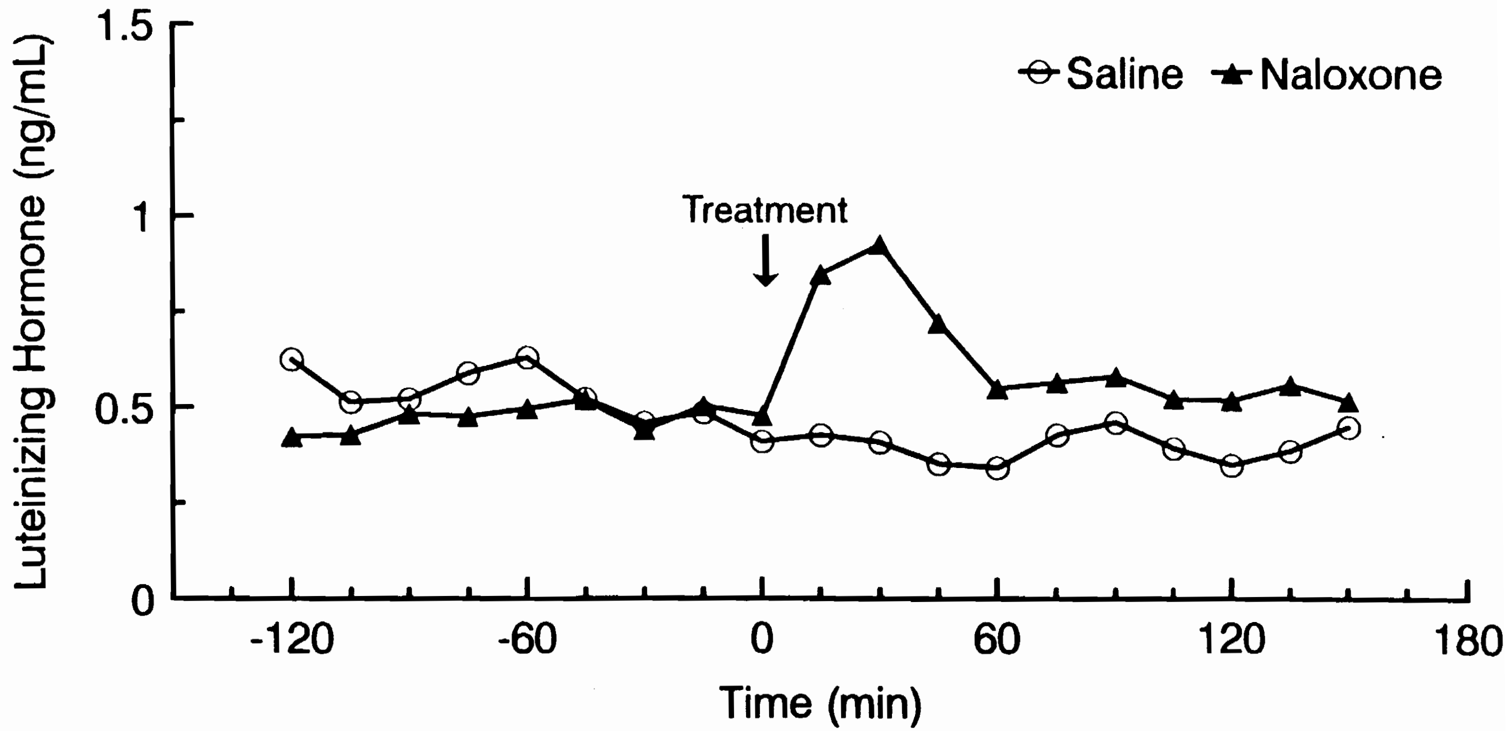

Figure 1. Mean serum luteinizing hormone concentrations in saline- (n=12) and naloxone (n=12) treated Holstein cows at day 15 postpartum. Naloxone (1 mg/kg BW) or saline was administered at time zero.

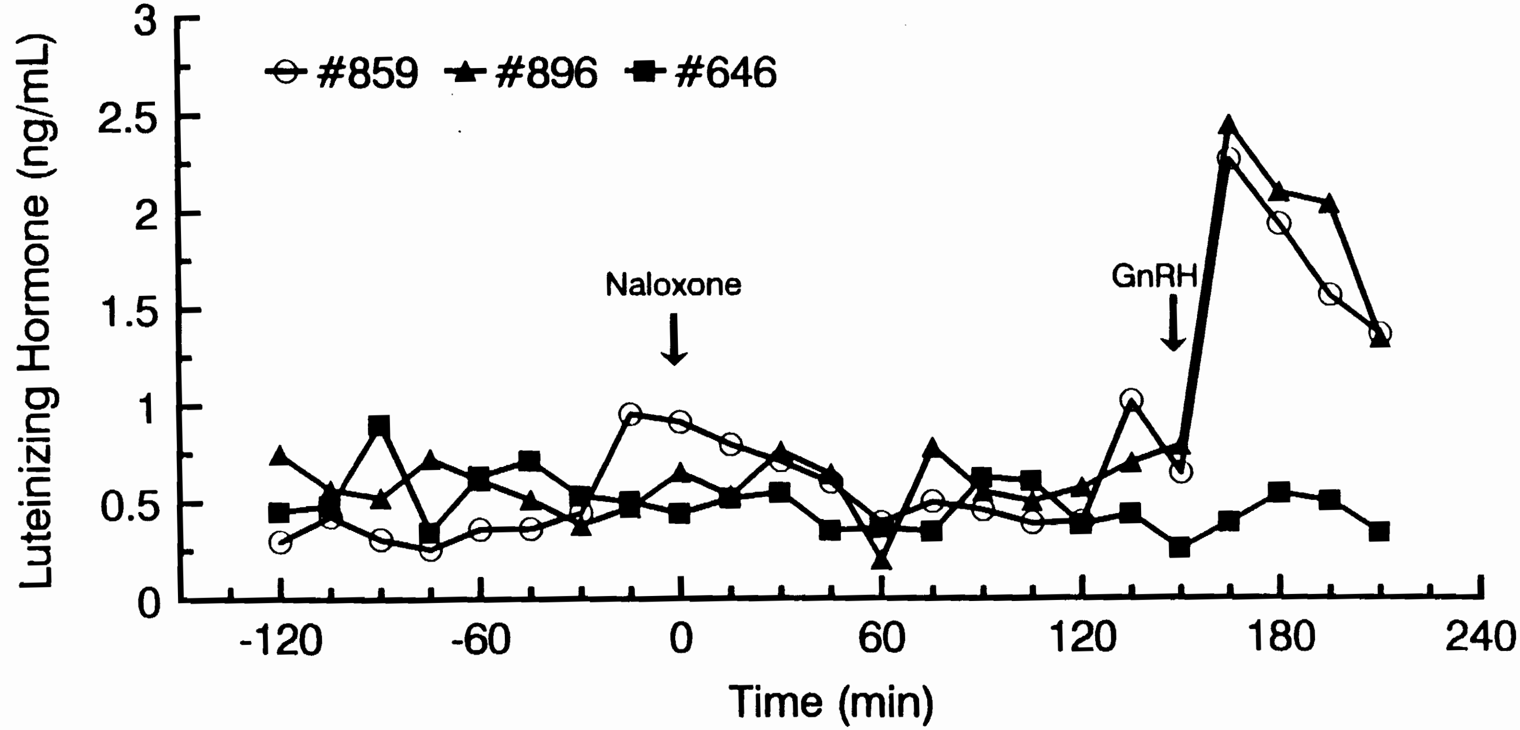

Figure 2. Mean serum luteinizing hormone concentrations in 3 Holstein cows (#859, #896, #646) which did not respond to naloxone (1 mg/kg BW) on day 15 postpartum.

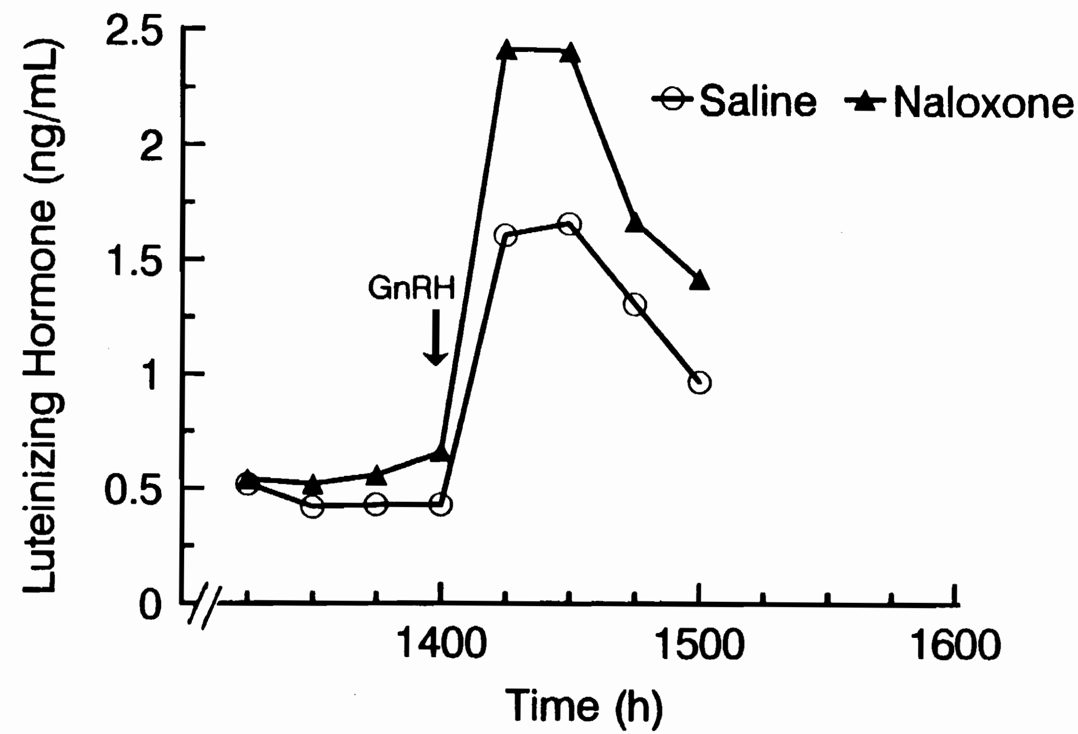

Figure 3. Mean serum luteinizing hormone concentration before and after gonadotropin releasing hormone (GnRH) injection (time=1400) in saline- and naloxone-treated primiparous Holstein cows on day 15 postpartum.

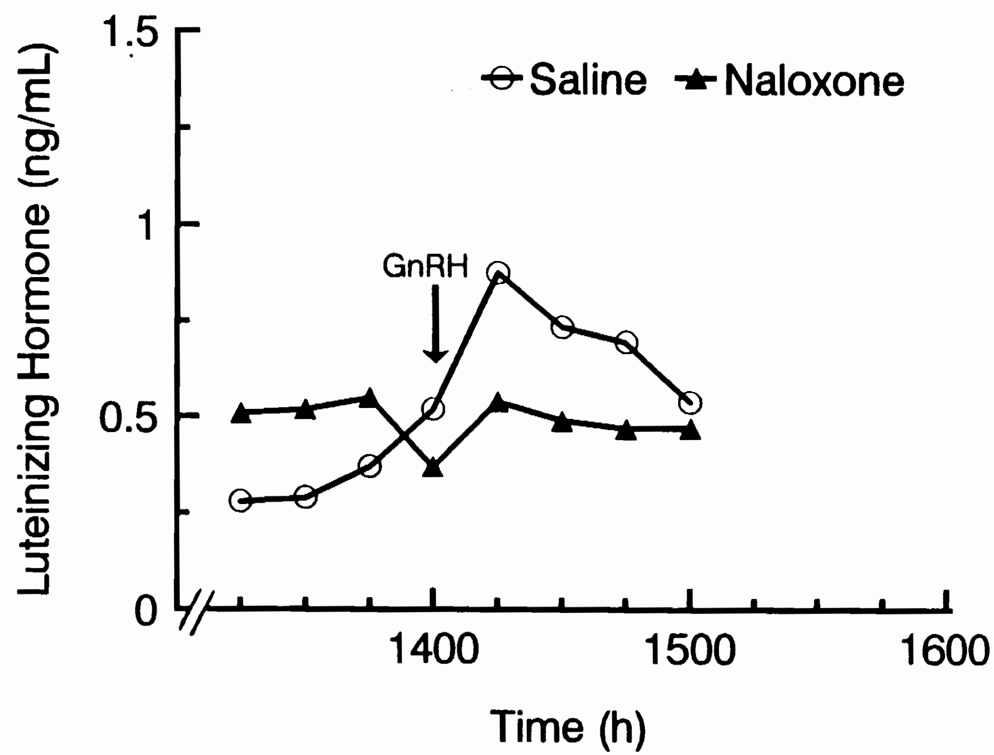

Figure 4. Mean serum luteinizing hormone concentration before and after gonadotropin releasing hormone (GnRH) injection (time=1400) in saline- and naloxone-treated multiparous Holstein cows on day 15 postpartum.

EXPERIMENT 2

The Effect of Naloxone on Luteinizing Hormone Secretion on Day 28 Postpartum in Holstein Cows

Results

Twelve of 27 cows had a corpus luteum on the day prior to the experiment. Serum progesterone concentrations of these twelve cows were greater than 1 ng/ml on the day of the experiment (d 28 postpartum). The remaining 15 cows did not have a detectable corpus luteum and serum progesterone concentrations of these cows were less than 1 ng/ml.

When LH data were analyzed for the effect of naloxone, parity and parity interaction with period and treatment were insignificant. Intravenous administration of naloxone resulted in an elevation of serum LH concentrations from .49 $\pm$ .13 ng/ml in period 1 to .93 $\pm$ .27 ng/ml in period 2 (Table 6). Nevertheless, this increase was not significant perhaps due to variation in basal LH concentrations between the animals. Serum LH concentrations declined to .63 $\pm$ .13 ng/ml during period 3 but did not return to the basal level. Saline injection did not result in any change in serum LH concentrations between the 3 periods (Table 6).

Serum LH concentrations of 4 cows were not affected by naloxone administration. Interestingly, each of these 4 cows had a corpus luteum and their serum progesterone concentrations were greater than 1 ng/ml. Because progesterone plays an important physiological role in the modulation of LH secretion (negative feedback mechanism), the serum LH profiles of all cows were examined based on their serum progesterone level at the time of the experiment. The cows (n=12) with serum progesterone concentrations greater than 1 ng/ml (HP_4) had a mean basal serum LH concentration of .26 ± .28 ng/ml whereas the cows (n=15) with serum progesterone concentrations less than 1 ng/ml (LP_4) had a mean basal serum LH of .64 ± .25 ng/ml. The difference in LH concentrations between these 2 groups approached significance ($P < .1$). In the LP_4 group, administration of naloxone (n=8) resulted in an apparent (non-significant) elevation of mean serum LH concentration from .66 ± .15 ng/ml in period 1, to 1.42 ± .33 in period 2 (Figure 5). Naloxone injection in HP_4 group cows (n=5) caused a small non-significant increase in mean serum LH concentration from .32 ± .06 in period 1, to .45 ± .13 in period 2. Serum LH concentration returned to basal level during period 3 (Figure 6). The increase in LH concentration during period 2 in the naloxone-treated HP_4 group was not as great as that seen during the corresponding period in the LP_4 group (an approximate 40% increase vs. an approximate 115% increase, respectively). Gonadotropin releasing hormone administration caused an increase ($P < .01$) in LH secretion in both saline- and naloxone-treated primiparous cows, but did not increase mean serum LH in multiparous cows (Table 7).

There was no difference in NEB or serum NEFA concentrations between primi- and multiparous cows. Net energy balance and serum NEFA concentrations of all cows on the day prior to the experiment (d 28 postpartum) had mean values of -4.6 ± .4 Mcal/d and 264 ± 21 μEQ/L, respectively (Table 8). Net energy balance tended to be less negative ($P > .1$) and serum NEFA concentrations tended to be lower ($P > .1$) at 4 weeks postpartum compared to 2 weeks postpartum (Table 8).

Discussion

Administration of naloxone did not significantly affect serum LH concentrations at day 28 postpartum in dairy cows (Figure 7). The lack of increase in LH secretion was more evident in cows with high progesterone concentrations than in those with low progesterone concentrations. These results are in agreement with the findings of Canfield and Butler (1991) in dairy cattle, but contrary to findings of Whisnant et al. (1986e) in beef cows. Whisnant et al. (1986b,e) suggested that opioidergic tone changes during the early postpartum period in beef cattle because lower doses of naloxone were required to evoke LH secretion on d 28 compared to d 15. Therefore, in that study it appears that the inhibitory effect of EOP on LH secretion wanes as days postpartum increases. This speculation may be supported by the fact that cows in experiment 1 (d 15 postpartum) had lower mean serum LH concentrations compared to the cows in LP_4 on d 28 (.5 $\pm$ .04 vs .64 $\pm$ .25). Thus, the failure of naloxone to significantly increase serum LH concentrations may be taken as evidence for removal of the opioid inhibition of LH secretion by d 28 postpartum.

The increase in LH secretion after naloxone injection in the HP_4 group was of lower magnitude compared to the LH increase in the LP_4 group (Figures 5 and 6). Progesterone modulates LH in cattle and is known to have a suppressive effect on LH secretion in mammals (Hansen and Convey, 1983). This negative feedback effect of progesterone is prominent during the luteal phase of the estrous cycle when blood progesterone concentration is high. Results of experiment 3, which immediately follows this experiment indicate that naloxone had no effect on LH secretion during the luteal phase of the estrous cycle. Thus, it is possible that in these cows (HP_4) the high progesterone level and its suppressive effect on LH secretion negated the stimulatory effect of naloxone and overrode the suppressing effects of EOP. Alternatively, a higher dose of naloxone might be needed to enhance LH secretion (Brooks et al., 1986b).

When naloxone administration does not alter LH secretion, the conclusions must be more circumspect. Opioid receptors may not be involved in modulation of LH secretion, or effects may be mediated by opioid receptor subtypes not antagonized by the dosage of naloxone used for the experiment. The failure of naloxone to stimulate LH release could also be due to a) insensitivity of pituitary LH cells to GnRH, b) insufficient pituitary stores of releasable LH and (or) c) large temporal variation in serum LH concentration in control animals so that naloxone-induced change is not significant (Malven, 1986).

The lack of significant increase in LH secretion by naloxone is less likely due to an insufficient dose of naloxone being used in this study because a dose of .5 mg/kg BW resulted in a significant increase in LH secretion on d 28 in postpartum beef cows (Whisnant et al. 1986e). Moreover, the same dose of naloxone used in this study (1 mg /kg BW) resulted in a significant increase in LH secretion on d 15 postpartum in these same cows in experiment 1.

Assuming that naloxone exerts its effect on LH secretion via release of GnRH from the hypothalamus and POA (Leshin et al. 1991), it can be argued that lack of significant increase in LH secretion after naloxone administration was due to insufficient quantities of GnRH to stimulate the anterior pituitary gland. However, this hypothesis may be rejected because two studies (Moss et al., 1988;Nett et al., 1988) indicated that in cattle, the hypothalamus and POA contained sufficient amount of GnRH to stimulate LH release from the pituitary gland at 15, 30 and 45 d postpartum.

Pituitary content of LH may be a limiting factor leading to lack of a significant increase in LH secretion by naloxone. However, this may not be the situation in this study because the same dose of naloxone caused a significant increase in LH secretion on d 15 postpartum in these same cows (experiment 1), and it has been shown (Nett et al., 1988) that pituitary content of LH was even less on d 15 postpartum than d 30 postpartum in cattle.

Another factor which may explain the failure of naloxone to induce significant LH release on d 28 postpartum is the possible reduction in sensitivity of the anterior pituitary LH cells to GnRH, which could be caused by a decrease in the number of receptors for GnRH (Nett, 1987). In other words, although GnRH release was induced by naloxone, there were not enough receptors for GnRH in the pituitary to stimulate LH secretion. To investigate this possibility, the competency of the pituitary to GnRH was examined in this experiment by injecting exogenous GnRH (3 μg) 2.5 h after naloxone or saline administration. Results indicated that the primiparous cows responded to this dose of GnRH with a significant increase in LH secretion (Figure 8), thus indicating that the sensitivity of the pituitary gland to GnRH was not reduced. Therefore, at least in the primiparous cows used in this study, the lack of significant increase in LH secretion following naloxone administration is likely not related to insensitivity of the anterior pituitary to GnRH. On the other hand, GnRH treatment had no effect on LH secretion in multiparous cows (Figure 8). Thus, it remains possible that the failure of naloxone to induce LH release in multiparous cows may be due to insensitivity of the anterior pituitary to GnRH.

An interaction between dietary energy level and EOP modulation of LH secretion in postpartum cows may exist because naloxone stimulated LH secretion in cows which were fed a high energy ration but not in cows on restricted energy diet (Holloway et al., 1987). Conversely, Canfield and Butler (1991) observed that naloxone did not affect LH secretion in cattle regardless of NEB. The means by which NEB and(or) NEFA interact with EOP to modulate the LH secretion is unknown. In the present experiment the absence of a significant LH response to naloxone may not be related to the NEB status or serum NEFA concentrations. The fact that naloxone did alter the serum LH concentration on d 15 postpartum (experiment 1) while the NEB and serum NEFA concentrations on that day were similar to d 28 postpartum, support this hypothesis.

In summary, naloxone was ineffective in causing a significant increase in LH secretion in dairy cows on d 28 postpartum. These results may be attributed to either the considerable variation in basal serum LH concentrations among the animals used in this study, so that the increase in LH secretion after naloxone administration was insignificant. However, when cows were separated based on their serum progesterone concentrations at the time of the experiment (d 28), those with low progesterone concentration exhibited an apparent increase in LH concentration after naloxone administration whereas cows with high serum progesterone concentrations did not. In latter group, opioid inhibition of LH secretion may be overridden by the suppressive effect of progesterone. Thus, perhaps the opioid inhibition of LH secretion wanes as days postpartum increases and(or) changes in serum progesterone due to onset of ovarian activity between d 15 to d 28 may play apart in this apparent insensitivity to naloxone-induced LH secretion.

Table 6. Mean serum luteinizing hormone concentrations[1] in Holstein cows treated with either saline or opioid antagonist (naloxone, 1 mg/kg) on day 28 postpartum.

Treatment group	No. cows	Period[2]	Serum luteinizing hormone concentration (ng/ml)
Saline	14	1	$.42 \pm .12$
		2	$.34 \pm .25$
		3	$.35 \pm .13$
Naloxone	13	1	$.49 \pm .13$
		2	$.93 \pm .27$
		3	$.63 \pm .13$

[1]. Least squares means, $\pm$ SEM.
[2]. Period 1=Mean concentration of 9 blood samples collected before naloxone or saline administration from 0930 to 1130;period 2= Mean concentration of 2 blood samples collected after naloxone or saline administration from 1145 to 1200;period 3=Mean concentration of 8 blood samples collected from 1215 to 1400.

Table 7. Mean serum luteinizing hormone concentrations[1] in primiparous and multiparous Holstein cows before and after gonadotropin releasing hormone (GnRH) administration on day 28 postpartum.

Parity	No. cows	Period[2]	Serum luteinizing hormone concentration (ng/ml)
Primiparous	14	Pre-GnRH	$.33 \pm .11^{a}$
		Post-GnRH	$.93 \pm .11^{b}$
Multiparous	13	Pre-GnRH	$.59 \pm .12$
		Post-GnRH	$.64 \pm .12$

[1]. Least squares means, ± SEM.
[2]. Pre-GnRH = Mean concentration of 4 blood samples collected 1 h prior to GnRH administration;post-GnRH = Mean concentration of 4 blood samples collected 1 h after GnRH administration.
[a,b] Means with different superscripts in the same parity group differ ($P < 0.01$).

Table 8. Mean serum non-esterified fatty acids (NEFA) concentration[1] and net energy balance[1] in Holstein cows at 15 and 28 days postpartum.

Days postpartum	No. cows	Serum NEFA concentration (μEQ/L)	Net energy balance (Mcal/d)
Day 15	27	307 $\pm$ 21	-5.6 $\pm$ .4
Day 28	27	264 $\pm$ 21	-4.6 $\pm$ .4

[1]. Least squares means $\pm$ SEM.

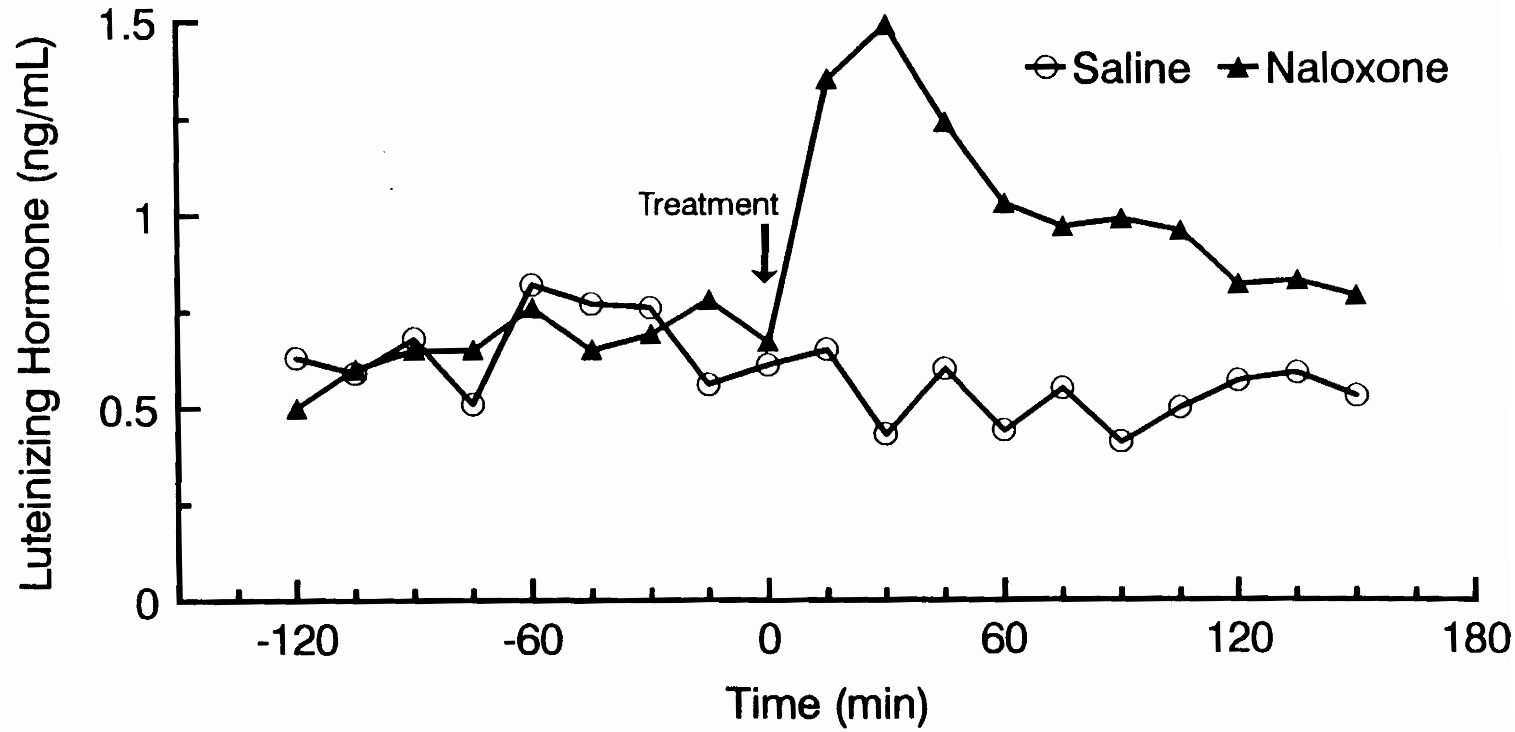

Figure 5. Mean serum luteinizing hormone concentrations in saline- (n=7) and naloxone (n=8) treated Holstein cows with serum progesterone concentrations less than 1 ng/ml on day 28 postpartum. Naloxone (1 mg/kg BW) or saline was administered at time zero.

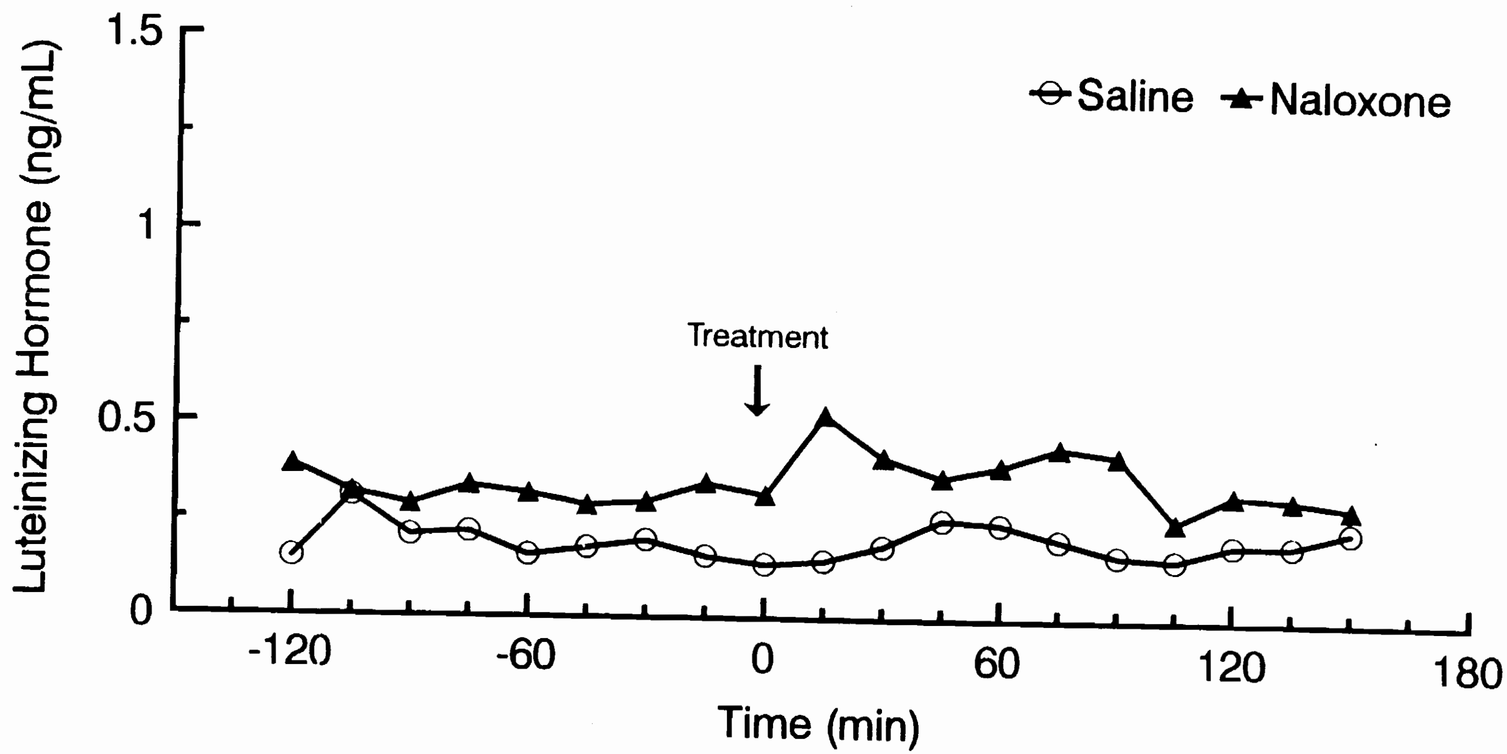

Figure 6. Mean serum luteinizing hormone concentrations in saline- (n=7) and naloxone (n=5) treated Holstein cows with serum progesterone concentrations greater than 1 ng/ml on day 28 postpartum. Naloxone (1 mg/kg BW) or saline was administered at time zero.

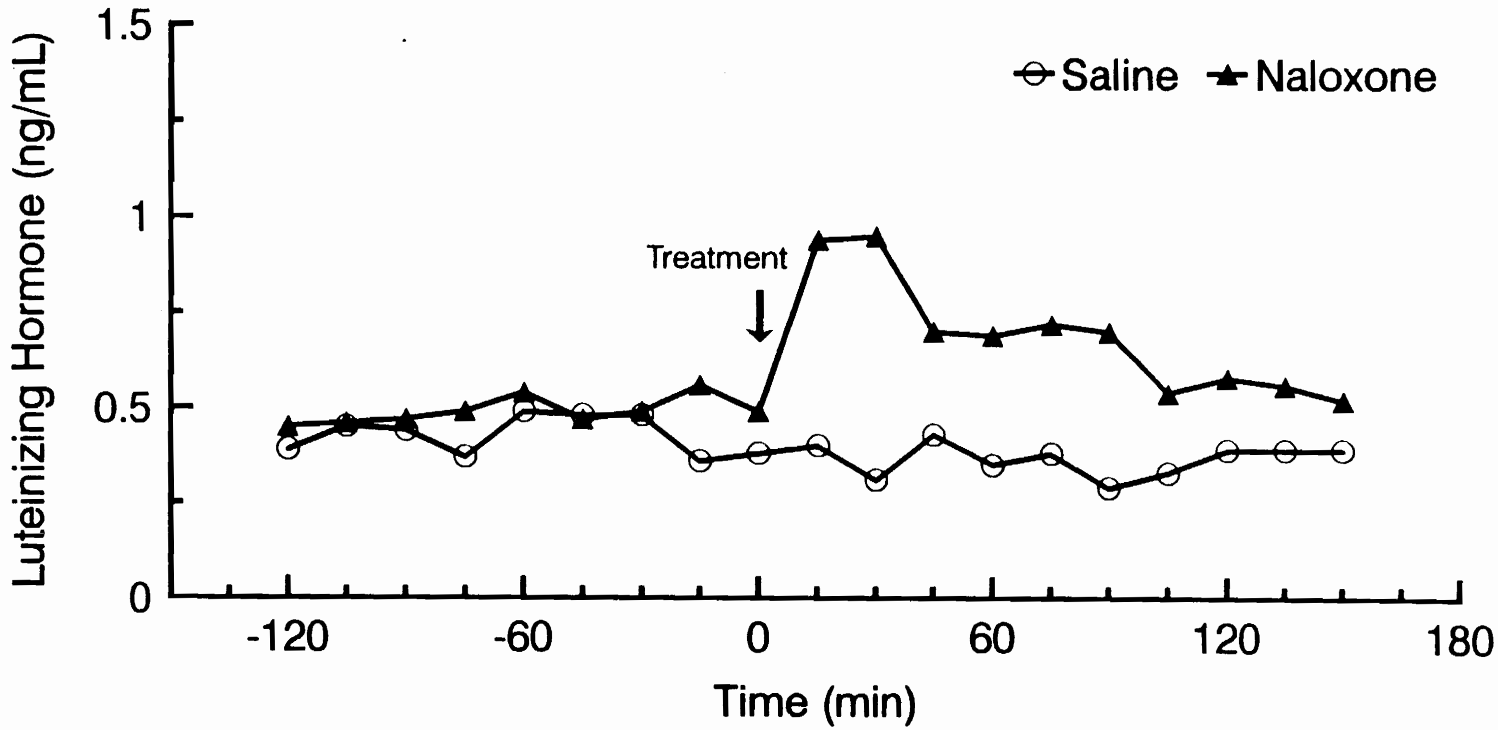

Figure 7. Mean serum luteinizing hormone concentrations in saline- (n=14) and naloxone (n=13) treated Holstein cows on day 28 postpartum. Naloxone (1 mg/kg BW) or saline was administered at time zero.

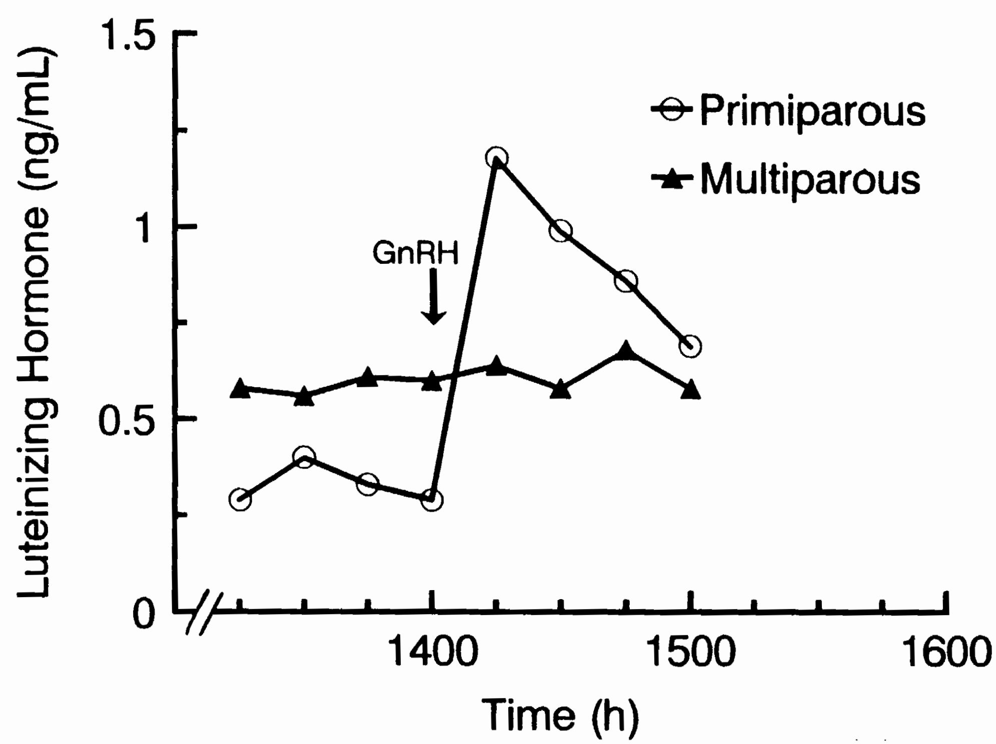

Figure 8. Mean serum luteinizing hormone concentration before and after gonadotropin releasing hormone (GnRH) injection (time=1400) in primi- and multiparous Holstein cows on day 28 postpartum.

EXPERIMENT 3

The Effect of Naloxone on Luteinizing Hormone Secretion in the Luteal Phase of the Estrous Cycle in Holstein Cows

Results

Results of ultrasonography on the day prior to the experiment indicated the presence of a corpus luteum in all cows. As was expected, serum progesterone concentrations of all cows were greater than 1 ng/ml before, and on the day of the experiment (d 10 and d 11 of the estrous cycle, respectively). Mean serum progesterone concentrations were greater ($P < .001$) during the luteal phase than follicular phase (experiment 4) of the estrous cycle and the means were $4.8 \pm .3$ ng/ml and $.5 \pm .3$ ng/ml, respectively (Table 9).

When LH data were analyzed for the effect of naloxone, parity, and parity interaction with period and treatment were insignificant. Mean serum LH concentrations for the three periods in both saline- and naloxone-treated groups are shown in Table 10. Mean basal serum LH concentrations (period 1) were $.18 \pm .02$ ng/ml and $.25 \pm .03$ ng/ml for the saline- and naloxone-treated groups, respectively. In the saline-treated group, serum LH concentrations did not differ in either periods 2 or 3 compared to period 1. In the naloxone-treated group, after naloxone administration, mean serum LH was increased, but not significantly, compared to the basal level (period 1).

Injection of GnRH (3 μg) increased mean LH concentrations in both saline- and naloxone-treated cows in period 4. Mean concentration of LH in period 4 (post-GnRH) was greater ($P < .05$) than during the pre-GnRH period (Table 11). There was no difference between primi- and multiparous cows in response to GnRH injection.

Discussion

There is evidence that both estradiol and progesterone act on the hypothalamus to modify the secretion of GnRH (Goodman, 1978:Clark et al., 1989). The responses to steroids are simple and rapid, but steroid receptors have not been found in GnRH cells (Thiery and Martin, 1991). Therefore, at least one other set of interneurones exist in the brain to mediate the effects of these ovarian hormones. There is evidence that dopaminergic, noradrenergic, serotinergic and opioidergic neurons may be involved. (Dailey et al., 1987). Opioids have been shown to play a part in the modulation of LH secretion during the reproductive cycle of non-farm species and these data provide strong evidence that opioid peptides mediate steroid feed back upon gonadotropin secretion (Quigley and Yen, 1980; Van Vuget et al., 1984). Also, it has been shown (Brooks et al., 1986b) that opioids play a role in LH secretion during different phases of the estrous cycle in the ewe. According to Haynes et al. (1988), the degree of opioidergic inhibition which can be demonstrated by the administration of opioid antagonists appears to be related to the particular steroid level at the time. In other words, the same dose of an opioid antagonist which is able to increase LH secretion in a specific phase of the estrous cycle may not be effective in another phase, because the steroid milieu has changed.

In the present experiment, mean basal serum LH concentration during the luteal phase was lower than during the follicular phase (Table 12). This suggests that cows were under the influence of negative feedback of progesterone. Opioid inhibition of LH secretion during the luteal phase of the estrous cycle of primi- and multiparous cows was not detectable in this study since there was no significant increase in LH secretion after naloxone injection (Figure 9). These findings are in agreement with other reports in multiparous beef cows (Mahmoud et al., 1989), dairy cows (Nanda et al., 1991), and cyclic heifers (Short et al., 1987). This result is contrary to the situation in yearling heifers (Mahmoud et al.,1989), female monkeys (Van Vugt et al., 1983), women (Quigley and Yen, 1980), and sheep (Brooks et al., 1986b) where naloxone was effective in increasing LH concentrations during the luteal phase of the reproductive cycle. Perhaps lactating dairy cows are different from other species in that the progesterone suppression of LH secretion during the luteal phase of the estrous cycle may not be mediated via an opioidergic mechanism (Short et al. 1987), or if that mechanism exists, it may be operating via different opioid receptors which naloxone is not capable of antagonizing. The latter hypothesis may be possible because naloxone has a higher affinity for μ subtype opioid receptors (Malven, 1986). However, in relatively high doses, naloxone binds with other receptors as well. The dose which was used in our study was similar to that used in the study by Mahmoud et al. (1989) and produced a similar result. Furthermore, in the study by Short et al. (1987), which reported similar results to this study, the opioid antagonist was quadazocine. This antagonist binds to at least three opioid receptors, μ, κ and δ (Ward, 1983). As a result, the opioid mediated mechanism for progesterone suppression of LH secretion via a different set of receptors does not appear to be a strong hypothesis.

Mahmoud et al. (1989) found that in the luteal phase of the estrous cycle, naloxone increased LH secretion only in yearling beef heifers and not in multiparous beef cows. Short et al. (1987) used older heifers compared to those that were used by Mahmoud et al., (1989) and observed no differences in LH secretion after administration of an opioid antagonist in the luteal phase of the estrous cycle. Mahmoud et al. (1989) suggested that in the heifer that has been undergoing ovarian cycles for at least several months, and in the cow, opioid inhibition of LH secretion during the luteal phase of the estrous cycle is not apparent. Thus, they suggested that EOP inhibition of LH secretion during the estrous cycle is either a parity- or age-dependent mechanism in this specie. Results of the present study support the hypothesis that opioid inhibition of LH secretion is not apparent during the luteal phase of lactating cow. Moreover, in the present study naloxone did not alter LH concentration in either primi- or multiparous cows. Thus, absence of LH response to naloxone in the luteal phase of the estrous cycle in lactating dairy cows was not dependent on parity.

Although there is no strong evidence from studies done in cows, the possibility that progesterone suppression of LH secretion is mediated via other interneurones seems logical. It has been shown (Deaver et al., 1987) that injection of dopaminergic antagonists during the luteal phase of the estrous cycle in sheep markedly increased LH pulse frequency. Moreover, it is possible that there is an interaction between opioids and other neuropeptides, and that opioids are not the only neuromodulator of LH. Some of the interaction between steroids and dopaminergic systems may also involve the opioidergic neurons (Thiery and Martin, 1991). Both β-endorphin and enkephalin inhibited the secretion of LH, and reduced the stimulatory effects of dopamine in male rats (Rotsztejn et al., 1978). Morphine, which suppresses LH secretion, reduced the release of noradrenalin from the brain nerve terminals in rats (Diez-Guerra et al., 1986) and naloxone, which increases LH secretion, increased noradrenaline turnover within the hypothalamus in rats (Rasmussen et al., 1988).

The lack of increase in LH secretion after naloxone administration could be due to the possibility that during the luteal phase of the estrous cycle opioidergic suppression was so great that the dose of naloxone (1 mg/kg) used in this study was insufficient to establish competitive inhibition. Such a possibility has been proposed by Brooks et al. (1986b), who observed that a high dose of naloxone was necessary to block the effects of opioids upon LH during the luteal phase in ewes, and Short et al. (1987), who found that an opioid antagonist did not increase LH concentration during the luteal phase of the estrous cycle in cyclic heifers. The dose of naloxone used in this study was higher, on a body weight basis, than the dose (approximately .5 mg/kg) administered to dairy cows during the luteal phase in the study by Nanda et al. (1991) which produced similar results to this study. Additionally, in anestrous beef cattle a dose of 0.5 mg/kg BW naloxone was sufficient to produce a significant response in LH secretion at d 42 postpartum (Whisnant et al., 1986b).

The lack of increase in LH secretion after naloxone administration in this study is less likely due to insensitivity of the pituitary LH cells to GnRH because all cows showed an increase in LH secretion after GnRH administration (Figure 10). Failure of naloxone to induce LH release is likely not related to insufficient pituitary stores of LH because Nett (1987) demonstrated that pituitary content of LH is established by d 30 postpartum in cattle.

Results of this study support previous findings and appear to indicate that the mechanisms which control LH secretion during the luteal phase of the estrous cycle in dairy cattle may be mediated via a different neural element and(or) that opioids may not be the only neuromodualtor of LH secretion in this phase of the estrous cycle.

Table 9. Mean serum progesterone concentrations[1] in Holstein cows in the luteal (experiment 3) and follicular phase (experiment 4) of the estrous cycle.

Phase of the estrous cycle	No. cows	Serum progesterone concentration (ng/ml)
Luteal	22	4.8 ± .3[a]
Follicular	20	.5 ± .3[b]

[1]. Least squares means ± SEM.
[a,b] Means differ ($P < .001$).

Table 10. Mean serum luteinizing hormone concentrations[1] in Holstein cows treated with either saline or opioid antagonist (naloxone, 1 mg/kg) in the luteal phase of the estrous cycle.

Treatment group	No. cows	Period[2]	Serum luteinizing hormone concentration (ng/ml)
Saline	11†	1	.18 ± .02
		2	.22 ± .03
		3	.22 ± .03
Naloxone	11†	1	.25 ± .02
		2	.39 ± .03
		3	.33 ± .03

[1]. Least squares means ± SEM.
[2]. Period 1=Mean concentration of 9 blood samples collected before naloxone or saline administration from 0930 to 1130;period 2=Mean concentration of 2 blood samples collected after naloxone or saline administration from 1145 to 1200;period 3=Mean concentration of 8 blood samples collected from 1215 to 1400.
† Primiparous cows (n=6), Multiparous cows (n=5).

Table 11. Mean serum luteinizing hormone concentrations[1] in Holstein cows before and after gonadotropin releasing hormone (GnRH) administration in the luteal phase of the estrous cycle.

Treatment group	No. cows	Period[2]	Serum luteinizing hormone concentration (ng/ml)
Saline	11†	Pre-GnRH	.24 ± .08[a]
		Post-GnRH	.55 ± .08[b]
Naloxone	11†	Pre-GnRH	.32 ± .08[a]
		Post-GnRH	.61 ± .08[b]

[1]. Least squares means, ± SEM.
[2]. Pre-GnRH=Mean concentration of 4 blood samples collected 1 h prior to GnRH administration;post-GnRH=Mean concentration of 4 blood samples collected 1 h after GnRH administration.
a,b Means with different superscripts in the same treatment group differ ($P < 0.05$).
† Primiparous cows (n=6), Multiparous cows (n=5).

Table 12. Mean basal luteinizing hormone concentrations[1] in Holstein cows in the luteal (experiment 3) and follicular phase (experiment 4) of the estrous cycle.

Phase of the estrous cycle	No. cows	Serum luteinizing hormone concentration (ng/ml)
Luteal	22	$.22 \pm .05^{a}$
Follicular	20	$.55 \pm .06^{b}$

[1]. Least squares means $\pm$ SEM.
[a,b] Means differ ($P < .001$).

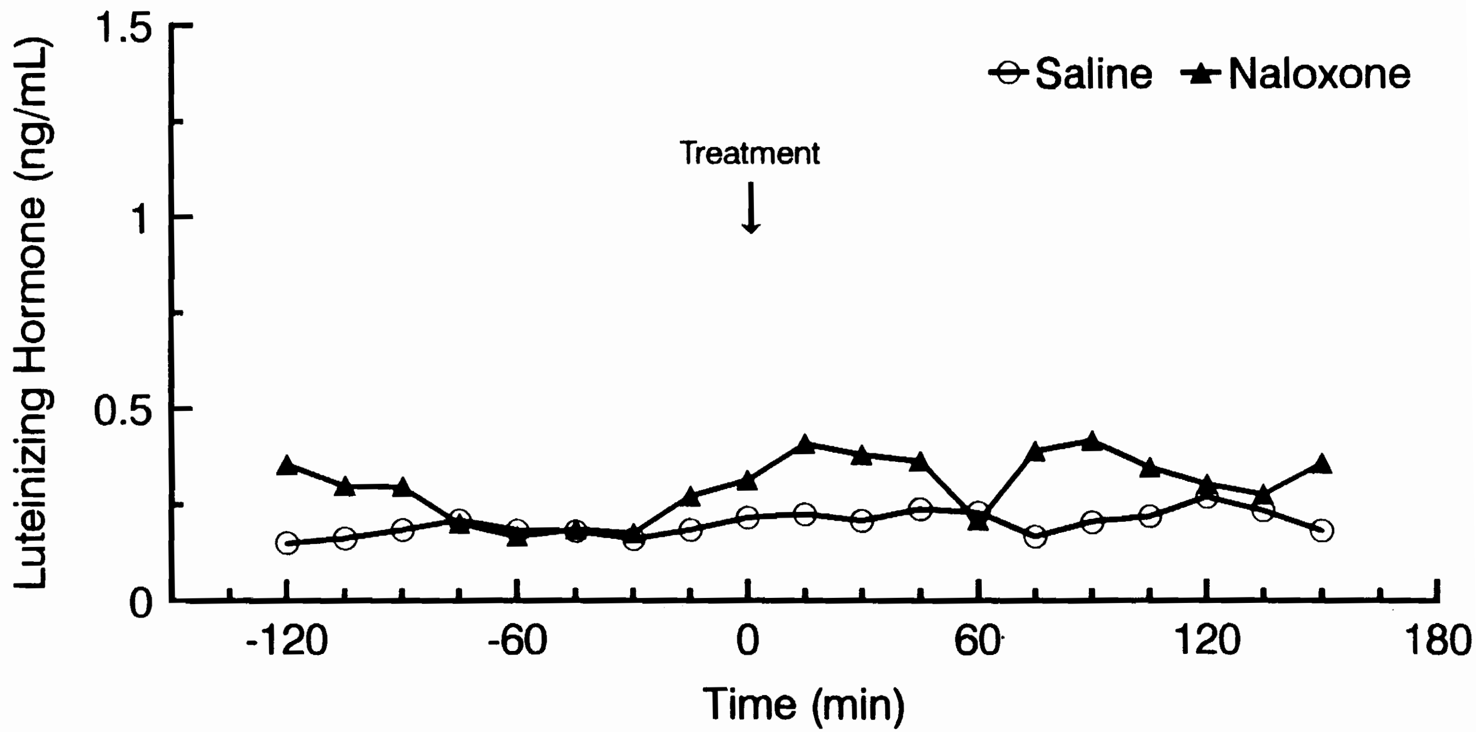

Figure 9. Mean serum luteinizing hormone concentrations in saline- (n=11) or naloxone (n=11) treated Holstein cows during the luteal phase of the estrous cycle. Naloxone (1 mg/kg BW) or saline was administered at time zero.

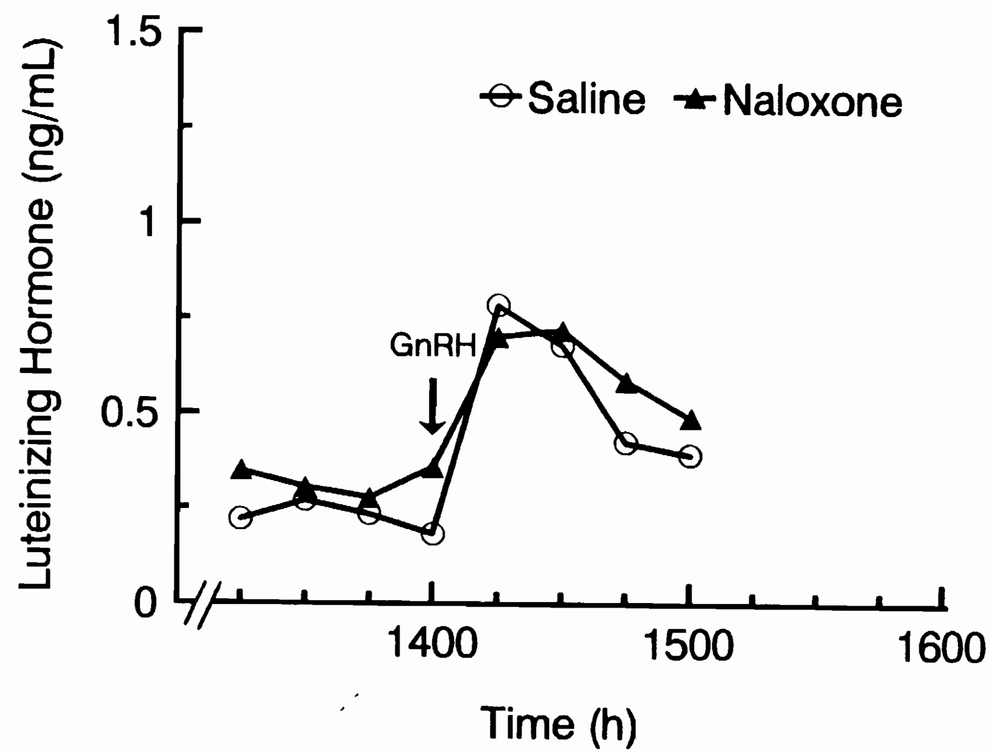

Figure 10. Mean serum luteinizing hormone concentration before and after gonadotropin releasing hormone (GnRH) injection (time=1400) in saline- and naloxone-treated Holstein cows in the luteal phase of the estrous cycle.

Experiment 4

The Effect of Naloxone on Luteinizing Hormone Secretion in the Follicular Phase of the Estrous Cycle in Holstein Cows

Results

On the day of the experiment, 36 h after $PGF_{2\alpha}$ administration , 20 of 22 cows had serum progesterone concentrations of less than 1 ng/ml. The twenty cows with serum progesterone concentration less than 1 ng/ml were considered to be in the follicular phase of the estrous cycle. The two cows that had serum progesterone concentrations greater than 1 ng/ml were considered not to be in the follicular phase and were excluded from the analysis. Serum progesterone concentration of these 20 cows was lower ($P < .001$) in the follicular phase compared to the luteal phase, prior to $PGF_{2\alpha}$ administration (Table 9). These results and the fact that there was no evidence of estrous activity prior to and on the day of the experiment verified that the experiment was conducted in the follicular phase of the estrous cycle. Estrus was observed in 16 of 22 cows at a mean of 80.5 $\pm$ 19 h after administration of $PGF_{2\alpha}$.

Mean basal LH concentration was greater ($P < .001$) during the follicular phase than during the corresponding period (period 1) in luteal phase cows (experiment 3)(Table 12). Mean basal LH concentration did not differ between primi-and multiparous cows.

Naloxone elicited an increase ($P < .05$) in serum LH level during the follicular phase of the estrous cycle and its effect on LH secretion was similar between primi- and multiparous cows. Prior to treatment (during period 1), mean serum LH concentrations were .47 $\pm$ .03 ng/ml and .62 $\pm$ .03 ng/ml for saline- and naloxone-treated groups, respectively (Table 13). In naloxone-treated cows, LH concentrations were increased ($P < .05$) to .87 $\pm$ .06 ng/ml in period 2 (15 and 30 min post-naloxone) compared to a mean value of .62 $\pm$ .03 ng/ml during the basal period (Table 13). In period 3, mean LH concentration returned to .53 $\pm$ .03 ng/ml and was similar to period 1. Serum LH concentrations did not change between the 3 periods in saline-treated cows (Table 13). Although the magnitude of LH response to naloxone was similar for both primi- and multiparous cows, time of response to naloxone was delayed 15 min in first-lactation cows compared to multiparous cows (Figures 11 and 12). One cow (#867) which had elevated serum LH concentration prior to treatment did not respond to naloxone with an increase in serum LH concentration, but the data from this cow was included in the analysis (Figure 13). Administration of GnRH increased serum LH in all cows (Figure 14). Mean LH concentration in period 4 (60 min post-GnRH) was greater as compared to the pre-GnRH period in both the saline- and naloxone-treated group ($P < .05$ and .01, respectively) (Table 14).

Sixteen of 22 cows exhibited estrus by 80.5 $\pm$ 19 h after administration of $PGF_{2\alpha}$ (2130 on the day of experiment 3). Two of the 6 remaining cows were those in which serum progesterone did not decline to below 1 ng/ml after $PGF_{2\alpha}$ injection.

Discussion

The use of $PGF_{2\alpha}$ to achieve luteolysis is a common reliable method. Louis et al. (1974) demonstrated that injection of $PGF_{2\alpha}$ caused a dramatic luteolysis in cows and decreased serum progesterone concentration to 50% of the values measured prior to $PGF_{2\alpha}$ administration within 12 hours. Administration of $PGF_{2\alpha}$ caused a change in the mean hormonal profile such that it was similar to cows during a naturally occurring follicular phase (Rahe et al., 1980). Chenauh et al. (1976) showed that the mean interval between $PGF_{2\alpha}$ injection and the onset of estrus (surge of estradiol) was 74.9 h. This method of luteolysis was also successfully used by Mahmoud et al. (1989).

Low serum progesterone (below 1 ng/ml), increased mean basal serum LH concentration (.55 $\pm$ .04 ng/ml) compared to luteal phase cows (.22 $\pm$ .04), and eventual occurrence of estrus indicated a change from the luteal to the follicular phase of the estrous cycle, and that LH secretion was under minimal effect of progesterone suppression.

Naloxone administration in the early follicular phase of the estrous cycle increased LH secretion in lactating dairy cows (Figure 11 and 12) suggesting that EOP played a role in suppression of LH during this phase of the estrous cycle. Increased release of LH in response to an opioid antagonist during the follicular phase of the cycle has been demonstrated in ewes (Brooks et al., 1986; Yang et al., 1988). Short et al. (1987), and Mahmoud et al. (1989) provided evidence that in heifers, opioid antagonists increased LH secretion in the follicular phase. In the latter study, because only heifers, and not cows, responded to naloxone, the authors suggested that EOP inhibition of LH secretion during this phase of the estrous cycle is a parity or age dependent mechanism in cattle.

Lack of LH response to naloxone has been reported in women in early but not late follicular phase (Quigley and Yen, 1980). Also, Nanda et al. (1991) reported the lack of LH response to naloxone injection alone in dairy cows during strong negative feedback of estradiol (late follicular phase). However, effects of an opioid agonist injection on LH secretion was significant, and the effect was reversed by injection of an opioid antagonist. These authors suggested that although opiates were capable of causing further LH suppression during the negative feedback phase of estradiol, the lack of LH response to naloxone alone might be an indication that opioids are not the main or only active LH modulators at this phase.

There are several points which may explain the lack of LH response to naloxone in the Nanda et al. (1991) study and significant LH response to naloxone in the present experiment. First, in the Nanda et al. (1991) study the dose of naloxone was lower (.5 mg/kg BW) than that used in the present experiment. That lower dose might not be sufficient to disinhibit the effect of opioids. Second, in that study, naloxone was administered 2-15 h before the start of the LH surge and under the strong influence of estradiol, presumably during the late follicular phase. In comparison, the cows in this experiment exhibited estrus at a mean interval of 44 $\pm$ 19 h from the start of blood sampling and the experimental period coincided with the early follicular phase. Alternatively, in that study naloxone was administered relatively close to the time of the pre-ovulatory surge of LH. Perhaps LH pulse frequency and thus overall mean LH concentration had already increased, and thus, naloxone was unable to further elevate serum LH concentrations.

It appears that the effect of opioid changes during lactation and the opiodergic depression of LH in the cow is more dramatic in early postpartum and decreases with time (Barb et al., 1991;Whisnant et al., 1986b). Mahmoud et al. (1989) reported that multiparous beef cows did not respond to naloxone (1 mg/kg BW) in the follicular phase of the estrous cycle. Unlike the present experiment, cows in that study were in mid-lactation. Thus, it is possible that the lack of LH response to naloxone in the follicular phase was due to the fact that opioid becomes less effective in suppressing LH over time.

Effect of naloxone on LH secretion was similar between primi- and multiparous cows in the present experiment. This evidence may suggest that response to naloxone and(or) opioid inhibition of LH secretion is independent of parity or age in lactating dairy cows in the follicular phase of the estrous cycle.

Pre-ovulatory peak of LH starts near the beginning of estrus and LH concentration reaches the highest level in blood within a few hours (Hendricks et al., 1970). Cow (#867), which did not respond to naloxone, had elevated basal LH prior to naloxone injection (Figure 13). This elevated level could not be due to the pre-ovulatory surge of LH because this cow exhibited estrus 48 hours after the experiment. A higher dose of naloxone may be needed to increase LH secretion in cows which have previously had elevated LH. Moreover, the lack of response to naloxone in this cow was likely not due to pituitary insensitiveness to GnRH because exogenous GnRH increased LH secretion in this cow (Figure 13).

As discussed, naloxone evoked LH secretion in the follicular phase but not in the luteal phase of the estrous cycle. The reason for this difference is not clearly understood, but it could be due to a different (i.e. other than opioids) mechanism of control for LH secretion in the luteal phase. Another possibility is that there may be differences in the opioid receptors modulating LH secretion. It has been observed that in whole brain homogenate of female rats, the number of μ type opioid receptors fluctuates during different phases of the estrous cycle (Maggi et al., 1993). This phenomenon may explain the dissimilar magnitude of the effects on LH exerted by naloxone in different phases of the estrous cycle (Maggi et al., 1993). However, Yang et al.(1988) found no differences in hypothalamic opioid receptor concentrations in sheep during either the follicular or the luteal phase of the estrous cycle.

The present experiment was not designed to elucidate the precise mechanisms by which estradiol and specific opioids interact together to modulate LH secretion. Leshin et al. (1991) indicated that POA and ME of the hypothalamus are the potential site of opioid-gonadotropin regulation in heifers and cows because they contain LHRH and opioid (β-endorphin) neurons. Also, opiate receptors are abundant in both regions. It has been shown that the β-endorphin family of opioids have a higher affinity for μ subtype receptors (Malven, 1986) and naloxone has a slightly greater affinity for μ receptors (Chang, 1984;Malven, 1986). Thus, one may speculate that the μ opioid receptor subtype is involved in modulation of LH secretion in the follicular phase in dairy cows.

Nevertheless, it is clear from these studies that, while opioid antagonist alone is not capable of overcoming the suppressive effects of progesterone on LH secretion during the luteal phase of the estrous cycle in dairy cattle, this is not the case during the follicular phase. Thus, it appears that in the absence of an active corpus luteum, EOP are at least partly involved in the modulation of LH release in postpartum dairy cows.

Table 13. Mean serum luteinizing hormone concentrations[1] in Holstein cows treated with either saline or opioid antagonist (naloxone, 1 mg/kg) in the follicular phase of the estrous cycle.

Treatment group	No. cows	Period[2]	Serum luteinizing hormone concentration (ng/ml)
Saline	9†	1	$.47 \pm .03$
		2	$.40 \pm .07$
		3	$.42 \pm .04$
Naloxone	11‡	1	$.62 \pm .03^{a}$
		2	$.87 \pm .06^{b}$
		3	$.53 \pm .03^{a}$

[1]. Least squares means, ± SEM.
[2]. Period 1=Mean concentration of 9 blood samples collected before naloxone or saline administration from 0930 to 1130;period 2=mean concentration of 2 blood samples collected after naloxone or saline administration from 1145 to 1200;period 3=Mean concentration of 8 blood samples collected from 1215 to 1400.
† Primiparous cows (n=6), Multiparous cows (n=3).
‡ Primiparous cows (n=6), Multiparous cows (n=5).
[a,b] means with different superscripts in the same treatment differ ($P < .05$).

Table 14. Mean serum luteinizing hormone concentrations[1] in Holstein cows before and after gonadotropin releasing hormone (GnRH) administration in the follicular phase of the estrous cycle.

Treatment group	No. cows	Period[2]	Serum luteinizing hormone concentration (ng/ml)
Saline	9†	Pre-GnRH	$.39 \pm .16^{a}$
		Post-GnRH	$.86 \pm .16^{b}$
Naloxone	11‡	Pre-GnRH	$.51 \pm .14^{c}$
		Post-GnRH	$1.15 \pm .14^{d}$

[1]. Least squares means, ± SEM.
[2]. Pre-GnRH = Mean concentration of 4 blood samples collected 1 h prior to GnRH administration;post-GnRH = Mean concentration of 4 blood samples collected 1 h after GnRH administration.
[a,b] Means with different superscripts in same treatment group differ ($P < .05$).
[c,d] Means with different superscripts in the same treatment group differ ($P < .01$).
† Primiparous cows (n=6), Multiparous cows (n=3).
‡ Primiparous cows (n=6), Multiparous cows (n=5).

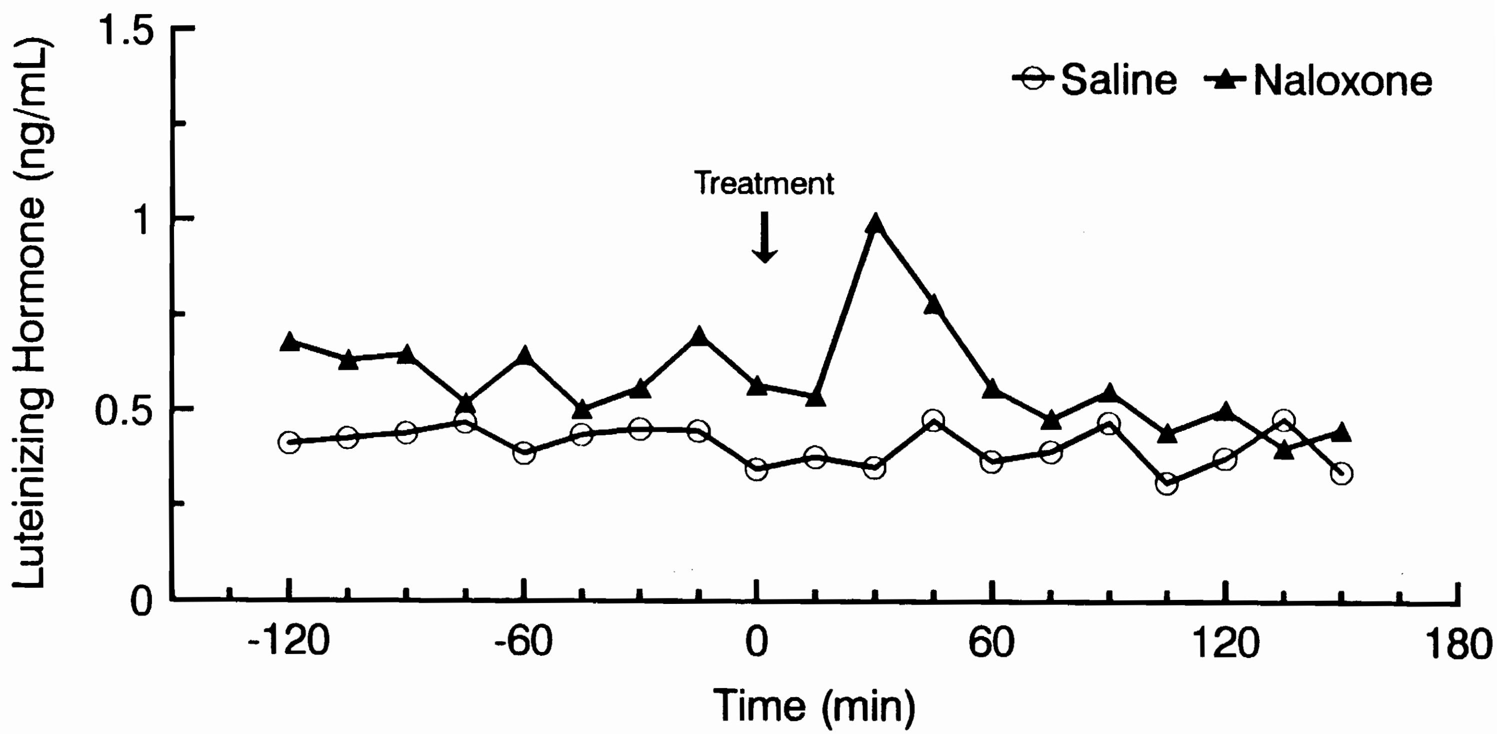

Figure 11. Mean serum luteinizing hormone concentrations in saline- (n=6) and naloxone (n=6) treated primiparous Holstein cows in the follicular phase of the estrous cycle. Naloxone (1 mg/kg BW) or saline was administered at time zero.

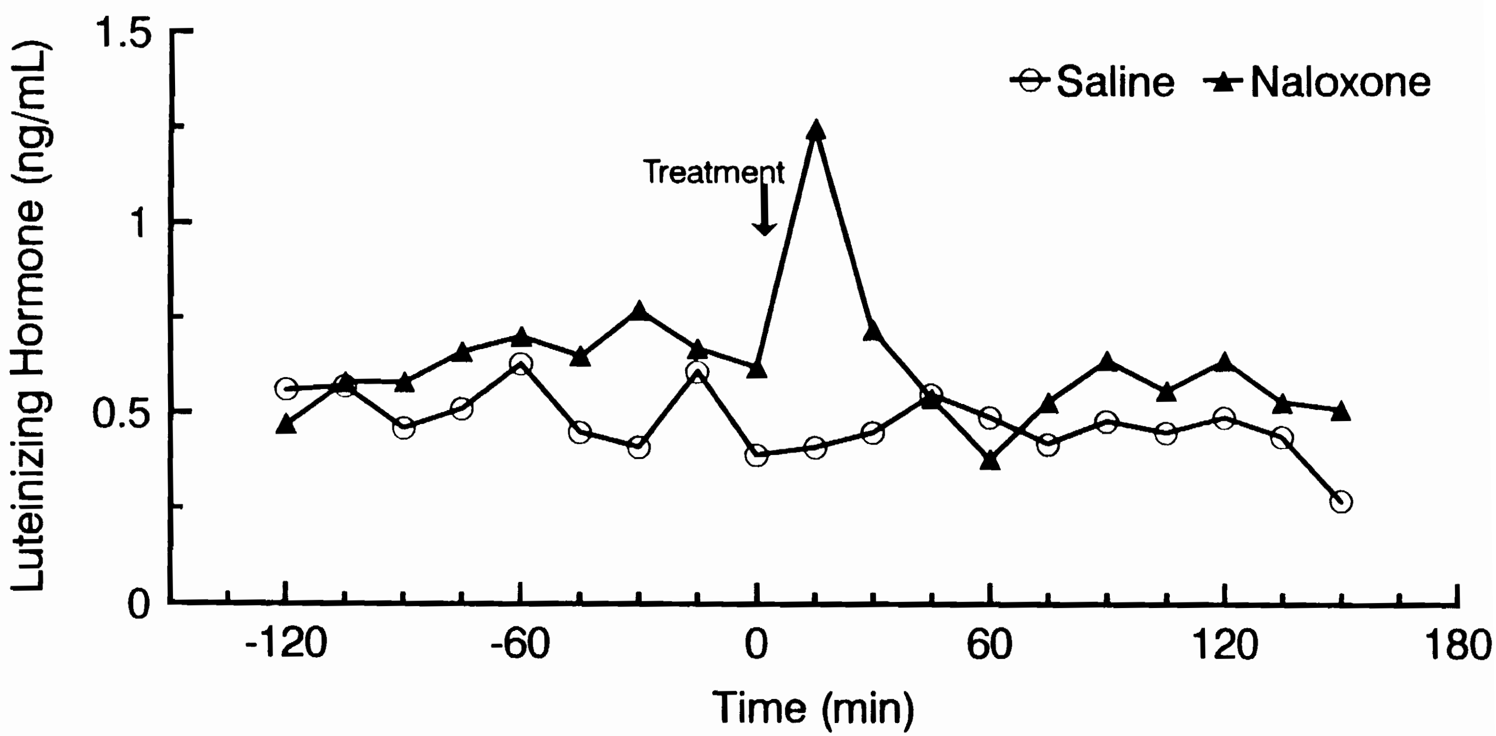

Figure 12. Mean serum luteinizing hormone concentrations in saline- (n=3) and naloxone (n=5) treated multiparous Holstein cows in the follicular phase of the estrous cycle. Naloxone (1 mg/kg BW) or saline was administered at time zero.

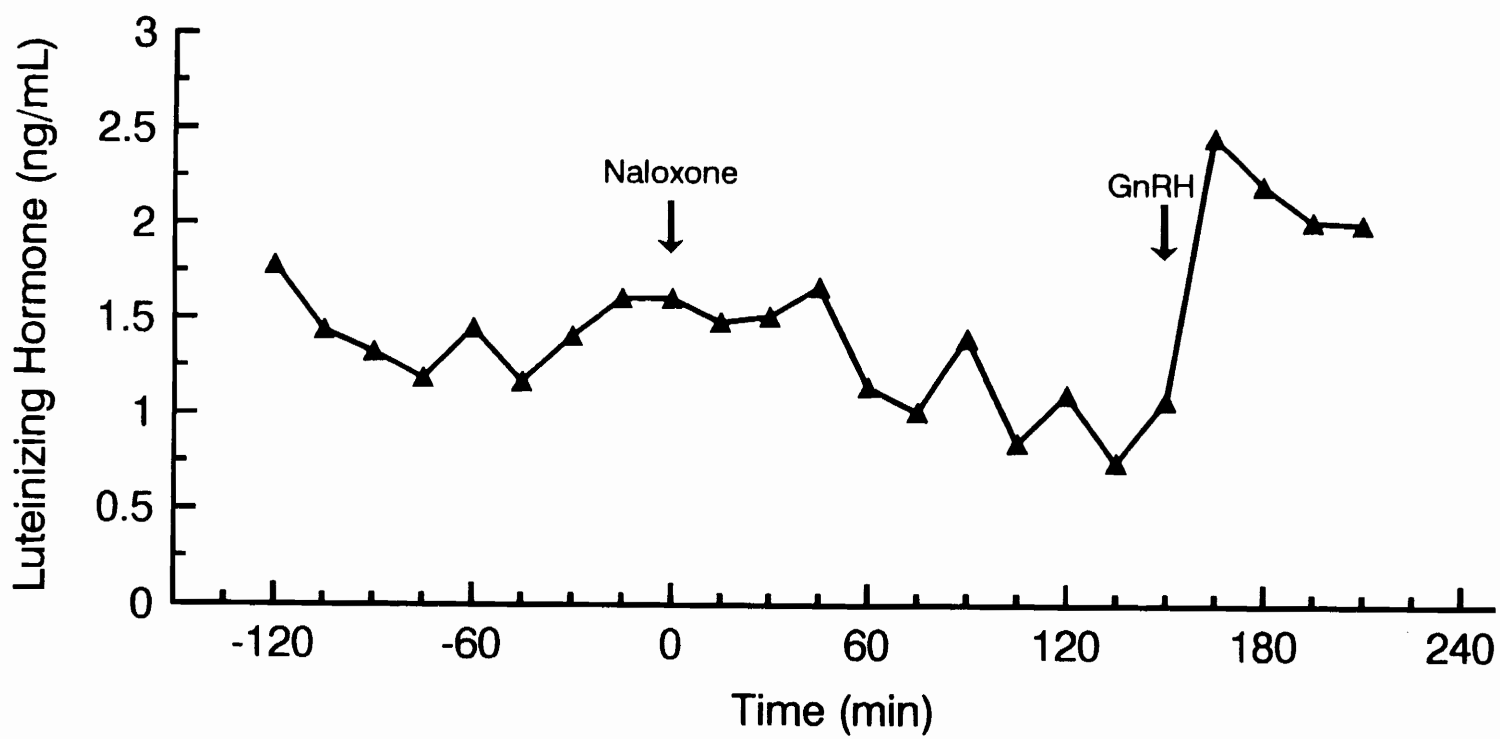

Figure 13. Mean serum luteinizing hormone concentrations before and after naloxone (1 mg/kg BW) administration in cow #867(▲) which did not exhibit an increase in serum LH concentrations but responded to GnRH injection. Gonadotropin releasing hormone (GnRH) was administered at time 150 minute.

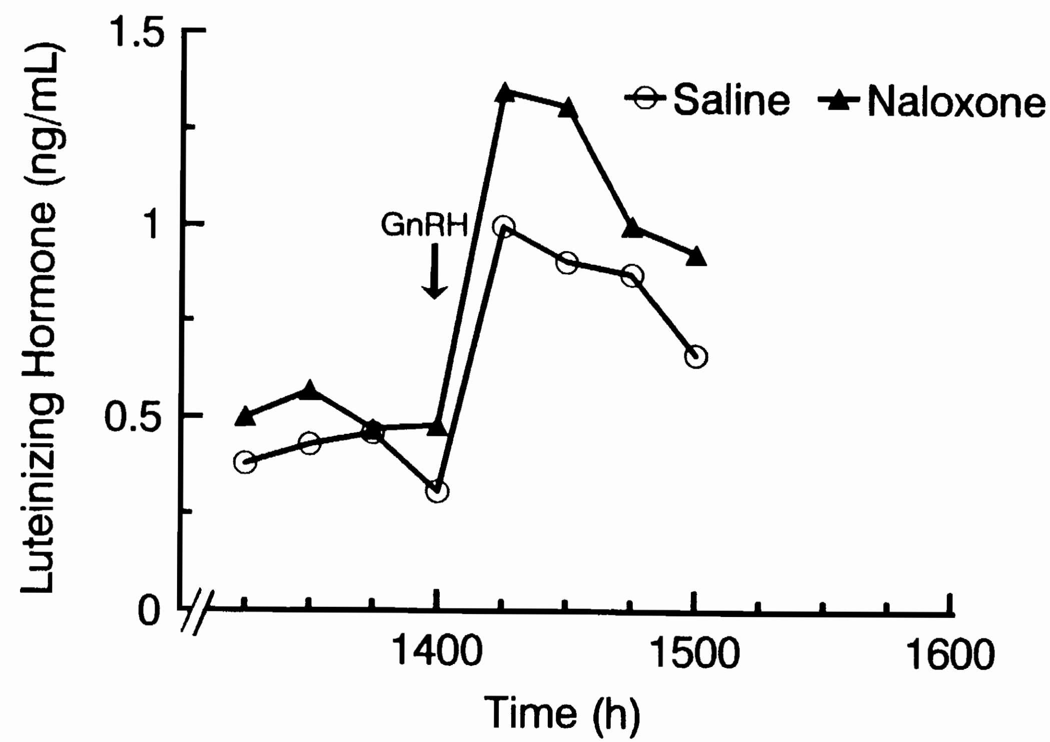

Figure 14. Mean serum luteinizing hormone concentration before and after gonadotropin releasing hormone (GnRH) injection (time=1400) in saline- and naloxone-treated Holstein cows in the follicular phase of the estrous cycle.

SUMMARY AND CONCLUSIONS

Circulating concentrations of LH are low during the early postpartum period in dairy cattle (Edgerton and Hafs, 1973). Pituitary LH secretion must return to a level similar to that observed in the cyclic cow before a normal estrous cycle can begin. However, the amount of LH secreted from the pituitary is insufficient to initiate cyclicity in the early postpartum period presumably due to infrequent discharge of GnRH into the hypothalamic-hypophysial portal circulation (Walters et al., 1982). Thus, prolongation of the postpartum period may be due to an alteration of the mechanism regulating GnRH release. In beef cattle it appears that EOP play a role in the modulation of LH by inhibiting the release of GnRH. Consequently, the anestrous postpartum period is prolonged in these animals. The results of experiment 1 and 2 provide evidence that systemic administration of an opiate antagonist, naloxone, increased LH concentration in dairy cows on d 15 postpartum, but not on d 28 postpartum. Although the lack of LH response to naloxone on d 28 postpartum must be interpreted with caution, the difference in LH response to naloxone between d 15 and d 28 postpartum may indicate that the ability of naloxone to affect LH secretion may change as days postpartum increases, perhaps due to changes in degree of inhibition by endogenous opioid peptides, and(or) changes in serum progesterone concentration due to onset of ovarian activity during postpartum period in dairy cows. Collectively these findings support the concept that EOP play a role, at least in part, in regulating LH secretion during the early postpartum period in dairy cattle. Clearly, use of opiate antagonists and (or) regulation of EOP activity in dairy cattle as a means of effective treatment to shorten the postpartum interval requires more basic information.

There is evidence that both estradiol and progesterone act on the hypothalamus to modify the secretion of GnRH (Clarke et al., 1989;Goodman, 1978). The responses to steroids are rapid. However, GnRH neurons lack intercellular receptors for steroids (Thiery and Martin, 1991). Therefore, at least one other set of interneurones exist in the brain to mediate the effects of these ovarian hormones. The observations in some farm animals and other species indicate that opioids are involved in LH modulation during the estrous cycle indicate that, in certain situations, opioid peptides may mediate the steroid feedback upon gonadotropin secretion. According to Haynes et al. (1989) the degree of opioidergic inhibition which can be demonstrated by the administration of opioid antagonists to cyclic animals appears to be related to the particular steroid which is exerting an effect at the time. The data from experiment 3 provide additional evidence that an opioid-mediated mechanism for the control of LH secretion during the luteal phase of the estrous cycle was absent, or at least overridden by the presence of progesterone. This appears to indicate that negative feedback of progesterone on LH during the luteal phase is not mediated via the opioidergic interneurones, or alternatively, the opioids may not be the only modulator of LH in this phase of the estrous cycle. There is additional evidence that in other species dopaminergic, noradrenergic, or serotinergic interneurones may be involved in the modulation of LH (Dailey et al., 1987). Currently there is not enough information to elucidate the neuroendocrine control of LH secretion during the luteal phase of the estrous cycle in dairy cows. Perhaps in the future, studies utilizing other biogenic amines, particularly noradrenaline, dopamine, and serotonin and their antagonists will provide further evidence and enhance our knowledge of neuroendocrine regulation of LH secretion during the luteal phase of the estrous cycle in dairy cattle.

Results of experiment 4 provide the first information concerning the positive effect of an opiate antagonist on the release of pituitary LH in dairy cattle during the early follicular phase of the estrous cycle. This appears to indicate that EOP play a role in suppression of LH during this phase of the estrous cycle. In other words, in the follicular phase of the estrous cycle when the negative feedback effect of progesterone on LH is minimal, the increase in LH secretion as a result of naloxone indicates that by removing the suppressive effect of EOP, no suppressive effect of estradiol on LH secretion was evident. Thus, the effect of this steroid on the hypothalamus to modify the secretion of GnRH may be mediated via opioidergic interneurones.

Ovulatory follicles must possess an enhanced capacity compared with other follicles to synthesize and release steroids to trigger physiological events (ovulation) necessary for reproduction (Ireland, 1987). Gonadotropins are required for the follicular growth, maturation, and subsequent development of an ovulatory follicle (Ireland, 1987). This is possible only when pulses of LH that are released into the circulation are of sufficient amplitude (Nett, 1987). If this hypothesis is true, then it is possible that EOP play an indirect role in the maturation of the follicles and subsequent ovulation in dairy cattle by regulating LH secretion. Nevertheless, the possible use of opioid antagonists to enhance LH secretion during this phase of the estrous cycle requires more basic knowledge about the endocrine and neuroendocrine mechanisms which regulate LH secretion.

Although naloxone is known to be most active at the μ receptor sites (Malven, 1986), the present study does not provide specific proof that the μ receptors were responsible for changes in LH secretion after naloxone injection. The administration of an opioid agonist with a high affinity for a specific receptor in combination with an opioid antagonist for the same receptor and subsequent measurement of the LH response would provide more definite information as to which family of opioids and which receptors are involved in modulating LH secretion. Endogenous opioid peptides are found in the POA and the ME of bovine, and LHRH release from these two areas has been induced by naloxone infusion (Leshin et al., 1991). However, the present studies do not define the specific area(s) in which opioid(s) inhibit LH secretion. The systemic infusion of naloxone over a period of time could provide evidence as to whether the increase in LH concentration after naloxone administration is due to increase in pulse frequency or pulse amplitude, and in part could provide additional information about the site of action of naloxone within the brain.

The findings of the present studies raise questions which provide opportunity for future investigations: What opioid(s) are involved in modulation of pituitary LH? What types of opioid receptors are being activated? What is the exact site of the action of exogenous opiate antagonist or endogenous opioids?

Nevertheless, the current studies do provide evidence that EOP are involved in modulation of LH secretion in early postpartum dairy cows. Further studies are necessary to investigate the possibility of altering EOP, or their effects, in order to restore earlier reproductive function in anestrous postpartum cattle.

LITERATURE CITED

Barb, C. R., R. R. Kraeling, G. B. Rampacek, and C. S. Whisnant. 1985. Opioid inhibition stimulates luteinizing hormone and prolactin secretion in the gilt. Domest. Anim. Endocrinol. 2:93.

Barb, C. R., R. R. Kraeling, and G. B. Rampacek. 1991. Opioid modulation of gonadotropin and prolactin secretion in domestic farm animal. Domest. Anim. Endocrinol. 8:15.

Barb, C. R., R. R. Kraeling, G. B. Rampacek, and C. S. Whisnant. 1986a. Influence of stage of the estrous cycle on endogenous opioid modulation of luteinizing hormone, prolactin and cortisol secretion in the gilt. Biol. Reprod. 35:1162.

Barb, C. R., R. R. Kraeling, G. B. Rampacek, and C. S. Whisnant. 1986b. Opioid inhibition of luteinizing hormone secretion in the post-partum lactating sow. Biol. Reprod. 35:368.

Barraclough, C. A., and C. H. Sawyer. 1955. Inhibition of the release of pituitary ovulatory hormones in the rat by morphine. Endocrinology. 57:329.

Bauman, D. E., C. J. Peel, W. D. Steinhour, P. J. Reynolds, H. T. Tyrrell, A. C. G. Brown, and G. L. Haland. 1988. Effect of bovine somatotrophin on metabolism of lactating dairy cows: influence on rates of irreversible loss and oxidation of glucose on nonesterified fatty acids. J. Nutr. 118:1031.

Berkowitz, B. A. In: A. N. Brooks et al.(Ed.). 1986. Endogenous opioid peptides and control of gonadotrophin secretion. Research in Vet. Sci. 41:285.

Blank, M. S. and D. L. Roberts. 1982. Antagonist of gonadotropin-releasing hormone blocks naloxone-induced elevations in serum luteinizing hormone. Neuroendocrinology. 35:309.

Blank, M. S., and D. R. Mann. 1981. Diurinal influences on serum luteinizing hormone responses to opiate receptor blockade with naloxone or to luteinizing hormone-releasing hormone in the immature female rat. Proc. Soc. Exp. Biol. 168:338.

Blumberg, H., H. B. Dayton. 1974. Naloxone, naltrexone, and related noroxymorphones. In: Malven (Ed.) Inhibition of pituitary LH release resulting from endogenous opioid peptides. Domest. Anim. Endocrinol. 3(3):135.

Bradbury, A. F., D. G. Smith, C. R. Snell, N. J. M. Birdsall, and E. C. Hulme. 1976. C fragment of lipotropin has high affinity for brain receptors. Nature. 260:793

Brooks, A. N., G. E. Lamming, and N. B. Haynes. 1986a. Endogenous opioid peptides and control of gonadotrophin secretion. Research in Vet. Sci. 41:285.

Brooks, A. N., G. E. Lamming, P. D. Lees, and N. B. Haynes. 1986b. Opioid modulation of LH secretion in the ewe. J. Reprod. Fertil. 76:693.

Bruni, J. F., D. Van Vugt, D. Marshall, and J. Meites. 1977. Effects of naloxone, morphine and methionine enkephalin on serum prolactin, luteinizing hormone, follicle stimulating hormone, thyroid stimulating hormone and growth hormone. Life Sci. 21:461.

Byerley, D. J. T. E. Kiser, J. K. Bertrand, and R. R. Kraeling. 1992. Release of luteinizing hormone after administration of naloxone in pre- and peripuberal heifers. J. Anim. Sci. 70:2794.

Canfield, R. W., and W. R. Butler. 1990. Energy balance and pulsatile LH secretion in early postpartum dairy cattle. Domest. Anim. Endocrinol. 7:323.

Canfield, R. W., and W. R. Butler. 1991. Energy balance, first ovulation and the effects of naloxone on LH secretion in early postpartum dairy cows. J. Anim. Sci. 69:740.

Carrer, H. F., and S. Taleisnik. 1972. Neural pathway associated with the mesencephalic inhibitory influence on gonadotropin secretion. Brain research. 38:299.

Carruthers, T. D., E. M. Convey, J. S. Kesner, H. D. Hafs, and K. W. Cheng. 1980. The hypothalamo-pituitary gonadotropic axis of suckled and non-suckled dairy cows postpartum. J. Anim. Sci. 51:949.

Carter, M. L., D. J. Dierscke, J. J. Rutledge, and E. R. Hauser. 1980. Effect of gonadotropin-releasing hormone and calf removal on pituitary-ovarian function and reproductive performance in postpartum beef cows. J. Anim. Sci. 51:903.

Chang, K. J. 1984. Opioid receptors: Multiplicity and sequelae of ligand-receptor interaction. In P. M. Conn (Ed.) The Receptors. Vol. 1. p 1-81. Academic Press, New York.

Chang, W. J., F. N. Thompson, L. A. Rund, L. S. Leshin, and T. E. Kiser. 1988. Effect of naloxone on luteinizing hormone secretion in prepubertal beef heifers. J. Anim. Sci. 66(suppl 1):409.(Abstr).

Chao, C. C., C. E. Moss, and P. V. Malven. 1986. Direct opioid regulation of pituitary release of bovine luteinizing hormone. Life Sci. 39:527.

Chenault, J. R., W. W. Thatcher, P. S. Kalra, R. M. Abrams, and C. J. Wilcox. 1976. Plasma progestins, estradiol, and luteinizing hormone following prostaglandin $F_{2\alpha}$ injection. J. Dairy Sci. 59:1342.

Cicero, T. J., T. M. Badger, C. E. Wilcox, R. D. Bell, and E. R. Meyer. 1977. Morphine decreased luteinizing hormone by an action on the hypothalamic pituitary axis. J. Pharmacol. Exp. Therap. 203:548.

Clarke, I. J., J. T. Cummins, M. E. Crowder, and T. M. Nett. 1989. Long term negative feedback effects of estrogen and progesterone on the pituitary gland of the long-term ovariectomized ewe. J. Endorinol. 120:207.

Conner, H. C., P. L. Houghton, R. P. Lemenger, P. V. Malven, J. R. Parfet, and G. E. Moss. 1990. Effect of dietary energy, body condition and calf removal on pituitary gonadotropins, gonadotropin-releasing hormones (GnRH) and hypothalamic opioids in beef cows. J. Anim. Sci. 7:403.

Cross, J. C., L. M. Rutter, and J. G. Manns. 1987. Effect of progesterone and weaning on LH and FSH responses to naloxone in postpartum beef cows. Domest. Anim. Endocrinol. 4:111.

Currie, W. D., and N. C. Rawlings. 1989. Fluctuation in respons- iveness of LH and lack of responsiveness of FSH to prolonged infusion of morphine and naloxone in the ewe. J. Reprod. Fertil. 86:359.

Currie, W. D., and N. C. Rawling. 1987. Naloxone enhances the LH but not FSH release during various phases of the estrous cycle in the ewe. J. Reprod. Fertil. 81:277.

Dailey, R.A., D. R. Deaver, and R. L. Goodman. 1987. Neurotransmitter regulation of luteinizing hormone and prolactin secretion. J. Reprod. Fertil. 34(Suppl):17.

Deaver, D. R. D. H. Keisler, R. A. Dailey. 1987. Effects of domperidone and thyrotropin releasing hormone on secretion of luteinizing hormone and prolactin during the luteal phase and following induction of luteal regression in sheep. Domest. Anim. Endocrinol. 4:95

Diez-Guerra, F. J., S. Augood, P. C. Emson, and R. G. Dyer. 1986. Morphine inhibits electrically stimulated noradrenaline release from slices of rat medial preoptic area. Neuroendocrinology. 43:89.

Dyer, R. G., S. Mansfield, H. Corbet, and A. D. P. Dean. 1985. Fasting impairs LH secretion in female rats by activating an inhibitory opioid pathway. J. Endocrinology. 105:91.

Dunshea, F. R., A. W. Bell, and T. E. Trigg. 1989. Relations between plasma non-esterified fatty acid metabolism and body fat mobilization in primiparous lactating goats. Br. J. Nutr. 62:51.

Edgerton, L. A., and H. D. Hafs. 1973. Serum luteinizing hormone, prolactin, glucocorticoid and progestin in dairy cows from calving to gestation. J. Dairy Sci. 56:451.

Ferin, M., W. B. Wehrenbergh, N. Y. Lam, E. J. Alston, and R. L. Van Wielde. 1982. Effects and site of action of morphine on gonadotrophin secretion in the female rhesus monkey. Endocrinology. 111:1652.

Fernandes, L. C., W. W. Thatcher, C. J. Wilcox, and E. P. Call. 1978. LH release in response to GnRH during the postpartum period of dairy cows. J. Anim. Sci. 46:443.

Gabriel, S. M., J. W. Simpkins, and S. P. Kalara. 1983. Modulation of endogenous opioid influence on luteinizing hormone secretion by progesterone and estrogen. Endocrinology. 113:1806.

Games, P. 1977. An improved t-table for simultaneous control on g contrast. J. Amer. Stat. Assoc. 72:531.

Gold, M. S., and A. C. Pottash. Naloxone and naltrexone: endorphin antihormones. 1982. Hormone antagonists. In: M. K. Agarwal (Ed.).p 671. Wlter de Gruyter. New York.

Goldstein, A. and A. Naidu. 1989. Multiple opioid receptors: ligand selectivity profiles and binding site signatures. Mol. Pharmocol. 36:265.

Goodman, R. L. 1978. A quantitative analysis of the physiological role of estradiol and progesterone in the control of tonic and surge secretion of luteinizinh hormone in the rat. Endocrinology. 102:142

Gosselinet, R. E., J. Blankstein, D. W. Dent, W. C. Hobson, G. B. Fuller F. I. Reyes, J. S. D. Winter, and C. Faiman. 1983. Effects of naloxone and enkephalin analog on serum prolactin, cortisol and gonadotrophins in the chimpanzee. Endocrinology. 112: 2168.

Gregg, D. W., G. E. Moss, R. E. Hudgens, and P. V. Malven. 1986. Endogenous opioid modulation of luteinizing hormone and prolactin secretion in postpartum ewes and cows. J. Anim. Sci. 63:838.

Grossman, A., and L. H. Rees. 1983. The neuroendocrinology of opioid peptides. Br. Medical Bulletin. 39:83.

Guillemin, R., T. Vargo, J. Rossier, S. Minick, N. Ling, C. Rivier, W. Vale, and F. Bloom. 1977. β-endorphin and adrenocorticotropin are secreted concomitantly by the pituitary gland. Science. 197:1367.

Guthrie, H. D., and D.J. Bolt. 1983. Changes in plasma estrogen, luteinizing hormone, follicle stimulating hormone, and 13-14-dihydro -15 keto prostaglandin $F_{2\alpha}$ during the blockade of luteolysis in pigs after human chorionic gonadotropin treatment. J. Anim. Sci. 57:993.

Hansen, W., and E. M. Convey. 1983. Physiology of the estrous cycle. J. Anim. Sci. 57(Suppl. 2):404.

Haynes, N. B., G. E. Lamming, K. P. Yang, A. N. Brooks, and A. D. Finnie. 1989. Endogenous opioid peptides and farm animal reproduction. Oxford Rev. Reprod. Biol. 11:111.

Henderson, G., J. Hughes, and H. W. Kosterlitz. 1972. A new example of morphine sensitive neuro-effector junction; adrenergic nuerotransmission in mouse vas deferens. Br. J. Pharmcol. 46:764.

Hendricks, D. M., J. F. Dicky, and G. D. Niswender. 1970. Serum luteinizing hormone and progesterone levels during the estrous cycle and early pregnancy in cows.

Biol. Reprod. 2:346.

Holloway, K. E., T. E. Kieser, F. N. Thompson, D. L. Boggs, L. S. Leshin, and L. A. Rund. 1987. Effect of metabolizable energy level on LH response to naloxone in postpartum beef cows. J. Anim. Sci. 65(suppl. 1):396 (Abstr).

Holt, V. 1986. Opioid peptide processing and receptor selectivity. Annu. Rev. Pharmacol. Toxicol. 26:59

Hughes, J., T. W. Smith. and H.W. Kosterlitz. 1975. Identification of two related pantapeptides from the brain with potent agonist activity. Nature. 258:577.

Ieiri, t., H. T. Chen, and J. Meites. 1980. Naloxone stimulation of luteinizing hormone release in prepubertal female rats; role of sertonergic system. Life Science. 26:1269.

Imura, H., K. Nakai, K. Nakao, S. Oki, I. Tanaki, H. Jingami, T. Tsukada, Y. Ikeda, M. Suda, and M. Sakamoto. 1983. Biosynthesis and distribution of opioid peptides. J. Endocr. Invest. 69:139.

Kalra, S. P., R. V. Gallo. 1983. Effect of intraventricular catecholamine on luteinizing hormone release in morphine treated rats. Endocrinology. 113:23.

Kalra, S. P., and J. W. Simpkins. 1981. Evidence for noradrenergic mediation of opioid effects on luteinizing hormone secretion. Endocrinology. 109:776.

Kineman, R. D., J. W. Crime, L. S. Leshin, G. B. Rampacek, R. R. Kraeling, and C. B. Barb. 1989. Localization of proopiomelanocrotin (POMC) immunoreactive neurons in the forebrain of the pig. Biol Reprod. 40:1119.

Kosterlitz, H. W. J. A. H. Lord, S. J. Paterson, and A. A. Waterfield. 1980. Effects of changes in structure of enlepha- lins and of narcotic analgesic drugs on their interactions with u and δ-receptors. Br. J. Pharmacol. 68:333.

Lakoski, J. M. and G. F. Gebhart. 1981. Attenuation of morphine's depression of serum luteinizing hormone by lesion in amygdala. Neuroendocrinology. 105:111.

Leshin, L. S., L. A. Rund, R. R. Kraeling, and T. E. Kiser. 1991. The bovine preoptic area and median eminence: sites of opioid inhibition of luteinizing hormone-releasing hormone secretion. J. Anim. Sci. 69:3733.

Leshin, L. S., L. A. Rund, J. W. Crim, and T. E. Kiser. 1988. Immunocytochemical localization of luteinizing hormone-releasing hormone and proopiomelanocortin neurons within preoptic area and hypothalamus of the bovine brain. Biol. Reprod. 39:1988.

Li, C. H., D. Chung. 1976. Isolation and structure of an untriakontapeptides with opiate activity from camel pituitary glands. Proc. Nat. Acad. Sci., USA. 73:1145.

Li. P. S. and Wagner. 1983. In vivo and in vitro studies on the effect of adrenocorticotrophic hormone or cortisol on the pituitary response to gonadotropin releasing hormone. Biol. Reprod. 29:25.

Louis, T. M., H. D. Hafs, D. A. Morrow. 1974. Intrauterine administration of prostaglandin $F_{2\alpha}$ in cows: progesterone, estradiol, LH estrus and ovulation. J. Anim. Sci. 38:347.

Maggi, R., D. Dondi, G. E. Rovati, L. Martini, F. Piva, and P. Limonta. 1993. Binding characteristics of hypothalamic mu opioid receptors throughout the estrous cycle in the rat. Neuroendocrinology. 58:366.

Mahmoud, A. I., F. N. Thompson, D.D. Peck, K. M. Mezinga, L.S. Leshin, R. A. Rund, J. A. Stuedemann, T.E. Kiser. 1989. Difference in luteinizing hormone response to an opioid antagonist in beef heifers and cows. Biol. Reprod. 41:431.

Malven, P. V. 1993. Miscellaneous Neuropeptides. In: P. V Malven (Ed.). Mammalian neuroendocrinology. p 64. CRC Press, Boca Raton, FL.

Malven, P. V. 1986. Inhibition of pituitary LH release resulting from endogenous opioid peptides. Domest. Anim. Endocrinol. 3(3):135.

Malven, P. V., D. F. B. Bossut, and M. A. Diekman. 1984. Effects of naloxone and electroacupuncture treatment on plasma concentrations of LH in sheep. J. Endocrinol. 101:75.

Malven, P. V. and R. E. Hudgens. 1987. Naloxone-reversible inhibition of luteinizing hormone in postpartum ewes: effects of suckling and season. J. Anim. Sci. 65:196.

Mansour, A., H. Khachaturian, M. E. Lewis, A. Akil, and S. J. Watson. 1987. Aoutoradiographic differentiation of mu, delta, and kappa opioid receptors in rat forebrain and midbrain. J. Neurosci. 7:2445.

Matteri, R. L., and G. P. Moberg. 1985. The effect of opioid petides on ovine pituitary gonadotropin secretion in vitro. Peptides. 6:957.

McCann, S. M. 1983. Role of peptides in the control of repro- duction. In:S. M. McCann, and D. S. Dhinsda (Ed.) Elsevier Biomedical. p 3. New York.

McCutcheon, S. N., D. E. Bauman. 1986. Effects of chronic growth hormone treatment on response to epinephrine and thyrotropin-releasing hormone in lactating cows. J. Dairy Sci. 69:44

Mcllwain, H., and H. S. Bachelard. 1985. Biochemistry and the central nervous system. 5th ed. p 288. Churchill Livingstone, Edinburgh.

Miller, K. F., J. K. Crister, O. J. Ginther. 1982. Inhibition and subsequent rebound of FSH secretion following treatment with bovine follicular fluid in the ewe. Theriogenology. 18:45

Moss, G. E., J. R. Parfet, C. A. Malven, R. D. Allrich, and M. A. Diekman. 1985. Pituitary concentration of gonadotropins and receptors for GnRH in suckled beef cows at various times after calving. J. Anim. Sci. 60:1985.

Nanda, A. S., W. R. Ward, and H. Dobson. 1989. Opioid modulation of tonic luteinizing hormone release in ovariectomized dairy cows. J. Vet. Therap. 12:397.

Nanda, A. S., W. R. Ward, and H. Dobson. 1991. Opioid involvement in LH release during the negative feedback effect of estradiol and progesterone in dairy cows. Reprod. Fertil. Dev. 3:709.

Nett, T. M. 1987. Function of the hypothalamic-hypophysial axis during the post-partum period in ewes and cows. J. Reprod. Fertil. 34:201.

Nett, T. M., D. Cermake, T. Barden, J. Manns, and G. D. Niswender. 1987. Pituitary receptors for GnRH and estrous cycle period. Domest. Anim. Endocrinol. 4:123.

Nett, T. M., D. Cermake, T. Barden, J. Manns, and G. D. Niswender. 1988. Pituitary receptors for GnRH and estradiol, and pituitary content of gonadotropins in beef cows. II. Changes during the postpartum period. Domest. Anim. Endocrinol. 5:81.

Nilaver, G. E. A. Zimmerman, R. Defendini, A. S. Liotta, D. T. Kreiger, and M. J.

Brownstein. 1979. Adrenocorticotropin and β-lipotropin in the hypothalamus. Localization in the same arcuate neurons by sequential immunocytochemical procedures. J. Cell Biol. 81:50.

NRC. 1989. Nutrient Requirements of Dairy Cattle (6th Ed.). National Academy Press, Washington, DC.

Panerai, A. E., A. Martini, F. Petraglia, A. M. Di Giulio, and P. Mantegazz. 1983. Opiates and their antagonists modulate luteinizing hormone acting outside the blood brain barrier. Life Science. 32:1751.

Packman, P. M., and J. A. Rothchild. 1976. Morphine inhibition of ovulation: reversal by naloxone. Endocrinology. 253: 563.

Peck, D. D., F. N. Thompson, A. Jernigan, and T. E. Kiser. 1988. Effect of morphine on serum gonadotropin concentrations in postpartum beef cows. J. Anim. Sci. 66: 2930.

Pert, C. B., and S. H. Snyder. 1973. Opiate receptors demonstration in nervous tissue. Science. 179:1011.

Peters, A. R., M. G. Pimentel, and G. E. Lamming. 1985. Hormone response to exogenous GnRH pulses in post-partum dairy cows. J. Reprod. Fertil. 75:557.

Piva, F., R. Maggi, P. Limonta, M. Motta, and L. Marttini. 1985. Effect of naloxone on luteinizing hormone, follicle stimulating hormone, and prolactin secretion in different phases of estrous cycle. Endocrinology. 117:766.

Quigley, M. E., and S. C. C. Yen. 1980. The role of endogenous opiates on LH secretion during the menstrual cycle. J. Clin. Endocrinol. & Metab. 51:179.

Rahe, C. H., R. E. Owen, J. L. Fleeger, H. J. Newton, and P.G. Harms. 1980. Pattern of plasma luteinizing hormone in the cyclic cow: dependence upon the period of the cycle. Endocrinology. 107:498.

Rasmussen, D. D., B. P. Kennedy, M. G. Ziegler, and T. M. Nett. 1988. Endogenous opioid inhibition and facilitation of gonadotropin-releasing hormone release from the median eminence in vitro: potential role of catocholamines. Endocrinology. 123:2916.

Ried R. L., J. D. Hoff, S. S. C. Yen, and C. H. Li. 1981. Effect of exogenous endorphin on pituitary hormone secretion and its disappearance rate in normal human subject. J. Clin. Enococrinol. & Metab. 52:1179.

Rotsztejn, W. H., S. V. Drouva, E. Pattou, and C. Kordon. 1978. Met-enkephalin inhibits *in vitro* dopamine induced LHRH release from mediobasal hypothalamus of male rats. Nature (Lond.). 274:281.

Rund, L. A., L. S. Leshin. F. N. Thompson, G. B. Rampacek, and T. E. Kiser. 1989. Influence of ovary and suckling on luteinizing hormone response to naloxone in postpartum beef cows. J. Anim. Sci. 67:1527.

Sawynok, J., C. Pinsky, and F. S. Labella. 1979. Minireview on the specificity of naloxone as an opiate antagonist. Life. Sci. 25:1621.

Schneider, P. G., and S. M. McCann. 1970. Mono- and indolamines and control of LH secretion. Endocrinology. 86:1127.

Short, R. E., A. N. Brooks, A. R. Peteres, and G. E. Lamming. 1987. Opioid modulation of LH secretion during the oestrous cycle of heifers. J. Reprod. Fertil. 80: 213.

Sirinathsinghji, D. J., S. L. Martini. 1984. Effects of bromocriptine and naloxone on plasma levels of prolactin, LH, and FSH during suckling in the female rat: Responses to gonadotropin releasing hormone J. Endocrinol. 100:175.

Spampinato, S., V. Locatelli, D. Cocchi, L. Vincentini, S. Bajusz, S. Ferri, and E. F. Muller. 1979. Involvement of brain serotonin in prolactin releasing effect of opioid peptides. Endocrinology. 105:163.

Sylvester, P. W., D. K. Sarkar, K. P. Briski, and J. Meites. 1985. Relation of gonadal hormones to differential LH response to naloxone in prepubertal male and female rats. Neuroendocrinology. 40:165.

Szekely, J. I. 1982. Opioid peptides and the neuroendocrine functions. In: J. I. Szekely and A. Z. Ronai (Ed.). Opioid Peptides. Vol.II. Pharmacology. p 207. CRC Press. Boca Raton, FL.

Takahara, J., J. Kageyama, S. Yunoki, W. Yakushiji, J. Yamauchi, N. Kageyama, and T. Ofuji. 1978. Effects of 2-bromo-alpha ergocryptine on β-endorphin induced growth hormone, prolactin, and luteinizing hormone release in urethane anesthetized rats. Life Science. 22:2205.

Trout, W. E. and P. V. Malven. 1988. Quantification of naloxone binding sites in brains from suckled beef cows during postpartum anestrus and resumption of estrous cycle. J. Anim. Sci. 66:954.

Thiery, J. C., and G. B. Martin. 1991. Neurophysiological control of the secretion of gonadotropin-releasing hormone and luteinizing hormone in the sheep. Reprod. Fertil. Dev. 3:137.

Van Vugt, D. A, N. Y. Lam, and M. Ferin. (1984). Reduced frequency of pulsatile luteinizing hormone secretion in luteal phase of rhesus monkey. Involvement of endogenous opiates. Endocrinology. 115:1095.

Walters, D. L., R. E. Short, E. M. Convey, R. B. Staigniller, T. G. Dunn, and C. C. Kaltenbach. 1982. Pituitary and ovarian function in postpartum beef cows. III. Induction of estrus, ovulation and luteal function with intermittent small-dose injections of GnRH. Biol. Reprod. 26:655.

Ward, S. J., A. K. Pierson, W. F. Michne. 1983. Multiple opioid receptor profile *in vitro* and *in vivo* of the potent opioid antagonist Win 44442-3. Life Science (suppl. 1). 33:303.

Webb, R., G. E. Lamming, N. B. Haynes, H. D. Hafs, and J .G. Manns. 1977. Response of cyclic and postpartum suckled cows to injection of synthetic LHRH. J. Reprod. Fertil. 50:203.

Whisnant, C.S. T. E. Kiser, R. N. Thompson, and C. R. Barb. 1986a. Influence of calf removal on the serum luteinizing hormone response to naloxone in the postpartum beef cow. J. Anim. Sci. 66(suppl 1):409.

Whisnant, C. S. T. E. Kiser, R. N. Thompson, and C. R. Barb. 1986b. Opioid inhibition of luteinizing hormone secretion during the postpartum period in suckled beef cows. J. Anim. Endocrinol. 3:49.

Whisnant, C. S., R. N. Thompson, T. E. Kiser, and C. R. Barb. 1986c. Effect of naloxone on serum luteinizing hormone , cortisol and prolactin concentrations in anestrous beef cows. J. Anim. Sci. 62:1340.

Whisnant, C. S., T. E. Kiser, R. N. Thompson, and C. R. Barb. 1986d. Naloxone infusion increases pulsatile luteinizing hormone release in postpartum beef cows. Domest. Anim. Endocrinol. 3:49.

Whisnant, C. S., T. E. Kiser, R. N. Thompson, and C. R. Barb. 1986e. Opioid inhibition of luteinizing hormone secretion during the postpartum period in suckled beef cows. J. Anim Sci. 63:1445.

Whisnant, C. S., R. L. Goodman. 1988. Effects of opioid antagonist on pulsatile luteinizing hormone secretion in the ewe vary with changes in steroid negative feedback. Biol. Reprod. 39:1032.

Williams, G. L., J. Kotwica, W. D. Slanger, D. K. Olson, J. E. Tilton, and L. H. Johnson. 1982. Effect of suckling on pituitary responsiveness to gonadotropin-releasing hormone through the early postpartum period in beef cows. J. Anim. Sci. 54:594.

Yang, K., N. B. Haynes, G. E. Lamming and A. N. Brooks. 1988. Ovarian steroid hormone involvement in endogenous opioid modulation of LH secretion in mature ewes during breeding and non-breeding seasons. J. Reprod. Fertil. 83:129.

APPENDICES

Appendix A. Table 1. Analysis of variance for the effect of naloxone treatment on serum luteinizing hormone concentration in Holstein cows on day 15 postpartum. (n=24)

Source of Variation	df	Mean Square	F Value	PR > F
Treatment (Tmt)*	1	288.279	2.20	0.1538
Parity*	1	142.957	1.09	0.3090
Parity×Tmt*	1	67.176	0.52	0.4805
Cow(Tmt×Parity)	20	131.192	32.93	0.0001
Period**	2	604.445	1.75	0.1874
Tmt×Period**	2	164.190	4.74	0.0142
Parity×Period**	2	9.994	0.29	0.7507
Parity×Period×Tmt**	2	16.095	0.47	0.6314
Period×Cow(Tmt×Parity)	40	34.603	8.69	0.0001
Error	384	3.984		

* Using type III MS for Cow(Tmt×Parity) as the error term
** using type III MS for Per×Cow(Tmt×Parity) as the error term

Appendix A. Table 2. Analysis of variance for the effect of gondaotropin releasing hormone treatment on serum luteinizing hormone concentration in Holstein cows on day 15 postpartum. (n=24)

Source of Variation	df	Mean Square	F Value	PR > F
Treatment (Tmt)*	1	178.78	1.95	0.1778
Parity*	1	1857.11	20.27	0.0002
Parity×Tmt*	1	128.89	1.41	0.2497
Cow(Tmt×Parity)	20	191.64	6.13	0.0001
Period**	1	2140.05	33.33	0.0001
Tmt×Period**	1	7.28	0.11	0.7390
Parity×Period**	1	1221.46	19.02	0.0003
Parity×Period×Tmt**	1	185.44	2.88	0.1051
Period×Cow(Tmt×Parity)	20	64.21	4.30	0.0001
Error	144	14.94		

* Using type III MS for Cow(Tmt×Parity) as the error term.
** using type III MS for Per×Cow(Tmt×Parity) as the error term.

Appendix A. Table 3. Analysis of variance for the effect of parity on net energy balance the at 15 days postpartum.

Source of variation	df	Mean Square	F Value	PR > F
Parity	1	4.816	0.40	0.5134
Residual Error	22	11.912		

Appendix A. Table 4. Analysis of variance for the effect of parity on serum non-esterified fatty acid concentrations at 15 days postpartum.

Source of variation	df	Mean Square	F Value	PR > F
Parity	1	1.031	0.06	0.8169
Residual Error	22	16.258		

Appendix B. Table 1. Analysis of variance for the effect of naloxone treatment on serum luteinizing hormone concentration in Holstein cows on day 28 postpartum. (n=27)

Source of Variation	df	Mean Square	F Value	PR > F
Treatment (Tmt)*	1	1156.97	1.44	0.2455
Progesterone (Prog)*	1	2292.38	2.85	0.1079
Parity*	1	591.93	0.73	0.4022
Tmt×Prog*	1	289.42	0.36	0.5560
Tmt×Parity*	1	91.06	0.11	0.7394
Parity×Prog*	1	842.70	1.05	0.3193
Tmt×Prog×Parity*	1	106.07	0.13	0.7200
Cow(Tmt×Parity×Prog)	19	805.46	121.01	0.0001
Period**	2	130.14	0.74	0.4826
Tmt×Period**	2	291.19	1.66	0.2032
Prog×Per**	2	75.27	0.43	0.6539
Parity×Period**	2	168.53	0.96	0.3913
Tmt×Prog×Per**	2	186.49	1.06	0.3350
Tmt×Parity×Per**	2	105.62	0.60	.5524
Parity×Prog×Per**	2	84.65	0.48	0.6205
Tmt×Parity×Prog×Per**	2	156.25	0.89	0.4183
Period×Cow(Tmt×Parity×Prog)	38	175.19	26.32	0.0001
Error	432	4821.47		

* Using type III MS for Cow(Tmt×Parity×Prog) as the error term.
** using type III MS for Per×Cow(Tmt×Parity×Prog) as the error term.

Appendix B. Table 2. Analysis of variance for the effect of gondaotropin releasing hormone treatment on serum luteinizing hormone concentration in Holstein cows on day 28 postpartum. (n=27)

Source of Variation	df	Mean Square	F Value	PR > F
Treatment (Tmt)*	1	715.46	0.73	0.4042
Progesteron (Prog)*	1	1334.13	1.36	0.2585
Parity*	1	134.08	0.14	0.7160
Tmt×Prog*	1	569.32	0.58	0.4560
Tmt×Parity*	1	180.36	0.18	0.6732
Parity×Prog*	1	654.69	0.67	0.4246
Tmt×Prog×Parity*	1	134.97	0.14	0.7151
Cow(Tmt×Parity×Prog)	19	983.08	130.50	0.0001
Period**	1	474.33	7.47	0.0132
Tmt×Period**	1	0.94	0.01	0.9045
Prog×Per**	1	25.70	0.40	0.5322
Parity×Period**	1	476.58	7.51	0.0130
Tmt×Prog×Per**	1	17.61	0.28	0.6044
Tmt×Parity×Per**	1	10.33	0.16	0.6912
Parity×Prog×Per**	1	1.85	0.03	0.8662
Tmt×Parity×Prog×Per**	1	38.08	0.60	0.4481
Period×Cow(Tmt×Parity×Prog)	19	63.47	8.43	0.0001
Error	162	7.53		

* Using type III MS for Cow(Tmt×Parity×Prog) as the error term.
** using type III MS for Per×Cow(Tmt×Parity×Prog) as the error term.

Appendix B. Table 3. Analysis of variance for the effects of days postpartum (day 15 and day 28) and parity on net energy balance. (n=27)

Source of Variation	df	Mean Square	F Value	PR > F
Parity*	1	65.15	2.35	0.1100
Cow(parity)	25	27.72	4.53	0.0002
Days	1	14.16	2.82	0.1053
Parity × Days	1	11.64	2.32	0.1400
Residual Error	25	5.01		

* Using type III MS for Cow(Parity) as the error term.

Appendix B. Table 4. Analysis of variance for the effects of days postpartum (day 15 and day 28) and parity on non-esterified fatty acids. (n=27)

Source of Variation	df	Mean Square	F Value	PR > F
Parity*	1	0.020	0.97	0.3340
Cow(parity)	25	0.021	1.65	0.1089
Days	1	0.025	2.06	0.1639
Parity × Days	1	0.041	3.34	0.0800
Residual Error	25	0.0124		

* Using type III MS for Cow(Parity) as the error term.

Appendix C. Table 1. Analysis of variance of the comparison of serum progesterone concentrations in the luteal and follicular phases of the estrous cycle.

Source of Variation	df	Mean square	F Value	PR > F
Parity*	1	0.494	0.19	0.6663
Cow(Parity)	20	2.58	1.18	0.0001
Phase of estrous cycle (Phase)	1	196.49	90.11	0.0001
Parity × Phase	1	0.34	0.16	0.6974
Residual Error	18	2.18		

* Using type III MS for Cow(Parity) as the error term.

Appendix C. Table 2. Analysis of variance for the effect of naloxone treatment on serum luteinizing hormone concentration in Holstein cows in the luteal phase of the estrous cycle (n=22)

Source of Variation	df	Mean Square	F Value	PR > F
Treatment (Tmt)*	1	92.28	2.95	0.1032
Parity*	1	5.60	0.18	0.6773
Parity×Tmt*	1	8.55	0.27	0.6076
Cow(Tmt×Parity)	18	31.31	14.68	0.0001
Period**	2	23.34	4.23	0.0225
Tmt×Period**	2	4.84	0.88	0.4249
Parity×Period**	2	10.29	1.86	0.1699
Parity×Period×Tmt**	2	3.59	0.65	0.5281
Period×Cow(Tmt×Parity)	36	5.52	82.590	0.0001
Error	352	2.13		

* Using type III MS for Cow(Tmt×Parity) as the error term.
** Using type III MS for Per×Cow(Tmt×Parity) as the error term.

Appendix C. Table 3. Analysis of variance for the effect of gonadotropin releasing hormone treatment on serum luteinizing hormone concentration in Holstein cows in the luteal phase of the estrous cycle (n=22)

Source of Variation	df	Mean Square	F Value	PR > F
Treatmen (Tmt)*	1	22.01	0.19	0.6695
Parity*	1	203.26	1.74	0.2038
Parity×Tmt*	1	10.65	0.09	0.7662
Cow(Tmt×Parity)	18	116.88	26.81	0.0001
Period**	1	408.58	13.42	0.0018
Tmt×Period**	1	0.43	0.01	0.9067
Parity×Period**	1	61.75	2.03	0.1715
Parity×Period×Tmt**	1	6.91	0.23	0.6396
Period×Cow(Tmt×Parity)	18	30.45	6.99	0.0001
Error	132	4.36		

* Using type III MS for Cow(Tmt×Parity) as the error term.
** Using type III MS for Per×Cow(Tmt×Parity) as the error term.

Appendix C. Table 4. Analysis of variance of the comparison of basal luteinizing hormone concentration in the luteal and follicular phases of the estrous cycle.

Source of Variation	df	Mean Square	F Value	PR > F
Parity*	1	15.78	0.25	0.6201
Cow(Parity)	20	62.21	27.47	0.0001
Phase of estrous cycle (phase)**	1	938.01	30.66	0.0001
Parity × Phase**	1	7.40	0.24	0.6288
Phase × Cow(Parity)	18	30.59	3.51	0.0001
Residual Error	336	2.26		

* Using type III MS for Cow(Parity) as the error term.
** Using type III MS for Day × Cow(Parity) as the error term.

Appendix D. Table 1. Analysis of variance for the effect of naloxone treatment on serum luteinizing hormone concentration in Holstein cows in the follicular phase of the estrous cycle. (n=20)

Source of Variation	df	Mean Square	F Value	PR > F
Treatment (Tmt)*	1	344.10	3.16	0.0946
Parity*	1	34.63	0.32	0.5808
Parity×Tmt*	1	0.62	0.01	0.9409
Cow(Tmt×Parity)	16	108.10	2.53	0.0001
Period**	2	42.48	4.70	0.0163
Tmt×Period**	2	51.51	5.69	0.0077
Parity×Period**	2	4.34	0.48	0.6232
Parity×Period×Tmt**	2	3.98	0.44	0.6480
Period×Cow(Tmt×Parity)	32	9.04	0.70	0.0001
Residual Error	320	3.35		

* Using type III MS for Cow(Tmt×Parity) as the error term.
** using type III MS for Per×Cow(Tmt×Parity) as the error term.

Appendix D. Table 2. Analysis of variance for the effect of gonadotropin releasing hormone treatment on serum luteinizing hormone concentration in Holstein cows in the follicular phase of the estrous cycle. (n=20)

Source of Variation	df	Mean Square	F Value	PR > F
Treatment (Tmt)*	1	145.51	0.81	0.3823
Parity*	1	7.24	0.04	0.8436
Parity×Tmt*	1	1.96	0.01	0.9183
Cow(Tmt×Parity)	16	180.29	25.78	0.0001
Period**	1	1165.91	14.76	0.0014
Tmt×Period**	1	103.02	5.69	0.5499
Parity×Period**	1	52.99	0.67	0.4248
Parity×Period×Tmt**	1	0.69	0.01	0.9262
Period×Cow(Tmt×Parity)	16	78.98	11.29	0.0001
Residual Error	120	6.99		

* Using type III MS for Cow(Tmt×Parity) as the error term.
** Using type III MS for Per×Cow(Tmt×Parity) as the error term.

VITA

Amin Ahmadzadeh, the son of Hashem and Mansoureh Ahmadzadeh, was born on May 22nd, 1964, in Mashhad,Iran. He graduated from High School in Mashhad, in 1982.

After working in the dairy industry for 5 years, the author came to the United States in 1987 and enrolled at Delaware Valley College, Doylestown, Pennsylvania. He received a Bachelor of Science degree, majoring in Dairy Science in 1992. In August of 1992, he began his masters program at Virginia Tech, and received a Master of Science degree in Dairy Science in physiology in December, 1994. In January of 1995, the author will begin his doctorate degree at Virginia Tech in animal physiology.

The author is a member of the American Dairy Science Association , Dairy Shrine, Delta Tau Alpha, and Gamma Sigma Delta Agriculture Honor Society.

Amin Ahmadzadeh