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EFFECTS OF SUCKLING ON POSTPARTUM OVULATION, LUTEINIZING HORMONE, FOLLICLE-STIMULATING HORMONE AND PROLACTIN IN HOLSTEIN COWS

presented by

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has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Dairy Science

Harold D. Hafs
Major professor

Date 12/22/78

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EFFECTS OF SUCKLING ON POSTPARTUM OVULATION, LUTEINIZING HORMONE, FOLLICLE-STIMULATING HORMONE AND PROLACTIN IN HOLSTEIN COWS

By

Terry David Carruthers

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Dairy Science

1979

ABSTRACT

EFFECTS OF SUCKLING ON POSTPARTUM OVULATION, LUTEINIZING HORMONE, FOLLICLE-STIMULATING HORMONE AND PROLACTIN IN HOLSTEIN COWS

By

Terry David Carruthers

Suckling-induced postpartum (PP) anovulation in beef cows prevents many cattlemen from attaining an economically desirable 12-month calving interval. How suckling inhibits PP ovulation is not known. Elucidation of this mechanism may lead to successful strategies for inducing early PP ovulation and estrous cycles in suckled cows.

The objective of this thesis was to explore possible endocrine mechanisms by which suckling could delay PP ovulation in cows. This research was conducted using dairy rather than beef cows because of availability and amenability to handling and sampling procedures.

Experiment I was designed to confirm effects of suckling on the PP intervals to ovulation and observed estrus in Holstein cows. Treatments consisted of nonsuckled controls milked twice daily (NS-2X, $n = 5$), suckled cows milked twice daily (S-2X, $n = 6$) and nonsuckled cows milked four times daily (NS-4X, $n = 5$). Calves were free to nurse except while the milking machine was attached and during periods of estrus detection. Mean intervals to first PP ovulation did not differ between NS-2X and NS-4X treatments (19.4 ± 3.3 vs 23.0 ± 5.3, $P > .25$), whereas S-2X cows ovulated later (38.7 ± 4.9 days, $P < .05$) than nonsuckled cows. The PP interval to first observed estrus was

prolonged in S-2X cows compared to NS-2X cows (50.2 ± 4.3 vs 39.0 ± 2.4 days, $P < .06$) but not compared to NS-4X cows (44.8 ± 6.1 days, $P > .25$).

On days 7 and 14 PP, blood was collected at frequent intervals for 4.5 h centered on the 1600 h milking. Serum was assayed for luteinizing hormone (LH) and prolactin, with selected samples assayed for total glucocorticoids, follicle-stimulating hormone (FSH) and estradiol-17β. Mean serum LH was lower in S-2X cows than in NS-2X or NS-4X cows (.7 vs 1.4 or 1.3 ng/ml, $P < .05$), but LH in NS-2X and NS-4X cows did not differ ($P > .25$). During the anovulatory period, LH was released in an episodic pattern. The reduced mean LH in suckled cows resulted from decreased frequency and amplitude of episodic LH peaks ($P < .05$). Mean serum LH concentrations increased from day 7 to 14 PP across all treatments (.9 vs 1.4 ng/ml, $P < .05$), due to increased frequency and amplitude of LH peaks. Basal and milking-induced serum prolactin was greater on day 14 than on day 7 PP, but was not affected by suckling. No effects of suckling were apparent on serum FSH, glucocorticoids, estradiol-17β or progesterone.

In Experiment II, I examined how suckling decreased episodic LH secretion in early PP cows. Treatments consisted of nonsuckled milked controls (NS-2X, n = 8) and milked cows suckled ad libitum by two calves (S-2X, n = 9). Blood was collected at 15-min intervals for 8 h on day 13 PP, then cows were killed on day 14 postpartum. Hypothalami and pituitaries were collected for in vitro studies.

Experiment II confirmed that suckling reduced the frequency and amplitude of episodic LH peaks. Suckling did not significantly

alter serum FSH, prolactin, progesterone, estradiol-17β or glucocorticoids. Hypothalamic content of gonadotropin-releasing hormone (GnRH) did not differ between S-2X and NS-2X cows on day 14 PP (118.2 ± 16.3 vs 111.4 ± 19.5 ng/hypothalmus, $P > .25$). Suckling did not affect the LH, FSH or prolactin content of pituitaries from cows killed 14 days postpartum.

Pituitary explants were challenged in vitro with GnRH (25 ng/ml) or K^+ (59 mM) in continuous-flow superfusion. Suckling reduced significantly LH but not FSH released in response to either GnRH or potassium. Because similar amounts of tissue were superfused and tissue LH contents were not different in suckled and nonsuckled cows, it appears that LH in pituitaries of suckled cows is less releasable than that of nonsuckled cows. Reduced LH secretion in suckled cows did not appear to result from a specific reduction in response to gonadotropin-releasing hormone.

In overview, delayed PP ovulation in suckled cows is associated with reduced episodic secretion of luteinizing hormone. The reduced LH secretion does not reflect depleted hypothalamic GnRH or pituitary LH content, but may result from reduced hypothalamic GnRH secretion leading to reduced LH mobilization and secretion.

To my wife Carolyn.

ACKNOWLEDGMENTS

I wish to express my appreciation to my major professor, Dr. H. D. Hafs, for his encouragement and advice during the course of my studies at Michigan State University. I am also grateful to the other members of my graduate committee, Drs. R. H. Douglas, J. L. Gill, G. L. Waxler and E. M. Convey, for their efforts on my behalf during my course of studies and the preparation of this thesis. I am especially indebted to Dr. E. M. Convey for his advice and assistance in designing and executing my research as well as for his valued friendship. My fellow graduate students, the faculty and staff of the Dairy Science Department have all contributed immensely to the success of my graduate studies at Michigan State and I am very grateful to them.

I wish to thank the Department of Dairy Science, Michigan State University for providing facilities and funding for my research and the Medical Research Council of Canada for the Fellowship which has supported me during my graduate studies.

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INTRODUCTION

Females of many species undergo a period of reproductive acyclicity following parturition. The length of this anovulatory period may vary dramatically and be influenced by many factors among which nutrition, season and lactational or suckling intensity have been demonstrated to be of major importance. In cows, it appears that suckling is the principle stimulus for a prolonged postpartum (PP) anovulatory period although concurrent nutritional and/or environmental factors may exacerbate the suckling effect.

A 12-month calving interval is associated with maximum economic return for both beef and dairy cows; however, current industry averages range from 13 to 14 months. Since a 12-month calving interval includes the 280-day bovine gestation there remains only 85 days during which ovulatory ovarian activity must be reinitiated and conception occur. In most management schemes, breeding does not occur until detection (by bull or man) of the first PP behavioural estrus; however, ovulation not estrus is the critical prerequisite to conception. This is particularily apparent in view of recent advances in ovulation synchronization and timed artificial insemination which have reduced or eliminated the need for estrus detection. Unfortunately, these techniques are of limited practical value in acyclic cows.

The aim of this thesis research was to examine the influence of suckling on bovine PP reproduction and endocrinology in order to elucidate the mechanism by which suckling delays the first PP ovulation. Understanding the basic mechanisms controlling the interval to first PP ovulation in cows and the manner by which suckling delays this ovulation is important to development of management schemes to optimize PP reproduction in cattle.

REVIEW OF LITERATURE

General

The PP intervals to first ovulation and first observed estrus in cattle have been summarized by Casida (1971) and more recently by Stevenson (1977). The reported PP intervals for milked, suckled and nonlactating beef and dairy cows are summarized in Table 1.

Mean intervals to first PP ovulation ranged from 16 to 71 days whereas first observed PP estrus was reported to have occured after mean intervals of 20 to 84 days. The percentage of cows which did not exhibit detectable estrous behavior at the first PP ovulation ranged from 25 to 100% (Hackett et al., 1973; Hartigan et al., 1974; King et al., 1976), reasons for these "silent" ovulations are unclear. However, the absence of appreciable concentrations of serum progesterone prior to the first PP ovulation may have been involved in the reduced estrual behaviour since pre-exposure to progesterone has been shown to increase the behavioural response to exogenous estrogens (Melampy et al., 1957). Estrus without subsequent ovulation was infrequent in cattle (Labhsetwar et al., 1963; Casida, 1971) and frequency of "silent" ovulations decreased with both increasing PP interval and number of preceeding ovulations (Casida and Wisnicky, 1950; Kidder et al., 1952; Stevenson and Britt, 1977a). Delays in PP rebreeding that result

Table 1. Intervals to first postpartum (PP) ovulation and estrus in cows: Literature review.

Reference	Cow type[a]	Lactation status[b]	Interval[c] (days) to PP	
			Ovulation	Estrus
Saidduddin et al., 1968	D	NL	----	30.0
Casida & Venzke, 1936	D	M-2X	41.0	----
Clapp, 1937	D	M-2X	----	46.0
Olds et al., 1949	D	M-2X	----	30.0
Herman & Edmondson, 1950	D	M-2X	----	57.0
Buch et al., 1955	D	M-2X	----	33.0
Trimberger & Fincher, 1956	D	M-2X	44.0	50.0
Fosgate et al., 1962	D	M-2X	30.0	48.0
Menge et al., 1962	D	M-2X	20.0	34.0
Morrow et al., 1968	D	M-2X	16.4	----
Saiduddin et al., 1968	D	M-2X	----	23.5
Morrow et al., 1969	D	M-2X	22.9	----
Moller, 1970b	D	M-2X	35.9	----
Edgerton & Hafs, 1973	D	M-2X	----	36.0
Williams et al., 1973	D	M-2X	18.0	55.0
Britt et al., 1974	D	M-2X	19.5	39.3
Manns & Richardson, 1976	D	M-2X	19.0	----
Stevenson, 1977	D	M-2X	17.6	26.8
Goodale et al., 1978	D	M-2X	19.1	38.2
Clapp, 1937	D	M-4X	----	69.0
Clapp, 1937	D	S-1	----	72.0
Saiduddin et al., 1968	D	S-1	-----	45.0
Moller, 1970b	D	S-2X	60.6	----
" "	D	S-3	61.8	----
" "	D	S-4	71.0	----
Graves et al., 1968	B	NL	----	30.7
McCartor, 1972	B	NL	----	36.7
England et al., 1973	B	NL	21.3	38.6
Bellows et al., 1974	B	NL	----	20.2
LaVoie et al., 1974	B	NL	----	30.0
Wiltbank & Cook, 1958	B	M-2X	36.0	54.0
Lasley & Bogart, 1943	B	S-1	----	80.0
Wiltbank & Cook, 1958	B	S-1	53.0	84.0
Warnick, 1955	B	S-1	----	61.0

Table 1 (cont.)

Reference	Cow type[a]	Lactation status[b]	Interval[c] (days) to PP	
			Ovulation	Estrus
Foote et al., 1960	B	S-1	61.5	81.0
Foote & Hunter, 1964	B	S-1	44.0	49.0
Foote & Saiduddin, 1964	B	S-1	38.0	46.0
Graves et al., 1968	B	S-1	----	73.0
Oxenreider, 1968	B	S-1	36.0	45.0
Brown et al., 1973	B	S-1	55.0	48.0
McCartor, 1972	B	S-1	----	67.0
England et al., 1973	B	S-1	43.3	65.0
Bellows et al., 1974	B	S-1	----	40.4
LaVoie & Moody, 1974	B	S-1	----	62.0
Corah et al., 1975	B	S-1	----	49.0
Humphrey, 1977	B	S-1	----	76.0
Clemente et al., 1978	B	S-1	----	41.0
Holness et al., 1978	B	S-1	----	70.5
Oxenreider, 1968	B	S-2	55.0	55.0

[a] D = dairy breed, B = beef breed.

[b] NL = nonlactating (weaned < 14 days PP), M = milked, S = suckled; 2X or 4X refers to number of milkings or sucklings per day and the single number after S refers to the number of calves per cow.

[c] Mean within experiment.

from undetected estrus are amenable to management procedures which improve the level of estrus detection (Esslemont, 1974; Britt, 1975; Stevenson and Britt, 1977b) or eliminate it entirely by use of timed insemination following ovulation synchronization (Inskeep, 1973; Lauderdale, 1974; Roche, 1976).

Ovulation is required for conception and there is little evidence (excluding cystic ovarian disease and pyometra) that ovarian ovulatory cyclicity is interrupted prior to conception once the first spontaneous PP ovulation has occurred. For these reasons, understanding the mechanisms controlling the first PP ovulation and the factors which influence the timing of this event are crucial to intelligent manipulation of bovine PP reproduction.

Various factors that have been suggested to influence PP intervals in cows will be reviewed in the next section.

Factors Influencing the Intervals to First Postpartum Ovulation and Estrus in Cows

Suckling

The effect of suckling on reproduction has been known for many years. Seyle and McKeown (1934) recognized that suckling affected the occurrence of estrus in rats. Clapp (1937) reported that cows which suckled their calves exhibited a longer period between calving and first estrus than milked cows. Based on the literature tabulated in Table 1, it can be seen that beef cows had mean PP intervals to ovulation ranging from 21.3 to 61.5 days compared to 16.4 to 71.0 days for dairy cows. Also, suckled cows

had mean PP anovulatory periods of 36.0 to 71.0 days whereas non-suckled (milked or nonlactating) cows ovulated 16.4 to 44.0 days postpartum. Nonsuckled beef cows may ovulate by day 7 PP (Han and Moody, 1974), an interval even shorter than that of milked dairy cows. Also, the suckled dairy cow may have a PP anovulatory period lasting 60 to 71 days (Moller, 1970b). In both beef and dairy cows suckling by two or more calves was more effective in delaying PP ovulation than suckling by a single calf (Moller, 1970b; Bellows et al., 1974). This may have resulted from an increased frequency of suckling. However, even once daily suckling by a single calf had more effect than twice daily milking (Moller, 1970b), suggesting that the influence of the calf may be more complex than frequency of milk removal alone.

In conclusion, in both beef and dairy cows, suckling by one or more calves will cause a major increase in the interval to the first PP ovulation.

Lactational Intensity

In milked cows, an increase in frequency of milking from twice to four times daily has been shown to increase the PP interval to estrus (Clapp, 1937; Wiltbank and Cook, 1958); effects on the PP interval to first ovulation have not been reported. Nonlactating PP cows exhibited first ovulation and estrus as early as day 7 PP (Han and Moody, 1974). Heifers which were mastectomized prior to calving exhibited first PP estrus earlier and presumably ovulated earlier than nonsuckled intact controls (LaVoie et al.,

1974; Short et al., 1976; Samuelson et al., 1977), suggesting a possible effect of the mammary gland itself on PP reproduction.

In summary, although milking will prolong the interval to first PP ovulation and estrus, its effects are much less marked than those of suckling, probably because milking mimics only a portion of the complex of stimuli associated with a suckling calf.

Nutrition

A number of studies have demonstrated that reduced energy intake in the face of recommended levels of other nutrients resulted in prolonged PP intervals to ovulation and estrus (Wiltbank et al., 1962, 1964; Wagner and Oxenreider, 1971; McCartor, 1972). Prepartum energy balance appears more critical than PP energy intake since cows subject to high prepartum energy levels followed by restricted PP intake (H:L) had shorter intervals to estrus than L:H, L:L or H:H (Wiltbank et al., 1962, 1964). This may have reflected the importance of body condition at calving in determining the body weight change PP as reported by Oxenreider and Wagner (1971) and the reported shorter PP anovulatory period in cows losing the least body weight during the early PP period (Menge et al., 1962).

Wiltbank and coworkers (1962) suggested that a reduction in dietary energy may reduce pituitary gonadotropin production and/or release. McClure (1968) speculated that low energy intake reduces circulating glucose concentrations and that lack of glucose may reduce gonadotropin production by the pituitary. A recent report by Radford et al. (1978) does not support this

concept since serum glucose concentrations did not differ between suckled and nonsuckled beef cows during the first 100 days postpartum. Suckled cows in this same experiment had lower serum concentration of LH and markedly prolonged PP intervals to ovulation and estrus when compared to nonsuckled controls.

It should also be noted that reduced energy intake did not delay onset of PP ovarian activity in milked cows (Gardner, 1969; Whitmore et al., 1974; Corah et al., 1974; Ray et al., 1974) or in nonlactating cows (Oxenreider and Wagner, 1971).

The effects of excess energy on PP ovulation and estrus are less definite. Wiltbank et al. (1964) reported that a diet supplying 150% of National Research Council energy requirements resulted in prolonged intervals to first PP estrus in suckled beef cows. Morrow et al. (1969b) increased the PP interval by about nine days in dairy cows fed "liberal" amounts of concentrate. However, Holness and coworkers (1978) were unable to influence PP reproduction in lactating beef cows fed 130% National Research Council energy requirements. In practical terms, overfeeding of cows to an extent that impairs PP reproduction is an unlikely occurrence.

Adequate nutrition, primarily pre- and postpartum energy intake, would appear to be important in achieving optimum PP reproductive performance in cattle. However, the literature does not suggest that inadequate energy intake is the major mechanism by which suckling causes prolonged PP anestrus.

Age and Parity

Parity has been reported to influence the interval to first estrus by some (Hammond and Sanders, 1923; Casida and Wisnicky, 1950) but not all investigators (Warnick, 1955; Foote et al., 1960). Older cows with more than four calvings tended to have slightly longer PP intervals than cows with two to four calvings. However, a more marked effect of parity was evident with first-calf heifers. The prolonged PP intervals to ovulation and estrus in young primiparous heifers may have reflected insufficient energy intake to meet the demands of growth and concurrent lactation (Menge et al., 1962).

It appears that age and parity have variable but relatively minor effects on PP reproduction which may in fact derive from confounding nutritional inadequacies.

Other Factors

Genetic variation in interval to first PP ovulation has been measured with a reported hereditability estimate of approximately .2 (Menge et al., 1962; Saiduddin et al., 1968). The interval to first estrus was prolonged in cows with genetically high milk production potential as compared to cows with genetically low production potential (Whitmore et al., 1974).

In contrast to other species such as the sheep, season appears to have minimal if any affect of PP anestrus in cattle. A few researchers have reported that cows calving in winter had longer intervals to first estrus than those calving during other seasons

(Chapman and Casida, 1937; Buch et al., 1955; Carman, 1955). However, other workers have been unable to support this finding (Herman and Edmondson, 1950; Wiltbank and Cook, 1958).

Periparturient diseases (dystocia, retained placenta, metritis, ketosis, severe mastitis) have been associated with delayed first PP ovulation (Callahan et al., 1971; Morrow, 1971). However, the delays were relatively minor and the results of Morrow may have been confounded with effects of milk yield, age and parity.

Summary

The intervals from parturition to first ovulation and estrus may vary greatly among cows, and a number of factors have been shown to influence them including suckling, lactational intensity, nutrition, age, parity, genetics and periparturient disease. Suckling appears to be the most effective inhibitor of PP ovulation in both beef and dairy cows, and although the suckling effect may be compounded by other concurrent factors as in underfed and growing heifers, it does not appear that suckling exerts its effect indirectly via the other factors discussed above.

The next section of this review will expand upon the reproductive physiology and endocinology of PP cows and the influence of suckling on this system.

Postpartum Reproductive Physiology and Endocrinology: Effects of Suckling

Normal PP physiology and endocrinology will be described for the major components of the reproductive system and the effects of suckling will be stressed. Emphasis will be on cows, with relevant information from other species cited where gaps exist in the literature on cattle.

Uterus and Cervix

Information regarding uterine involution in cows has been reviewed (Morrow et al., 1969a; Moller, 1970a). The majority of reports have been based upon rectal palpation of uterine size, tone and fluid content. These researchers have found that gross involution of the uterus and cervix was completed by 20 to 25 days PP unless complicated by pathological conditions. Based on histological criteria, other researchers concluded that uterine involution was essentially complete by 20 to 30 days postpartum (Gier and Marion, 1968; Wagner and Hansel, 1969). Most authors have also concluded that involution takes about 5 days longer in multiparous than in primiparous cows (Rasbeck, 1950; Morrow et al., 1966).

Wiltbank and Cook (1958) reported that suckling delayed uterine involution whereas Wagner and Hansel (1969) concluded that suckling had no effect on involution. In studies based on uterine histology as well as gross characteristics, Riesen et al., (1968) and Lauderdale et al. (1968) reported that suckling significantly

increased the rate of uterine involution in dairy and beef cows, respectively.

Although suckling has been shown to hasten uterine involution in some studies (possibly thru the release of oxytocin), there is no evidence that the uterus limits the onset of PP ovarian ovulatory cyclicity in suckled cows. Also, based on histological examination of the uterus on day 20 or 70 after calving in ovariectomized (24 h PP) and intact animals, Sawhney et al. (1966) concluded that the ovaries had little influence on the uterus during the early PP period.

Adrenals

Glucocorticoids measured in serum of PP dairy cows milked twice daily were found to average 3.2 ± .3 ng/ml from 1 to 8 weeks postpartum (Edgerton and Hafs, 1973). Milking resulted in a significant increase in total serum glucocorticoids (Smith et al., 1972; Koprowski and Tucker, 1973). However, the glucocorticoid response to milk removal did not differ between cows that were suckled and those that were milked (Wagner and Ozenreider, 1971). Wagner and Hansel (1969) reported that adrenals of suckled cows had lower contents of progesterone and cortisol than did adrenals of milked cows and suggested reduced synthesis or increased secretion of these steroids in suckled cows. Adrenal hypertrophy was not seen in the suckled cows as might be expected if chronic hyperstimulation had occurred, and in a subsequent study (Wagner and Oxenreider, 1971) suckling did not affect adrenal size or steroid content.

In serum collected from cows over a 24-h period, glucocorticoids increased to >10 ng/ml more frequently in milked and suckled cows than in nonlactating cows (Wagner and Oxenreider, 1971), suggesting that possibly the frequency of suckling-induced adrenal stimulation could be involved in suppression of PP ovarian activity.

The adrenal glands have been shown to secrete both glucocorticoids and progesterone (Short, 1960; Resko, 1968); however, there is no evidence that adrenal progesterone secretion is increased by the presence of a suckling calf in the PP cow.

I conclude that although adrenal secretion is stimulated by suckling, the adrenals do not appear to be principle mediators of suckling's effects on PP reproduction in cattle.

Ovaries

Corpus Luteum of Pregnancy

The corpus luteum of pregnancy regresses rapidly during the few days immediately prior to and following parturition in cows. Little if any functional capability exists following parturition as measured by gross size on rectal palpation (Morrow et al., 1966), progesterone content (Labhsetwar et al., 1964; Oxenreider, 1968), histological structure (Wagner and Hansel, 1969) or serum progesterone concentrations (Erb et al., 1968: Stabenfeldt, 1969; Edgerton and Hafs, 1973; Corah et al., 1974). Suckling has no influence on the corpus luteum of pregnancy and PP anestrus is not a result of prolonged maintenance of the pregnancy corpus luteum.

Follicular Growth

Follicular development reached a nadir at the time of parturition with only scattered small follicles present on the ovaries (Labhsetwar et al., 1964). Following calving there was rapid resumption of follicular growth, and developing follicles were present on the ovaries within a few days postpartum (Cole and Hughes, 1946; Marion and Gier, 1968; Saiduddin et al., 1968). Labhsetwar et al. (1964) reported that follicles of mature size had developed by day 21 PP, and follicular fluid weight increased as early as day 10 PP then remained constant thru day 30 postpartum (Saiduddin et al., 1968).

These authors found no effects of suckling on ovarian follicular fluid weight on days 10, 20, or 30 postpartum. Wagner and Oxenreider (1971) reported that suckled cows averaged 16.0 ± 3.3 days until follicles grew to >10mm in diameter, significantly longer than nonlactating cows which averaged 9.0 ± 1.6 days. Milked cows were intermediate at 13.0 ± 1.4 days.

In conclusion, there appears to be morphologically adequate follicular development in cows by as early as one week PP, and suckling delays follicular growth only about one week. Apparently follicular growth up to at least the preovulatory stage is not markedly slowed by suckling, and retarded follicular growth does not appear responsible for the prolonged PP anovulatory period in suckled cows. This suggests that follicle-stimulating hormone (FSH) secretion is not imparied in suckled cows but rather that

luteinizing hormone (LH) secretion is affected since ovulation, and not follicular growth, is delayed.

Postpartum Ovarian Steroid Secretion

One to two days prior to parturition serum progesterone decreased precipitously from >5 ng/ml to <1 ng/ml at the time of calving (Stabenfeldt et al., 1970; Henricks et al., 1972; Edgerton and Hafs, 1973; Smith et al., 1973). In some studies, progesterone remained low or undetectable from calving until corpus luteum formation following the first PP ovulation (Arije et al., 1974; Echternkamp and Hansel, 1973; Stevenson, 1977). Several other investigators have reported transitory (3 to 5 day) increases (>1 ng/ml) in serum progesterone concentration which return to baseline immediately before the first apparent PP estrus (Pope et al., 1969; Donaldson et al., 1970; Tribble et al., 1973; Corah et al., 1974; LaVoie et al., 1976; Humphrey et al., 1976; Castenson et al., 1976). Based on visual inspection of the ovaries, Castenson et al. (1976) concluded that in seven of eight beef heifers the pre-estrus progesterone increase they observed had been due to a corpus luteum formed following a prior "silent" ovulation. To a less marked degree, the corpus luteum lifespan has been reported to be shorter during the first PP estrous cycle than in subsequent cycles (Morrow et al., 1966; Marion and Gier, 1968; Pope et al., 1969; Corah et al., 1974; Stevenson, 1977). The source of the transitory progesterone increase was not definitively determined in the other studies. However, Humphrey et al. (1976)

collected blood at 6-h intervals and were unable to show an increase in estradiol or a preovulatory-like LH surge before the pre-estrus progesterone increase. They speculated that the source of the progesterone may have been partially luteinized anovulatory follicles. In support of this view, Wagner and Hansel (1969) described a thickening and infolding of granulosa cells in large follicles which approached but did not reach maturity in PP cows. They suggested that this phenomenon may have represented unsuccessful or partial luteinization.

Although the majority of investigators who observed a transient progesterone increase prior to first estrus used suckled beef cows, this phenomenon has been described in nonlactating beef and milked diary cows; therefore it does not appear to be a direct results of suckling.

Other progestogens in serum have not been extensively characterized during the bovine PP period although in one report by Tribble et al. (1973) serum concentration of 20β-hydroxypregn-4-ene-3-one was found to be increased prior to the first PP LH surge and 17-hydroxypregn-4-ene-3,20-dione concentration peaked at or near standing estrus. The study was limited to three primiparous nonlactating heifers.

Estradiol-17β, estrone and total estrogen concentrations increased linearly in serum during the last 25 to 30 days of gestation. These estrogens were derived from the placenta and not the ovary (Henricks et al., 1972; Stellflug, 1972; Edqvist et al., 1973; Smith et al., 1973). Following a peak immediately prior to

parturition, estrogen concentrations in serum declined to low values by four to eight days postpartum (Robinson et al., 1970; Arije et al., 1974; Echternkamp and Hansel, 1973; Smith et al., 1973; Corah et al., 1974; Stevenson, 1977). Serum estrogen levels remained relatively low during the PP interval until immediately prior to the first PP ovulation when they increased to typical proestrus concentrations of 12 to 25 pg/ml (Henricks et al., 1972; Echternkamp and Hansel, 1973; Humphrey et al., 1976; Stevenson, 1977). The low amplitude fluctuations in estrogen secretion during the PP period may have reflected follicular growth and atresia occurring at this time.

Humphrey (personal communication, 1978) measured serum estrone concentrations in PP suckled beef cows and found discrete peaks which lasted one to three days and occurred at approximately 10- to 14-day intervals from calving until first ovulation. These peaks of estrone may represent waves of follicular growth occurring in the absence of adequate concentrations of luteinizing hormone. In support of this view, Nadaraja and Hansel (1976) reported that the major estrogen secreted in heifers passively immunized against LH was estrone.

No reports in the literature have demonstrated a direct influence of suckling on serum estrogen concentrations during the PP anovulatory period.

Profiles of ovarian steroids in serum of suckled cows have not been demonstrated to differ from those in nonsuckled cows, except that the interval from parturition to the acute preovulatory

follicular growth and estradiol-17β secretion is prolonged in suckled cows.

Ovarian Responsiveness to Exogenous Gonadotropins

Casida et al. (1943) demonstrated that the ovaries responded with follicular growth and ovulation to exogenous gonadotropins injected early in the PP period of suckled beef cows. Oxenreider (1968) was also able to induce ovulation in lactating beef cows within one week of calving using a combination of pregnant mares serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG). A single intravenous injection of HCG on days 5, 15 or 25 PP in suckled Hereford cows caused a reduction in the interval to first ovulation from 47 days in saline controls to 29 days in HCG-treated cows (Foote et al., 1966). However, it appeared that the HCG-induced corpus luteum had a shorter than normal lifespan in some cows and there was a failure to reovulate in some animals following regression of the induced corpus luteum. Porcine-FSH and HCG injections induced corpora lutea formation in 90% of anestrus PP beef cows when treatment was commenced 30 to 40 days PP (Rovira et al., 1978). Wettemann et al. (1978a) found that the ovaries of anestrous beef cows on day 45 PP secreted both estradiol and progesterone in response to PMSG injection. Echternkamp (1978) was unable to demonstrate an influence of suckling on ovarian steroid secretion following PMSG treatment on day 42 PP in beef cows.

Fertility was increased in suckled cows treated with PMSG following synchronization with short-term progesterone treatment (Mulvehill and Sreenan, 1977); however, earlier work by Brown et al. (1972) found no effects of exogenous gonadotropins on fertility following treatments with progesterone and estradiol valerate in PP cows.

In summary, only limited direct comparisons of ovarian response to exogenous gonadotropins have been made between suckled, milked and nonlactating PP cows. However, since the ovaries of suckled cows will respond to exogenous gonadotropic stimulation with follicular growth and ovulation very shortly after parturition, it would appear that suckling's effect on PP reproduction does not result from inability of the ovaries to respond to an appropriate gonadotropic stimulus.

Effects of Exogenous Steroids on Postpartum Intervals

Attempts to reduce the interval to first ovulation, estrus and conception with progestogens and/or estrogens have been made with varying degrees of success (Ulberg and Lindley, 1960; Foote and Hunter, 1964; Saiduddin et al., 1968; Hill et al., 1972; Brown et al., 1972; Britt et al., 1974; Lavoie and Moody, 1974; Cupin et al., 1975). From these and other studies it would appear that short-term (7 to 14 day) progestogen administration with or without estrogen was the most consistently effective treatment for reducing the PP intervals to estrus and conception in beef and dairy cows.

Searle Agriculture Inc. (Elburn, Il.) has widely promoted its Synchro-Mate-B (SMB) treatment for synchronization of ovulation and estrus in PP beef cows. The SMB treatment consists of an implant (6 mg of the synthetic progestogen Norgestomet) placed subcutaneously in an ear for 9 days plus an intramuscular injection of 3 mg Norgestomet and 6 mg estradiol valerate on day one of the implant. This treatment is frequently used in combination with 24- or 48-h temporary weaning at the time of implant removal and timed insemination 48 to 60 h thereafter. This series of procedures has been found to increase the percentage of treated cows conceiving early in the breeding season (Mares et al., 1977; Smith et al., 1977; Kaltenbach et al., 1978). Walters and coworkers (1977) reported that SMB was relatively ineffective in thin anestrus beef cows and that complete weaning in conjunction with SMB was more effective than only 48-h weaning after SMB. Also, in the study of Mares et al. (1977), the cows which were classified as noncyclic or anestrus had pregnancy rates 15 to 20% lower than those of cyclic cows.

I conclude that although short-term progesterone treatment will synchronize cycling beef and diary cows, it is only partially effective at inducing cyclic activity in anestrus beef cows and its efficacy is markedly impaired by high suckling intensity and/or low energy intake.

Hypothalamo-Pituitary Axis

Hypothalamus

The major factors controlling secretion of the pituitary gonadotropins LH and FSH as well as prolactin are the hypothalamic-releasing factors which are secreted by modified neurons within the medial-basal hypothalamus and delivered to the anterior pituitary via the hypothalamo-hypophyseal portal circulation. The historical development and current concepts of the neuroendocrine theory are beyond the scope of this thesis; however, they have been extensively reviewed (Convey, 1973; Schally et al., 1975, 1976; Arimura, 1976).

To date, the evidence suggests that secretion of LH and FSH is controlled by a single stimulatory hypothalamic-releasing factor, the decapeptide gonadotropin-releasing hormone (GnRH; Schally et al., 1975). First isolated from hypothalami of swine (Schally et al., 1971) and sheep (Amoss et al., 1971), the amino acid sequence of GnRH has been identified and synthesized (Matsuo et al., 1971). Species differences in its structure have not been reported. The ability of GnRH and/or its synthetic analogues to release both LH and FSH has been reviewed (Convey, 1973; Schally et al., 1975, 1976; Arumura, 1976).

Zolman and Convey (1972) demonstrated that GnRH caused LH release from bovine pituitary tissue in vitro using a continuous-flow superfusion system. In vivo, both heifers and bulls secreted LH in a dose-dependent manner in response to increasing amounts of intravenously injected GnRH (Zolman et al., 1973, 1974). FSH

release in response to synthetic GnRH injection has been demonstrated in cows (Akbar et al., 1973). Gonadotropin secretion in response to exogenous GnRH administered to PP cows and the effects of GnRH on PP intervals to ovulation and estrus will be reviewed later.

Gonadotropin-releasing hormone has been localized and quantified in the bovine hypothalamus (Estes et al., 1977) and median eminence (Kizer et al., 1976). Estes and coworkers (1977) found GnRH content to be greatest in hypothalamic sections corresponding to the median eminence and pituitary stalk with lesser concentrations located bilaterally in the anterior hypothalamus. Comparisons of hypothalamic GnRH content and localization in suckled and nonsuckled cows have not been reported; however, hypothalami of lactating PP rats have less GnRH bioactivity than those of nonlactating PP rats (Minaguchi and Meites, 1967).

The possibility that suckling affects hypothalamic control of LH secretion has been suggested by two types of experiments. First, suckling has been demonstrated to reduce the post-ovariectomy increases in serum LH and FSH in PP rats (Smith and Neill, 1977) and rhesus monkeys (Weiss et al., 1976). Echternkamp (1978) recently reported that the frequency of episodic LH secretion and the minimum LH concentration observed on day 30 PP was greater in nonsuckled than in suckled beef heifers which had been ovariectomized on day three postpartum. If the post-ovariectomy gonadotropin increase represents predominantly an increased hypothalamic secretion of GnRH then these studies suggest that suckling may act to

inhibit GnRH release. Secondly, Short et al. (1974) reported that intramuscular injection of estradiol benzoate (10 mg) tended to be less effective in stimulating LH surges in suckled beef heifers at 2 weeks PP than in nonsuckled controls; however, this trend was not evident at 4, 6 or 8 weeks postpartum. A recent study by Radford et al. (1978) demonstrated a more consistent failure of suckled anestrus PP cows to respond to estrogen injections (500 μg estradiol benzoate in oil) with a LH surge at 6 weeks PP as compared to 14 weeks postpartum. Comparisons between suckled and nonsuckled cows in this experiment are questionable since nonsuckled cows had ovulated and therefore been exposed to corpus luteum progesterone secretion prior to the estrogen challenge whereas suckled cows had not ovulated nor been exposed to elevated progesterone concentrations. Although results in the cow are somewhat inconsistent, there is a suggestion that suckling may act to prevent estrogen-induced LH surges, an effect that probably involves inhibition of hypothalmic GnRH secretion.

Anterior Pituitary Gland

Hormone Content

Early reports on gonadotropins in the PP cow were concerned primarily with anterior pituitary content as measured by bioassay. Pituitary LH content has been reported to be low at parturition and to increase PP (Labhsetwar et al., 1964; Saiduddin and Foote, 1964; Quevedo et al., 1967; Saiduddin et al., 1968; Wagner, Saatman and Hansel, 1969). Pituitary FSH content peaked at parturition and

then decreased following calving (Labhsetwar et al., 1964; Saiduddin et al., 1968). Pituitary FSH content increased again to concentrations equivalent to those in late pregnancy by day 15 of the first and subsequent PP estrous cycles (Saiduddin et al., 1968). Thus, the decline in pituitary FSH during PP anestrus may have resulted from decreased steroid feedback at this time. Secretion of FSH appears to be adequate at this time as reflected by the observed PP resumption of follicular growth. Suckling did not affect pituitary LH or FSH content in beef or dairy cows (Labhsetwar et al., 1964; Saiduddin et al., 1968).

Pituitary prolactin bio-activity as measured by the pigeon crop assay did not differ when measured on day 1, 10, 20 or 30 PP in dairy cows (Riesen et al., 1968), nor on day 3 or 15 following the first PP estrus in beef cows (Lauderdale et al., 1968). In the above studies suckled dairy but not beef cows had a slight but significant decrease in pituitary prolactin activity when compared to nonsuckled controls.

Thyroid-stimulating hormone content varied from 3.1 to 4.2 USP mU/mg anterior pituitary tissue in PP cows and differences due to suckling or PP interval were not significant (Wagner et al., 1969).

In conclusion, pituitaries of suckled cows do not appear markedly different in pituitary hormone content from those of non-suckled cows and would not appear to be the limiting factor in the onset of PP reproductive cyclicity.

Anterior Pituitary Hormones in Serum of Postpartum Cows

With the development of specific and sensitive radioimmunoassays for anterior pituitary hormones, recent studies have concentrated on the characterization of their serum profiles during the PP period.

Serum LH concentrations were low during late pregnancy and immediately PP in samples collected at daily or weekly intervals (Erb et al., 1971; Arije et al., 1974; Edgerton and Hafs, 1973; Ingalls et al., 1973). Concentrations of LH increased within one to two weeks PP in serum of nonsuckled cows (Echternkamp and Hansel, 1973; Edgerton and Hafs, 1973; Ingalls et al., 1973; Randel et al., 1976). When blood was collected at 15- to 60-min intervals, distinct but brief increases in serum LH were reported to occur prior to the first PP preovulatory LH surge (Schams et al., 1972; Humphrey et al., 1976; Stevenson, 1977; Goodale et al., 1978). These brief episodic releases of LH resulted in average peak serum concentrations of 1.5 to 4 ng/ml which declined to baseline within 15 to 30 min (Stevenson, 1977; Goodale et al., 1978). Controlled studies on the effects of suckling on episodic release of LH have not been reported; however, their onset appears to be delayed in the suckled beef cow (Humphrey et al., 1976).

Only limited information is available on serum FSH concentrations in cattle during any reproductive stage due to a lack of satisfactory radioimmunoassays. Recently, Dobson (1978a, b) described changes in serum FSH concentrations during the bovine

estrous cycle and PP period in milked dairy cows. Postpartum concentrations of FSH were similar to those occurring during the luteal phase of the estrous cycle. Schallenberger (1977) has reported on serum FSH during the PP period and described wavelike peaks of FSH occuring at 12- to 14-day intervals. Neither concentration nor pattern of FSH secretion was affected by suckling in this study. Characteristics of these two FSH radioimmunoassays and the physiological results reported await confirmation in other laboratories.

Control of prolactin secretion in cattle has been reviewed by Karg and Schams (1974). A distinct peak of prolactin occured around parturition and then concentrations decreased to prepartum baseline values by 2 to 3 days postpartum (Schams and Karg, 1969; Johke, 1971; Ingalls et al., 1973; Karg and Schams, 1974). Milk removal, either by suckling or milking, was one of many extrinsic stimuli shown to cause acute prolactin release in cows (Johke, 1969, 1970; Karg and Schams, 1970; Fell et al., 1971; Tucker, 1971; Koprowski and Tucker, 1971). The abrupt PP decrease in serum prolactin seen in cows is distinct from the sustained PP hyperprolactinemia seen in suckled rats (Amenomori et al., 1970), goats (Johke et al., 1971) and sheep (Kann et al., 1977). The difference probably arises from differences in suckling intensity and frequency since restricted suckling caused a rapid fall in serum prolactin in these species (Amenomori et al., 1970; Johke et al., 1971).

There is no conclusive evidence of a role for prolactin in controlling bovine reproductive processes with the exception of mammogenesis and lactogenesis. On the contrary, Hoffman et al. (1974) were unable to demonstrate a luteotrophic action of prolactin in the cow, and inhibition of prolactin secretion with the ergot alkaloid CB-154 (2-bromo-α-ergocryptine-methansulphonate, Sandoz, Switzerland) did not affect the estrous cycle of cows (Karg and Schams, 1974). In addition, recent studies on PP cows have used CB-154 to suppress endogenous prolactin without effects on the response to estrus synchronization at 50 days PP (Kaltenbach et al., 1977; Williams and Ray, 1978) or the PP interval to estrus (Clemente et al., 1978; Gimenez et al., 1977; Williams and Ray, 1978). Also, Clemente et al. (1978) were unable to influence occurrence of the first PP estrus by passive immunization of suckled cows with prolactin antiserum.

Suckling may selectively delay the episodic release of LH in PP cows without altering circulating concentrations of follicle-stimulating hormone. However, controlled contrasts between suckled and nonsuckled cows are lacking and the mechanism by which suckling affects LH and FSH secretion is not clear. Serum prolactin may be increased marginally in suckled cows but does not appear to be involved in suckling-induced PP anestrus in cattle.

Effects of GnRH on Gonadotropins and Postpartum Intervals

Pelletier (1976) has reviewed the influences of GnRH on LH and FSH secretion in domestic animals including cows. Treatment

of dairy cows with GnRH caused release of LH (Kaltenbach et al., 1974; Zolman et al., 1974; Kesler et al., 1977). Ovulation followed by recurrent estrous cycles occurred when GnRH was given 14 to 20 days after calving in dairy cows (Schams et al., 1973; Britt et al., 1974; Manns and Richardson, 1976; Garverick et al., 1978). Dairy cows responded to GnRH injection with reduced LH release during the first 1 to 2 weeks PP possibly reflecting low pituitary LH stores (Kesler et al., 1977). The LH response was linearly related to the log of the GnRH dose injected into cycling beef cows suckling calves (Schams et al., 1974; Webb et al., 1977). In anestrus suckled beef cows, maximal LH release in response to GnRH injection increased until day 20 PP; a pattern similar to that observed for early PP dairy cows (Irvin et al., 1977; Webb et al., 1977). A single GnRH injection caused anestrus beef cows to undergo follicular growth and luteinization or ovulation as indicated by progesterone secretion. However, these cows frequently failed to establish normal ovulatory cycles following this brief increase in serum progesterone (Fonseca et al., 1977; Irvin et al., 1977; Wettemann et al., 1978a). A second GnRH treatment 10 to 14 days after the first resulted in ovulation and initiation of cyclic activity in a high proportion of these cows (Fonseca et al., 1977; Webb et al., 1977).

Lishman et al. (1977) were unable to increase the LH released in response to 300 μg of GnRH in 30-day PP suckled beef heifers by priming injections of FSH nor was the interval to first estrus reduced. In a study using 24-day PP beef cows, 32-h but not

24-h weaning prior to 300 μg of GnRH caused an increase in LH released as compared to nonweaned controls. The PP interval to ovulation was not affected and exceeded 70 days (Inskeep et al., 1977). Fertility in suckled beef cows synchronized with SMB treatment and weaned between implant removal and insemination was not improved by injection of 125 μg GnRH at 30 h post implant removal (Kaltenbach et al., 1978). Estrogen enhanced the LH release in response to GnRH infusion (Ellicott et al., 1978) or injection (Irvin et al., 1978) in early PP beef cows. Hyperprolactinemia induced by multiple injections of thyrotropin-releasing hormone did not effect the GnRH-induced LH release in ewes (Kann et al., 1976) or beef cows (Nancarrow and Radford, 1976).

Follicle-stimulating hormone release in response to GnRH has not been described for either suckled or nonsuckled PP cows.

Direct comparisons of LH release in response to GnRH have not been reported for suckled versus nonsuckled cows during the early PP period. Echternkamp (1978) was unable to demonstrate an influence of suckling on LH secretion in response to 7, 150 or 300 μg of GnRH injected on day 42 PP in beef heifers. Since the pituitary of the suckled cow responds to GnRH with LH secretion as early as 1 to 2 weeks PP, well before the first spontaneous PP ovulation occurs, the depressed serum LH and delayed ovulation does not appear to reflect pituitary insensitivity to GnRH but rather may be a result of decreased GnRH release from the hypothalamus.

Summary

Suckling has an inhibitory effect on PP reproduction in beef and dairy cows that appears to be discrete from that of other factors such as nutrition. The end result of this inhibition is a prolonged PP annovulatory and anestrus interval in suckled cows which leads to calving intervals that exceed the optimum 12 months. The mechanism(s) by which the suckling stimulus or complex of stimuli delays ovulation is unknown. Neither the uterus nor adrenals appears to play a major role in inhibition of ovulation by suckling although adrenal secretion is stimulated by suckling. Ovaries of suckled anestrus cows will ovulate in response to exogenous or endogenous gonadotropins, suggesting that their failure to ovulate spontaneously is secondary to inadequate gonadotropin secretion.

Serum prolactin in suckled cows may be elevated over that of controls; however, this hyperprolactinemia is not great nor has it been demonstrated to have antigonadotropic actions in the cow. It must be remembered that results obtained in PP sheep, primates and rats may not extrapolate well to the PP cow since prolactin has been demonstrated to play a significant role in PP anestrus in the former species but not in the cow.

Changes in FSH secretion have not been well characterized in the PP cow but do not appear to be involved in suckling-induced anestrus. This view is supported by the presence of follicular growth in early PP nonsuckled and suckled cows and by similar

pituitary FSH content in nonsuckled and suckled cows. In addition, limited available data suggests that concentrations of FSH in serum of suckled cows are apparently similar to values observed during the bovine estrous cycle.

In contrast, serum LH concentrations are low in early PP cows and although definitive studies have not been reported, suckling appears to delay onset of episodic LH secretion. Since pituitaries of suckled cows secrete reduced amounts of LH in response to exogenous GnRH only during the first 1 to 2 weeks PP, suckling-induced depression of serum LH after this time may reflect a decreased pituitary stimulation as a result of suckling. The major trophic factor involved in acute release of LH is believed to be hypothalamic gonadotropin-releasing hormone. Although the hypothalamic distribution of GnRH has been reported for the bovine, no studies in cattle have reported the effects of suckling on the content or distribution of GnRH in the hypothalamus. Hypothalamic GnRH bioactivity was found to be reduced by suckling in PP rats. The possibility of a suckling-induced hypothalamic dysfunction, failure to release GnRH in response to stimuli such as estrogen injection or ovariectomy, has been suggested in studies on suckled ewes, monkeys and rats as well as in cattle.

Since dairy cattle were readily available and the literature suggested that suckling effectively increased the PP anovulatory period in dairy as well as beef cows, Experiment I was designed with the following objectives:

1. to determine the suitability of the PP dairy cow as a model system for studying PP anovulation and anestrus caused by a suckling calf and

2. to characterize the effects of suckling on PP intervals and serum concentrations of reproductively significant hormones.

Based on the results of Experiment I and the literature reviewed, a second experiment was designed to investigate the hypothesis that suckling-induced depression of serum LH was a result of altered hypothalamic GnRH content or release. More specificly the objectives of Experiment II were to determine the effects of suckling by two calves on:

1. hypothalamic GnRH content and gross distribution,

2. pituitary LH, FSH and prolactin content,

3. *in vitro* pituitary secretion of LH and FSH in response to GnRH or elevated K^+ concentrations,

4. serum profiles of LH, FSH and prolactin, as well as

5. estimates of ovarian and adrenal activity by measurement of serum estradiol-17β, progesterone and glucocorticoid concentrations.

It was felt that these experiments would lead to an increased understanding of both the level at which and the mechanism by which the suckling stimulus acts to delay the first PP ovulation in cow. This would be important in designing logical and effective management regimes for controlling PP reproduction in cows.

MATERIALS AND METHODS

General

Blood Collection and Handling

Blood samples were collected as indicated in each experiment by either percutaneous puncture of the coccygeal artery or vein using a 20 G needle or via jugular vein cannulae (Medical polyvinyl tubing, sive v-10; Bolab Inc., Derry, N.H.) which were inserted at least 12 h prior to sample collection. Blood samples were allowed to clot at room temperature for 4 to 8 h then stored at 4 C for 24 to 48 h before harvesting serum. After centrifuging at 2000 x g for 30 min, serum was decanted into 12 x 75 mm culture tubes, capped and stored at -20 C until assayed.

Assays

Detailed protocols for the competitive binding assays used in this research as well as the validations of the follicle-stimulating hormone (FSH) and estradiol-17β radioimmunoassays are contained in Appendices A thru G.

In overview, luteinizing hormone (LH), prolactin, progesterone and gonadotropin-releasing hormone (GnRH) were measured using previously described double antibody radioimmunoassays (Convey et al., 1976; Tucker, 1971; Convey et al., 1977; Estes et al., 1977). Total serum glucocorticoids were determined by competitive protein binding assay as described by Smith et al. (1973).

Bovine FSH was quantified by a homologous radioimmunoassay using antiserum (rabbit anti-bovine FSH-B2) and highly purified bovine FSH (FSH-HS-2) generously provided by Dr. K. W. Cheng.[1] The assay was performed as previously described (Cheng, 1978) with three modifications. First, the purified bovine FSH was iodinated using chloramine-T rather than the lactoperoxidase method used by Cheng. The iodination method has been described (McCarthy, 1978) and consisted of reacting 5 μg FSH, 1 mCi $Na^{125}I$ and 5 μg chloramine-T for 120 sec at 25 C in a total volume of 30 μl of phosphate (.5M) buffered (pH 7.4) saline. The reaction was quenched with 12.5 μg sodium metabisulfate (2.5 μg/μl in water) and 100 μl transfer solution (16% sucrose and 1% KI in water). Products were separated immediatley on a .8 x 15 mm column of Biogel P-60 (BioRad Laboratores, Richmond, CA) using phosphate (.01M) buffered (pH 7.0) saline (.14M) containing .1% bovine serum albumin (BSA) as eluant. The second modification was in using a total sample volume of 500 μl for all standards and unknowns. Finally, normal rabbit serum (excess gamma globulin required for double antibody precipitation) was included with the first antibody rather than being added separately at the time of second antibody addition.

Estradiol-17β was assayed using antiserum (rabbit anti-estradiol-17β-11β-hemisuccinate: BSA, #930) and ^{125}I labelled

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[1]University of Manitoba, Winnipeg, Manitoba, Canada.

estradiol-17β-11α-tyrosine methyl ester as tracer.[2] Radioiodination and assay procedures were similar to those described by England et al. (1974) and are detailed in Appendix G. Notable modifications included use of diethyl ether for serum extraction, rather than benzene, and chromatography using commercially prepared columns (Quik-Sep Extended LH-20 Columns, Isolab Inc., Akron, OH).

Protein content of tissue extracts was determined by dye-binding assay (BioRad Protein Assay; RioRad Laboratores, Chemical Division, Richmond, CA) as described by Bradford (1976).

Statistical Analysis

Radioimmunoassay results were calculated using a Fortran language interactive program on the Michigan State Univeristy time-share computer system. Raw data were first corrected for background and expressed as percent of total binding or zero standards. Then standard curve means were fitted to a third order polynomial equation using least squares methods. Unknown concentrations were estimated by solving this equation for each unknown. Appropriate corrections were made for volume assayed, dilutions and extraction efficiency.

Statistical analyses of serum hormone concentrations were performed as described by Gill (1978a, b). Double split-plot

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[2]Antiserum and unlabelled estradiol-tyrosine methyl ester conjugate were provided by Dr. K. T. Kirton of The Upjohn Co., Kalamazoo, MI.

analysis of variance with repeated measures over time was the basic model used. Treatment group, sampling period and sampling time (within period) were the main effects. Homogeneity of variance was tested and data transformed to logarithms where appropriate. Treatment means were compared using appropriate tests: f-tests of orthogonal contrasts, Scheffé's interval, Student's-t, etc.

Experiment I: Effects of Suckling on Postpartum Intervals and Serum Hormone Concentrations in Holstein Cows

For reasons of availability and manageability, the dairy cow was more amenable than the beef cow for studies on postpartum (PP) reproductive endocrinology which involve frequent blood sampling and animal handling. Based on this premise, the rationale behind this experiment was to determine the suitability of the suckled Holstein cow as a model for studying the mechanisms of suckling-induced PP anovulation, a problem of economic importance in the less easily studied beef cow. The experiment had these specific objectives:

1. to characterize the PP intervals to ovulation and estrus,
2. to measure the chronic and acute fluctuations in serum gonadotropins, prolactin and steroids during the early PP period in our experimental population of milked Holstein cows, and
3. to determine the effects of a single suckling calf on these functional and endocrine parameters.

Multiparous Holstein cows from the Michigan State University dairy herd were assigned at calving to one of three treatment

groups. Cows in treatment I were nonsuckled controls milked at 12-h intervals (NS-2X) and those in treatment II were suckled ad libitum by one calf and milked at 12-h intervals (S-2X). Treatment III cows were not suckled and were milked at 6-h intervals (NS-4X). All animals calved in individual box stalls and at approximately 24 h PP nonsuckled cows (I and III) were moved to tie stalls located in the same barn. Suckled cows (II) remained in their box stalls with their calves and were tied in the same manner as the nonsuckled cows. Beginning on day two PP, all cows were milked in their stalls using a portable milking machine and vacuum pump. Calves were free to nurse at all times except while the milking machine was attached and during periods of estrus detection.

All cows were fed individually a ration of corn silage (30% dry matter), concentrate mix (11 to 15% crude protein), alfalfa hay (88% dry matter) and haylage (60% dry matter) calculated to meet 100% NRC (1971) requirements for lactating cows. Animals were not involved in concurrent nutrition experiments.

Estrous detection consisted of visual observation from 0600 to 0800 h and from 1700 to 1800 h during which periods the cows were penned outdoors with a minimum of five other cows and a testosterone-treated teaser heifer (Kiser et al., 1977; Stevenson and Britt, 1977b). Each animal was also fitted with a Mate Master rump-mounted heat detector (Stevenson and Britt, 1977). Estrus was defined as standing to be mounted by other cows or the teaser heifer.

Ovaries were palpated per rectum twice weekly to record follicular growth, ovulation and corpus luteum development. Prior to each palpation a 10-ml tail vein blood sample was collected; later this serum was assayed for progesterone content and results were used in conjunction with palpation data to determine time of first ovulation. For statistical analysis, time of first ovulation was set at 48 h prior to the first increase in progesterone to greater than 1 ng/ml for more than two consecutive samples.

At weekly intervals from day 7 PP until day 28 PP or first ovulation whichever was earliest, blood samples were collected from indwelling jugular cannulae at frequent intervals for 4.5 h centered on the 1600 h milking. Serum from these samples was assayed for luteinizing hormone and prolactin, with selected samples from each period also assayed for total glucocorticoids, follicle-stimulating hormone and estradiol-17β.

Experiment II: Effects of Suckling on the Hypothalamo-Pituitary Gonadotropic Axis of Early Postpartum Holstein Cows

Based on Experiment I, this experiment was designed to determine the mechanisms by which suckling affected the hypothalamo-pituitary luteinizing hormone (LH) axis.

The in vivo portion was essentially a modification of Experiment I with suckling intensity increased by use of two calves and frequent blood sampling only on day 13 postpartum. Its objective was to confirm the relationships between suckling and serum hormone concentrations observed in Experiment I.

The rationale for the in vitro studies was to define the level(s) at which the suckling stimuli alter LH secretion by measuring serum, pituitary and hypothalamic components of the LH control system within each animal of the suckled and nonsuckled treatments. A direct functional assessment of the anterior pituitary was obtained by measuring the LH release in response to maximal doses of gonadotropin-releasing hormone (GnRH) and elevated potassium ion (K^+). Receptor-mediated LH release as demonstrated by response to GnRH was compared to LH released by 59 mM K^+ and used to evaluate the functional adequacy of the pituitary gonadotrophs' GnRH receptors.

In Vivo

Healthy, pregnant Holstein cows were purchased in April, 1977 and maintained on pasture until they calved during May. Supplemental feed consisting of alfalfa hay and haylage: corn silage (1:2) was provided prepartum. At parturition, cows were assigned alternately to be suckled ad libitum by two calves (n=9, S-2X) or nonsuckled as controls (n=8, NS-2X) with each suckled and subsequent nonsuckled cow being considered as one treatment pair for future sampling and slaughter assignment.

From calving until slaughter on approximately day 14 PP all cows were handled as follows. Within 2 to 4 h of calving, cows were moved from pasture to tie stalls and calves were removed from nonsuckled dams and fostered onto the respective suckled cow of that treatment pair. Calves were restricted to the area of

their dams by fences enclosing a 2 x 5 m area with the cow tied at one end. Animals were maintained under natural photoperiod and fed a ration of alfalfa hay, corn silage and 11 to 14% crude protein concentrate mix to meet or exceed 100% NRC requirements. Water was supplied ad libitum. In an attempt to minimize differences in nutritional drain caused by lactation (i.e. suckled cows possibly producing less milk than nonsuckled cows), cows in both groups were machine-milked at 0600 and 1800 h daily; milk weights were recorded at each milking. Body weights were recorded during the early afternoon on days 4, 8 and 12 PP, as well as on the morning of slaughter. Neither feed nor water were restricted prior to weighing or slaughter and cows were milked at the scheduled time on the morning of slaughter.

Tail vein blood was collected prior to each morning milking. On day 12 PP cows were fitted with jugular cannulae. Jugular blood was sampled at 15-min intervals from 0815 to 1200 and from 2015 to 2400 h on day 13 PP for characterization of serum LH, prolactin, and follicle-stimulating hormone (FSH) profiles.

Treatment pairs, consisting of one suckled and one nonsuckled cow, were sampled together and then killed between 0700 and 0800 h on day 14 PP in the Michigan State Univeristy Meat Science abbatoire. Animals were stunned using a captive bolt pistol and then rapidly exsanguinated.

In Vitro

Tissue Collection

Hypothalami with pituitary stalks attached were collected within 22 ± 1.5 min of stunning, frozen on Dry Ice then stored at -60 C until trimmed, extracted and extracts assayed by radioimmunoassy for gonadotropin-releasing hormone content.

Whole pituitaries were removed from the sella turcica and sectioned midsagittally, then the anterior pituitary tissue was dissected free and weighed. One half of the anterior pituitary was frozen on Dry Ice and stored at -60 C until extracted and extracts assayed for LH, FSH and prolactin content. The remaining half was sliced 1 mm thick in the saggittal plane using a Staddie-Riggs microtome and diced with a scalpel blade into 1 mm^3 pieces. Diced tissue was placed in tissue culture medium (TC 199, Appendix H) until used in the superfusion studies. All pituitary tissue preparation was carried out at room temperature (20 to 24 C).

Ovaries and uteri were collected at slaughter and evaluated for gross ovarian activity and degree of uterine involution.

Extraction of LH, FSH and Prolactin from Pituitary Tissue

Pituitary tissue (150-200 mg) was weighed and placed in 15-ml plastic tubes with .5 ml of carbonate (.05 M) buffer containing 18.6 g/liter (ethylenedinitrilo)-tetracetic acid disodium salt (Na_2EDTA,pH10.0), and homogenized with a polytron tissue homogenizer (three 5-sec intervals at highest speed). Homogenate was transferred to a 50-ml plastic screw cap tube and the small

tube and homogenizer were washed with four 5-ml washes of buffer. Each wash was transferred to the 50 ml tube. Homogenates were shaken overnite at 4 C, the pH was reduced to 8.5 with 1 N HCl and the extracts centrifuged at 2000 x g for 30 minutes. Supernates were decanted and frozen. No measure of extraction efficiency was made, and protein content of the extracts was determined (Bradford, 1976). Supernates were diluted 10 to 1000X with the appropriate assay buffer and then dilution duplicates (10,20,40, 80,160µl) assayed for LH, FSH and prolactin. Results were expressed as total content (mg hormone/pituitary) and as concentration (µg hormone/mg tissue).

Hypothalamic Tissue Trimming and GnRH Extraction

Frozen hypothalami were trimmed and divided into three pieces designated preoptic (PO), anterior hypothalamic (AH) and mediobasal hypothalamic (MBH) which also included the pituitary stalk. Anterior-posterior and lateral boundaries are shown in Figure 1, a ventral veiw of the bovine brain; dorso-ventrally the trimmed tissue was 5 to 6 mm deep. Tissue blocks were kept frozen during and after trimming.

GnRH was extracted as follows. Tissue blocks were weighed, placed in 15-ml plastic centrifuge tubes with 5 ml (PO and AH) or 10 ml (MBH) of cold 2N acetic acid and homogenized by 2 x 30 sec bursts of a polytron tissue homogenizer (Type PT 10 OD; Brinkmann Instruments, Westbury, N.Y.). The homogenizer blade was rinsed

POA = Preoptic Area
AHA = Anterior Hypothalamic Area
MBH = Medial Basal Hypothalamus
MB = Mammillary Bodies

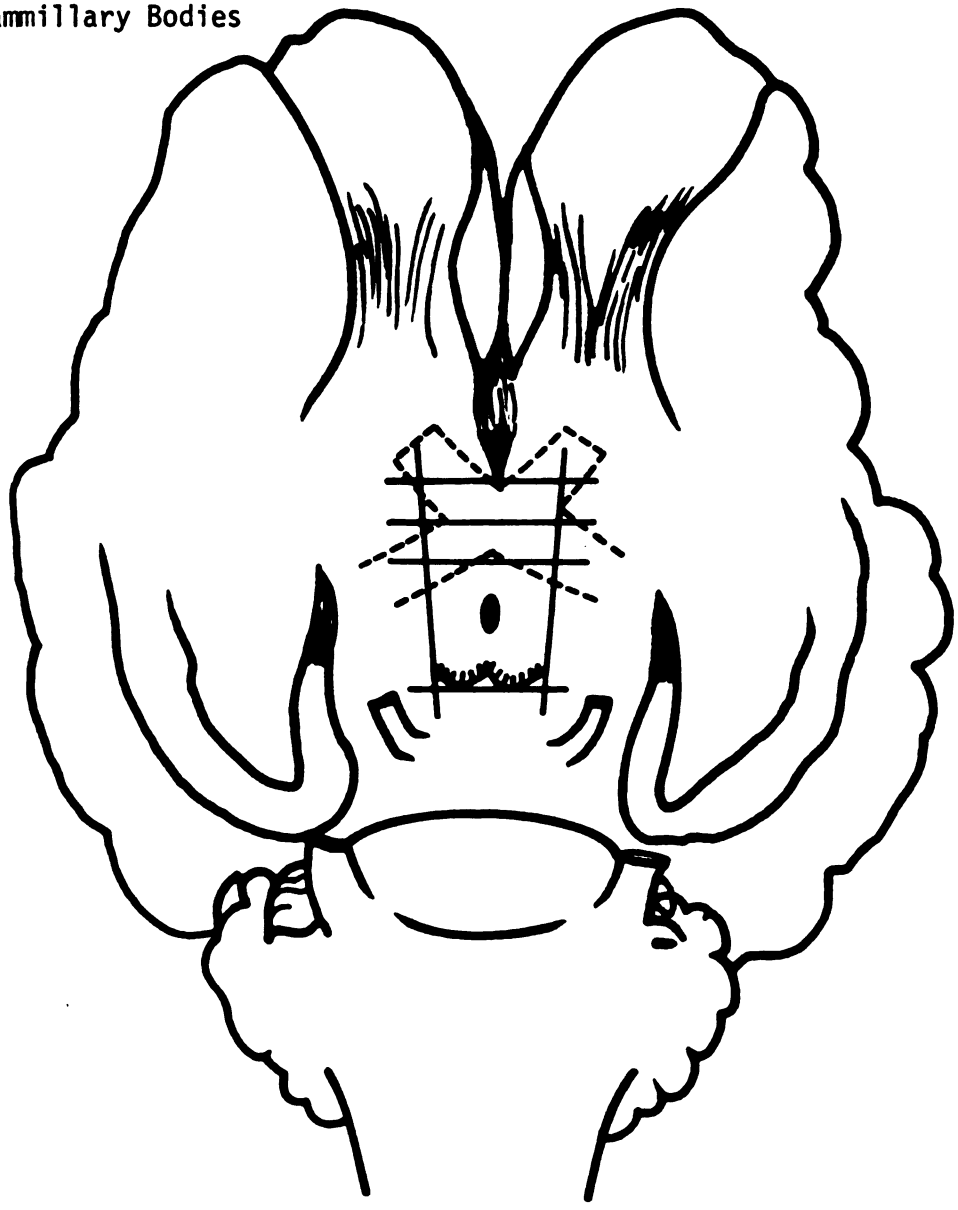
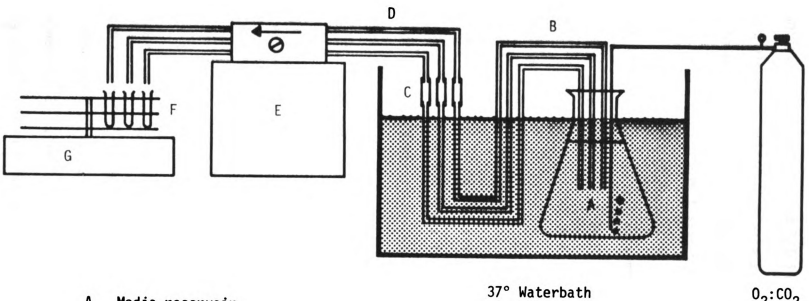


Figure 1. Diagram of the ventral surface of the bovine brain with the dissection boundaries (anterior-posterior and lateral) of the hypothalamic tissue blocks removed for extraction and radioimmunoassay of gonadotropin-releasing hormone.

with 5 ml of 2N acetic acid which was pooled with the homogenate. Prior to homogenization, 10,000 cpm of ^{3}H-GnRH (19.6 Ci/mM, Abbott Laboratories) was added to each tube for determining recovery. Individual homogenates were frozen until all tissue was processed. Then homogenates were thawed and centrifuged for 10 min at 10,000 x g in a refrigerated centrifuge (Sorvall RC-2B). Supernates were decanted into 20-ml glass scintillation vials, frozen, lyophilized and stored at -20 C. Prior to assay, the extracts were reconstituted to original volume with phosphate (.01M) buffered (pH 7.0) saline containing .1% (w/v) pigskin gelatin (#5247; Eastman Kodak G., Rochester,N.Y.). An aliquot was counted in a liquid scintillation spectrophotometer (Model 6860 Mark I., Nuclear, Chicago) for calculation of procedural losses.

Pituitary Superfusion Method

A multichannel in vitro superfusion system was used to compare the LH and FSH responses of anterior pituitary tissue from suckled and nonsuckled cows to GnRH or elevated K^{+} concentration. A schematic diagram of the system used is shown in Figure 2; it is similar to that used by Zolman and Convey (1972). Media were maintained at 37 C in a thermoregulated waterbath and continuously gassed with O_2:CO_2 (95:5). Medium was drawn from the reservoir (A) to the tissue chambers (C) thru efferent tubings (B; Tygon tubing, 1/32 inch I.D. x 3/32 inch O.D.; Arthur H. Thomas Co., Philadelphia, PA) which were also submerged in the waterbath. The chambers consisted of 4-cm lengths of larger bore



A. Media reservoir
B. Efferent tubings
C. Tissue chambers
D. Afferent tubings
E. Peristaltic pump
F. Media fractions
G. Fraction collector

Figure 2. Schematic diagram of the apparatus used for in vitro superfusion of bovine pituitary explants.

Tygon tubing (1/8 inch I.D. x 1/4 inch O.D.) into which were placed 4 to 6 pieces of anterior pituitary tissue (≈50 mg). Gauze mesh was used to prevent tissue from occluding or entering the afferent tubes (D) which passed from the tissue chambers thru a peristaltic pump (E; Model 1201; Harvard Apparatus, Milles, Mass.) and were positioned so that spent media fractions (F) were collected on a fraction collector (G). A flow rate of .37 ± .03 ml/min/channel was used for all superfusions and fractions were collected for 5-min intervals.

The superfusion protocol consisted of loading tissue into the chambers within 90 min of stunning the donors, a preliminary equilibration with TC 199 media for 1 h, and continued equilibration for 1 h with Krebs-Ringer bicarbonate (KRB) buffer (pH 7.2) containing .1% glucose. Then fractions of spent medium were collected for a 30-min baseline period, a 30-min treatment period and finally a 90-min post-treatment period. Sixteen fractions/channel were saved to be assayed for LH and FSH. These corresponded to the 5-min intervals beginning at -30, -20, -10, 0, 5, 10, 15, 20, 25, 35, 45, 55, 70, 85, 100 and 115 min relative to the start of the 30-min treatment period. Pre- and post-treatment medium was KRB with .1% glucose and 5.9 mM K^+ (KRB-5.9) and medium was changed during the treatment period to either KRB-5.9 plus 25 ng/ml GnRH (KRB-GnRH) or KRB with .1% glucose and 59 mM K^+ (KRB-59). All media were at 37 C, pH 7.2 and gassed before and during use with O_2:CO_2 (95:5). Both treatments were intended to elicit maximal

gonadotrophic responses; KRB-GnRH via a receptor-mediated chain of events and KRB-59 by a more direct cell membrane depolarization. Tissue from each cow was replicated with four channels each for KRB-GnRH and KRB-59 and each superfusion contained tissues from one suckled and one nonsuckled cow. Following the 90-min post-treatment period, tissue was removed from the chambers, blotted gently and weighed.

Freezing and degassing resulted in an increased pH of the stored medium samples and formation of a precipitate which dissolved when samples were neutralized by addition of one or two drops of 1N HCl prior to assay.

RESULTS AND DISCUSSION

Experiment I: Effects of Suckling on Postpartum Intervals and Serum Hormone Concentrations in Holstein Cows

Animal Characteristics and PP Intervals to Ovulation and Estrus

Descriptive characteristics of the Holstein cows used in Experiment I are in Table 2; the three treatment groups were non-suckled controls-milked twice daily (NS-2X, n=5), suckled ad libitum-milked twice daily (S-2X, n-6) and nonsuckled-milked four times daily (NS-4X, n=5). All multiparous cows from the Michigan State University dairy herd which calved between January 2, 1976 and April 13, 1976 were assigned alternately to one of the three treatments. A total of 23 cows calved during this interval, three cows were discarded from the S-2X group as a result of calf morbidity and four other animals (three from NS-4X and one from NS-2X) were excluded because of periparturient disease (1 cystic ovaries, 1 severe mastitis, 2 unresponsive metritis cases). With the exception of one animal in each treatment, all cows were three years old with their second calf. Based on the immediately preceeding lactation record and the genetic group classification, cows in the NS-2X treatment would appear to be higher producing than those in the S-2X or NS-4X groups. However, since potential for milk production is felt to be negatively correlated with postpartum

Table 2. Characteristics of suckled (S) and nonsuckled (NS) Holstein cows milked twice (2X) or four times (4X) daily: Experiment I.

Treatment group	Cow	Calving date	Parity	Genetic group[a]	Production (ME,kg)[b]	Body wt[c]	Body wt change[d]
NS-2X	1318	1-2	2	H	6705	615	-2.1
(n=5)	1334	1-9	2	H	8738	551	2.5
	1142	2-17	5	H	7471	650	-5.6
	1368	3-1	2	H	6993	541	-4.6
	1356	3-2	2	L	7194	550	-7.9
				$\bar{x}$	7420	581	-3.5
				SE	352	22	1.8
S-2X	1308	1-17	2	L	8444	645	-4.8
(n=6)	1314	1-22	2	L	4881	564	-2.2
	1322	1-31	2	L	6415	608	-0.2
	1347	2-5	2	H	5670	516	0.0
	1365	3-30	2	L	6342	545	-1.3
	1196	4-13	4	L	7896	673	.3
				$\bar{x}$	6608	592	-2.2
				SE	548	25	1.3
NS-4X	1358	1-24	2	L	6034	557	-5.7
(N=5)	1352	2-15	2	H	6437	548	-5.4
	1195	2-24	3	L	8153	681	-1.9
	1341	3-5	2	H	6886	475	-1.0
	1355	3-7	2	L	5547	564	-0.4
				$\bar{x}$	6611	565	-2.9
				SE	444	33	1.1

[a]High (H) or low (L) genetic potential for milk production.

[b]Lactation record (1975; adjusted to mature equivalent).

[c]Body weight (kg) at approximately 7 days postpartum.

[d]Percent change in body weight between days 7 and 28 postpartum.

(PP) intervals to ovulation and estrus, this possible difference between the treatment groups would be expected to reduce not enhance differences caused by suckling or increased milking frequency. No differences in body weight on day 7 PP or in body weight change between days 7 and 14 PP were apparent between treatments. The weight loss in cows on all treatments would indicate a negative energy balance, a common finding in dairy cows during early lactation.

The intervals from parturition to first PP ovulation and observed behavioural estrus are summarized in Table 3. The average interval in days to first PP ovulation did not differ significantly between NS-2X and NS-4X cows (19.4 ± 3.3 vs 23.0 ± 5.3 days, $P > .25$), whereas S-2X cows had a mean interval to first ovulation of 38.7 ± 4.9 days which was significantly greater than nonsuckled cows ($P < .05$). The overall average interval to PP ovulation for nonsuckled cows (21.2 days) agrees with that reported for M-2X dairy cows (mean = 25.3 days; range = 16.4 to 44.0 days) as summarized in Table 1 and is similar to the 21.3 days reported by England et al. (1973) for nonlactating beef cows. The interval to first PP ovulation in the suckled cows (38.7 days) is shorter than that reported by Moller (1970b) for dairy cows nursing multiple calves (64.5 days). However, if the intervals for suckled cows are expressed as percentages of the intervals for nonsuckled controls within each experiment, the results are remarkably similar (181% vs 180%). The interval to first ovulation in S-2X cows also lies

Table 3. Intervals to first postpartum ovulation and estrus in suckled (S) and nonsuckled (NS) Holstein cows milked twice (2X) or four times (4X) daily.

Treatment	Interval[a] to first postpartum	
	Ovulation	Observed Estrus
NS-2X (n=5)	19.4 ± 3.3	39.0 ± 2.4
S-2X (n=6)	38.7 ± 4.9[b]	50.2 ± 4.3[c]
NS-4X (n=5)	23.0 ± 5.3	44.8 ± 6.1

[a]Days, $\bar{x}$ ± SE.

[b]S-2X > NS-2X and NS-4X, $P < .05$.

[c]S-2X > NS-2X but not NS-4X, $P < .06$.

within the range of mean intervals reported for suckled beef cows in Table 1 (36.0 to 61.5 days).

The PP interval to first observed estrus was increased in S-2X cows compared to NS-2X cows (50.2 ± 4.3 vs 39.0 ± 2.4 days, $P < .06$), but not significantly compared to NS-4X cows (44.8 ± 6.1 days, $P > .25$). Nonsuckled cows (NS-2X vs NS-4X) did not differ significantly in the mean interval to first observed estrus. Across all treatments, interval to first estrus was longer than interval to first ovulation ($P < .01$ by paried t-test) as would be expected since 81% of the cows ovulated one or more times before showing estrous behaviour.

The high incidence of silent ovulations during the early PP period is in agreement with the literature (Hackett et al., 1973; Hartigan et al., 1974; King et al., 1976). The occurrence of estrus associated with the first PP ovulation in three of six S-2X cows versus none of ten NS cows may reflect the greater PP interval to ovulation in this treatment group since increasing PP interval has been associated with reduced frequency of silent ovulation (Casida and Wisnicky, 1950; Stevenson, 1977).

I conclude that the presence of a calf allowed to suckle ad libitum delays the first PP ovulation in Holstein cows milked twice daily. Suckling also causes an increase in the interval to the first PP estrus. Increasing the milking frequency from twice to four times daily did not significantly alter the PP intervals to ovulation or estrus in this experiment.

Serum Hormone Concentrations

Luteinizing Hormone

Serum from jugular vein blood collected at 15-min intervals from 1400 to 1800 h on days 7 and 14 PP was assayed for LH content. Analysis of variance was performed using a double split-plot model with repeat measure over time; main effects were treatment group, period (day PP sampled) and time of sample. There was no significant treatment by period interaction. Mean serum LH concentration was lower in S-2X cows than in NS-2X or NS-4X (.7 vs 1.4 ng/ml, $P < .05$) but NS-2X and NS-4X did not differ significantly (1.4 vs 1.3 ng/ml, $P > .25$). Mean serum LH concentration increased significantly between day 7 and day 14 PP across all three treatments (.9 vs 1.4 ng/ml, $P < .05$).

Figures 3, 4 and 5 present the individual serum LH profiles on days 7 and 14 PP for five cows from the NS-2X, S-2X and NS-4X treatments. Episodic releases (peaks) of LH were apparent upon inspection of plotted data and were identified by visual appraisal since no appropriate and valid statistical method of selection was available. Increases which were considered as peaks are marked with asterisks. Data for one randomly selected cow from the six S-2X animals were not presented for expediency in drawing the graphs.

Characteristics of the serum LH profiles are summarized in Table 4 and Figure 6. The following criteria were calculated for each 4.5-h sampling period; mean LH concentration (ng/ml), coefficient of variation of the LH concentration (%), frequency of

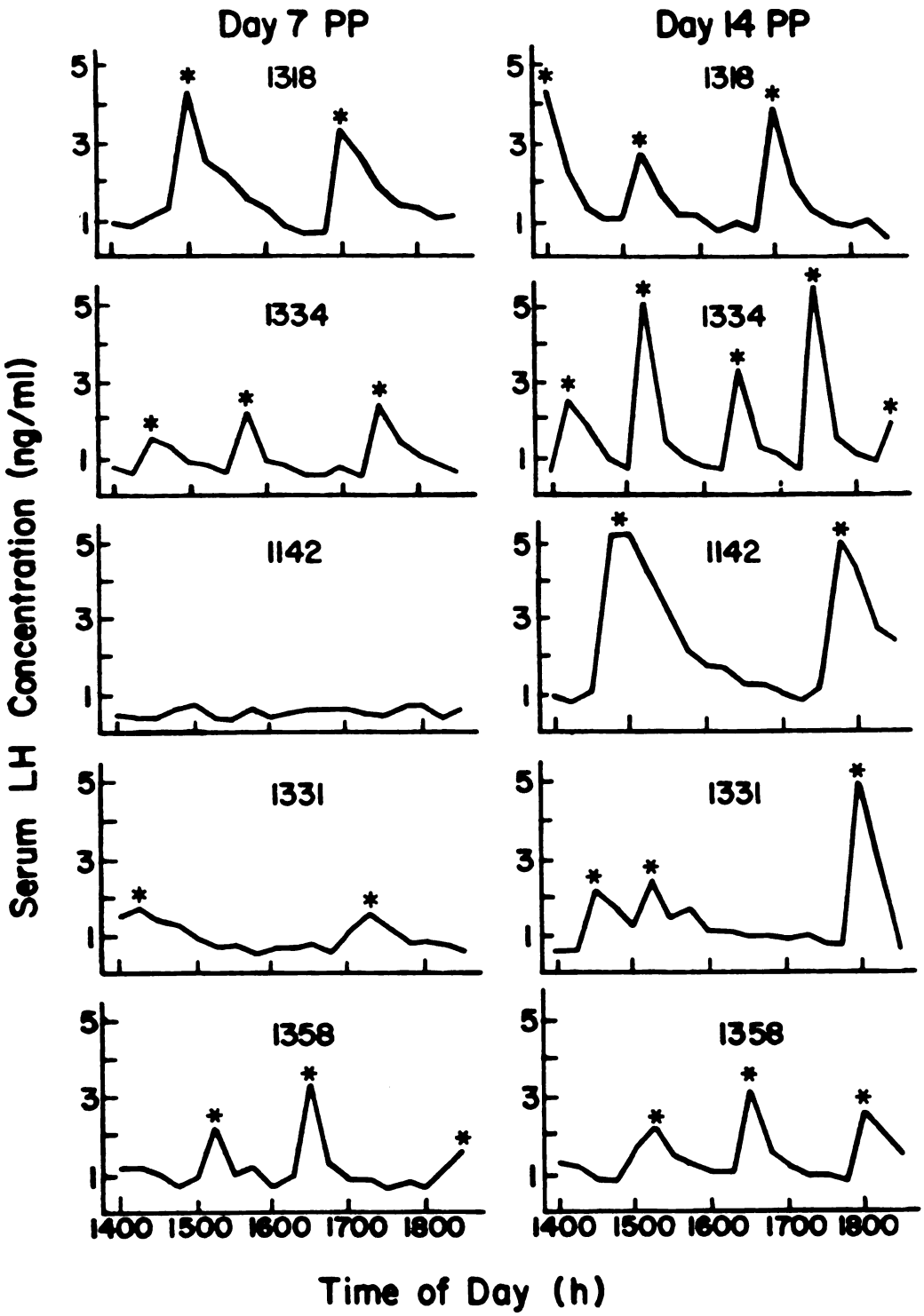


Figure 3. Luteinizing hormone profiles in serum of nonsuckled twice daily milked (NS-2X) cows on days 7 and 14 postpartum, (* = designated peak).

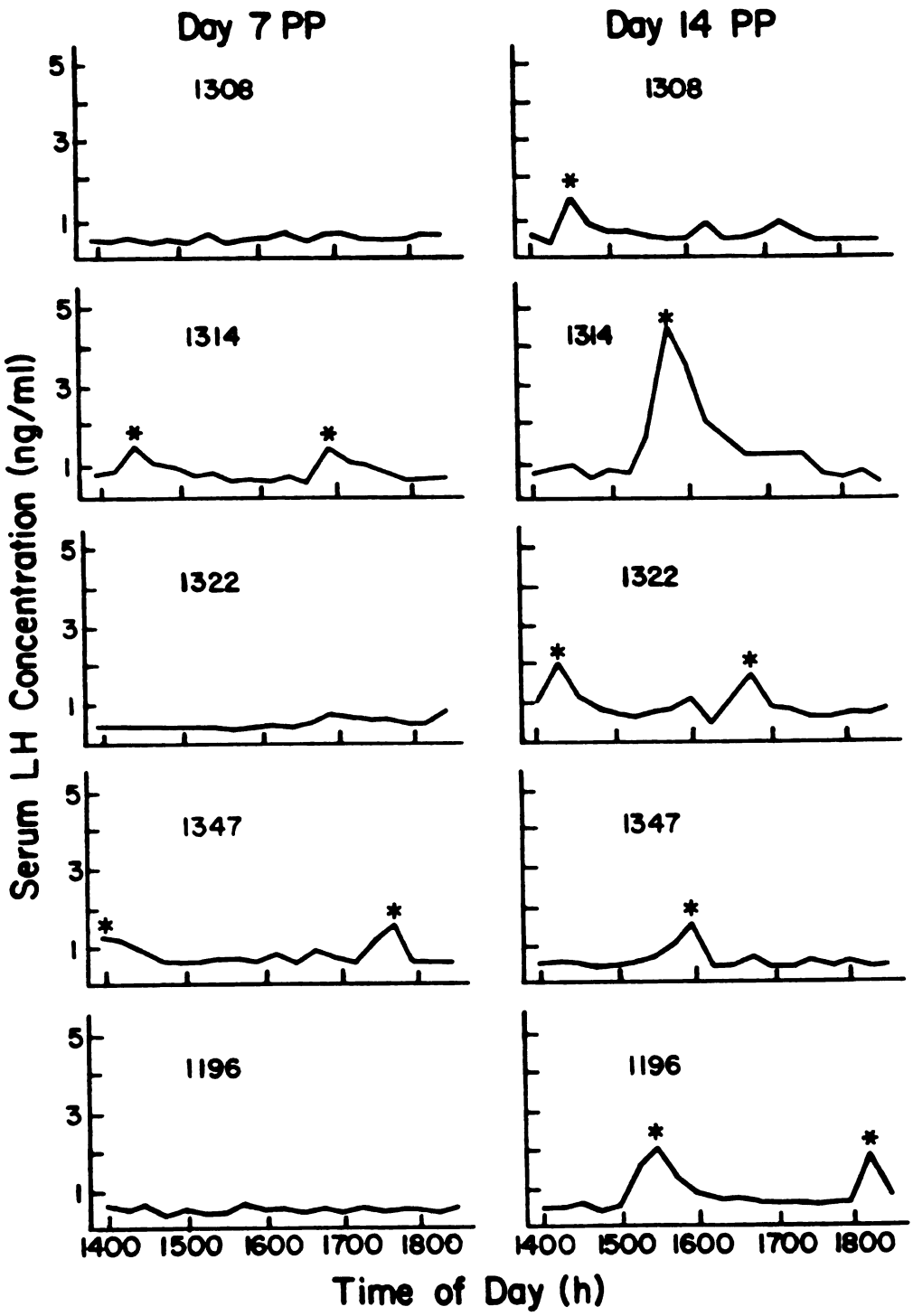


Figure 4. Luteinizing hormone profiles in serum of suckled twice daily milked (S-2X) cows on days 7 and 14 postpartum (* = designated peak).

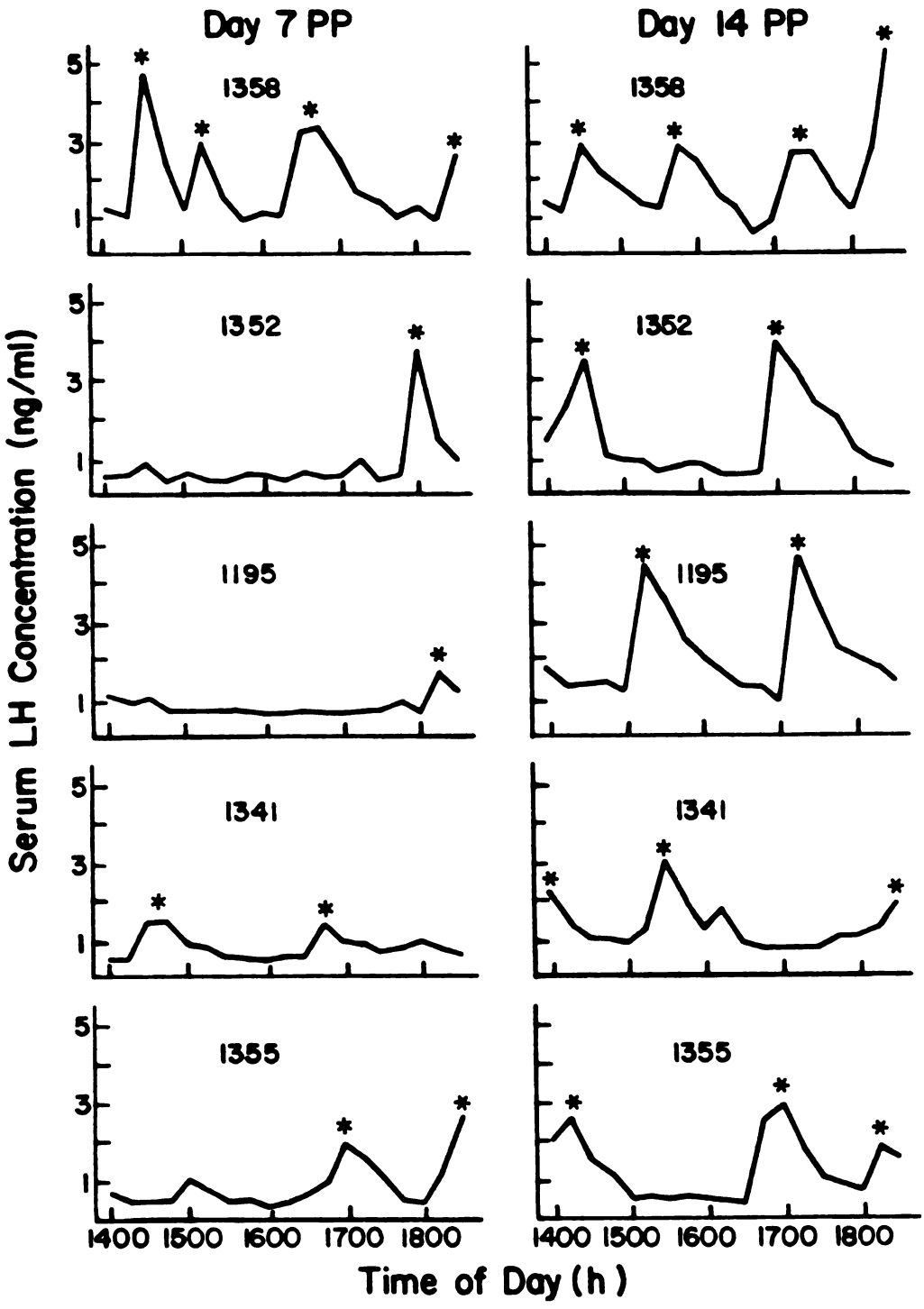


Figure 5. Luteinizing hormone profiles in serum of nonsuckled four times daily milked (NS-4X) cows on days 7 and 14 postpartum, (* = designated peak).

Table 4. Summary characteristics of serum LH profiles in suckled (S) and nonsuckled (NS) cows on days 7 and 14 postpartum (PP).

	NS-2X (n = 5)		S-2X (n = 6)		NS-4X (n = 5)	
LH	Day 7 PP	Day 14 PP	Day 7 PP	Day 14 PP	Day 7 PP	Day 14 PP
Mean[a]	1.0 ± .2	1.7 ± .4	.6 ± .1	.9 ± .1	1.0 ± .2	1.6 ± .2
% CV[b]	45 ± 8	70 ± 7	28 ± 5	57 ± 7	58 ± 13	59 ± 5
Peak frequency[c]	2.0 ± .5	3.2 ± .5	.7 ± .4	1.5 ± .2	2.0 ± .5	2.8 ± .4
Peak amplitude[d]	2.4 ± .5	3.5 ± .5	1.4 ± .1	2.3 ± .4	2.4 ± .5	2.9 ± .3

[a]Average (ng/ml) of 19 samples collected at 15-min intervals over a 4.5-h period.

[b]Percent coefficient of variation of the LH concentration in the 19 samples.

[c]Number of visually determined LH peak/cow/4.5-h sampling period.

[d]Average LH peak amplitude (ng/ml) including baseline.

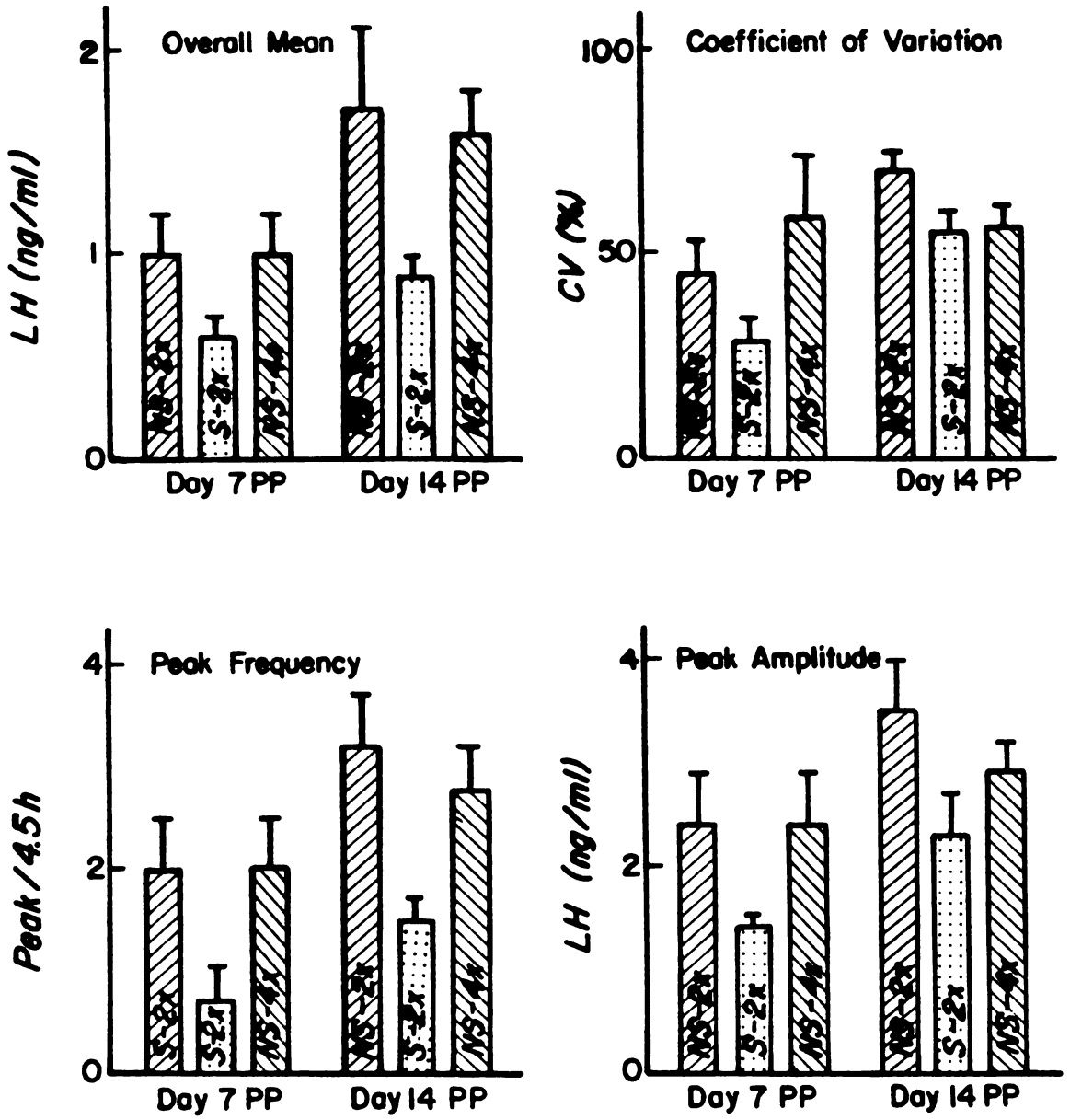


Figure 6. Characteristics of serum luteinizing hormone (LH) to profiles in suckled (S) and nonsuckled (NS) cows milked twice (2X) or four times (4X) daily.

LH peaks (number/4.5 h) and mean amplitude of episodic LH peaks (ng/ml). Incomplete peaks were included and baseline concentrations were not subtracted from peak amplitudes.

Frequency of milking did not affect the characteristics of the LH profiles in nonsuckled cows (NS-2X vs NS-4X). The reduced mean LH concentration in suckled cows resulted from decreased frequency ($P < .05$) and amplitude ($P < .05$) of the episodic LH peaks as compared to nonsuckled cows. Coefficient of variation for LH concentration tended to be lower in suckled than in nonsuckled cows on day 7 PP, but not on day 14 postpartum.

Luteinizing hormone mean concentration and coefficient of variation increased ($P < .01$) between day 7 and day 14 PP in all treatments as a result of increased ($P < .01$) frequency and amplitude of episodic LH peaks. These results indicate that the stimuli provided by a single calf allowed to suckle ad libitum inhibits or delays the onset of pulsatile LH secretion during the early PP period in Holstein cows.

Episodic or pulsatile secretion of LH has been reported in PP milked dairy cows (Stevenson, 1977; Goodale et al., 1978) and suckled beef cows (Corah et al., 1975; Humphrey, 1977). In agreement with these studies, the present study demonstrated increased frequency and amplitude of the episodic LH releases as the PP interval increased with maximum LH secretory activity occurring prior to the first PP ovulation.

This thesis is the first report of a direct comparison of LH secretory profiles in suckled and nonsuckled cows. The

observation that one effect of suckling is to delay resumption of pulsatile LH secretion following parturition supports the hypothesis that delayed PP ovulation in suckled cows is a result of inhibited LH secretion.

Follicle-Stimulating Hormone

Serum concentrations of FSH were measured in pooled serum from each of five NS-2X and six S-2X cows on days 7 and 14 postpartum. Mean serum concentrations of FSH in suckled cows (43.3 ± 4.9 and 42.9 ± 7.3 ng/ml) on days 7 and 14 PP did not differ significantly from those in nonsuckled cows (48.0 ± 10.1 and 63.0 ± 12.5 ng/ml). No significant effect of day PP was observed.

These FSH concentrations are within the range of those reported recently by Dobson (1978a, b) for milked dairy cows. Follicle-stimulating hormone does not appear to limit resumption of ovarian activity in PP cows nor does suckling appear to delay PP ovulation by decreasing circulating FSH concentrations.

Prolactin

Prolactin was assayed in all samples taken on days 7 and 14 PP for cows in the NS-2X and S-2X treatments. Analysis of variance revealed no effect of suckling and no suckling by time interactions, however both period (day 7 vs day 14 PP) and period by time interaction were significant ($P < .01$). Since no treatment by period or treatment by period by time interactions were significant, results were pooled within treatment (Figure 7) and within period (Figure 8). The elevated but declining baseline

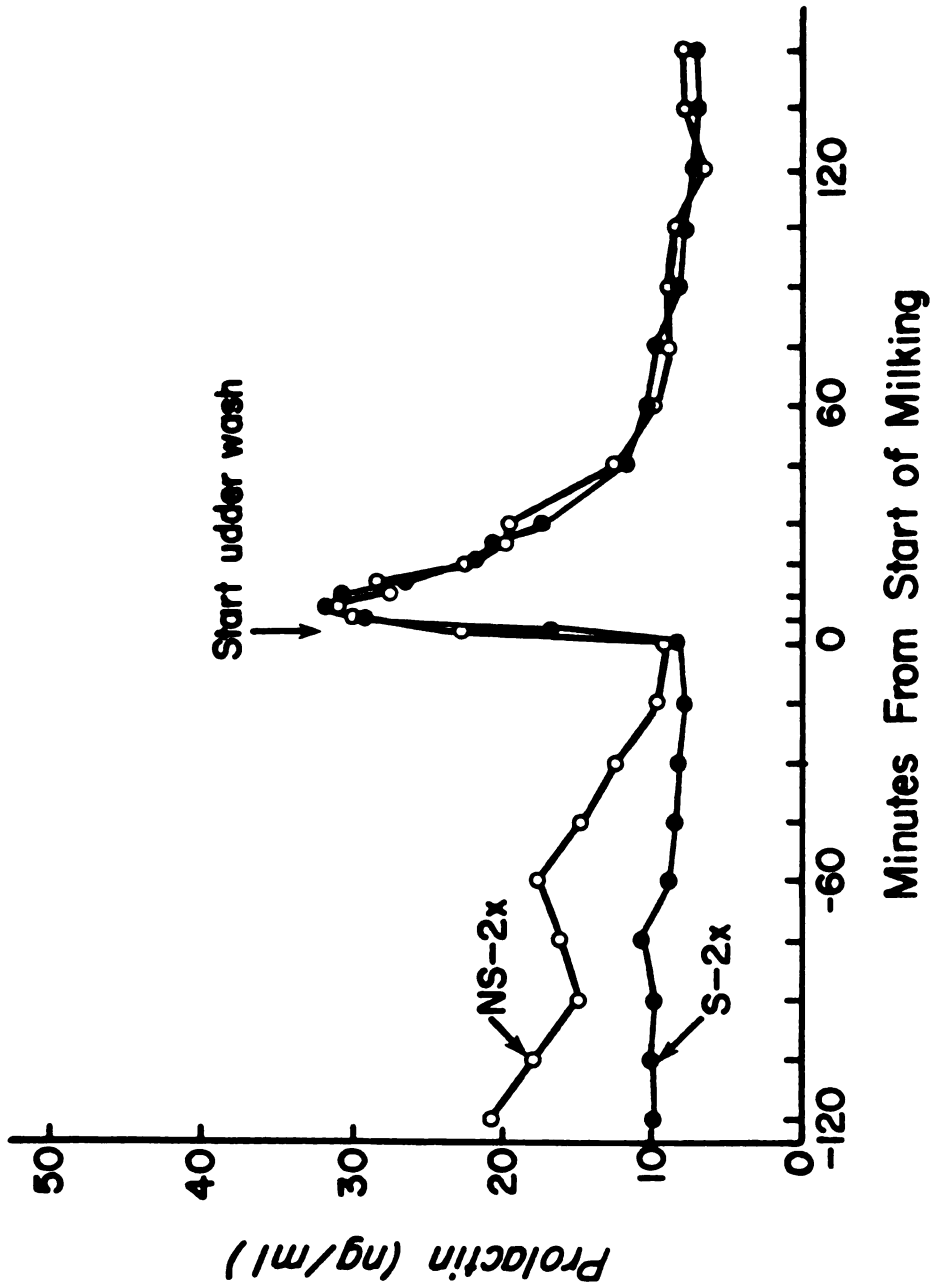


Figure 7. Prolactin in serum of suckled (S-2X) and nonsuckled (NS-2X) postpartum (day 7 and 14 pooled) Holstein cows for 2 h before and after start of afternoon milking.

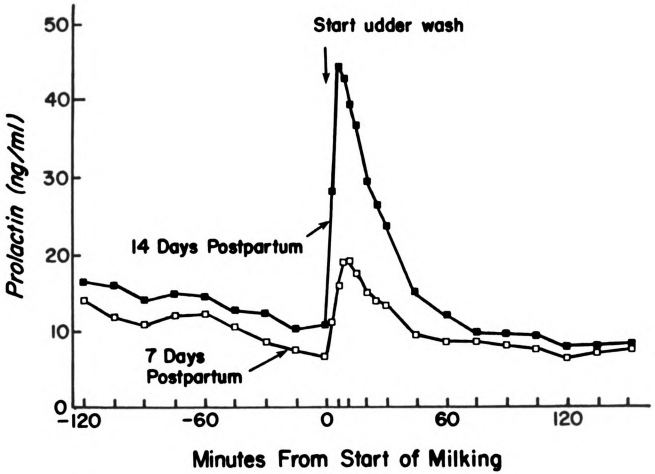


Figure 8. Prolactin in serum of Holstein cows (suckled and nonsuckled treatments pooled) for 2 h before and after start of afternoon milking on days 7 and 14 postpartum.

(Figure 7) in the NS-2X cows during the 2 h before milking probably reflects disturbance to these cows caused by movement of adjacent cows to and from the milking parlor, as well as feeding which occurred during this time. The S-2X cows, housed in box stalls at the opposite end of the barn, were physically removed from most of this disturbance.

Average response to the milking stimulus and post-milking concentrations of prolactin were almost identical in nonsuckled and suckled cows. These results do not support a role for prolactin as a mediator of suckling's inhibition of PP ovulation in cows since the stimuli provided by one calf did not elevate either the basal or milking-induced release of prolactin but did increase the PP interval to ovulation. In both suckled and nonsuckled cows, basal and milking-induced release of prolactin increased between day 7 and day 14 PP (Figure 8); this may reflect recovery of the pituitary from depletion caused by the massive prolactin secretion associated with parturition (Ingalls et al., 1973; Karg and Schams, 1974).

Progesterone, Estradiol-17β and Glucocorticoids

Progesterone, estradiol-17β and total glucocorticoids were measured in serum collected on days 7 and 14 PP from cows in the NS-2X and S-2X treatments; results are summarized in Table 5. Both progesterone and estradiol-17β concentrations were low and did not differ significantly between suckled and nonsuckled cows nor between day 7 and 14 postpartum. Premilking serum

Table 5. Serum concentrations ($\bar{x}$ ± SE) of progesterone, estradiol-17β and glucocorticoids in suckled (S-2X) and nonsuckled (NS-2X) cows on days 7 and 14 postpartum (PP).

	NS-2X (n = 5)		S-2X (n = 6)	
Hormone	Day 7 PP	Day 14 PP	Day 7 PP	Day 14 PP
Progesterone[a] (ng/ml)	.2 ± .1	.2 ± .1	.2 ± .1	.2 ± .1
Estradiol-17β[a] (pg/ml)	2.7 ± .6	3.1 ± .9	2.1 ± .6	2.7 ± .7
Glucocorticoids[b] (ng/ml)	5.6 ± .9	3.8 ± .6	2.7 ± 1.2	6.7 ± 2.2

[a]Average of two samples/cow/day.

[b]Average of five samples/cow/day collected prior to the start of milking at 1600 hours.

concentrations of glucocorticoids as shown in Table 5 did not differ between treatments or day PP ($P > .2$). The peak milking-induced glucocorticoid release was greater on day 14 than on day 7 PP (12.9 ± 2.8 vs 5.9 ± 1.5 ng/ml, $P < .05$), although nonsuckled and suckled cows did not differ significantly either on day 7 PP (6.9 ± 1.6 vs 5.2 ± 2.5 ng/ml) or day 14 PP (10.5 ± 2.6 vs 14.18 ± 4.7 ng/ ml).

These results do not support a direct role for the ovarian (progesterone and estradiol-17β) or adrenal (total glucocorticoids) steroids as mediators of suckling's effect on LH secretion or on delay of PP ovulation and estrus.

Summary

This experiment demonstrates that stimuli provided by a single calf allowed to suckle ad libitum approximately doubles the interval to first PP ovulation in milked dairy cows. Suckling significantly increased the interval to first PP observed estrus; however, the effect was less dramatic than on ovulation because there tended to be a lower incidence of ovulation without observed estrus in the suckled cows. Increased frequency of milking did not alter the PP intervals to ovulation or estrus.

Suckling resulted in delayed PP resumption of episodic LH secretion; both LH peak amplitude and frequency were reduced resulting in lower mean and coefficient of variation for serum LH concentration. Serum concentrations of FSH, prolactin, progesterone, estradiol-17β and total glucocorticoids did not differ

significantly between suckled and nonsuckled cows. However, basal prolactin and milking-induced prolactin and glucocorticoid concentrations were greater on day 14 than on day 7 PP in both treatments.

It would appear that suckling suppresses episodic LH secretion during the PP period and this in turn may be responsible for the increased interval to first PP ovulation in these cows.

Experiment II: Effects of Suckling on the Hypothalamo-Pituitary Gonadotropic Axis in Early Postpartum Holstein Cows

Animal Characteristics and Slaughter Data

Charateristics of Holstein cows used in Experiment II are summarized in Table 6. No information was available on past milk production or on reproductive histories of these animals. Treatment means for day PP killed, body weight at slaughter and percent body weight change between day 4 PP and slaughter did not differ significantly between nonsuckled (NS-2X, n=8) and suckled (S-2X, n=9) cows.

Milk removed by twice daily machine milking of all cows during the two weeks between calving and slaughter averaged 20.8 ± 1.1 kg/day for nonsuckled cows and 5.9 ± 1.7 kg/day for cows suckled ad libitum by two calves.

Suckling frequency was estimated by observation during the periods of intensive blood sampling; each cow was observed for an average of 26 ± 4 h and the calculated suckling frequency was

Table 6. Characteristics of suckled (S-2X) and nonsuckled (NS-2X) postpartum (PP) Holstein cows used in Experiment II.

Treatment	Cow	Calving Date	Parity[a]	Body condition[b]	Day PP killed	Slaughter wt (kg)	% wt change[c]
NS-2X	818[d]	5-1	0	1	15	389	4.6
(n = 8)	821	5-7	1	2	16	482	-5.4
	607	5-28	1	3	16	515	-0.4
	609[e]	5-14	1	3	12	441	---
	611	5-21	0	1	17	514	-0.6
	825	5-7	1	4	12	527	.0
	615	5-13	0	1	12	405	-1.0
	822	5-16	0	2	16	427	2.1
		$\bar{x}$	.5	2.1	14.5	462	-0.1
		SE	.2	.4	.8	19	1.2
S-2X	816	5-7	0	2	16	494	-0.8
(n = 9)	820	5-13	1	2	13	516	5.1
	819	5-27	0	2	15	450	.8
	608	5-24	1	1	14	595	-1.2
	616	5-29	0	1	15	444	-0.9
	612	5-6	0	2	13	432	2.6
	824	5-1	1	2	15	509	1.3
	817	5-10	1	2	15	518	-1.3
	612	5-16	0	3	16	461	-2.0
		$\bar{x}$	.4	1.9	14.7	491	.4
		SE	.2	.2	.4	17	.8

[a]Heifer = 0, cow = 1.

[b]Subjective evaluation: thin = 1, fair = 2, good = 3, fat = 4.

[c]Percent body weight change = (slaughter wt - day 4 PP wt) X 100/day 4 PP wt.

[d]818 was the smallest heifer and had the largest calf, required assistance and retained placenta for 36 hours.

[e]609 had breech presentation which required assistance.

9.9 ± 1.0 suckling episodes/24 h. This result assumes that the suckling activity observed from 0800 to 1200 and 2000 to 2400 h is representative of the remainder of the day.

Based upon uterine measurements and subjective appraisal of uterine content and endometrium on day 14 PP, there was a tendency for suckled cows to have smaller more involuted uteri. Uteri of nonsuckled cows appeared to contain a greater volume of fluid contents and more inflammatory-like changes such as severe endometrial hyperemia and purulent material than those of suckled cows.

Ovaries of both suckled and nonsuckled cows showed evidence of follicular growth. The majority of follices were small to medium in size (<10 mm diameter) although in five of seven suckled cows and four of six non-suckled cows at least one ovary contained a follicle >10 mm in diameter. No luteal tissue was detected in any cow except for the regressed corpus luteum of pregnancy on the ovary ipsilateral to the previously gravid uterine horn. Differences in ovarian status due to suckling were not apparent although detailed evaluation of ovarian weight, follicular fluid weight and ovarian histology or steroid contents was not performed.

Serum Hormone Concentrations

Luteinizing Hormone

Luteinizing hormone was measured in serum collected at 15-min intervals between 0815 to 1200 and 2015 to 2400 h on the

day before slaughter, approximately day 13 postpartum. Secretory profiles of LH were characterized as described in Experiment I and results are summarized in Table 7 and Figure 9. No change in LH secretion was associated with the time of day at which samples were collected for characterization of the secretory profile. However, suckled cows had lower ($P < .05$) mean serum LH concentrations (.9 ± .1 ng/ml) than nonsuckled controls (1.6 ± .3 ng/ml). The lower mean LH concentration resulted from both peak frequency and amplitude being reduced ($P < .05$) in suckled vs nonsuckled cows (.9 ± .2 peaks/4 h, 1.8 ± .1 ng/ml vs 2.1 ± .2 peaks/4 h, 3.1 ± .9 ng/ml).

Follicle-Stimulating Hormone

Follicle-stimulating hormone was assayed in serum from blood collected at 0815, 0915, 1015, 1115, 2015, 2115, 2215, and 2315 h on day 13 PP, results are shown in Figure 10. There was no significant difference between morning and evening samples nor did suckled cows differ much from nonsuckled cows (67.3 ± 6.9 vs 59.1 ± 4.6 ng/ml, $P > .3$). Serum from tail vein blood samples collected on day 7 and 13 PP had average FSH concentrations of 65.2 ± 7.6 and 66.6 ± 12.6 ng/ml for suckled cows and 56.2 ± 6.3 and 66.1 ± 7.8 ng/ml for nonsuckled cows. There were no significiant differences due to day PP or suckling. These results are in agreement with those of Dobson (1978a, b) for milked PP Holstein cows and support the contention that FSH secretion is not

Table 7. Characteristics of LH secretory profiles in suckled (S-2X) and nonsuckled (NS-2X) cows on day 13 postpartum.

	NS-2X (n = 8)		S-2X (n = 9)	
LH	AM	PM	AM	PM
Mean[a]	1.6 ± .3	1.6 ± .3	.9 ± .1	.9 ± .1
% CV[b]	39 ± 11	37 ± 6	36 ± 7	34 ± 5
Peak frequency[c]	2.1 ± .4	2.1 ± .3	.8 ± .2	1.1 ± .3
Peak amplitude[d]	3.4 ± 1.1	3.8 ± .7	1.9 ± .2	1.7 ± .1

[a]Average (ng/ml) of 16 samples/cow collected over a 4-h period.

[b]Percent coefficient of variation of the LH concentration.

[c]Mean number of visually determined LH peaks/cow/4-h sampling period.

[d]Average LH peak amplitude (ng/ml) including baseline.

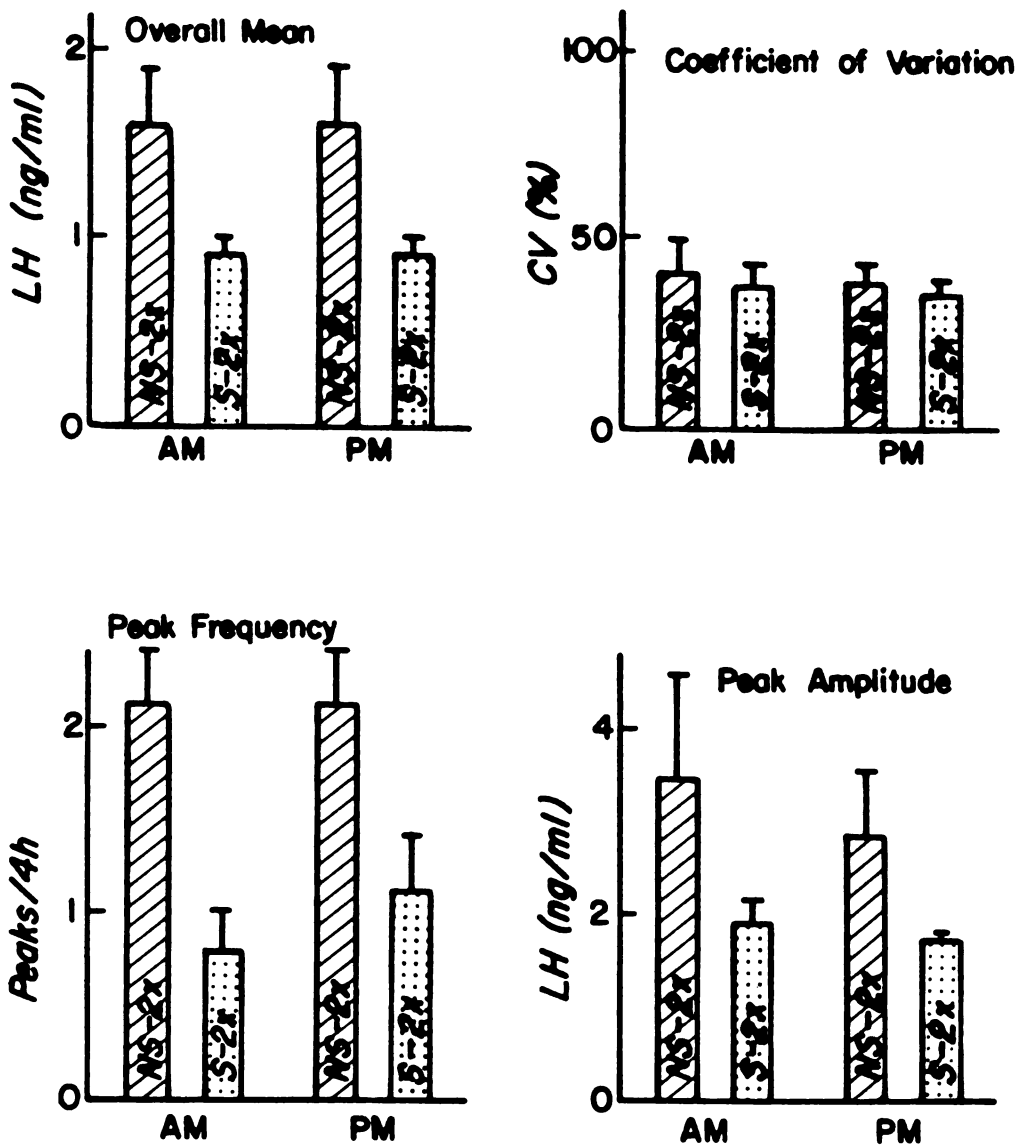


Figure 9. Characteristics of serum luteinizing hormone (LH) profiles in suckled (S-2X) and nonsuckled (NS-2X) cows on day 13 postpartum.

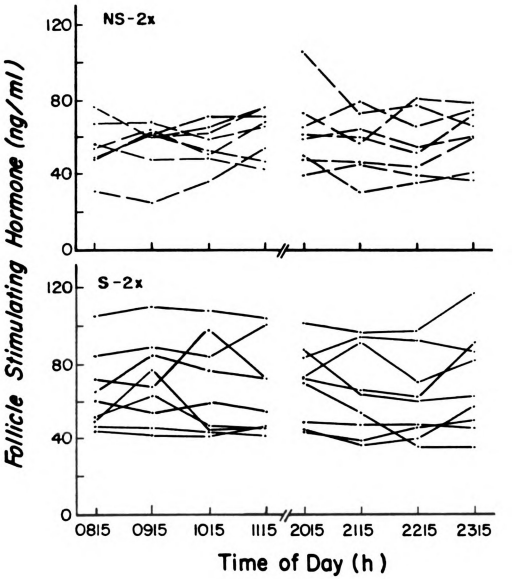


Figure 10. Concentrations of follicle-stimulating hormone in serum of suckled (S-2X) and nonsuckled (NS-2X) cows on day 13 postpartum.

a limiting factor in resumption of PP ovulatory ovarian cycles in suckled or nonsuckled cows.

Prolactin

Serum concentrations of prolactin on day 13 PP are shown in Figure 11 for nonsuckled and suckled cows. No differences were apparent as a result of time of day sampled or the suckling treatment. Suckling did not appear to result in consistent increases in serum prolactin in the first and second samples collected after an observed episode of suckling when compared to the sample taken immediately prior to the start of suckling (63.6 ± 8.6 and 67.4 ± 9.3 vs 56.1 ± 8.1 ng/ml, n = 24). Sampling at 15-min intervals may have been too infrequent to accurately detect a suckling-induced prolactin release. Reports of suckling-induced prolactin release in cattle (Johke, 1969, 1970; Karg and Schams, 1970) have involved calf removal and then replacement for suckling challenge; response to continuous calf presence and ad libitum suckling has not been reported. Goodman (personal communication, 1978) has found that the presence of a calf that was not allowed to nurse resulted in lower basal and thyrotropin-releasing hormone-induced prolactin secretion in milked PP Holstein cows. The significance of this finding is unclear, but it suggests that presence of the calf influences secretion of prolactin without involving mammary stimulation, a concept that also may be applicable to other pituitary hormones such as luteinizing hormone.

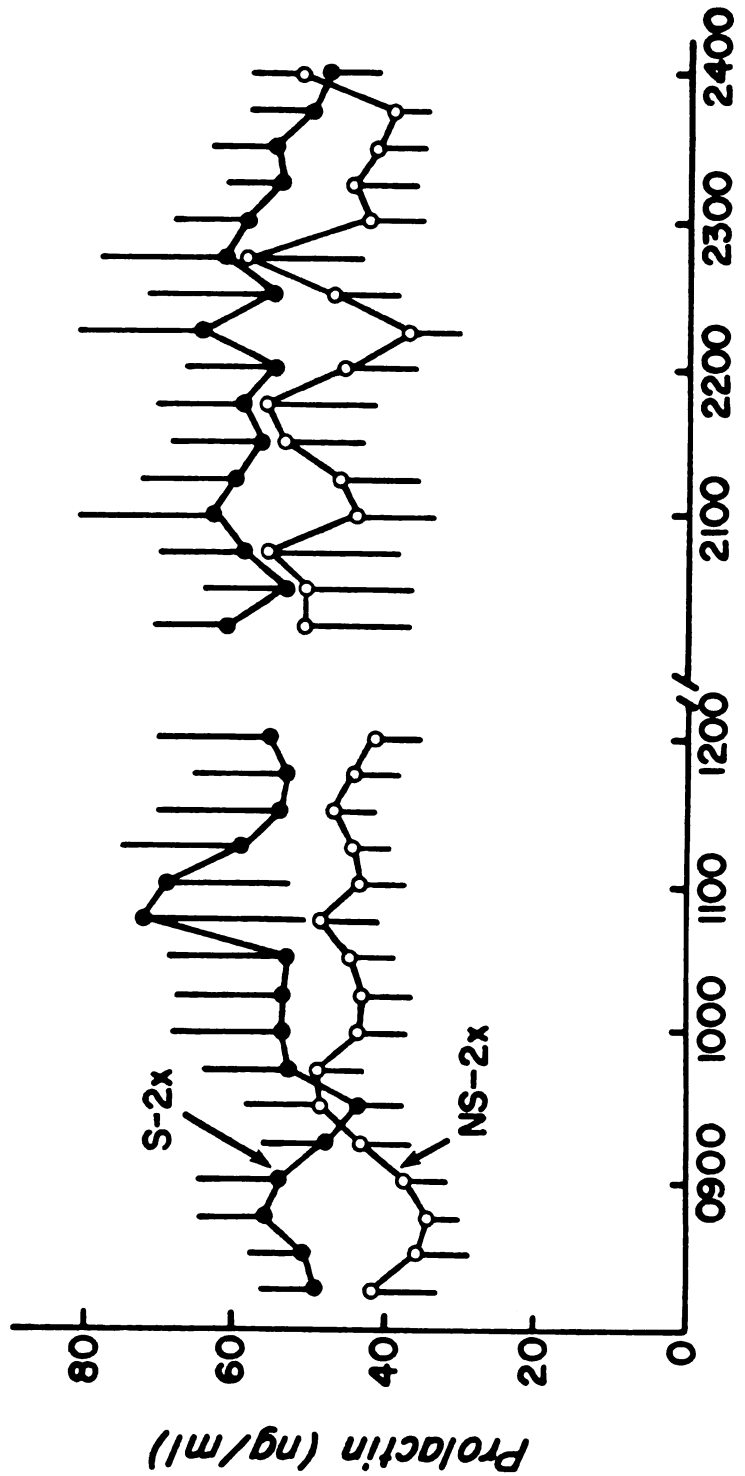


Figure 11. Mean concentrations of prolactin in serum of suckled (S-2X) and nonsuckled (NS-2X) cows on day 13 postpartum.

Progesterone, Estradiol-17β and Glucocorticoids

Progesterone concentrations in serum declined to <.5 ng/ml by day one PP and remained at or below this level throughout the experiment. This is consistent with the lack of active luteal tissue in the ovaries of these cows at slaughter.

Estradiol-17β was measured in serum collected on days 4, 8, 12 and 13 PP; two samples were collected on day 13 PP at 1200 and 2400 hours. Means for suckled and nonsuckled cows are summarized in Figure 12. Estradiol concentrations were low and did not differ between suckled and nonsuckled cows, nor did they appear to change significantly over the period from day 4 to day 13 postpartum.

Total serum glucocorticoids were measured in samples collected at 0815, 0915, 1015, 1115, 2015, 2125, 2215 and 2315 h on day 13 postpartum. No time of day effect was observed nor did mean serum glucocorticoid concentrations differ significantly ($P > .2$) in suckled and nonsuckled cows ($6.6 \pm .8$ vs 5.1 ± 1.2 ng/ml). Serum concentrations of glucocorticoids were only slightly higher in this experiment than those reported for the first 8 weeks PP in milked Holstein cows (Edgerton and Hafs, 1973). These results also agree with the work of Wagner and Oxenreider (1971) who reported no difference between mean serum concentrations of glucocorticoids in milked and suckled cows. These results do not support the view that suckling-induced changes in adrenal

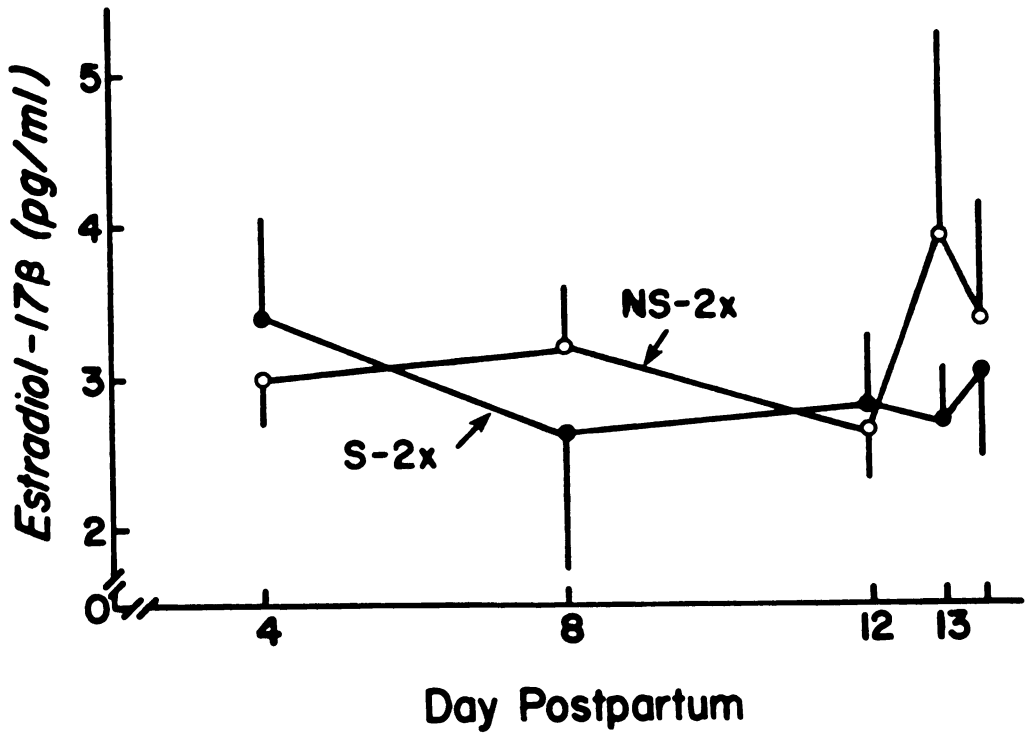


Figure 12. Serum concentrations of estradiol-17β in suckled (S-2X) and nonsuckled (NS-2X) cows from day 4 to 14 postpartum.

glucocorticoid secretion are responsible for the depressed LH secretion and delayed PP ovulation in suckled cows.

Summary

Mean serum hormone concentrations in suckled and non-suckled cows on day 13 PP are summarized in Table 8. The only significant change in serum hormones associated with suckling was a reduction in frequency and amplitude of episodic LH secretion which was reflected in decreased mean serum LH concentration. These results do not support a role for increased serum prolactin or glucocorticoids in suckling-induced suppression of episodic LH secretion, nor does ovarian progesterone or estradiol-17β secretion appear to be directly involved.

Hypothalamic Gonadotropin-Releasing Hormone (GnRH) Content

Hypothalamic content of GnRH is summarized in Table 9. There was no significant difference in the total hypothalamic GnRH content of suckled and nonsuckled cows (118.2 ± 16.3 vs 111.4 ± 19.5 ng/hypothalamus, $P > .2$), nor did suckling affect the gross hypothalamic distribution of gonadotropin-releasing hormone. The distribution of GnRH was similar to that reported by Estes et al. (1977) with >95% of the total content confined to the medial-basal hypothalamus and pituitary stalk. The bilateral anterior or preoptic concentration of GnRH reported by Estes et al. (1977) was not apparent in this study. The large size of the preoptic tissue block may have resulted in a dilution effect that masked the

Table 8. Serum hormone concentrations ($\bar{x}$ ± SE) in suckled (S-2X) and nonsuckled NS-2X) Holstein cows on day 13 postpartum.

Serum hormone	Treatment		Significance level
	NS-2X (n = 8)	S-2X (n = 9)	
LH (ng/ml)	1.6 ± .3	.9 ± .1	P < .01
FSH (ng/ml)	59.1 ± 4.6	67.3 ± 6.9	N.S.
Prolactin (ng/ml)	46.2 ± 8.9	59.6 ± 13.4	N.S.
Progesterone (ng/ml)	.09 ± .02	.13 ± .03	N.S.
Estradiol-17 (pg/ml)	3.0 ± .4	2.9 ± .3	N.S.
Glucocorticoids (ng/ml)	5.1 ± 1.2	6.6 ± .8	N.S.

Table 9. Content and gross distribution of hypothalamic GnRH in suckled (S-2X) and nonsuckled (NS-2X) cows killed on day 14 postpartum.

Hypothalamic area	Treatment group	
	NS-2X (n = 8)	S-2X (n = 9)
Preoptic[b,e]	3.9 ± .8 ng[a]	4.2 ± .4 ng
Anterior[c,e]	2.9 ± .4 ng	3.8 ± .5 ng
Medial basal[d,e]	104.6 ± 18.6 ng	110.1 ± 15.5 ng
Total	111.4 ± 19.5 ng	118.2 ± 16.3 ng

[a]Ng/tissue block, $\bar{x}$ ± SE.

[b]+10 to +15 mm anterior to the center of the pituitary stalk.

[c]+6 to +10 mm anterior to the center of the pituitary stalk.

[d]Mamillary bodies to +6 mm anterior to the center of the pituitary stalk.

[e]All blocks extended laterally on each side to the lateral sulci or 6 mm whichever was least and to a depth of 5 to 6 mm.

relatively discrete regions defined previously by assay of individual 500 micron serial sections.

To my knowledge, this thesis is the first report of hypothalamic GnRH content in early PP cows or of direct comparison of hypothalamic GnRH in suckled and nonsuckled ruminants of any species. Although tissue content is difficult to interpret without knowledge of secretion rates, these findings suggest that reduced episodic LH secretion in suckled cows does not result from inadequate stores of hypothalamic gonadotropin-releasing hormone.

Anterior Pituitary Gland

Weight and Hormone Content

Mean anterior pituitary weights and total contents and tissue concentrations of LH, FSH and prolactin in suckled and nonsuckled cows are summarized in Table 10. As demonstrated previously using bioassay procedures (Labhsetwar et al., 1964; Saiduddin et al., 1968), suckling did not affect pituitary content of LH or follicle-stimulating hormone. The trend towards greater FSH content in suckled cows would not appear to result from decreased secretion since serum FSH concentrations were at least as great in suckled cows as in nonsuckled cows 1 day before slaughter. Unlike the report of Riesen et al. (1968), there was no decrease in pituitary prolactin content or concentration as a result of suckling. The possibility of a change in the bioactivity:radioimmunoactivity ratio as a result of suckling cannot be excluded, but to my knowledge this has not been examined in suckled animals.

Table 10. Content of LH, FSH and prolactin in anterior pituitaries of suckled (S-2X) and nonsuckled (NS-2X) cows killed on day 14 postpartum.

Criteria	Treatment group		Significance level
	NS-2X (n = 8)	S-2X (n = 9)	
LH			
mg[a]	2.3 ± .3	2.9 ± .3	N.S.
µg/mg[b]	1.2 ± .1	1.3 ± .1	N.S.
FSH			
mg	17.9 ± 3.2	24.3 ± 1.5	P < .1
µg/mg	9.8 ± 1.9	10.8 ± .8	N.S.
Prolactin			
mg	45.8 ± 11.5	53.1 ± 10.1	N.S.
µg/mg	23.9 ± 5.0	25.5 ± 4.4	N.S.
Pituitary weight			
g	1.9 ± .1	2.3 ± .2	P < .12

[a]mg of hormone/anterior pituitary

[b]µg of hormone/mg of anterior pituitary tissue (wet weight)

It does not appear that suckling causes decreased pulsatile LH secretion by reducing the total pituitary stores of LH as measured by radioimmunoassay.

In Vitro Superfusion: Response to Gonadotropin-Releasing Hormone and K^+

Luteinizing hormone secretion from superfused pituitary pieces challenged with 25 ng/ml GnRH or 59 mM K^+ is shown is Figure 13. Analysis of variance showed that the main effects of treatment (NS-2X vs S-2X) and time were highly significant ($P < .01$) as were the time by treatment and challenge (GnRH vs K^+) by time interactions. There was no significant main effect of challenge nor were treatment by challenge or treatment by challenge by time interactions significant ($P > .5$). These results may be summarized by the following points.

1. less LH was secreted from pituitaries of suckled cows than from those of nonsuckled cows in response to *in vitro* GnRH (25 ng/ml) or K^+ (59 mM) challenge,
2. GnRH challenge resulted in secretion of amounts of LH similar to those secreted in response to K^+ challenge in both suckled and nonsuckled cow pituitaries, and
3. the time-course of LH release was more prolonged following 30-min exposure to GnRH than after similar exposure to elevated K^+ in both suckled and nonsuckled cow pituitary tissue.

Since pituitary content of LH in suckled cows did not differ from that in nonsuckled controls, the reduced *in vitro* secretion of LH in response to both specific and non-specific stimuli may reflect a reduction in the "releasable portion" of the

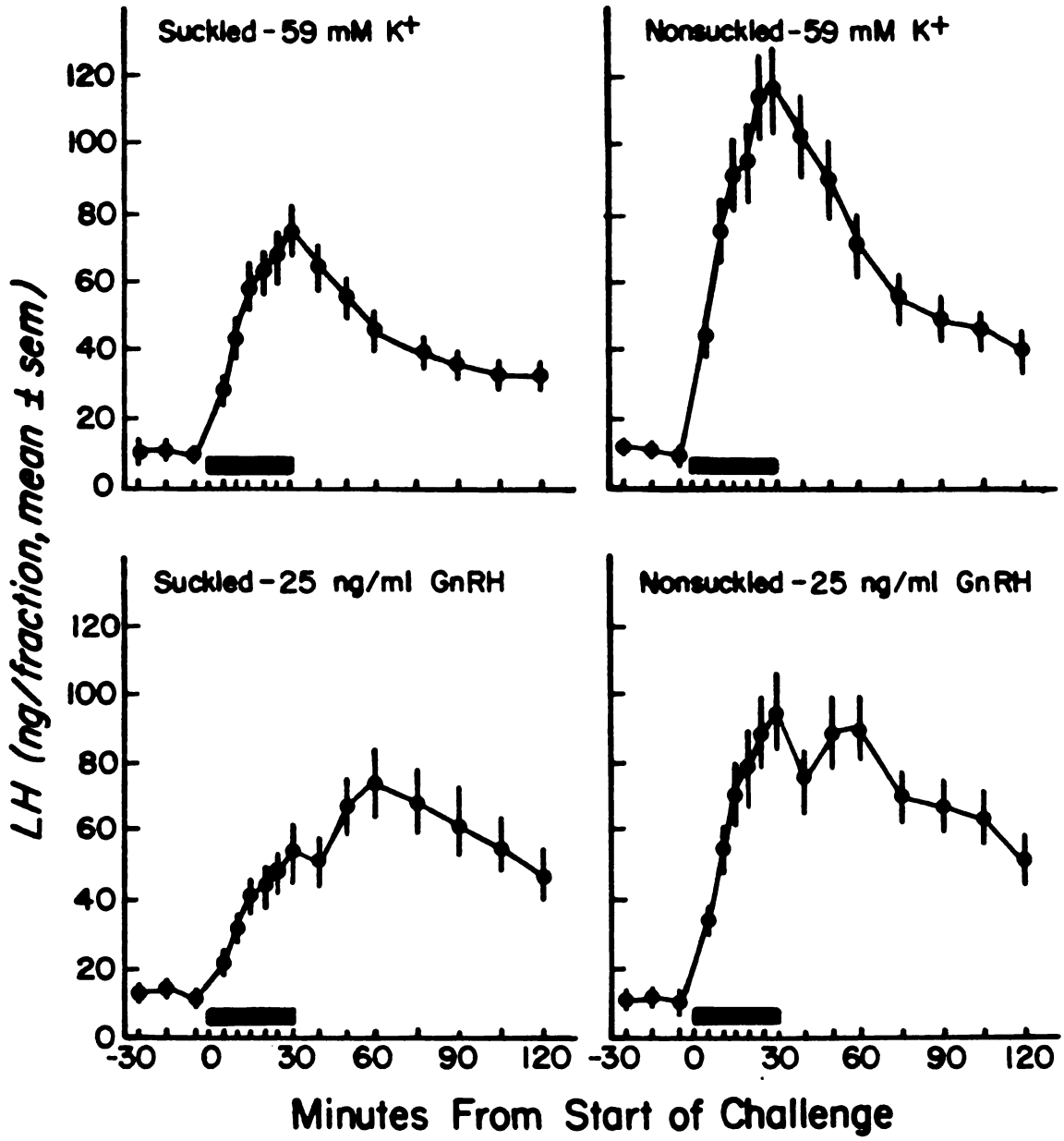


Figure 13. In vitro secretion of luteinizing hormone (LH) in response to gonadotropin-releasing hormone (GnRH) or K^+ challenge (solid bar) by pituitary pieces from suckled and nonsuckled cows on day 14 postpartum.

LH found in pituitaries of suckled cows. Based on work by Zolman (1973) and Kesner (personal communication), the duration of exposure and concentrations of both GnRH and K^+ used in this experiment were assumed to be greater than those which would elicit maximal LH secretion. This assumption was not tested. However, the lack of a significant challenge effect supports the concept that both stimuli caused maximal LH secretion of a common releasable pool. Zolman (1973) reported that the effects of GnRH and K^+ on LH secretion *in vitro* were not additive, a finding also suggestive of a single releasable LH pool. The lack of a treatment by challenge interaction would suggest that suckling's reduction of the releasable LH pool is not a specific effect on GnRH receptors unless the amount of releasable LH is a direct reflection of the number of GnRH receptors present. The more prolonged release of LH following GnRH challenge is consistent with a receptor mediated mechanism in comparison with the electrochemical membrane depolarization caused by elevated K^+ concentration.

Follicle-stimulating hormone content was assayed in the fractions collected beginning -5, 25, 55, 85 and 115 min relative to the start of the *in vitro* challenge. The only significant main effect was time ($P < .001$) indicating that FSH was secreted in response to *in vitro* challenge but that the amount released was not affected by the type of challenge (GnRH vs K^+) or the *in vivo* treatment (NS-2X vs S-2X). The only significant interaction was time by challenge ($P < .001$) resulting from less abrupt and more prolonged FSH release following GnRH than K^+ stimulation, a

finding consistent with the receptor vs electrochemical mediation of the two stimuli. Time-course of FSH release or response to *in vitro* challenge was not affected by suckling treatment *in vivo*. Results of *in vitro* FSH response to GnRH and K^+ in suckled and non-suckled cows on day 14 PP are summarized in Figure 14.

Summary

Results in this experiment confirm the finding in Experiment I that suckling causes a suppression of episodic LH secretion in early PP Holstein cows as reflected by decreased overall mean and reduced frequency and amplitude of episodic LH release. Although the sampling procedures differed slightly from those in Experiment I (i.e. no milking challenge during sampling; morning and evening sampling periods) and suckling intensity was doubled (two calves), I found no effect of suckling on serum concentrations of FSH, prolactin, progesterone, estradiol-17β and glucocorticoids. These results agree with those of Experiment I.

The reduced *in vivo* secretion of LH in suckled cows was not a result of decreased hypothalamic GnRH stores, nor was total pituitary LH content lower in suckled cows. However, as demonstrated by the *in vitro* superfusion results, there appeared to be a reduction in the releasable portion of the LH stores in the pituitaries of suckled cows, but suckling did not affect FSH released by *in vitro* challenge. Comparison of the LH and FSH responses to GnRH and K^+ would support a receptor mediated action of GnRH.

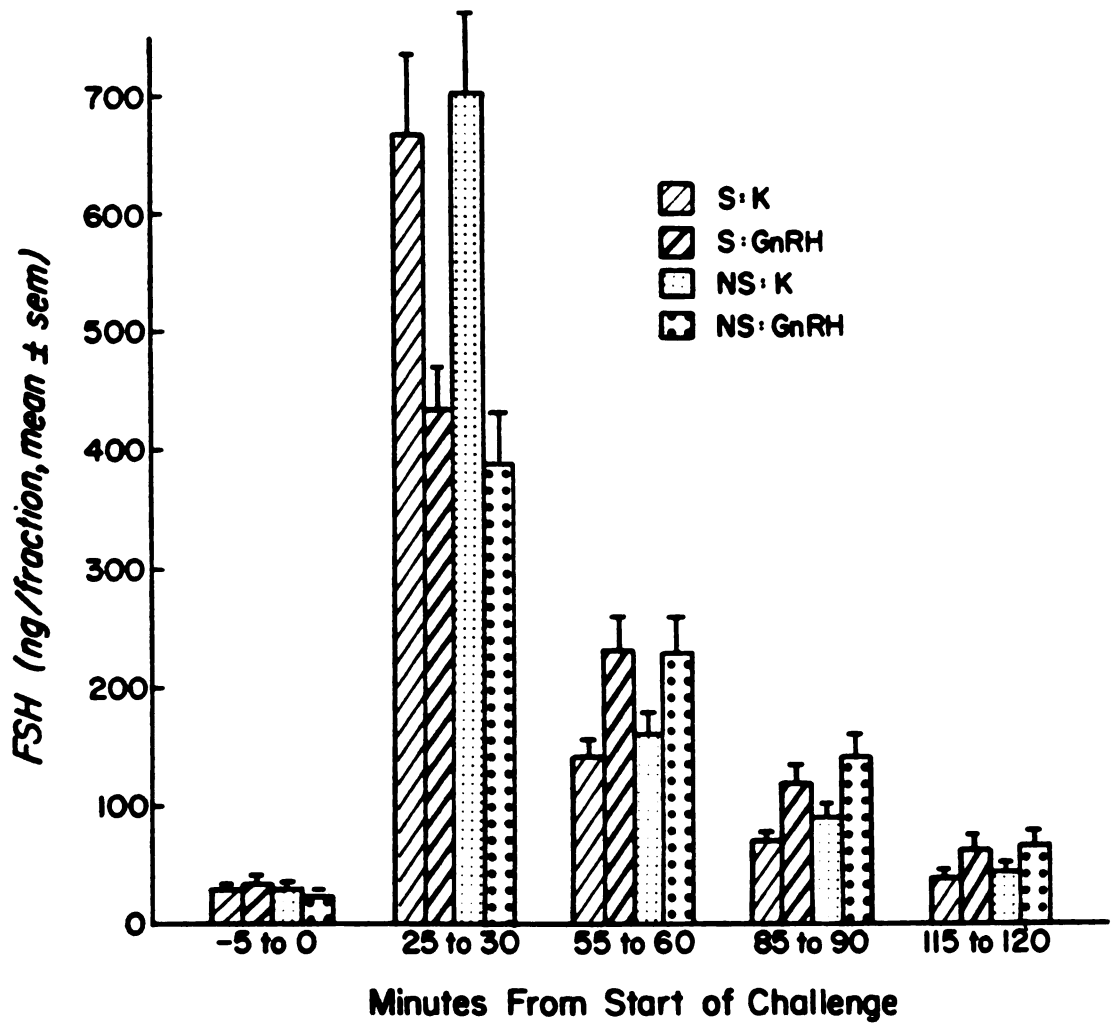


Figure 14. In vitro secretion of follicle-stimulating hormone (FSH) in response to gonadotropin-releasing hormone (GnRH) or K^+ challenge (0 to 30 min) by pituitary pieces from suckled (S) and nonsuckled (NS) cows on day 14 postpartum.

GENERAL DISCUSSION

In Experiment I, the stimulus provided by one suckling calf significantly prolonged the postpartum (PP) intervals to ovulation and observed estrus in Holstein cows. These results are in agreement with those from previous reports summarized in Table 1 and support the view that suckling acts as a significant inhibitor of PP reproduction in dairy cows. In Experiment I, four times daily milking did not influence PP intervals to ovulation or estrus when compared to controls milked twice daily. Clapp (1937) reported that the first PP estrus was delayed by four times daily milking. However, he did not monitor ovulation. The disparity between these experiments may represent managemental factors (nutritional status, machine vs hand-milking, etc.) which affected the response to four times daily milking or factors that affected the level of estrus detection (frequency of observation, use of heat detection aids, etc.). The results of Experiment I suggest that frequency of milk removal may not be the critical factor in suckling's influence in PP reproduction. However, four times daily milking may still have represented a relatively low milk removal frequency in comparison with the suckling frequency of a single calf penned with the cow.

The high incidence of ovulation without observed estrus in Experiment I is in agreement with the literature for PP cows

(Hackett et al., 1973; Hartigan et al., 1974; King et al., 1976). The apparently higher frequency of estrus with first PP ovulation in suckled versus nonsuckled cows (50% vs 0%) contrasts with the report by Moller (1970b) that suckled cows had a greater incidence of first PP ovulations without observed estrus than nonsuckled cows; reasons for this difference are unknown. The results in Experiment I may reflect the greater PP interval to first ovulation in the suckled cows since increasing PP interval has been associated with a reduced incidence of silent ovulations (Casida and Wisnicky, 1950; Stevenson, 1977).

The only detected endocrine change associated with suckling's delay of PP ovulation in Experiment I was a reduction in serum LH in the suckled cows. Randel et al. (1976) reported that suckled beef cows had lower serum LH concentrations than nonsuckled cows during the first 7 days PP. Samples were collected once daily in these experiments.

Secretion of LH in an episodic pattern has been reported in PP milked dairy cows (Stevenson, 1977; Goodale et al., 1978) and suckled beef cows (Corah et al., 1975; Humphrey, 1977). These studies have demonstrated increased frequency and amplitude of these episodic LH releases with increasing PP interval. Their findings are supported by Experiment I, where serum LH on day 14 PP had greater overall mean concentration, as a result of more frequent and larger LH peaks, than on day 7 postpartum. Suckling-induced reduction in mean serum LH concentration in both Experiments I and II resulted from a decreased frequency and amplitude of

episodic secretion on both days 7 and 14 postpartum. Thus, the results of Experiments I and II are the first reports of a direct comparison of the influence of suckling on acute secretory LH profiles in intact cows, beef or dairy. Since ovulation is a response to LH and depressed LH secretion is associated with the delayed PP ovulation in suckled cows, this suggests that suckling inhibits PP ovulation by inhibiting the resumption of episodic LH secretion.

Although less fully characterized than LH secretion, FSH secretion was not altered by the interval PP or by suckling in either Experiment I or II. In conjunction with the findings that ovarian follicular growth and pituitary FSH content were similar on day 14 PP in suckled and nonsuckled cows (Experiment II), these results support the concept that FSH is not limiting to PP ovulation in suckled cows. These findings also indicate a degree of specificity in suckling's influence on pituitary gonadotropin secretion.

Hyperprolactinemia induced by suckling has been implicated in PP anovulation in rats, ewes, monkeys and women but not in cows, nor has manipulation of serum prolactin concentrations been effective in altering the intervals to first PP ovulation or estrus in cows (see literature review). Suckling did not significantly increase either milking-induced (Experiment I) or basal (Experiments I and II) prolactin secretion, and this further refutes a role for prolactin in regulation of bovine reproduction.

Concentrations of serum progesterone, estradiol-17β and glucocorticoids were not affected by suckling in either Experiments

I or II, and agree well with values reported in the literature (Stevenson, 1977; Goodale et al., 1978). Progesterone concentration was probably too low to exert any physiological influence (<.5 ng/ml) throughout these experiments. Serum estradiol-17β concentrations were consistently low (2 to 6 pg/ml), and although estrogen has been shown to enhance LH response to GnRH in cattle in vivo (Beck and Convey, 1976) and in vitro (Padmanabhan et al., 1978), it does not appear that suckling acts to inhibit LH secretion by altering serum estradiol concentrations. Neither the literature (Wagner and Oxenreider, 1971) nor the results of these experiments support a role for adrenal glucocorticoids in suckling's effects on PP ovulation or LH secretion.

Minaguchi and Meites (1967) reported that hypothalamic GnRH and pituitary LH content were lower in lactating PP rats than in nonlactating PP rats. However, in Experiment II no differences were found in the total content or gross distribution of radioimmunoassayable GnRH in suckled and nonsuckled cows killed 14 days after calving. Total pituitary LH stores also were similar in suckled and nonsuckled cows. These results suggest major species differences in LH regulation during the PP period and dictate caution in extrapolation of experimental results from rodents to cows.

The key point from the in vitro superfusion study in Experiment II is that pituitaries from suckled cows release a smaller portion of their total pituitary LH content than those from nonsuckled cows in response to GnRH or K^+ challenge. This

suggests that suckled cows have smaller releasable pools of LH even though their total pituitary content of LH is similar to that of nonsuckled cows. The fact that both GnRH and K^+ in doses presumed to be maximal caused release of equivalent amounts of LH would suggest that GnRH receptors were not the limiting factor in release of LH by the pituitaries of suckled cows.

An important concept is the recent finding in dairy cows that one injection of GnRH apparently results in a transient increase in the amount of LH that can be released by a second similar injection of GnRH at 90 but not 360 min following the first injection (Foster, 1978). The *in vitro* releasable pools of LH in suckled and nonsuckled cows may reflect different *in vivo* exposures to gonadotropin-releasing hormone.

Pituitaries of nonsuckled cows must be more actively synthesizing and releasing LH because serum LH concentrations are greater than in suckled cows yet pituitary contents are similar. There is no evidence that suckling has changed the clearance rate of luteinizing hormone. Rather, the increased LH in nonsuckled cows is a result of more frequent and larger acute releases of the hormone. These results therefore suggest that pituitaries of nonsuckled cows receive a greater net stimulus to secrete LH or conversely that suckling results in decreased net stimulus to secrete luteinizing hormone. Given the fact that steroid feedback does not appear to be involved (i.e. no differences in measured serum steroid concentrations between suckled and nonsuckled),

suckling would most likely affect acute LH secretion by inhibiting episodic GnRH secretion from the hypothalamus.

In conclusion, a tentative hypothesis for suckling's effects on PP ovulation in the cow is as follows. Suckling stimuli act via the hypothalamus to inhibit episodic release of GnRH and possibly also inhibit GnRH release induced by estrogens. Reduced episodic GnRH release results in decreased episodic LH secretion and reduced LH synthesis, and the low frequency of GnRH stimulation of the pituitary may not allow enhancement or priming of the releasable LH pool to occur. Low frequency and amplitude of LH secretion may be responsible for failure of follicles to progress through the final preovulatory development required for estradiol secretion and ovulation.

Much of this hypothesis is speculative yet it provides a possible explanation for the results of this research and a framework upon which to further study the mechanism(s) by which the suckling stimulus influences PP reproduction in cows. Direct measurement of hypothalamic GnRH release, synthesis and/or turnover rate in cows would be required to prove this hypothesis and is unlikely to be achieved in the foreseeable future. The response of suckled PP cows to frequent small pulses of GnRH is one possible indirect approach to testing the hypothesis that GnRH secretion is limiting in suckled cows. Further understanding of the factors controlling follicular development and atresia will also shed light on how the pattern of LH secretion may be limiting to the maturation of preovulatory follicles.

SUMMARY AND CONCLUSIONS

The results of Experiments I and II have generated the following observations concerning the effects of suckling on PP ovulation and LH secretion in the Holstein cow.

1. Suckling by one or more calves significantly prolonged the interval to first PP ovulation, an effect not duplicated by four times daily milking.

2. Suckled cows had delayed PP resumption of episodic LH secretion without concurrent changes in secretion of FSH, prolactin, estradiol-17β, progesterone or glucocorticoids.

3. Pituitary contents of LH, FSH and prolactin were not significantly different in suckled and nonsuckled cows killed on day 14 postpartum.

4. Suckling did not change total hypothalamic content or gross distribution of gonadotropin-releasing hormone.

5. Pituitaries from suckled cows had less releasable LH than those from nonsuckled cows when challenged *in vitro* with GnRH or elevated concentrations of potassium ion.

6. Suckling did not affect the FSH released by pituitary tissue challenged *in vitro* with K^+ or GnRH.

7. Pituitary tissue (nonsuckled or suckled origin) released equivalent amounts of both LH and FSH in response to either K^+ (59 mM) or GnRH (25 ng/ml) challenge.

8. The time-course of LH and FSH secretion following *in vitro* challenge with GnRH was more prolonged than following K^+ challenge, possibly reflecting the receptor mediation of pituitary stimulation by gonadotropin-releasing hormone.

Based on these summary points, I conclude that suckling by one or two calves will significantly prolong the PP annovulatory and anestrus intervals in dairy cattle. Associated with this PP anovulation in suckled cows is a delayed resumption of episodic or pulsatile LH secretion. Evidence that suckling inhibited LH secretion via changes in circulating hormone feedback or depletion of hypothalamic GnRH and/or pituitary LH stores was not found. The reduction in the releasable pituitary LH stores in suckled cows would suggest reduced pituitary activity. In light of these results and the current literature, I hypothesize that the reduced pulsatile LH secretion in suckled cows results from a direct (probably neural) inhibition of hypothalamic stimulation of pituitary LH secretion. The most likely mechanism is reduced hypothalamic GnRH secretion.

This thesis suggests that episodic LH secretion may be important in the resumption of PP ovulatory cyclicity in cows and that suckling may inhibit PP reproduction via inhibition of episodic LH secretion. Therefore, the success of treatment regimes designed to induce early PP cyclicity in suckled cows may depend upon inducing or mimicking this LH secretory pattern.

APPENDICES

APPENDIX A

RADIOIMMUNOASSAY FOR BOVINE LUTEINIZING HORMONE (LH)

APPENDIX A

RADIOIMMUNOASSAY FOR BOVINE LUTEINIZING HORMONE (LH)

(Convey et al., 1976. Proc. Soc. Exp. Biol. Med.151:84)

1. General

Assay buffer was phosphate (.01 M) buffered (pH = 7.0) saline (.14 M) containing .1% Knox gelatin (PBS-K). Each unknown serum was assayed at dilution duplicates of 200 and 300 µl, superfusion media were assayed in duplicate at 10,20,40,80 and 160 µl as were pituitary extracts after predilution (10- to 1000-X) with assay buffer. Easy assay contained three or four sets of standards which each consisted of a background tube (first antibody blank), two total count tubes (^{125}I-LH only), four total binding tubes (zero standards), a 12-tube set of standards (NIH-LH-B8) range - .015 to 5.0 ng/tube) and dilution duplicates of low (postpartum cow) and high (ovex heifer) standard sera. A total volume of 500 µl was obtained in all tubes by addition of assay buffer.

2. First Antibody (guinea pig anti-bovine LH)

Two hundred µl of anti-LH (1:1,000,000 in PBS:EDTA with 1:400 guinea pig control serum) were added to each tube except background tubes which received 1:400 guinea pig control serum in

PBS:EDTA without first antibody and total count tubes which received nothing. Tubes were vortexed gently then covered and incubated at 4 C for 24 hours.

3. Tracer (^{125}I-bLH-LER-1072-2)

Method of radioiodination of purified bovine LH was essentially that of Niswender et al., 1969. Endo. 84:1166; except that Na^{125}I was used and the column of Bio-Gel P60 was coated with egg white albumin. One hundred µl of ^{125}I-LH (20,000 cpm) in PBS-K were added to each assay tube and incubation continued at 4 C for 24 hours.

4. Second Antibody (ovine anti-guinea pig gamma globulin)

Two hundred µl of a precipitating dilution (1:15 to 1:25 in PBS:EDTA) of second antibody were added to all but the total count tubes and after vortexing, incubation was continued at 4 C for 72 hours.

5. Precipitation

Following final incubation, 3 ml of cold PBS was added to each tube (except total count tubes) to dilute the unbound ^{125}I-tracer. Centrifugation at 2,000 x g for 30 min in a refrigerated centrifuge was used to precipitate the antibody bound radioactivity. The supernatant was decanted, fluid adherent to the rim of the tube was removed by rinsing inverted tubes in running water, blotting and allowing tubes to dry before turning upright. The bound ^{125}I in the precipitate was then quantified in an automatic gamma counter. Samples were counted for 10 min or a total of 4,000 counts, whichever occurred first.

APPENDIX B

RADIOIMMUNOASSAY FOR BOVINE PROLACTIN

APPENDIX B

RADIOIMMUNOASSAY FOR BOVINE PROLACTIN

(Tucker, 1971. J. Anim. Sci. 32(Suppl. 1):137)

1. General

Assay buffer was phosphate (.01 M) buffered (pH 7.0) saline (.14 M) containing 1% bovine serum albumin (PBS-BSA). Sera and diluted pituitary extracts were assayed in dilution triplicates of 10,20 and 40 µl; total sample volume was made to 500 µl with assay buffer. Four standard sets were included in each assay; they were similar in composition to those in the LH assay except that the reference standards consisted of 14 tubes (NIH-PRL-B3, range = .1 to 4.0 ng/tube) and a single high standard serum assayed at volumes of 10 to 320 µl/tube.

2. First Antibody (guinea pig anti-bovine PRL)

One hundred µl of anti-PRL (1:30,000 in PBS:EDTA with 1:400 guinea pig control serum) was added to each tube. Background tubes received antibody-free control serum. Tubes were vortexed gently then covered and incubated at 4 C for 24 hours.

3. Tracer (^{125}I-NIH-PRL-B3)

Method of radioiodination of bovine PRL was essentially that of Niswender et al., 1969. Endo 84:1166; except that Na^{125}I was used for labelling. One hundred µl of ^{125}I-PRL (20,000 cpm)

in PBS-BSA was added to every tube and incubation was continued at 4 C for 24 hours.

4. Second Antibody - as in LH assay (Appendix A) except volume of 100 µl.

5. Precipitation - as in LH assay (Appendix A).

APPENDIX C

EXTRACTION AND RADIOIMMUNOASSAY OF PROGESTERONE

APPENDIX C

EXTRACTION AND RADIOMMUNOASSY OF PROGESTERONE

(Convey et al., 1977. J Anim. Sci. 46:792)

Extraction

1. Duplicate aliquants of serum (200 µl) were pipetted into disposable culture tubes (16 x 100mm).
2. To account for procedural loss, a third aliquant from a representative number of unknowns (10 per assay) were added to a culture tube (16 x 100 mm) which contained 2,500 cpm ^{3}H-1,2,6,7-progesterone (New England Nuclear; 80 to 100 Ci/mM; repurified by column chromatography) in methanol. Twenty-five hundred cpm of ^{3}H-progesterone were also added to scintillation vials (4 per assay) for total counts (100% recovery tubes).
3. Tubes containing serum ^{3}H-progesterone were vortexed (10 sec) then labelled and endogenous progesterone were allowed to equilibrate for 30 min at room temperature.
4. Serum was extracted with 3 ml nanograde benzene:hexane (1:2) by vortexing for 30 sec and was then stored at -20 C for at least 1 h to freeze the aqueous phase.

   Sovent extracts destined for assay were decanted into disposable culture tubes (12 x 75 mm).

Extracts for procedural loss were decanted into scintillation vials.

5. Recovered radioactivity was averaged to determine a single recovery correction factor to account for procedural losses for all samples.
6. For comparison among assays, standard sera with high and low progesterone concentrations were assayed in triplicate in each assay.

Radioimmunoassay

1. Progesterone (Sigma Chemical Co.) for standards (.05, .10, .15, .20, .25, .30, .40, .60, .80 and 1.0 ng/tube) was pipetted into disposable assay tubes from a stock solution of 10 ng/ml in absolute methanol and three sets included in each assay.
2. Standards and serum extracts were evaporated to dryness in a vacuum oven (28-30 inches vacuum) at a temperature less than 50 C.
3. First antibody[1] (200 µl) diluted 1:3,000 in PBS-K[2] containing 1:100 normal rabbit serum was added to each tube, vortexed briefly and incubated at room temperature for 2 hours.
4. Then, 200 µl PBS-K containing 5,000 cpm of ^{3}H-progesterone were added to each tube, vortexed briefly and incubated at 4 C for 24 hours.

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[1]First antibody = MSU #74 rabbit anti-progesterone-human serum albumin.

[2]Phosphate (.01 M) buffered saline (pH 7.4) saline (.14 M) with .1% Knox gelatin.

5. Second antibody (400 µl; MSU ovine anti-rabbit gamma globulin) at a 1:15 dilution in PBS-K were added to each tube and after vortexing tubes were incubated at 4 C for 48 hours.
6. All assay tubes were centrifuged at 2,500 x g for 15 min at 4 C and a .5 ml aliquant of supernatant was diluted with 5 ml scintillation fluid (3a70B Preblend, RPI Corp., Elk Grove Village, Illinios) then counted in a liquid scintillation spectrophotometer.

APPENDIX D

RADIOIMMUNOASSAY OF GONADOTROPIN-RELEASING HORMONE (GnRH)

APPENDIX D

RADIOIMMUNOASSAY OF GONADOTROPIN-RELEASING HORMONE (GnRH)

(Estes et al., 1977. Biol. Reprod. 17:706)

Radioimmunoassay:

1. General

Assay buffer was phosphate (.01 M) buffered (pH = 7.0) saline (.14 M) containing .1% pigskin gelatin (PBS-PSG). Reconstituted hypothalamic extracts were assayed in duplicate at 5,10, 25,50,100 and 200 ul; medial basal hypothalamic extracts were prediluted 1:10 with assay buffer. Four sets of 13 tubes containing 1.5 to 200 pg/tube of synthetic GnRH (Abbot Laboratores) were included in the assay along with appropriate total count, total binding and background tubes. Total standard and unknown volumes were adjusted to 500 ul with assay buffer; all unknowns were included in a single assay. The assay was carried out in 10 x 75 mm polyvinyl culture tubes (Falcon Plastics Co.).

2. First Antibody[1] (rabbit anti-GnRH, #R42)

Two hundred ul of anti-GnRH (1:40,000 in PBS:EDTA with 1:400 normal rabbit serum) were added to all but total count and background tubes. Tubes were vortexed and incubated at 4 C for 24 hours.

[1]Antibody supplied by Dr. Terry Nett, Colorado State Univ., Fort Collins, Co.

3. Tracer (^{125}I-GnRH)

Radioiodination of synthetic GnRH was by the chloramine-T method as described by Nett et al. (1973) and outlined below. One hundred ul of ^{125}I-GnRH (20,000 cpm) was added to each tube, tubes were vortexed and incubation continued at 4 C for 24 hours.

4. Second Antibody (ovine anti-rabbit gamma globulin, MSU #388-11/12/75)

Each assay tube received 200 µl of a 1:25 dilution of second antibody and after vortexing, incubation was continued at 4 C for an additional 72 hours.

5. Precipitation - as in LH assay (Appendix A) except that radioactivity was counted for 10 min or 10,000 counts, whichever occurred first.

Iodination Procedure:

1. Column preparation

Expand Sephadex LH-20 (Pharmacia Fine Chemicals) in PBS and evacuate at room temperature overnight.
Pour LH-20 into disposable glass column (10 x 150 mm).
Coat column with 2 ml PBS-PSG.
Wash column with PBS for two hours.

2. Iodination

To 1 mCi Na^{125}I (New England Nuclear) add:
25 µl .5 M phosphate buffer
3 µg GnRH in 20 µl water
20 µg chloramine-T in 10 µl water.
Mix for 45 seconds at room temperature.
Add 100 µg sodium metabisulfite in 50 µl water.
Add 100 µl transfer solution.
Transfer to LH-20 column.
Collect 45 fractions at 3-min intervals in 12 x 75 mm glass culture tubes containing 1 ml of PBS-PSG, monoiodinated GnRH recovered in fractions 40 to 60.

APPENDIX E

EXTRACTION AND COMPETITIVE PROTEIN BINDING RADIOASSAY OF TOTAL GLUCOCORTICOIDS

APPENDIX E

EXTRACTION AND COMPETITIVE PROTEIN BINDING RADIOASSAY OF TOTAL GLUCOCORTICOIDS

(Smith et al., 1973. J. Anim. Sci. 36:391)

Extraction

1. Duplicate aliquants of serum (.2 ml) were pipetted into disposable culture tubes (16 x 100 mm).
2. To account for recovery, a third aliquant from representative unknowns (10 per assay) was added to a tube containing 2,000 cpm ^{3}H-1,2,6,7-cortisol (New England Nuclear; 91 Ci/mM; repurified by column chromatography) in methanol. Tritiated cortisol was also added to four scintillation vials per assay for total counts (100% recovery counts).
3. Tubes containing serum and ^{3}H-cortisol were mixed by vortexing briefly and allowed to equilibrate for 30 min at room temperature.
4. Serum was extracted by vortexing with 2 ml iso-octane (2,2,4-trimethyl pentane) for 1 min then stored at -20 C for at least 1 h to freeze the aqueous phase. Organic supernatant was discarded and samples allowed to thaw.
5. Thawed aqueous phase was re-extracted by vortexing with 2 ml methylene chloride for 1 min and stored at -20 C for at least 1 h to freeze the aqueous phase. Extracts were decanted into

disposable assay culture tubes (12 x 75 mm). Extracts from aliquants for recovery losses were decanted into scintillation vials. Recovered radioactivity was averaged to determine a single correction factor to account for recovery efficiency in all samples.

Protein Binding Assay

1. Cortisol (Sigma Chemical Co.) for sets (3 per assay) of standards (.05, .10, .15, .20, .25, .50, 1.00, 1.50, 2.00 and 2.50 ng/tube) was pipetted into disposable assay tubes from a stock solution of 10 ng/ml in absolute methanol and included in each assay. Thus, total unknown glucocorticoids were calculated in terms of cortisol.
2. Standards and serum extracts were evaporated to dryness in a vacuum oven (28-30 inches vacuum) at a temperature less than 50 C.
3. Then, 1 ml of 1.25% dog plasma[1] with 11,000 to 12,000 cpm/.5 ml of ^{3}H-cortisol was added to each tube. Tubes were vortexed briefly and stored at 4 C for 18 hours.

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[1] Dog plasma (Colorado Serum Co.) was diluted to 2.5% in glass distilled water and mixed with 60 g Florisil (120 mg/ml diluted dog plasma; 80 mesh, Matheson, Coleman and Bell) for 3 h to strip endogenous steroids. The suspension was centrifuged at 2,800 rpm for 15 min and the supernatant fluid volume as doubled with glass distilled water to give a 1.25% solution. Tritiated cortisol was added to the plasma to give about 11,000 to 12,000 cpm/.5 ml and stored at 4 C for up to 1 month.

4. Assay tubes were placed in ice and allowed to equilibrate for 15 min then .5 ml of 1% dextran T70 (Pharmacia) and .5% carbon decolorizing neutral norit charcoal (Fisher Scientific) in glass distilled water was added to each tube.
5. Contents were allowed to incubate in ice for 5 min, then centrifuged at 2,000 x g for 15 min at 4 C. A .5 ml aliquant of the supernatant fluid was diluted with 5 ml of scintillation fluid (3a70B Preblend, RPI Corp., Elk Grove Village, Illinois) for quantification of radioactivity in a liquid scintillation spectrophotometer.

APPENDIX F

RADIOIMMUNOASSAY OF BOVINE FOLLICLE-STIMULATING HORMONE (FSH)

APPENDIX F

RADIOIMMUNOASSAY OF BOVINE FOLLICLE-STIMULATING HORMONE (FSH)

(modified from: Cheng, K.-W., 1978. J. Endocr. 77:185)

Assay Protocol:

1. General

Assay buffer was PBS-BSA (see Appendix B). Serum and superfusion media were assayed at dilution duplicates of 100 and 200 µl, pituitary extracts were diluted 1:50 then assayed in duplicate at 5,10,20,40 and 80 µl. Each assay contained four replicate standard sets, each consisting of total count tubes, total binding tubes, background or nonspecific binding tubes, a standard curve of NIH-FSH-B1 (1 to 256 ng/tube) and two standard sera curves with 20 to 320 µl/tube. Total sample and standard volumes were made to 500 µl by addition of assay buffer.

2. First Antibody (rabbit anti-bovine FSH, rabFSH-B2[1])

Two hundred µl of anti-bovine FSH (1:160,000 in PBS-BSA with 1:400 normal rabbit serum) were added to all but the total count and background tubes (normal rabbit serum only). Tubes were vortexed and incubated at 4 C for 24 hours.

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[1]Antibody supplied by Dr. K.-W. Cheng, University of Manitoba, Winnipeg, Manitoba, Canada.

3. Tracer (^{125}I-bFSH-HS-2)

Higher purified bovine FSH[2] (Cheng, 1976) was iodinated by the chloramine-T method using modifications described by McCarthy (p. 78). Two hundred µl of ^{125}I-FSH (20,000 cpm) were added to each assay tube and incubation continued at 4 C for a further 24 hours.

4. Second Antibody (ovine anti-rabbit gamma globulin, MSU#388-6/16/77)

One hundred µl of a 1:10 dilution in PBS-BSA were added to each tube and incubation continued for 72 hours.

5. Precipitation - as in LH assay (Appendix A).

Assay Validation:

Total binding and nonspecific binding averaged 21.4 ± 1.1% and 1.2 ± .1% ($\bar{x}$ ± SE, n = 8), respectively, of the total count tubes. The 80,50 and 20% intercepts of the standard curve were 4.6 ± .4, 25.0 ± 1.0 and 120.7 ± 6.2 ng of NIH-FSH-B1/tube (n = 8). Sensitivity of the assay was approximately 2 ng/tube of FSH-B1 equivalents as determined by the lower limits of the 95% C. . on the total binding tubes. Figure F.1 demonstrates parallelism between serial dilutions of various sera, media, pituitary extracts and the reference standard (NIH-FSH-B1). Major cross reactants in the assay are summarized in Table F.1; further cross reactivities

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[2]Purified bovine FSH (bFSH-HS-2) supplied by Dr. K.-W. Cheng.

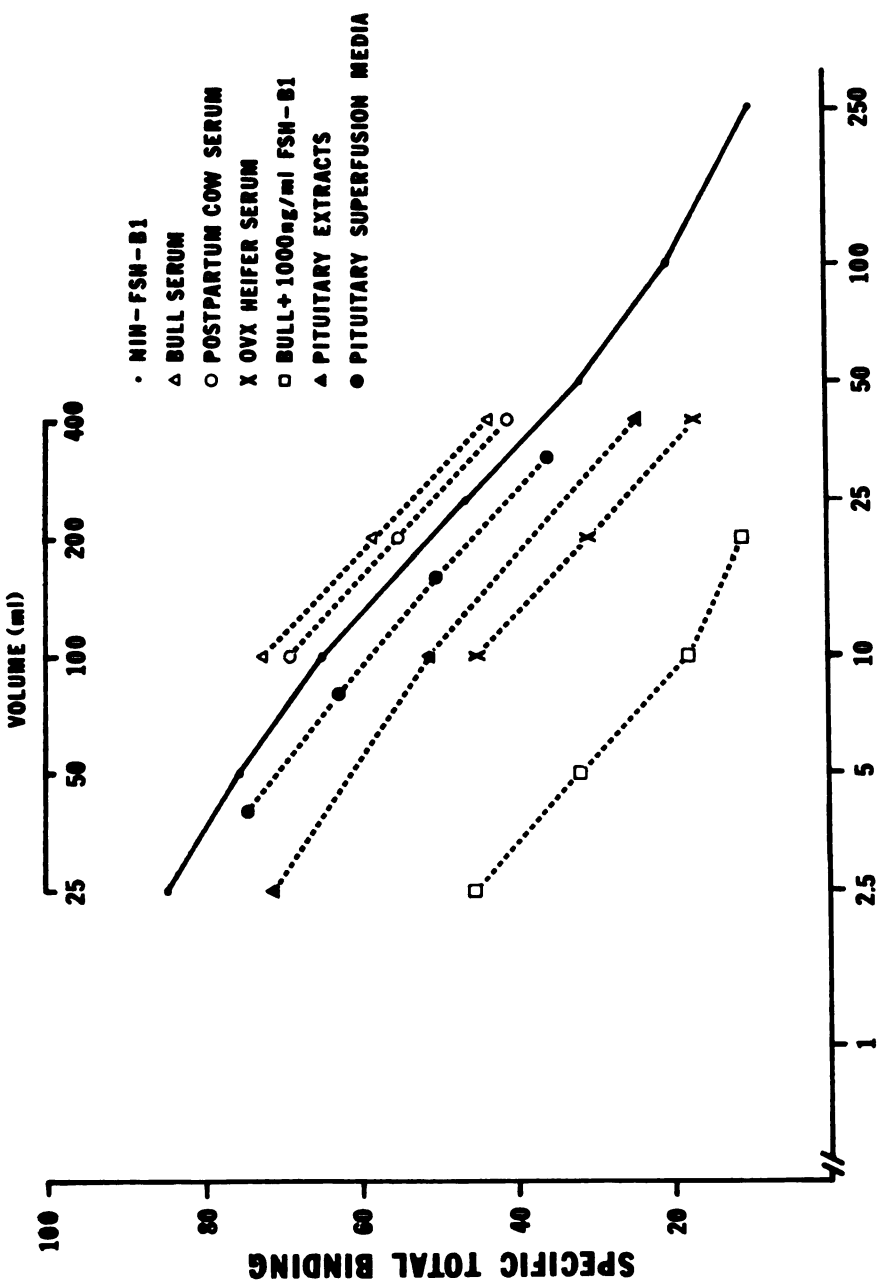

Figure F.1. Parallelism of serum, superfusion media, pituitary extracts and follicle-stimulating hormone reference preparation (NIH-FSH-B1) in the bovine follicle-stimulating hormone radioimmunoassay.

Table F.1. Cross reaction of other bovine pituitary hormones in the follicle-stimulating hormone radioimmunoassay.

Preparation[a]	Relative Immunoreactivity[b]
bFSH-LER-1072[c]	100.0%
FSH-NIH[d]-B1	9.0%
TSH-NIH-B6	5.5%
LH-NIH-B8	.4%
GH-NIH-B17	N.D.[e]
Prolactin-NIH-B4	N.D.

[a]FSH = follicle-stimulating hormone; LH = luteinizing hormone; TSH = thyroid-stimulating hormone; GH = growth hormone.

[b]Calculated at 50% specific binding.

[c]Purified bovine FSH provided by Dr. L. E. Reichert.

[d]NIH hormone preparations provided by Endocrine Study Section, Hormone Distribution Program, National Institutes of Health.

[e]Less than 10% inhibition of specific binding by 1000 ng.

have been characterized for this antibody by Cheng (1978). Table F.2 shows the recovery of 1000 ng/ml of NIH-FSH-B1 added to bull serum containing 68 ng/ml of endogenous activity. Recovery averaged 96.1% for volumes of serum from 10 to 160 microliters. High (133.5 ng/ml) and low (57.1 ng/ml) standard sera had inter-assay C.V. of 15.8 and 15.1% respectively, with intraassay C.V. of 11.2 and 13.2%.

Table F.2. Assay recovery of FSH[a] added to bull serum.

Serum volume assayed (µl)	Immunoassayable FSH (ng/ml)		Recovery (%)
	Total	Corrected[b]	
10	9.2	8.5	85.1
20	20.8	19.5	97.3
40	42.5	39.8	99.5
80	81.4	76.0	95.0
160	176.5	165.5	103.3
Average			96.1

[a]FSH = follicle-stimulating hormone, NIH-B1, 1 µg added per ml of bull serum.

[b]Corrected for endogenous FSH activity of 68.0 ng/ml in bull serum.

APPENDIX G

EXTRACTION, CHROMATOGRAPHY AND RADIO-IMMUNOASSAY OF ESTRADIOL-17β

APPENDIX G

EXTRACTION, CHROMATOGRAPHY AND RADIO-IMMUNOASSAY OF ESTRADIOL-17β

(modified from England et al., 1974. J. Clin. Endocr. Metab. 38:42)

Extraction

1. Individual recoveries were monitored for each quality control and unknown sample using ^{3}H-(2,4)-17α-estradiol (Amersham Corp., 42 Ci/mM) as an internal standard. Tritiated estradiol-17α (5,000 cpm in 20 µl of phosphate (.01 M) buffered (pH 7.0) saline (.14 M) containing .1% Knox gelatin (PBS-K)) was added to each extraction tube (15-ml conical bottom glass tubes with teflon stoppers) plus four scintillation vials. An additional 80 µl of PBS-K was added to the scintillation vials.
2. Samples were pipetted into extraction tubes, vortexed and allowed to equilibrate with the internal standard at room temperature for at least 1 hour.
3. Diethyl ether (10 ml; Mallinckrodt Inc., anesthesia grade) from a freshly opened can was added to each extraction tube and after stoppering, extraction was performed by shaking in a horizontal position for 45 min at 60 oscillations/min on a platform shaker.
4. Following extraction, phases were separated by centrifugation (10 min at 1000 x g) and the aqueous phase was frozen by

immersing in a Dry Ice: methanol bath; then, the ether phase was decanted into 12-ml conical bottom glass tubes.

5. Extracts were evaporated to dryness in a vacuum oven (28 inches vacuum, 45 C) and sides of tubes rinsed once with 1 ml of fresh ether.
6. Quality control standards included a water blank, serum blank (ovariectomized cow), added mass standard (serum plus 10 pg/ml of unlabelled estradiol-17β) and high standard serum (estrus cow). One such set of standards was inclued at the beginning and end of each assay.
7. Up to 32 unknown samples were run in each assay, serum volumes were between .5 and 3.0 ml depending upon the expected estradiol concentrations.

Chromatography

1. Extracts were chromatographed thru Sephadex LH-20 columns (QS-44 Quik-Sep Extended LH-20 Columns, Isolab Inc., Akron, OH.). These commercially prepared columns are 8 mm ID x 253 mm long and contain 1000 mg of Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden).
2. Freshly mixed, glass distilled benzene:methanol (90:10 v/v, Burdich and Jackson Lab. Inc., Muskegon, MI.) was used as column solvent. Columns were prepared for use by equilibrating with 25 ml of solvent then samples were transferred from the conical drying tubes to the columns using a 9 inch Pasteur

pipette (one/sample) and three aliquots of column solvent (300, 200 and 200 µl).

3. Unwanted materials were eluted from the columns by an additional 5.5 ml of solvent following which a 4.2-ml fraction containing the estradiol peak was collected into a 12 x 75 glass culture tube. The column solvent was evaporated in a drying oven and the residue redissolved in 600 µl of PBS-K.
4. Columns were washed with an additional 40 ml of column solvent before reuse or storage in a glass cylinder filled with column solvent.

Radioimmunoassay

1. The column fractions in PBS-K were aliquoted using a Hamilton syringe, 2 x 200 µl into 12 x 75 mm culture tubes for assay and 1 x 100 µl into a scintillation vial for determination of recovery of internal standard. Scintillation fluid (5 ml; 3a70B Preblend, RPI Corp., Elk Grove Village, Il.) was added to scintillation vials and radioactivity quantified in an automatic liquid scintillation spectrophotometer.
2. Assay buffer was PBS-K and total sample volume was 200 µl; each assay consisted of four total count tubes, eight total binding tubes (zero standards), four nonspecific binding tubes (500 pg/tube of estradiol-17β) and three standard curves of estradiol-17 (stock solution = .32 ng/ml in PBS-K, curve range = .32 to 64.0 pg/tube) in addition to duplicate assay tubes from each of up to 40 samples (quality control and unknowns).

3. Two hundred µl of first antibody[1] (1:200,000 in PBS-K) were added to each tube, mixed by gentle vortexing and assay incubated at room temperature (≈22 C) for 90 min then placed in an icebath for 30 minutes.
4. One hundred µl of ice cold tracer[2] (^{125}-I-estradiol-17β-11α-tyrosine methyl ester; 20,000 cpm/100 µl) were added to each tube, mixed and incubation continued on ice for 45 minutes. Estradiol conjugate was radioiodinated using the chloramine-T method as described for this preparation by England et al. (1974).
5. One ml of dextran T70 (.025%, Pharmacia Fine Chemicals, Uppsala, Sweden)-coated charcoal (.25%) in ice cold PBS was added to all but the total count tubes, vortexed briefly and allowed to incubate on ice for 30 min then transferred to cold carriers and centrifuged (10 min, 2,000 x g, 4 C) to precipitate the charcoal. Supernates containing antibody-bound ^{125}I-tracer were immediately decanted into 12 x 75 mm tubes and capped.
6. Radioactivity in total count tubes and supernates was quantified in an automatic gamma radiation spectrophotometer. Each tube was counted for 10 min or 10,000 counts, whichever occurred first.

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[1]Rabbit anti-estradiol-17β-11β-hemisuccinate-bovine serum albumin, #930 supplied by Dr. K. T. Kirton, The Upjohn Co., Kalamazoo, MI.

[2]Uniodinated estradiol conjugate supplied by Dr. K. T. Kirton, The Upjohn Co., Kalamazoo, MI.

7. Estradiol-17β immunoreactivity/assay tube was corrected for serum volume extracted and recovery efficiency off the column to give estradiol concentration in pg/ml of serum.

General

1. All glassware except pipettes and columns was oven baked at 600 F for 4 h before use, a process that significantly decreased the occurrence elevated water and serum blanks.

Assay Validation

1. In 10 assays, total binding (zero standards) averaged 34.1 ± 1.9% of the total counts added and nonspecific binding (excess cold standard) averaged 3.1 ± .3%. Standard curve intercepts (80%, 50% and 20%) averaged 2.4 ± .2, 9.7 ± .7 and 42.6 ± 3.3 pg/ml respectively in these 10 assays.
2. Table G.1 summarizes the values obtained for the water blanks, serum blanks (ovariectomized cow) and high serum standard (estrus cow). Unknowns were not corrected for either water or serum blanks.
3. Insufficient assays were available for calculation of the interassay coefficient of variation (CV). However, the intraassay CV for the ovariectomized and estrus standard sera in assays containing samples from the experiments in this thesis were 23.2% and 11,3% respectively.
4. Procedural losses ranged from 5 to 25% as calculated from the ^{3}H-estradiol-17α recovery and sera were corrected individually.

Table G.1 Mean (± SE) concentrations of quality control standards in estradiol-17β radioimmunoassay.

Quality control standard	n	Assayed estradiol-17β
Distilled water	30	.4 ± .1 pg/tube
Ovariectomized cow serum	28	1.1 ± .2 pg/ml
Estrus cow serum	20	20.7 ± 2.3 pg/ml

These losses included those due to adsorption to glass, extraction inefficiencies, and chromatography and transfer losses. No correction was made for the mass of ^{3}H-tracer in the tubes since 60,000 cpm of the estradiol-17α was found to cause less than 5% inhibition of the total binding tubes in the assay.

5. Cross reactivity of some important steroids in the estradiol-17β assay are summarized in Table G.2.
6. Recovery of estradiol-17β added to ovariectomized cow serum (10 pg/ml) was 91.5 ± 1.5% (n = 10) when corrected for 1.1 pg/ml of endogenous immunoreactivity in the ovariectomized cow serum.

Table G.2. Cross reaction of major steroids in the estradiol-17β radioimmunoassay.

Steroid	Relative immunoreactivity[a]
Estradiol-17β	100.0%
Estrone	4.04%
Estriol	.12%
Progesterone	.004%
Testosterone	.004%
Dihydrotestosterone	.004%
Corticosterone	N.D.[b]
Cortisol	N.D.
Androstenediol	N.D.
Cholesterol	N.D.

[a]Calculated at the 50% inhibition level on the standard curve.

[b]Less than .001% cross reaction.

APPENDIX H

STOCK SOLUTIONS AND MIXING RATIOS FOR MAKING KREB'S RINGER BICARBONATE BUFFERS USED IN PITUITARY SUPERFUSIONS

APPENDIX H

STOCK SOLUTIONS AND MIXING RATIOS FOR MAKING KREB'S RINGER BICARBONATE BUFFERS USED IN PITUITARY SUPERFUSIONS

(from: Zolman, 1973)

Table H.1 lists the stock solutions and their compositions. All solutions were made using double-distilled water and were stored at 4 C until used to make the appropriate buffers. The glucose solution was sterilized by filtration thru a Nalgene filter unit (.2 m pore size, No. 120-0020, Nalge Sybron Corp., Rochester, N.Y.), and all stock solutions were remade at weekly intervals.

The volumes of individual stock solutions required to make 1.32 liters of either 5.9 mM or 59 mM K^+ Kreb's Ringer bicarbonate buffer are listed in Table H.2. The $NaHCO_3$ stock solution must be gassed with CO_2 for at least 1 h before use and the final buffer solutions must be gassed with O_2:CO_2 (95:5) for at least 30 min and equilibrated to 37 C before and during use. Gonadotropin-releasing hormone challenge medium was obtained by adding 1 ml of PBS-BSA containing 6.25 μg gonadotropin-releasing hormone (GnRH) to 249 ml of KRB-5.9; 1 ml of PBS-BSA without GnRH was added to the KRB-59.

Table H.1. Stock solutions used for Kreb's Ringer bicarbonate buffer.

Solution	Reagent[a]	Percent (v/b)
I	NaCl	.90%
II	KCl	1.15%
III	$CaCl_2$	1.22%
IV	KH_2PO_4	2.11%
V	$MgSO_4 \cdot 7H_2O$	3.82%
VI	$NaHCO_3$	1.30%
VII	Glucose	5.00%

[a]Reagent grade chemicals purchased from Mallinckrodt Chemical Works, St. Louis, MO.

Table H.2. Volumes of stock solutions required for 1.32 liters of Kreb's Ringer bicarbonate buffer containing 5.9 mM K^+ (KRB-5.9) or 59 mM K^+ (KRB-59).

Solution	Volume	
	KRB-5.9	KRB-59
I	1000	550
II	40	490
III	30	30
IV	10	10
V	10	10
VI	210	210
VII	20	20

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