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UNIVERSITY OF ALBERTA

OVARIAN FUNCTION IN DAIRY COWS

BY

BRIAN JAMES CAMERON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled OVARIAN FUNCTION IN DAIRY COWS submitted by BRIAN JAMES CAMERON in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in ANIMAL REPRODUCTION.

.....

J.J. Kennelly, Supervisor

.....

J.G. Manns

.....

G.R. Foxcroft

.....

Date: 19

Dedication

To my wife Corinne,

son Colin and

daughter Jean-Christy

ABSTRACT

The overall objective of this thesis was to evaluate certain factors that may modulate ovarian function and physiological changes that result from ovarian activity in dairy cows. Specific objectives were to evaluate the role of insulin-like growth factor-I (IGF-I) in preovulatory bovine follicles following growth hormone (GH) treatment. GH treatment of 20.6 mg/d for 5 d prior to ovariectomy elevated circulating and follicular fluid IGF-I concentrations and circulating GH over saline controls. Granulosa cell preparations were hybridized to IGF-I, IGF Type-I receptor, and GH receptor antisense RNA riboprobes. Four species of IGF-I mRNA, three GH receptor transcripts, and four IGF Type-I receptor mRNAs were detected in all granulosa cell preparations. GH treatment failed to effect IGF-I, IGF Type-I receptor, or GH receptor mRNA abundance. These results provide evidence that IGF-I is produced in bovine granulosa cells, that GH may act directly on these cells and that IGF-I may have an autocrine and(or) paracrine mode of action in the bovine preovulatory follicle.

In a second experiment, vaginal and vulval electrical admittance values relative to changes in plasma concentrations of estradiol, LH, and progesterone were investigated. Circulating estradiol was positively correlated with the LH surge and standing estrous behavior. Pooled probe admittance values were positively correlated with elevated estradiol concentrations in both intact and ovariectomized cattle. There was a 2 to 3 day latent response in the maximal vaginal admittance correlation to peak circulating estradiol concentrations. This response was observed regardless of circulating concentrations of progesterone. These results

indicate that estradiol is likely the driving force behind elevated vaginal admittance. Progesterone indirectly affects vaginal admittance by modulating ovarian estradiol production. Development of a vulval probe, which is capable of consistently measuring tissue admittance holds promise as an estrous detection aid.

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I. INTRODUCTION

Reproductive performance is a major factor influencing milk production in dairy cows, and consequently, the profitability of a dairy herd. Progress toward enhancing the efficiency of milk production is closely tied to reproductive performance (Britt, 1979; Laben et al., 1982). Selection for high producing cows, aided by the advent of artificial insemination in the 1950's, sire selection, and current embryo and biotechnology research, have contributed to a substantial increase in the milk production potential of the dairy cow. This has inspired many researchers to investigate methods of maintaining or improving reproductive performance in the high producing dairy cow. Overall herd profitability is closely related to our success in controlling and(or) improving reproductive performance. Controlling reproductive performance in turn hinges on our ability to understand the biology of bovine reproduction. Research on reproductive endocrinology (Stevenson and Britt, 1979; Walters and Schallenberger, 1984), physiology (Fonseca et al., 1983), health (Oltenacu et al., 1984), behavior (Hurnik et al., 1975), and nutrition (Weaver, 1987; Knutson and Allrich, 1988) have contributed to our understanding of this area. The development of monoclonal antibodies (Mondschein et al., 1989), embryo transfer and micromanipulation (Pashen, 1987), and molecular biology techniques continue to expand this field of study.

Two developments in particular dramatically enhanced our ability to study reproduction in cattle and other species. The first was the ability

to synthesize prostaglandins, the naturally occurring hormones, that cause luteolysis (Pike, 1970; Corey, 1971). About the same time the hypothalamic gonadotrophin releasing hormone (GnRH) which alters the synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) was isolated, identified as a decapeptide and is also now available as the native molecule or as super-active agonists or antagonists (for reviews see Schally et al., 1973; Vale et al., 1973). The interactions between the hypothalamic-pituitary axis in releasing GnRH and appropriate gonadotrophin signals to stimulate ovarian development and luteal function and the luteolytic actions of uterine prostaglandins causing the demise of the corpus luteum are well documented and form the endocrine basis for the 21-day bovine estrus cycle (for review see Peters and Ball, 1987).

A properly functioning endocrine system is essential for normal reproductive function and the ovary is a key component of this system. Simply in terms of the neural-endocrine interactions within the hypothalamic-pituitary-ovarian axis, the secretion of ovarian steroids (estrogen, progesterone), and peptides (inhibin, follostatin) have important feedback actions at both the hypothalamic and pituitary levels. The importance of the ovaries to the overall endocrine regulation of reproduction is illustrated in the cessation of reproductive function which follows ovariectomy. The ovariectomized model has been used for many years (Asdell et al., 1945; Katz et al., 1980; Rund et al., 1990) to study various aspects of bovine reproduction in the absence of endogenous ovarian hormone production. For instance, using exogenous hormonal treatments, this model permits detailed study of feedback activity within

the hypothalamic-pituitary-gonadal axis (Schoenemann et al., 1985).

The ovary serves two other key functions. First the production of ovarian steroid hormones maintains the development of the genital tract, facilitates the migration of the early embryo, and secures the embryo's successful implantation and development in the uterus. The second is the cyclical production of fertilizable ova. Therefore, normal ovarian function is clearly a prerequisite for optimal reproductive performance in dairy cows.

The ovarian steroids progesterone and estrogen are the primary mediators of ovarian action on reproduction. Progesterone is secreted from the corpus luteum during the luteal phase of the bovine estrous cycle. High concentrations of progesterone during this phase exert a negative feedback inhibition on LH release (Karsch, 1987). Uterine endometrial secretions, preparation of the uterus for implantation, and maintenance of pregnancy are also under the direct control of progesterone.

Estrogen tends to act in balance with progesterone such that the ratio of the two steroids directs the estrous cycle, and hence, reproduction. Prior to ovulation, circulating progesterone concentrations fall, altering LH release which in turn enhances estrogen synthesis by the developing follicles (Stumpf et al., 1989). Once a dominant follicle emerges, circulating estrogen concentrations increase dramatically (Amsterdam and Rotmensch, 1987). These events culminate in the preovulatory estrogen peak which produces a number of physiological changes in the cow. These include initiation of sexual behavior (Glencross et al., 1981), increased physical activity (Kiddy, 1977),

decreased cervical mucus viscosity (Glover and Scott Blair, 1953), production of estrus related pheromones (Klemm et al., 1987), and changes in the electrical properties of the genital tract (Aizinbud et al., 1984). The sequential changes from progesterone to estrogen dominance is however important and progesterone is known to act synergistically with estrogen to promote estrous behavior (Carrick and Shelton, 1969).

The follicle is the ovarian compartment that enables the ovary to fulfil its dual function of gametogenesis and steroidogenesis. Prior to ovulation, the granulosa cells of antral follicles are undergoing rapid growth and replication (Amsterdam and Rotmensch, 1987). Hormones such as LH, FSH, inhibin, and numerous growth factors are known to play a role in the modulation of folliculogenesis. One growth factor of particular interest is insulin-like growth factor-I (IGF-I). IGF-I has been shown to be the primary mediator of growth hormone's enhancement of growth and lactation in mammals (Froesch et al., 1985; Isaksson et al., 1985). Numerous studies have also implicated IGF-I as being involved in many aspects of folliculogenesis and steroidogenesis (Adashi et al., 1985; Holly and Wass, 1989). However, the exact mode of IGF-I action in ovarian granulosa cell growth and function remains to be elucidated.

The objective of this research was to characterize the presence of IGF-I, IGF Type-I receptor and growth hormone receptor mRNA in preovulatory bovine granulosa cells with and without exogenous growth hormone treatment. In addition, the hypothesis that estrogen and progesterone are responsible for changes in the vaginal and vulval tissue was tested in intact and ovariectomized cattle.

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II. EFFECTS OF BOVINE GROWTH HORMONE ON INSULIN-LIKE GROWTH FACTOR-I (IGF-I), IGF TYPE-I RECEPTOR, AND GROWTH HORMONE RECEPTOR GENE EXPRESSION IN BOVINE GRANULOSA CELLS

INTRODUCTION

The ovary is an endocrine gland that plays a central role in reproductive function in mammals. Numerous key hormonal modulators of ovarian activity include follicle stimulating hormone (FSH), luteinizing hormone (LH), prostaglandins, inhibin, oxytocin, and the ovarian steroids progesterone and estrogen (for reviews see Amsterdam and Rotmensch, 1987; Tonetta and diZerega, 1989). Recently, several other hormones have been implicated as regulators of ovarian granulosa cell function. These include growth hormone (GH) (Hsu and Hammond, 1987b), insulin (Holtorf et al. 1989), epidermal growth factor (Dorrington et al., 1987), and insulin-like growth factors I and II (IGF-I, IGF-II) (Adashi et al., 1985; Froesch et al., 1985).

Development of recombinant bovine GH has provoked considerable research interest in its use as a method of enhancing milk production in dairy cows (Miller et al., 1980). Several studies have examined the effect of GH injection on the reproductive performance of dairy cows (Hadsell et al., 1988; Morbeck et al., 1989; Moseley 1989; Simpson et al., 1989; McLeod et al., 1990; Schemm et al., 1990). However, most of these experiments have measured gross indicators of reproductive performance such as calving interval, services per conception, days to first service or circulating hormone concentrations. Information about the molecular

and cellular mechanisms underlying GH action is lacking and is required to fully understand the implications of GH treatment on reproductive processes in dairy cattle.

IGF-I has been shown to be the primary mediator of the growth promoting actions of GH in mammals (Schwander et al., 1983; Froesch et al., 1985). This growth factor was originally thought to be exclusively produced in the liver in response to GH. Recent research has shown molecular evidence for IGF-I synthesis in numerous nonhepatic tissues (D'Ercole et al., 1984; Lund et al., 1986; Murphy et al., 1987a) including the rat uterus (Murphy et al., 1987b) and ovary (Oliver et al., 1989; Hernandez et al., 1989). Circulating IGF-I concentrations are GH-dependant with virtually all of the plasma IGF-I bound to binding proteins (Daughaday and Rotwein, 1989). In addition, a recent study has demonstrated an association among circulating IGF-I, negative energy balance and increased luteal function (Spicer et al., 1990). Follicular fluid concentrations of IGF-I tend to increase with follicle size in cattle (Spicer et al., 1988) and with PMSG treatment in pigs (Hammond et al., 1988); concentrations are also positively correlated with follicular fluid estrogen concentrations and are higher than circulating IGF-I in dominant, but not cohort, human follicles (Eden et al., 1989).

Several granulosa cell culture studies have shown that IGF-I is important in ovarian function (Veldhuis and Demers, 1985; Veldhuis and Furlanetto, 1985). IGF-I treatment in vitro increases granulosa cell steroidogenesis and differentiation by enhancing FSH-induced aromatase activity (Davoren et al., 1985; Dorrington et al., 1987), cAMP accumulation (Veldhuis et al., 1985; Adashi et al., 1986) and synthesis

of cholesterol side-chain cleavage cytochrome P-450 (Veldhuis et al., 1986). This potent mitogen has also been shown to induce LH receptors (Davoren et al., 1986), increase estrogen and progesterone synthesis, as well as promote granulosa cell replication (Dorrington et al., 1987). In addition, IGF-I production from granulosa cells is enhanced by growth factors (Mondschein and Hammond, 1988), GH (Hsu and Hammond, 1987b), estrogen, progesterone, FSH and LH (Hammond et al., 1985; Hsu and Hammond, 1987a). Local short loop positive feedback systems seem to include estrogen and IGF-I. The presence of GH receptor mRNA in bovine ovaries has been reported (Tanner and Hauser, 1989), and IGF-I has been shown to bind to high affinity, low capacity granulosa cell receptors in the rat. These specific receptors are termed IGF Type-I receptor because they possess a rank order of affinity of IGF-I > IGF-II > insulin (Rechler and Nissley, 1985; Adashi et al., 1989). Although these observations support the hypothesis that IGF-I has an autocrine and(or) paracrine role in the ovary (Holly and Wass, 1989) and that the ovary may be directly influenced by GH, no molecular evidence of IGF-I, IGF Type-I receptor and GH receptor presence in bovine granulosa cells has yet been presented.

Therefore, the objective of this trial was to evaluate the role of IGF-I in preovulatory bovine granulosa cells in animals treated with exogenous GH. This was accomplished by identifying and characterizing the mRNA for IGF-I, IGF Type-I receptor, and GH receptor in granulosa cells from preovulatory follicles.

MATERIALS AND METHODS

Seven non-lactating, Holstein cows were assigned, based on parity, to receive either 20.6 mg/d bovine GH[1] (n=4) or saline (n=3) s.c. for 5 consecutive d prior to ovariectomy. Animals were fed 25 kg/d of a complete mixed diet containing 90% forage and 10% concentrate. All cows received synchronizing injections of prostaglandin $F_{2\alpha}$[2] (PGF) 12 and 2½ d prior to ovariectomy. Tail vein blood samples were collected daily into heparinized vacutainers[3] from all cows from 20 d before to seven d after ovariectomy for IGF-I and GH analysis. Additional blood samples were collected every 6 h from the fourth GH injection until ovariectomy for GH analysis only. Blood was centrifuged at 1,500 X g for 10 min at room temperature and plasma was stored at -20° C.

Standing mid-flank bilateral ovariectomies were performed approximately 60 h following the second PGF injection. The largest follicle of each ovary pair was cut open allowing the follicular fluid to drain into a petri dish. The granulosa cells were scraped into the dish using a sterile, looped wire. This mixture was centrifuged at 1,000 X g for 10 min at room temperature. Follicular fluid and granulosa cells were separated, frozen in liquid nitrogen and stored at -70° C. The preovulatory follicle from one GH-treated cow ruptured during ovariectomy and a second GH-treated cow failed to respond to the PGF (a corpus luteum instead of a follicle). A third GH-treated cow had two large follicles

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[1]American Cyanamid, Princeton, NJ. Lot No. AC6081-134D02.

[2]Lutalyse, Upjohn, Tuco, Orangeville, ON.

[3]Becton Dickinson and Co., Rutherford, NJ.

which were processed separately but the resulting values were pooled prior to statistical analysis. Therefore, follicle data from two GH-treated cows and three saline treated cows were used.

All granulosa cell preparations and control tissues were homogenized in 4.0 M gaunidine isothiocyanate. Total RNA was isolated by ultracentrifugation using a SW 41-Ti[4] rotor at 30,000 rpm for 23 h over a cushion of 5.7 M cesium chloride as described by Chirgwin et al. (1979). The concentration of RNA was estimated by absorbance at 260 nm. Total RNA was fractionated based on size on 1% (w/v) agarose denaturing gels. Following electrophoresis, RNA was transferred to nylon membranes. The quantity of RNA loaded and transferred was confirmed by UV light visualization of ethidium bromide-stained RNA. A 0.24 - 9.49 kilobase (kb) RNA ladder[5] was used as a size marker. Transferred RNA was fixed to the membrane by baking under vacuum for 2 h at 80° C.

A 643 base pair GH receptor cDNA insert fragment, contained in a rabbit GH receptor cDNA clone (Leung et al., 1987), was subcloned into pGEM-3[6]. A 748 base pair hIGF-I receptor cDNA insert, contained in human IGF Type-I receptor cDNA clone (Ullrich et al., 1986), was also subcloned into pGEM-3. The IGF-I probe was obtained using clone pGEM-1/hIGF-IB (Rotwein, 1986). GH receptor, IGF-I and IGF Type-I receptor radiolabeled ([^{32}P] CTP) antisense RNA probes were generated from linearized (Bam H1, Pvu-II and Eco-RV respectively) plasmid DNA using T7 (receptors) or SP6

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[4]Beckman Instruments Inc., Mississauga, ON.

[5]Bethesda Research Laboratories, Gaithersburg, MD.

[6]Promega, Madison, WI.

(IGF-I) RNA polymerase (Krieg and Melton, 1987). Unincorporated [^{32} P] CTP was removed by spun column chromatography. The concentration (4 x 10^6 dpm/ml) of probe was the same for each hybridization.

Prehybridization was in a buffer containing 60% (v/v) deionized formamide, 10% (w/v) dextran sulfate, 1% (w/v) sodium dodecyl sulfate (SDS), 1X SSPE (.18 mol NaCl/l, 10 mmol sodium phosphate/l (pH 7.4), 1 mmol EDTA/l), .5% (w/v) blotto (low fat milk powder) and 500 ug/ml sonicated denatured salmon sperm DNA. Prehybridization was for 3 h at 50° C. Hybridization was in the same buffer as prehybridization for 17 h at 50° C. Hybridized membranes were rinsed in 2X standard saline citrate (SSC) (1 X SSC is .15 mol NaCl/l, 15 mmol trisoduim citrate/l, pH 7.0) at room temperature (22° C) and then washed in 2X SSC containing 0.1% SDS for 15 min at room temperature. They were then washed in 0.2X SSC containing 1% SDS for 15 min at 70° C; and finally rinsed in 0.2X SSC at room temperature.

Membranes were exposed to x-ray film at -70° C with intensifying screens. Two membranes were probed with the IGF-I riboprobe and then stripped by washing twice in 0.1X SSC containing 0.1 % SDS for 20 min at 95° C. Complete stripping of the probe was confirmed by autoradiographic exposure for 3 d. Both membranes were subsequently reprobed with the IGF Type-I receptor riboprobe. Autoradiographs were subjected to densitometry to quantify mRNA abundance.

IGF-I in plasma and follicular fluid was separated from IGF-I binding proteins by acid gel filtration using a previously published procedure (Zapf et al., 1981) with modifications by Daughaday et al. (1986). The RIA for IGF-I was carried out in borosillicate glass tubes

using the non-equilibrium technique of Furlanetto et al. (1977). The assay buffer contained .05% Tween 20 as a substitute for bovine serum albumin. An aliquot of the gel filtrate of plasma and follicular fluid was dried by vacuum centrifugation in the assay tubes and resuspended in 100 ul of assay buffer to obtain a 1:600 dilution.

Standards (0.10 to 3.0 ng/ml synthetic IGF-I[7]) and unknowns were preincubated with the antiserum[8] (1:18000 final dilution) for 24 h at 4° C. Recombinant IGF-I[9] was used for iodination ([^{125}I][9]), by the chloramine-T method described by Zapf et al. (1981). Approximately 10,000 cpm [^{125}I] IGF-I in 100 ul of assay buffer was added to the assay tubes and the incubation continued for another 24 h at 4° C. Separation of antibody-bound and free [^{125}I]IGF-I was accomplished by adding 100 ul goat antirabbit gamma globulin[10] (diluted 1:25 in assay buffer), and 100 ul of normal rabbit serum (diluted 1:150 in assay buffer). After 24 h at 4° C the tubes were centrifuged (3000 X g, 15 min, 4° C), the supernatant aspirated, and the precipitate counted in a gamma counter. The gel filtrate of each sample was tested in triplicate and samples were assayed in a single assay. Intraassay coefficient of variation of two dry cow control samples averaged 8.2%.

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[7]Bachem Fine Chemicals, Torrance, CA. Lot No. 588C.

[8]IGF-I antiserum UBK 487 generously provided by J.J. Van Wyk and L.E. Underwood, University of North Carolina, NC. and distributed by the N.I.A.D.D.K.

[9]Bachem Fine Chemicals, Torrance, CA. Lot NO. ZE426.

[10]Gibco Canada Inc., Burlington, ON.

The plasma GH samples were analyzed using a previously described radioimmunoassay (de Boer and Kennelly, 1988). Standards and plasma were assayed in triplicate and all plasma samples were analyzed in one assay. Intraassay coefficient of variation of dry and early lactation control plasma samples averaged 8.8%.

Abundance of mRNA for each of the three probes: IGF-I, GH receptor, and IGF Type-I receptor were subjected to one-way analyses of variance with treatment (GH and saline) as the main effect. The number of abundance values for each of the two treatments were IGF-I (GH n=2, saline n=3), GH receptor (GH n=2, saline n=2) and IGF Type-I receptor (GH n=2, saline n=3).

Circulating IGF-I concentrations prior to ovariectomy and follicular fluid IGF-I concentrations were determined for the three saline treated and the two GH treated cows with follicles. Mean follicular fluid IGF-I concentrations were calculated for the cow with two follicles. The IGF-I data were subjected to two one-way analyses of variance. The first analysis used treatment (GH and saline) within IGF-I source (circulation and follicular fluid) as the main effect. The second analysis used IGF-I source within treatment as the main effect.

Daily GH and IGF-I plasma concentrations for the experimental period were partitioned into two periods: Pretreatment (Day -20 to -5 prior to ovariectomy); and Postreatment (6 d following ovariectomy). Cow numbers were n=4 for the GH treatment group and n=3 for the saline group. The data from each period for each hormone (GH and IGF-I) were analyzed using a split-plot analyses of variance with the whole plots being treatment (GH and saline) and the split-plots being days within the period. Sources of

variation for the whole plots were Treatments (T, t=2) and Cows within Treatments (C/T, c=4 for GH and c=3 for saline). The C/T mean square was used as the valid error term for testing treatments.

Circulating GH concentrations from the fourth GH injection until ovariectomy for the two treatments (GH and saline) were compared using the Kolmogorov-Smirnov two-sample test (Sokal and Rohlf, 1981). Circulating IGF-I concentrations for the six days following the first GH injection for the two treatments were also compared using the Kolmogorov-Smirnov test.

Pearson's correlation coefficients between IGF-I and GH daily concentrations, for the five days from the first GH injection to ovariectomy, were computed within each treatment (GH and saline).

RESULTS

The riboprobe hybridized to several transcripts in all granulosa cell preparations (Plate 1). Four species of IGF-I mRNA (4.1, 2.7, 1.7 and 1.0 to 0.7 kilobases (kb)) were identified in all granulosa cell preparations; two of these (4.1 and 2.7 kb) were also detected in bovine and rat liver. Both liver preparations showed one additional larger transcript of 7.7 kb. The GH receptor probe hybridized to three mRNA transcripts of 9.2, 4.3 and 2.3 kb in all granulosa cell preparations. One major and two minor transcripts of 4.2, 12.5 and 6.6 kb, respectively, were detected in rabbit liver total RNA. Four similar sized transcripts for the IGF Type-I receptor (11.3, 6.2, 4.7, and 3.3 kb) were observed for both granulosa cells and human placenta total RNA. There was no significant difference in abundance between GH-treated and control

preparations for IGF-I, GH receptor, or IGF-Type-I receptor.

Follicular fluid IGF-I concentrations at ovariectomy and circulating IGF-I concentrations, measured approximately 24 h earlier, in GH-treated cows were 225 and 192 % of control animals respectively (Figure 1). Within either GH or saline treated cows, there were no differences ($P > .05$) between circulating and follicular fluid IGF-I concentrations. Circulating GH and IGF-I concentrations between treatment groups were not different ($P > .05$) during the Pretreatment and Postreatment periods. Circulating GH concentrations increased in response to GH treatment (GH, 10.9 ± 1.4 ng/ml vs Saline, 4.0 ± 0.2 ng/ml, $P = .004$) from the fourth GH injection to ovariectomy (Figure 2). Circulating IGF-I concentrations increased in GH-treated cows (GH, 486 ng/ml vs Saline, 313 ng/ml, $P = .031$) during the six days following the first GH injection (Figure 3). Circulating IGF-I and GH concentrations were positively correlated in GH-treated ($r = .93$, $P = .025$) but not saline-treated ($r = -.38$, $P = .528$) cows during the 5 d GH injection period. Both saline and GH-treated IGF-I circulatory concentrations appeared to decrease at ovariectomy before stabilizing at preinjection levels 2 d later (Figure 3).

DISCUSSION

This study identified and characterized IGF-I, IGF Type-I receptor and GH receptor mRNAs in preovulatory bovine granulosa cells using northern hybridization analysis. Additionally, circulating GH, circulating IGF-I, and follicular fluid IGF-I concentrations were determined relative to GH treatment.

Circulating IGF-I concentrations have been shown to increase in response to exogenous GH in numerous species including the cow (Isaksson et al., 1985; Glimm et al., 1988). In this study, circulating IGF-I showed a marked increase in response to GH treatment. Within three days following the final GH injection, circulating IGF-I concentrations had returned to preinjection levels. GH treatment increased follicular fluid IGF-I and circulating IGF-I concentrations in a similar manner.

Follicular fluid IGF-I concentrations have been found to be comparable to circulating IGF-I concentrations in cohort, but significantly higher than circulating IGF-I in dominant (preovulatory) human follicles (Eden et al., 1989). In this study of preovulatory follicles only, no significant difference was observed between follicular fluid IGF-I and circulating IGF-I concentrations (Figure 1) measured approximately 24 h earlier. Circulating IGF-I decreased between this sampling point and 48 h later in both GH-treated and saline-treated cows as feed was restricted for 24 h prior to ovariectomy. This may have contributed to the reduction in circulating IGF-I concentrations (Froesch et al., 1985). Thus, actual circulating IGF-I concentrations may have been lower than follicular fluid IGF-I concentrations at ovariectomy. Another possibility is that bovine follicular fluid IGF-I concentrations are merely a reflection of circulating IGF-I concentrations which in turn reflect hepatic IGF-I synthesis.

The hybridization of the IGF-I riboprobe to specific mRNA transcripts in all bovine granulosa cell preparations confirms previous observations in rat granulosa cells (Oliver et al., 1989). Many in vitro studies have also shown that IGF-I release from cultured granulosa cells

is influenced by various trophic ovarian hormones and the physiological state of the ovary. The IGF-I mRNA transcripts detected in this study are similar in size to IGF-I species characterized in other mammalian tissues (Rotwein, 1986; Hynes et al., 1987; Murphy et al., 1987b) including the rat ovary (Oliver et al., 1989).

The relative abundance of IGF-I mRNA in GH-treated and saline granulosa cell preparations was not significantly different. This observation differs from studies with rat liver (Hynes et al., 1987), uterus (Murphy et al., 1987b), and skeletal muscle (Turner et al., 1988) where GH treatment resulted in greater abundance of IGF-I mRNA. In the rat, GH required diethylstilbestrol co-treatment to increase ovarian IGF-I mRNA abundance (Hernandez et al., 1989). In the rat uterus, estrogen treatment increased IGF-I mRNA abundance independent of GH status, but GH increased abundance of hepatic IGF-I mRNA (Norstedt et al., 1989). The apparent absence of a GH effect on IGF-I mRNA abundance in the present study indicates that GH induced IGF-I synthesis in bovine ovaries may not be manifested at the level of IGF-I gene transcription or mRNA turnover in granulosa cells.

Two separate studies have characterized high affinity, low capacity IGF Type-I cell surface receptors on rat (Davoren et al., 1986) and swine (Veldhuis and Furlanetto, 1985) granulosa cells. Our IGF Type-I receptor riboprobe results confirm these observations in bovine granulosa cells. The largest band is comparable to the IGF Type-I receptor mRNA transcript of 11.0 kb detected in human, rat and mouse tissues (Ullrich et al., 1986; Werner et al., 1989). Our probe failed to hybridize to insulin receptor mRNA which Ullrich et al. (1986) reported to have four major transcripts

ranging from 10.3 to 6.7 kb.

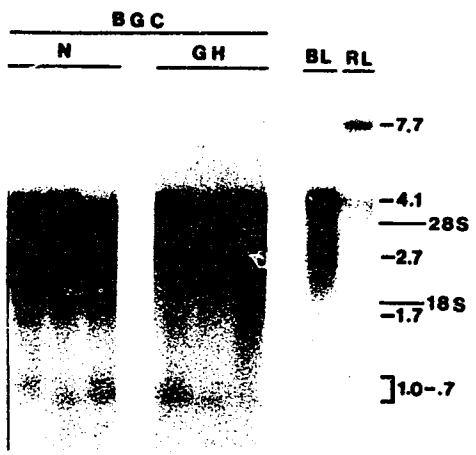
In all granulosa cell preparations the GH receptor riboprobe hybridized to three mRNA species which are similar to previously reported GH receptor mRNA transcripts detected in rat liver (Baumbach et al., 1989). The other GH receptor mRNA transcripts detected in rabbit liver corresponded to similar sized GH receptor mRNAs reported in rabbit liver (Leung et al., 1987), mouse liver, and mouse adipose tissue (Smith et al., 1988). No difference was observed in GH receptor mRNA abundance between GH-treated and saline granulosa cell preparations. The abundance of GH receptor mRNA has been shown to be influenced by GH treatment, diabetes, and nutritional status, and to be tissue specific (Hughes and Friesen, 1985; Bornfeldt et al., 1989). A direct effect of GH on cultured porcine granulosa cell production of IGF-I and progesterone has been demonstrated by Hsu and Hammond (1987b). These observations, taken together with our evidence for GH receptor mRNA, suggest that GH may act directly on the bovine granulosa cells.

SUMMARY

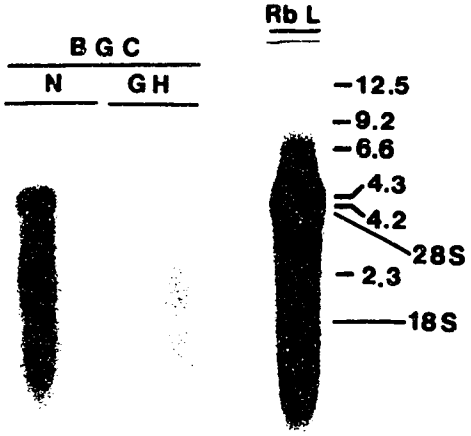
Local IGF-I synthesis was confirmed in preovulatory bovine granulosa cells. IGF Type-I receptor and GH receptor mRNA were also identified and characterized in the same cells. Circulating and follicular fluid IGF-I concentrations were increased following GH treatment. These results provide direct evidence of a possible paracrine and(or) autocrine model for IGF-I action in preovulatory bovine granulosa cells. Further studies with larger animal numbers to characterize this model should provide additional insight on the control of ovarian function in the dairy cow.

Plate II-1. Northern hybridization analysis of total RNA using antisense mRNA radiolabeled probes for IGF-I (left panel), GH receptor (middle panel), and IGF Type-I receptor (right panel). Lanes show bovine preovulatory granulosa cells (BGC) from normal (N, 20 ug RNA / lane) and growth hormone-treated (GH, 20 ug) nonlactating Holstein cows, rat liver (RL, 10 ug), bovine liver (BL, 10 ug), rabbit liver (Rb L, 10 ug), and human placenta (HP, 30 ug). RNA transcript sizes (kilobases) indicated to the right of each panel. Ribosomal 18S and 28S bands are also indicated.

IGF-I



GH Receptor



Type-I Receptor

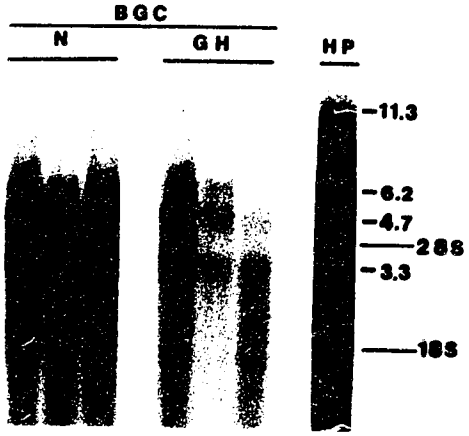


Figure II-1. Circulating (C) and follicular fluid (FF) IGF-I concentrations (ng / ml) from saline (S, ///) and growth hormone-treated (GH, \\\\) cows. * Sig. increase ($P < .005$) GHC over SC. ** Sig. increase ($P < .01$) GHFF over SFF. Bars are SEM.

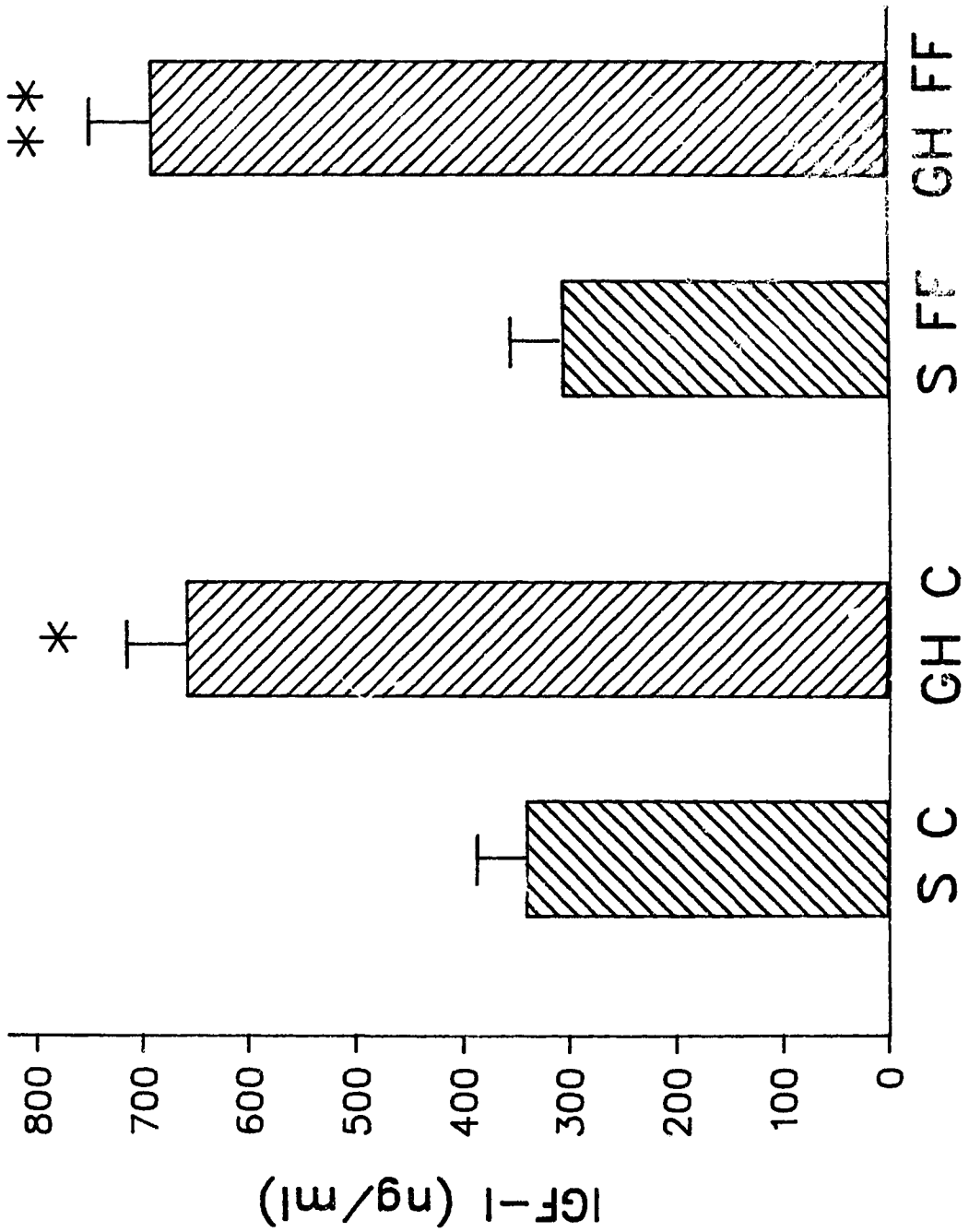

IGF-I (ng/ml)
800
700
600
500
400
300
200
100
0
S C
GH C
S FF
GH FF
*
**

Figure II-2. Plasma GH concentrations from saline (◆, n=3) and growth hormone-treated (■, 20.6 mg / d, n = 4) nonlactating Holstein cows. Sampling time is hours after the last two GH injections (indicated by arrowheads) prior to ovariectomy (OV). Bars indicate SEM.

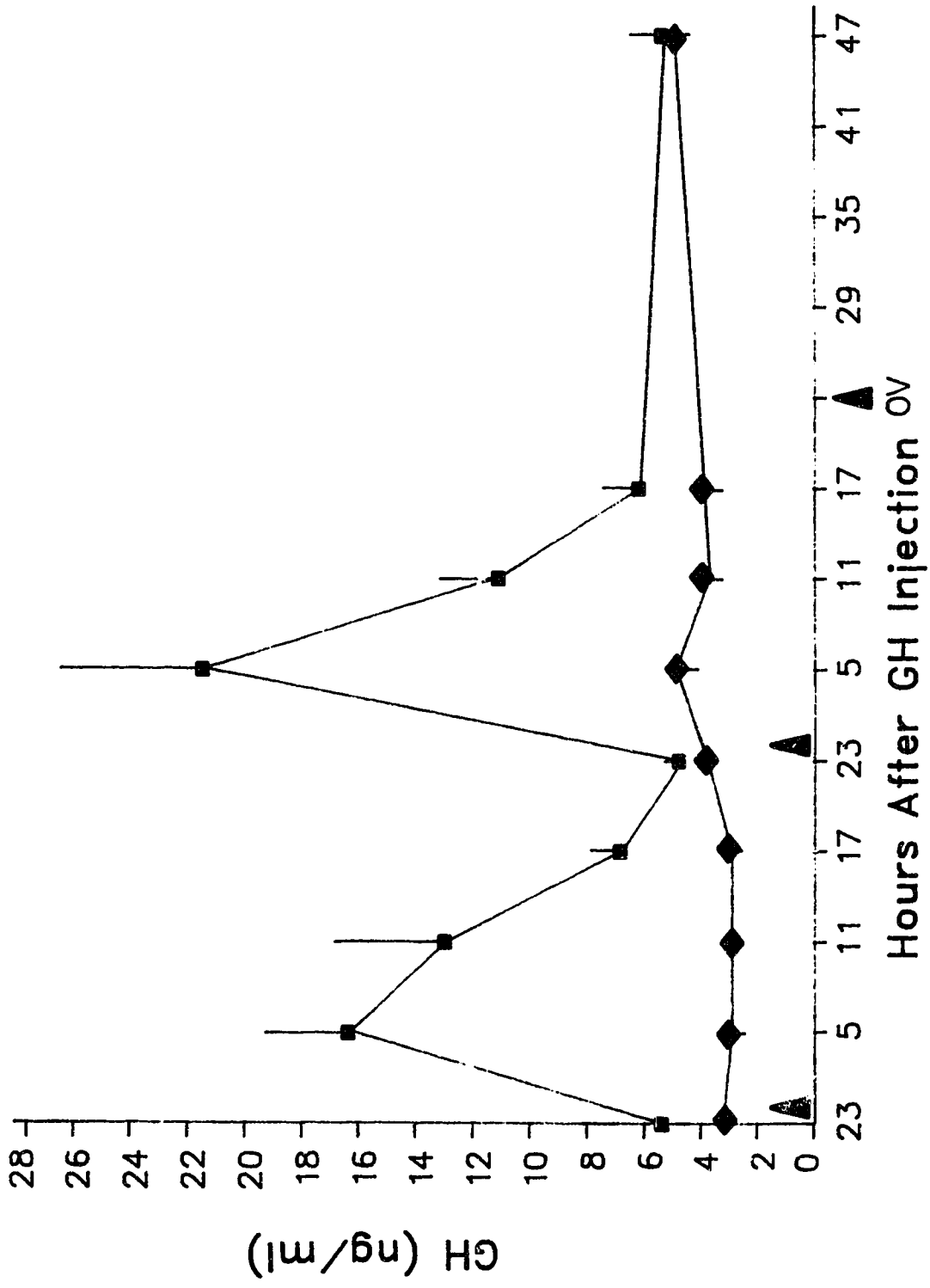
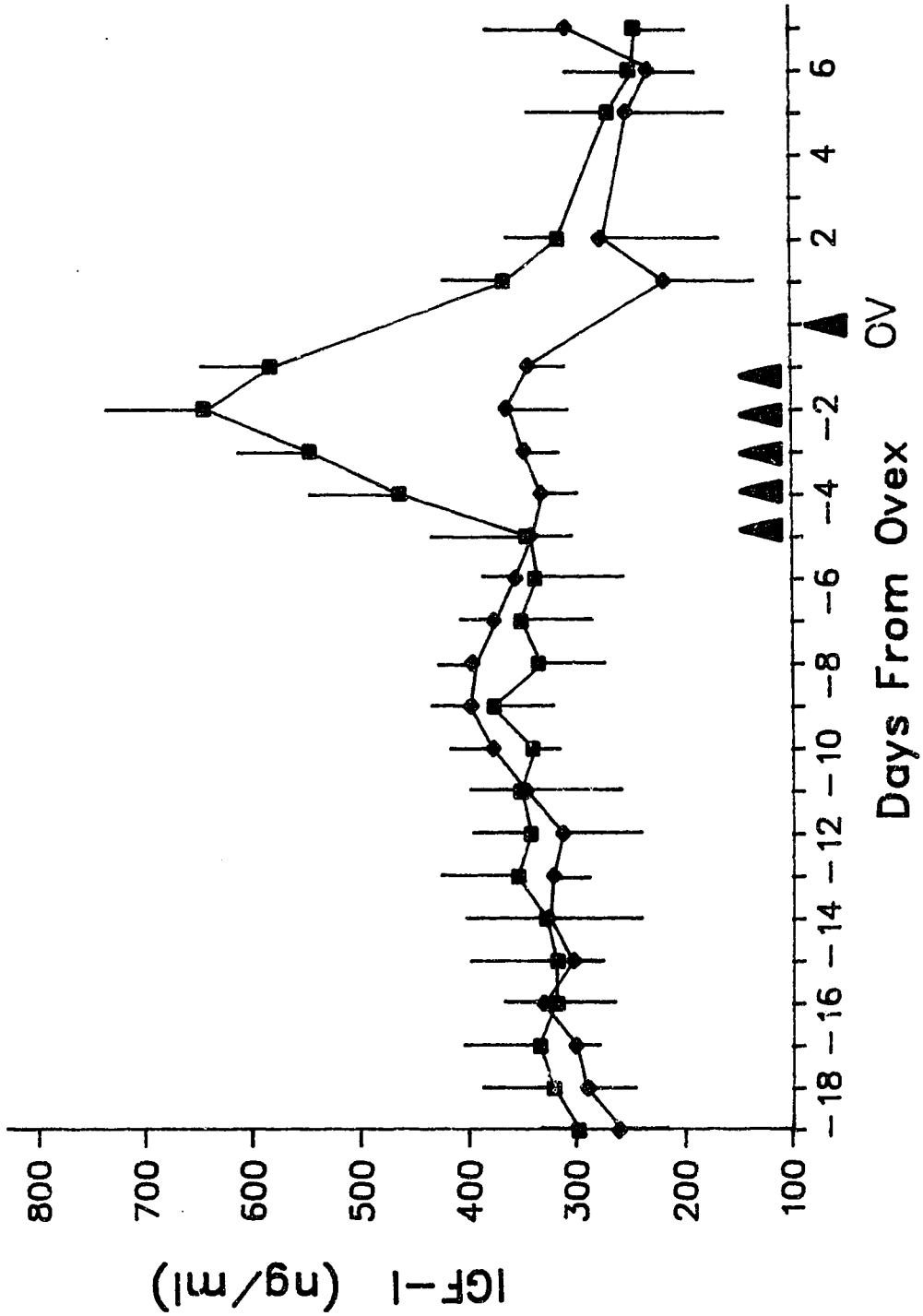

GH (ng/ml)
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
23
5
11
17
23
5
11
17
29
35
41
47
Hours After GH Injection OV

Figure II-3. Plasma IGF-I concentrations from saline (◆, n=3) and growth hormone-treated (■, 20.6 mg / d, n = 4) nonlactating Holstein cows. Sampling time is days relative to ovariectomy (OV). Arrowheads indicate GH injections. Bars indicate SEM.


IGF-I (ng/ml)
800
700
600
500
400
300
200
100
-18 -16 -14 -12 -10 -8 -6 -4 -2
OV
2 4 6
Days From Ovex

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III. EFFECTS OF PROGESTERONE AND ESTRADIOL ON VAGINAL AND VULVAL ELECTRICAL ADMITTANCE MEASURED WITH A VAGINAL PROBE IN CATTLE

INTRODUCTION

Efficient estrous detection is of primary importance in a dairy herd breeding program. In herds where cows can interact continuously (e.g. freestall or loose housing barns) visual determination of standing estrous behavior can be enhanced with heat mount detectors (Stevenson and Britt, 1977), androgenized females (Kiser et al., 1977), or 24-h surveillance equipment (King et al., 1976). The combination of visual observation and these techniques has permitted producers to achieve a higher percentage of estrous detection and hence, improved breeding efficiency (Sawyer et al., 1986). However, the stress associated with increased milk production and larger herd size makes effective estrous detection a greater challenge for dairy farmers. Furthermore, many herds are housed in tie-stalls or cows are not permitted to interact during inclement weather. In these situations, estrous detection aids such as cow-side milk progesterone tests (Schiavo et al., 1975), pedometers (Kiddy, 1976), phermone-detecting dogs (Kiddy et al., 1978), remote temperature sensing devices (Zartman and DeAlba, 1981) or vaginal probes (Aizinbud et al., 1984) have potential application. The challenge in all cases is to estimate the time of ovulation to maximize breeding efficiency.

The concept that cows undergo a characteristic set of behavioral and physiological changes prior to ovulation has been well established (Hurnik et al., 1975; Stevenson and Britt, 1979; Lewis and Newman, 1984; Walters and Schallenberger, 1984). These changes include follicular development and maturation (Ginther et al., 1989), hormone induced estrous behavior (Esslemont et al., 1980), and alterations in the bovine genital tract (Blazquez et al., 1987a,b) and its secretions (Bazer et al., 1987). Extensive research effort has been devoted to studying the changes in the genital tract as estrus approaches. The scope of this research has included characterizing the cervico-vaginal mucus viscosity (Glover and Blair, 1952), ferning pattern (Noonan et al., 1975), chemical properties (Lamond and Shanahan, 1969), and nuclear magnetic resonance properties (Merilan, 1983).

Since the mid 1960's, the electrical properties of the vagina and vulva have been studied using manually inserted and, more recently, surgically implanted probes (Aizinbudas and Dovil'tis, 1966; Aizinbud et al., 1984). The manually inserted metallic probes are reported to measure the electrical resistance or conductance of the vaginal lumen mucus surrounding the probe (Gartland et al., 1976; Marshall et al., 1979). Changes in probe profiles have been shown to correspond with estrous behavior (Heckman et al., 1979) and the preovulatory LH peak (Schams et al., 1977; Aboul-Ela et al., 1983). However, the results of field trials using manually inserted probes have proven to be too variable to accurately predict the peak of behavioral estrus (Carter and Duffy, 1980).

Variations in mucus to probe contact, electrode design, and probe placement within the genital tract (Marshall et al., 1979) have led to the

development of implanted electrodes. Vaginal and vulval tissue resistance, conductance and impedance, as measured by electrodes implanted in the tissue submucosa, have been shown to reflect tissue hydration changes associated with estrus in cattle (Feldmann et al., 1978; Aizinbud et al., 1985) and sheep (Adam et al., 1981). Tissue hydration, as measured by biopsy and implanted probes, was reported to increase during the estrous cycle (Smith et al., 1989).

Estrogen and progesterone are two ovarian steroid hormones that are considered to be central in the control of reproductive function and therefore, the estrous cycle. The rise in vaginal thermal conductance, following estradiol treatment, was thought to be a result of increased vaginal blood flow rate (Abrams et al., 1973). Vaginal pH and temperature (Lewis and Newman, 1984) and numerous characteristics of cervico-vaginal secretions (Glover and Scott Blair, 1953) were also found to vary around estrus. These observations suggest that changing estrogen and(or) progesterone concentrations around estrus influence the genital tract properties studied.

Ovariectomized cows and sheep have been used as models to study the influence of ovarian steroid hormones on various physiological and endocrinological parameters in the absence of endogenous secretion. For example, exogenous estradiol induces estrous behavior and an LH surge in ovariectomized heifers. However progesterone pretreatment is required to maintain the same level of induced activity following subsequent estradiol challenges (Carrick and Shelton, 1969).

Despite many advances in our understanding of the hormones controlling estrous behavior, the exact biological events underlying

changes in the genital tract during estrus remain to be elucidated. Thus, the objective of this study was to determine the relationships between endogenous progesterone, estradiol, LH, and vaginal tissue admittance. Additionally, the relationships between exogenous progesterone, estradiol, and vaginal and vulval tissue admittance in ovariectomized cows was examined.

Materials and Methods

Experiment 1. Ovariectomized Cows.

Animals

Six non-lactating, Holstein cows were assigned equally, on the basis of parity, to one of two treatment groups (progesterone or control). Animals were housed in tie-stalls and were fed 25 kg/d (as fed basis) of a complete mixed diet containing 90% forage and 10% concentrate. Free choice trace mineral salt was provided and animals received monthly vitamin ADE[1] injections. The estrous cycles of all cows were synchronized with two injections of prostaglandins $F_{2\alpha}$[2] (PGF) 11 d apart. Cows were ovariectomized bilaterally approximately 60 h following the second injection of PGF.

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[1]Pfizer, Montreal, QU. Lot 902 29009.

[2]Lutalyse, Upjohn, Tuco, Orangeville, ON.

Progesterone Implants

Subcutaneous implant therapy was designed to simulate luteal (treatment) or follicular (control) progesterone concentrations. Silastic tube[3] progesterone implants were constructed and implanted as described by Rajamahendran et al. (1982). Each implant was filled with 2 g progesterone[4] and incubated in PBS for 24 h at 37° C prior to implantation. The surface area of each implant was approximately 60 cm^2. All progesterone implants were weighed before implantation and following removal from the cow. At ovariectomy, each cow was implanted with one progesterone implant which was removed 8 d later (Figure 1). On the same day, cows were reimplanted with either two progesterone implants each or two empty silastic tubes in a crossover design with two 24 d periods.

Estradiol Challenges

Estradiol benzoate[5] was diluted in corn oil[6] to a concentration of 87.5 ug/ml oil. During each progesterone treatment period both progesterone implanted and control cows received three challenges of estradiol benzoate (Figure 1). The injection schedule was designed to mimic the follicular phase rise in estradiol during the estrous cycle. Each injection contained 175 ug estradiol benzoate in two ml oil. Three injections, comprising the first challenge, were given im at 12 h intervals to each cow commencing 72 h following double progesterone

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[3]Dow Corning, Midland, ON. Cat. No. 601-505.

[4]Fisher Scientific, Fairlawn, NJ. Lot No. 880726.

[5]Intervet, Milton, Cambridge, UK.

[6]Mazola, Etobicoke, ON.

implantation. The second challenge was initiated 16 d following double progesterone implantation and consisted of three injections, as above. The third challenge was a single injection 3 d following the second challenge.

Hormone Analysis

Blood samples for progesterone and estradiol analysis were collected daily throughout the experiment, following probing, from the tail vein into heparinized vacutainers. Blood samples for luteinizing hormone determination were collected every 6 h commencing 12 h prior to the first two estradiol benzoate challenges for a total of 96 h (challenge 1) or 6 d (challenge 2). Blood samples were centrifuged within 30 min of collection at 1,500 X g for 10 min at room temperature, plasma extracted, and stored at -20° C.

Plasma progesterone concentrations were determined using a previously described radioimmunoassay (Rawlings et al., 1984) with minor modifications. Briefly, progesterone was extracted from 100 ul of plasma and buffer standards using 4 ml petroleum ether. Antiserum[7] was diluted to a working dilution of 1:30,000. Iodinated progesterone[8] was added to unknown and standard[4] at approximately 10,000 cpm/tube. Assay sensitivity was .1 ng/ml. Inhibition curves for pooled (pregnant and estrus) control bovine plasma were parallel to both the buffer and extracted buffer standard curves. Progesterone concentrations of all unknowns were calculated using the extracted buffer curve. Intraassay and interassay

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[7]Progesterone antiserum (Rabbit A-18) purchased from N. Rawlings, University of Saskatchewan, Saskatoon, SK.

[8]Amersham Chemicals, Cat. No. IM-140.

coefficients of variation were 17.2 % and 19.6 % respectively.

Established radioimmunoassays were used to determine plasma concentrations of estradiol[9] (Rowell and Flood, 1988) and LH[9] (Rawlings et al., 1984). Intraassay coefficient of variation for LH was 17.5%. Intraassay and interassay coefficients of variation for estradiol were 13.7% and 20.8%, respectively.

Estrous Behavior

Cows were moved to a dirt corral for estrous detection, with observations being made by the same individual, for 30 min at 6 h intervals, from the first injection of each estradiol benzoate challenge until no standing estrus was observed for two consecutive periods. All attempted mounts, mounts, stand refusals, stands, and animal identifications were recorded for each period. Duration of standing behavior was determined as the midpoint between the two estrous detection periods when a cow started standing to the midpoint between the two periods when standing ceased.

Vaginal Probe

All cows were probed with a vaginal electrical admittance probe[10], designed to measure electrode surface admittance relative to the redox status of the surrounding medium. Admittance, the inverse of impedance, is a measure of electrical current between two electrodes in an alternating current circuit. Vitreous carbon was used to construct the electrodes to ensure that the admittance measured was from the electrode

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[9]Estradiol and LH analysis performed by Endocrine Lab Services, University of Saskatchewan, Saskatoon, SK.

[10]Venture Managers Prutec Ltd., UK.

surfaces and not the surrounding medium. This material also registers changes in admittance relative to the redox status of the surrounding medium.

The probe was constructed from inert plastic to a diameter of 35 mm with two sets (tip and rear) of four vitreous carbon electrodes injection moulded and sealed continuously with the probe's surface (Figure 2). Each electrode measured 18 mm wide and 24 mm long. Both sets of four electrodes were equally spaced around the circumference of the probe. The tip set were located 23 mm and the rear set 75 mm from the probe's tip. The electrodes were wired to measure admittance at three positions: either across opposite tip electrodes; between adjacent tip electrodes; or between the tip and rear electrodes. The probe was programmed to take readings once a second for 15 s from the three electrode positions.

Probings were conducted once daily commencing 20 d prior to ovariectomy for 103 d. Probe readings measuring electrode surface admittance (u Amps) at 1 and 50 kHz, using 200 mV, were taken at both vaginal (full insertion depth of approximately 38 cm) and vulval (partial insertion depth of approximately 13 cm) locations. Baseline probe readings were monitored daily, before and following use, in .9% saline. No corrections were made for baseline variation.

At probing, external genitalia were washed with a 2.5% Dettol[11] solution and a lubricant[12] was liberally applied to the vulval lips and the probe's tip prior to insertion. The probe was disinfected with a hot

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[11]Recitt & Coleman, Lachine, QU. Lot 0168.

[12]K-Y Gel, Johnson & Johnson, Lot 05499.

2.5% Dettol solution between cows, but not between insertions within a cow. Cow order and probe insertion sequence were randomly determined each day. Probe orientation for vaginal and vulval location readings was consistent throughout the trial with all cows being probed by the same individual. Tissue admittance values were recorded on a datalogger[13] and downloaded to a microcomputer for analysis. Mean admittance values from each 15 s time block were averaged for each electrode position (tip opposite, tip adjacent and tip to rear) and frequency (1 and 50 KHz). All reproductive tracts were examined by a veterinarian following 96 d of probing using a Caslic vaginal speculum.

Statistical Analysis

Plasma progesterone, LH, and estradiol concentrations, and probe admittance values were analyzed separately using analyses of variance. The experimental plan was a split-plot with the whole plots being a crossover design (Petersen, 1985) and the split-plots being periods. The data were analyzed using the ANOVA or GLM procedure (SAS, 1982) with all the split-plot variation going into residual error. Sources of variation were Groups (G, g=2), Cows within Group (C/G, c=3), Progesterone Treatment (T, t=2), T by G, and T by C/G. The mean square for T by C/G was used as the error term for significance testing of Progesterone Treatment.

Preliminary analysis of standing estrous behavior response to estradiol challenges revealed that only one cow responded to the third challenge. Therefore, the estrous event data following this challenge were not included in analyses of variance. The sum of each estrous event

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[13]CR-10, Campbell Scientific, Edmonton, AB.

for thirteen estrous detection periods following the first injection of each of the first two estradiol challenges were calculated. These data were subjected to analyses of variance as above with additional sources of variation including Estradiol Challenges (E, e=2), E by G, E by T, and E by T by G. Residual error mean square was used as the error term for significance testing of Estradiol Challenges.

Pearson's correlation coefficients of daily probe admittance values at vaginal and vulval locations within progesterone treatments (P_4+ and P_4-), and also between progesterone treatments within vaginal and vulval locations for the different electrode combinations and frequencies were computed.

Although initial analyses indicated no significant progesterone treatment effect for any of the estrous events and LH concentrations, a significant progesterone treatment effect was observed for estradiol concentration. Study of the estradiol data indicated this difference was essentially due to one cow in the first period following ovariectomy. No known reason could be determined for this difference. Therefore, estrous events, estradiol and LH data were pooled across treatments, on a daily basis, for correlation analyses. Correlation coefficients were calculated to relate circulating estradiol, LH, and standing estrous behavior.

Vaginal admittance values pooled across treatments were paired on a daily basis with circulating estradiol concentrations for calculation of correlation coefficients.

Weights of silastic tubes containing progesterone before and after each implantation period were analyzed using a split-plot analyses of

variance. The number of days implanted was the whole plots and the before and after weights were the split-plots.

Experiment 2. Intact Beef Heifers.

In order to facilitate the interpretation of data from experiment 1, data made available from a commercially-sponsored trial (Dr. G. Foxcroft, personal communication) have also been analysed. In this experiment conducted at the Faculty of Agricultural Sciences, University of Nottingham, U.K., twenty post-puberal, Hereford X Holstein heifers were housed in a single yard and treated for 12 d with a progesterone releasing intravaginal device (PRID)[14] . Estrus was synchronized in all heifers by im injection of PGF[15] 11 d following PRID removal.

Each heifer was probed once daily, with an earlier prototype of the vaginal probe used in experiment one, for 32 d from PGF injection. This probe was designed to measure the electrode surface admittance relative to the redox status of the surrounding medium across only one pair of electrodes. The probe was fully inserted into the vagina and programmed to measure admittance at 100, 150, and 200 mV using 50 KHz. Daily jugular blood samples were collected over the same 32 d period for progesterone and estradiol determination. A subgroup of six heifers was fitted with indwelling jugular cannulae, using a non-surgical technique, on the second day following PGF treatment. Blood samples were collected every 4 h for 72 h following cannulation in order to characterize the time of the pre-

[14]CEVA Ltd., Watford, UK.

[15]Estrumate, Coopers Agropharm Inc., Willowdale, ON.

ovulatory LH surge. All animals were restrained in a squeeze for probing and blood sampling. Progesterone, estradiol and LH concentrations were determined using established radioimmunoassays.

Daily means and standard error of means were computed for probe admittance values at 200 mV, and circulating estradiol, LH, and progesterone concentrations. Pearson's correlation coefficients were calculated between daily progesterone and estradiol with probe admittance means at all three voltages across the 32 d experimental period.

RESULTS

Experiment 1.

Visual inspection of all genital tracts following continuous probing for 96 d revealed no evidence of tissue irritation or bruising in five of the six cows. One cow appeared to have a minor bruise on her cervix. Periodically, all cows had clear vaginal discharge which was consistent with the mucus observed at the cervix.

Mean plasma progesterone concentrations, prior to and following ovariectomy, for control and progesterone implanted cows are shown in Figure 3. Although plasma progesterone concentrations were elevated significantly for progesterone implanted cows (.60 ± .03 ng/ml vs .26 ± .03 ng/ml $P < .0001$), the increase is unlikely to have biological significance. This conclusion is based on the fact that the progesterone implants failed to produce a sustained increase in the plasma progesterone concentrations above the level of 1 ng / ml which is considered necessary to influence the estrous cycle (Hobson and Hansel, 1972).

Estrous behavior events, following the first two estradiol challenges, are summarized in Table 1. Progesterone treatment failed to alter the frequency or duration of any of the estrous events measured. Estradiol challenges resulted in significantly more attempted mounts and stand refu. l after the first than after the second estradiol challenge.

Mean stands per cow for each 30 min estrous detection period for the preovariectomy and postovariectomy periods are presented in Figures 4 and 5, respectively. There is a clear temporal relationship between the circulating estradiol and LH concentrations and stands per cow in the ovariectomized cows (Figure 5). Correlation coefficients comparing estradiol concentrations with LH and mean stands per cow for the first and combined second and third estradiol challenge periods are listed in Table 2. The highly significant relationship between estradiol and standing activity is further strengthened by shifting the standing data back 6 h.

Correlation coefficients between vaginal and vulval probe admittance values within progesterone treatments (P_4+ and P_4-) and between progesterone treatments within vaginal and vulval location during the 24 d experimental period are listed in Table 3. Vaginal and vulval probe admittance values were poorly correlated within progesterone treatments. Admittance values within vaginal (but not vulval) position between progesterone treatments were positively correlated. Vulval values were extremely variable and therefore were not used for further analysis. Conversely, vaginal values were consistent and show similar trends at all electrode positions and at both frequencies (data not shown). Tip opposite admittance values at 50 KHz were selected as representative

vaginal probe readings for comparison with behavioral data and hormone profiles.

Vaginal probe admittance values and circulating progesterone and estradiol concentrations, relative to PGF injections and prior to ovariectomy are plotted in Figure 4. The probe admittance profile shows an increase followed by a decrease of equal magnitude during the period when progesterone is below 1 ng / ml. Mean estradiol concentrations are quite variable during this period due to the variable return to estrus following one PGF injection.

Circulating estradiol, LH, standing estrous events, and vaginal probe admittance values for the post-ovariectomy period are shown in Figure 5. The probe admittance profile shows an increase approximately 3 d following each of the estradiol peaks. The first increase is followed by a decrease of similar magnitude before stabilizing. Probe admittance values rise continuously following the second estradiol challenge. Correlation coefficients of the relationship between estradiol concentrations and probe admittance values increased as the estradiol curve was shifted forward 1, 2, and 3 d ($r = .10$, $P = .64$; $r = .39$, $P = .06$; and $r = .67$, $P = .0004$), respectively.

Experiment 2.

Probe admittance values at 200 mV from the intact heifers used in experiment 2 are plotted with circulating estradiol, progesterone and LH concentrations for the 32 d following PGF in Figure 6. These probe values display a periestrous peak followed by a decline of similar magnitude at approximately 4 and 25 d following PGF injection. Two smaller midcycle

increases and declines, at days 13 to 18 and 22 to 24, appear to follow smaller increases in estradiol concentrations at days 10 and 21 respectively. The day 18 nadir was present in 18 of the 20 heifer probe profiles (data not shown). Correlation coefficients between probe admittance values and circulating progesterone and estradiol concentrations are shown in Table 4. Shifting the estradiol curve forward by one and two days increases the correlation between estradiol and probe admittance at all three voltages. Conversely, circulating progesterone was strongly negatively correlated to probe admittance values at all three voltages.

DISCUSSION

Previous studies (Rajamahendran et al., 1982; Roche, 1974) have shown a long-term release of progesterone (peak > 3 ng/ml, 20 d > 1 ng/ml) from implant treatments similar to those used in the present study. Rate of steroid release through silicone rubber (Dziuk and Cook, 1966) and silastic (Roche, 1974) devices is proportional to surface area, inversely related to wall thickness, and can be complicated by the structure of the steroid. In this study, implant weights did not change ($P > .05$) from implantation to removal suggesting that the steroid did not leave the silastic tubes (data not shown). This may be the result of a fibrinous network encapsulating the implants and not permitting progesterone release (Chien, 1978). An additional factor which may have contributed to the low circulating progesterone response is that Rajamahendran et al. (1982) implanted 250 kg prepuberal heifers in contrast to the 680 kg cows used

in this study. Pretreatment with progesterone has been shown to increase estradiol induced estrous behavior in ovariectomized cattle (Carrick and Shelton, 1969). However, in our study there was no progesterone treatment effect ($P > .05$) on standing behavior presumably due to the failure of the progesterone implants to substantially elevate plasma progesterone concentrations.

The probe used in experiment 1 was designed for vaginal, not vulval, use. Probe admittance means for vaginal and vulval positions were poorly correlated within treatment (Table 3). Given the anatomical differences of the anterior vagina and the vulva, consistent tissue contact with the probe electrodes in both vaginal and vulval positions is extremely unlikely. McCaughey and Patterson (1981a,b) found the electrical resistance of the anterior vagina to be the optimal probing site in the bovine genital tract. However, other studies have shown a close relationship between vaginal and vulval electrical measurements (Adam et al., 1981; Aizinbud et al., 1984; Smith et al., 1989) using implanted electrodes. Development of an alternative probe design more closely fitted to the vulva's shape might enhance the reliability of admittance values from this position.

Each set of three estradiol benzoate injections in the first two challenges consisted of a total dose of 525 ug of estradiol. Previous studies have established a cumulative dose of 500 ug of estradiol as an approximate threshold for triggering estrous behavior in cattle (Katz et al., 1980; Cook et al., 1986). The estradiol benzoate treatments produced estrous behavior similar to previous studies with ovariectomized (Walton and King, 1984) and intact (Hurnik et al., 1975) cattle. The

frequency of stands per cow in response to each estradiol benzoate challenge (Table 1) was within the range of 12.7 and 36.2 stands per animal observed by Walton and King (1984). The third estradiol challenge only stimulated a standing estrus in one animal (Figure 5). No estrous response was observed in intact cows following a smaller-estradiol peak 5 d after ovulation (Glencross et al., 1973). Presumably the estradiol threshold was not surpassed or progesterone secretion from the developing corpus luteum suppressed the estrous response. Cows in the present study did not appear to become refractive to repeated estradiol benzoate injections, in the absence of progesterone, as was found in sheep (Robinson, 1954) and in a previous experiment with cattle (Carrick and Shelton, 1969).

The estrogen induced changes in cattle behavior are thought to be mediated via estrogen receptors in the neural regulatory centres (Glencross et al., 1981). Studies on uterine physiology in rats (Hsueh et al., 1976) and sheep (Rexroad, 1981a,b) have established that estrogen treatment enhances the cytoplasmic binding of estrogen and that progesterone modulates the presence of both estrogen and progesterone receptors. Therefore, it seems reasonable that the physiological changes observed in the genital tract may reflect responses to increased estrogen binding in vaginal, vulval and uterine tissues.

Estradiol treatment of ovariectomized females has also been shown to induce an LH surge similar to the preovulatory LH surge in intact females (Short et al., 1973; Stumpf et al., 1989). This is accomplished, at least in part, by an increase in the concentration of LH releasing hormone receptors in the pituitary (Schoenemann et al., 1985). However,

unlike endogenous progesterone in the intact cow, exogenous progesterone does not suppress estradiol induced estrous behavior or LH surge in ovariectomized cows (Short et al., 1979). In the present study, there was no difference in LH concentrations in both progesterone and control groups following estradiol benzoate challenges. Probe value changes relative to the preovulatory LH surge have been reported by Schams et al. (1977) and Aboul-Ela et al. (1983). The observed increase in probe admittance appears to be a latent response relative to the LH surge and estradiol peak.

Peters (1989) reported that vaginal resistance measurements showed a variable association with milk progesterone changes following PGF treatment. In experiment 1, mean vaginal probe admittance values increased followed by a decline after the first PGF injection as circulating progesterone decreased to baseline levels (Figure 4). In the ovariectomized cows, pooled mean vaginal probe admittance values increased following estradiol benzoate treatment. These appeared to be a 2 to 3 d latent response similar to the observations of Lewis et al. (1989) who found minimal Z-statistic for vulval resistance and maximal tissue hydration to occur after 1 to 2 days of increased estrogen and 3 to 4 days of low (< 1 ng/ml) progesterone. Our probe admittance response to elevated estradiol is consistent with previous studies which have found an increase in conductance (Feldmann et al., 1978) or a decrease in resistance (Heckman et al., 1979) and impedance (Aizinbud et al., 1984) of vaginal tissue in the periestrous period.

In experiment 2, vaginal probe admittance values at 200 mV showed a characteristic rise and fall following each elevation of plasma

estradiol (Figure 6). Rectal ultrasound technology has permitted the detailed study of follicular growth and the characterization of two or three follicular waves during the estrous cycle (Ginther et al., 1989). The nonovulatory follicle produces low levels of estrogen (Glencross et al., 1973) which may alter the genital tract tissue admittance, in the absence of estrous behavior. Shifting the estradiol curve ahead by one and two days increased the positive correlation with vaginal level admittance at all voltages (Table 4). Circulating progesterone was negatively correlated to probe admittance at all voltages suggesting an inverse relationship between tissue level admittance and progesterone. It is more likely that progesterone indirectly exerts its influence on vaginal admittance by suppressing the circulating estrogen concentrations.

SUMMARY

Estradiol appears to increase vaginal tissue level admittance, as measured by our probe, in both intact and ovariectomized cattle. There was a two to three day delay in the probe admittance response to estradiol as demonstrated by the high correlation between probe admittance values and circulating estradiol concentrations that are shifted 2 and 3 days. This latency of response detracts from the predictive potential of the probe studied. Admittance value peaks appeared to be suppressed during the luteal phase of the intact cattle studied. Based on these observations, we conclude that the genital tract electrical admittance properties are likely directly increased by estradiol and indirectly influenced by progesterone concentrations.

TABLE III-1. MEAN ESTROUS EVENTS FOR EACH PROGESTERONE TREATMENT AND THE FIRST TWO ESTRADIOL CHALLENGES. EXP.1

		ESTROUS EVENTS[1]				
VARIABLE	n	AM	M	NS	S	DUR
Progesterone[2]						
P4	12	6.8	17.3	8.7	13.5	21.5
Control	12	9.7	19.0	8.7	22.8	25.5
SEM		.40	.61	.27	.97	1.03
P =		.21	.61	.52	.12	.47
Estradiol[3]						
First EB	12	10.5	19.1	10.5	19.1	26.5
Second EB	12	5.9	17.3	5.9	17.3	20.5
SEM		1.03	2.83	1.12	4.23	3.02
P =		.01	.66	.02	.77	.20
Interaction[4]						
P =		.40	.54	.66	.25	.81

[1]Mean events per cow for the 13 estrous detection periods following estradiol injection. AM = Attempted Mounts, M = Mounts, NS = Stand Refusals, S = Stands, and DUR = Standing Duration in hours.

[2]Progesterone Treatment effect. All cows received either two P_4 implants or two placebos each for 24 d

[3]Effect of estradiol benzoate (EB) challenges one and two.

[4]Interaction effect between progesterone treatment and estradiol challenge.

TABLE III-2. PEARSON'S CORRELATION COEFFICIENTS BETWEEN ESTRADIOL, LH AND STANDING ESTROUS BEHAVIOR FOLLOWING ESTRADIOL CHALLENGES (EXP. 1).[1]

	E2[2]	LH[3]	EB[4]	EB -6 h[5]
E2	----	.842 (0.0087)	.711 (.0482)	.939 (.0005)
LH	.430 (.1240)	----	.472 (.0760)	.876 (.0001)
EB	.727 (.0032)	.347 (.0761)	----	.719 (.0025)
EB -6 h	.767 (.0014)	.679 (.0001)	.807 (.0001)	----

[1]Coefficients and (probabilities) above diagonal from first estradiol challenge; below diagonal from second and third estradiol challenges combined.
[2]Circulating estradiol concentration.
[3]Circulating LH concentration.
[4]Mean stands per cow per 30 min estrous detection period.
[5]Mean stands per cow shifted 6 h backwards.

TABLE III-3. PEARSON'S CORRELATION COEFFICIENTS BETWEEN PROBE INSERTION DEPTHS AND BETWEEN PROGESTERONE TREATMENTS (EXP. 1).

	Frequency[1]					
	1			50		
	Electrode Combinations[2]					
Comparison[3]	TO	TA	TR	TO	TA	TR
Vaginal vs Vulval						
(P_4+)	-.20	.30	.22	-.01	.34	.48*
(P_4-)	-.41*	-.43*	-.15	.39	.40	-.11
P_4+ vs P_4-						
Vaginal	.80***	.64**	.65**	.72***	.60*	.43*
Vulval	.22	.02	.14	-.05	.09	.51*

[1]Probe admittance at 1 or 50 KHz.
[2]Probe admittance read between opposite tip electrodes (TO), between adjacent tip electrodes (TA), or between tip and rear electrodes (TR).
[3]Vaginal (full insertion) versus Vulval (partial insertion) for progesterone (P_4+) and control (P_4-) groups. Also P_4+ versus P_4- for Vaginal and Vulval.
* $P < .05$
** $P < .001$
*** $P < .0001$

TABLE III-4. PEARSON'S CORRELATION COEFFICIENTS BETWEEN PROBE ADMITTANCE VALUES AND PROGESTERONE AND ESTRADIOL (EXP. 2).

	Voltage[2]			
Hormone[1]	100	150	200	Sig[3]
P_4	- .68	- .69	- .66	.0001
E_2	.58	.62	.63	.005
E_2 + 1 d	.70	.73	.72	.0001
E_2 + 2 d	.78	.79	.77	.0001

[1] P_4 = plasma progesterone, E_2 = plasma estradiol, or E_2 profile shifted ahead by one (+ 1 d) or two (+ 2 d) days.
[2] Probe admittance volatges (mV).
[3] Significance indicated for all voltages with each hormone.

Figure III-1. Progesterone and estradiol treatment schedule. Groups A and B are n = 3 treatment groupings. 1 X P4 = One 2 g progesterone implant, 2 X P4 = Two 2 g progesterone implants, Control is two empty implants. Day 0 = ovariectomy. All implants were removed / implanted on days 8, 32, 40, and 64. Estradiol benzoate injections (175 ug in 2 ml oil) are indicated by * . Days coincide with progesterone scale.

PROGESTERONE IMPLANTS

Group A (n = 3)	1 X P4	2 X P4	1 X P4	Control	
Group B (n = 3)	1 X P4	Control	1 X P4	2 X P4	
DAY	0	8	32	40	64

ESTRADIOL INJECTIONS

Estradiol	* * *				* * *		*	
DAYS	8	11	12		25	26	29	31
	40	43	44		57	58	61	64

Figure III-2. Vaginal admittance probe.

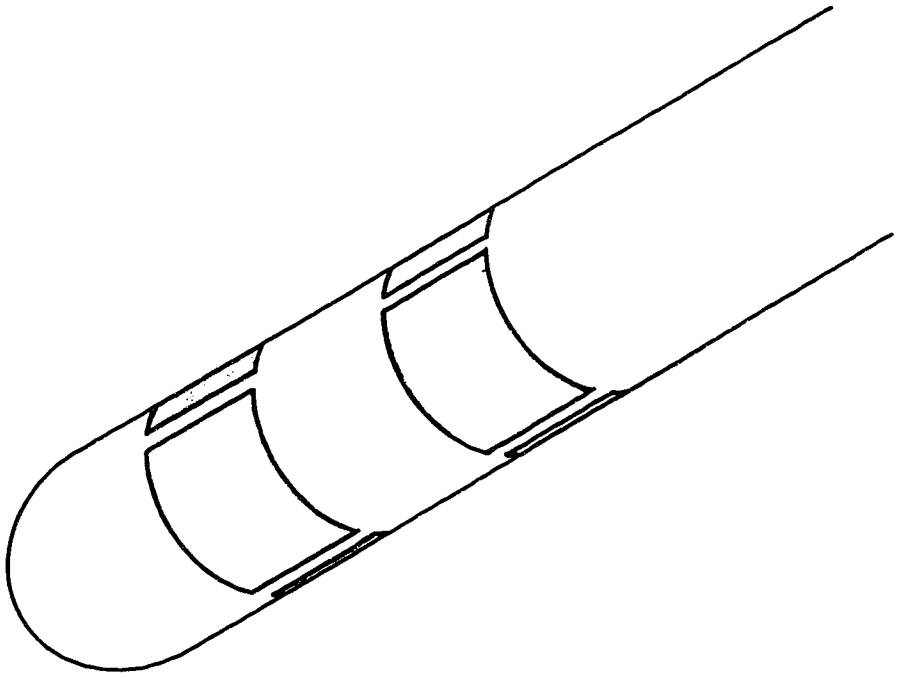


Figure III-3. Mean progesterone concentrations (ng / ml) over the experimental period. Prostaglandin (PG) injections synchronized estrus prior to ovariectomy (OV) for pretreatment cows (n = 6, ■). All cows received one progesterone implant at ovariectomy then two progesterone (Treated, n = 6, ◆) or two placebo (Control, n = 6, ——) implants at P4 for 24 d. Bars indicate SEM.

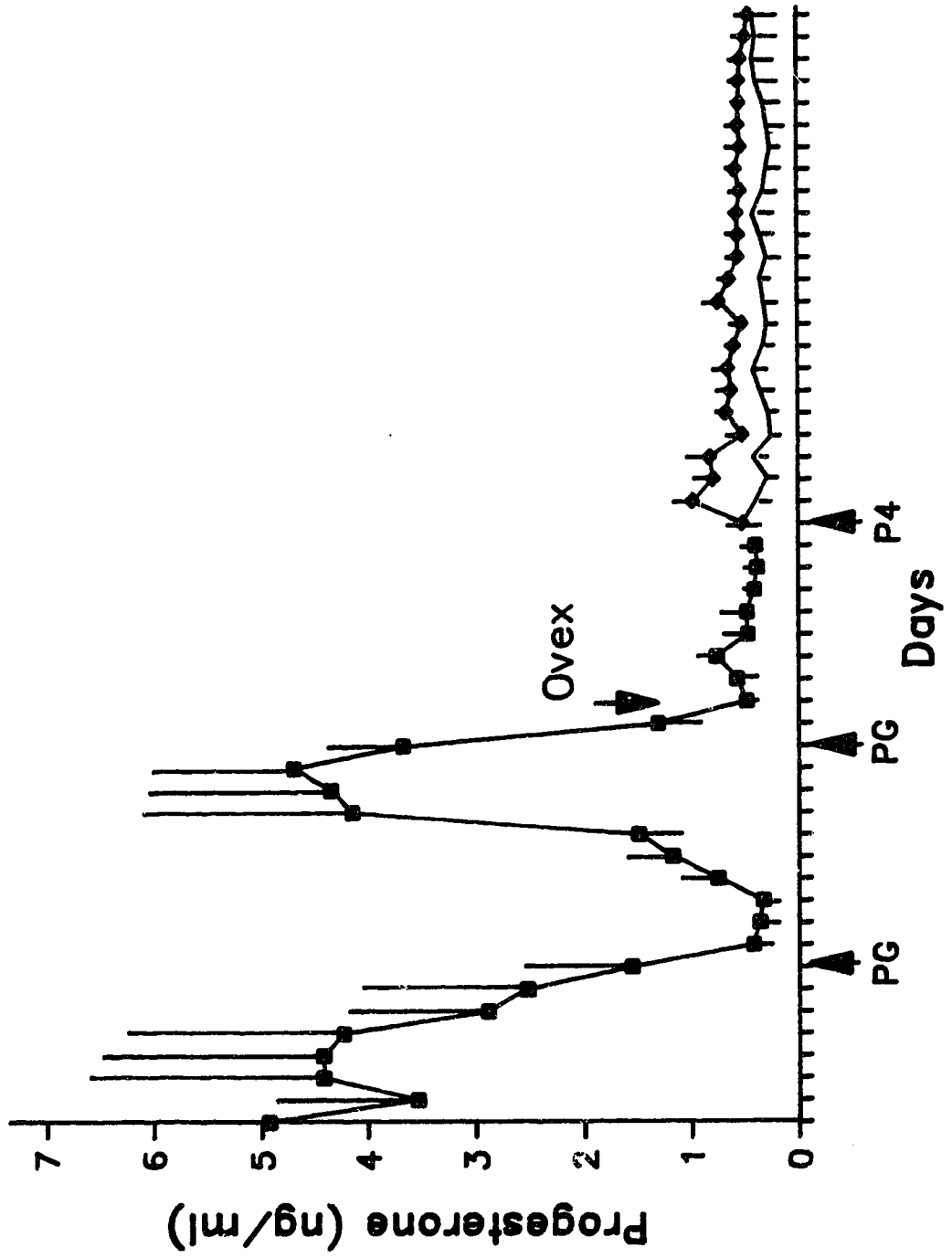

Progesterone (ng/ml)
0
1
2
3
4
5
6
7
Ovex
PG
PG
P4
Days

Figure III-4. Mean vaginal probe admittance values (u Amps, 50 KHz, tip opposite) plotted with pooled plasma estradiol (pg/ml), estrus standing frequency, and progesterone (ng/ml) concentrations prior to ovariectomy. Synchronizing prostaglandin injections (arrowheads) are 11 d apart. Bars indicate SEM.

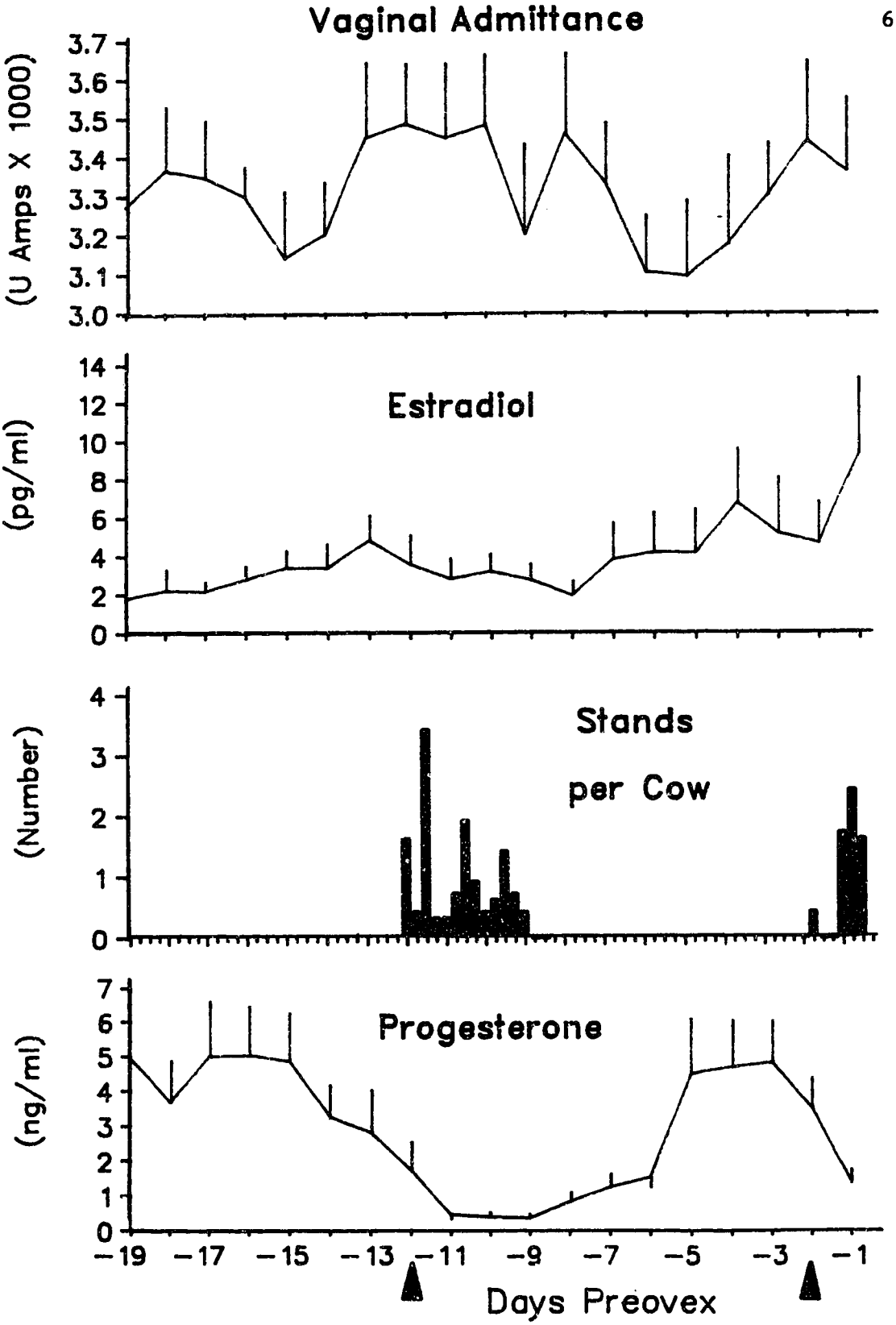

Vaginal Admittance
(U Amps X 1000)
3.7
3.6
3.5
3.4
3.3
3.2
3.1
3.0
Estradiol
(pg/ml)
14
12
10
8
6
4
2
0
Stands
per Cow
(Number)
4
3
2
1
0
Progesterone
(ng/ml)
7
6
5
4
3
2
1
0
−19 −17 −15 −13 −11 −9 −7 −5 −3 −1
Days Preovex

Figure III-5. Mean vaginal probe admittance values (u Amps, 50 KHz, tip opposite, daily) plotted with pooled plasma estradiol (pg/ml, every 12 h) and LH (ng/ml, every 6 h) concentrations and estrous standing frequency (every 6 h). Estradiol benzoate injection challenges are shown with arrowheads. Bars indicate SEM.

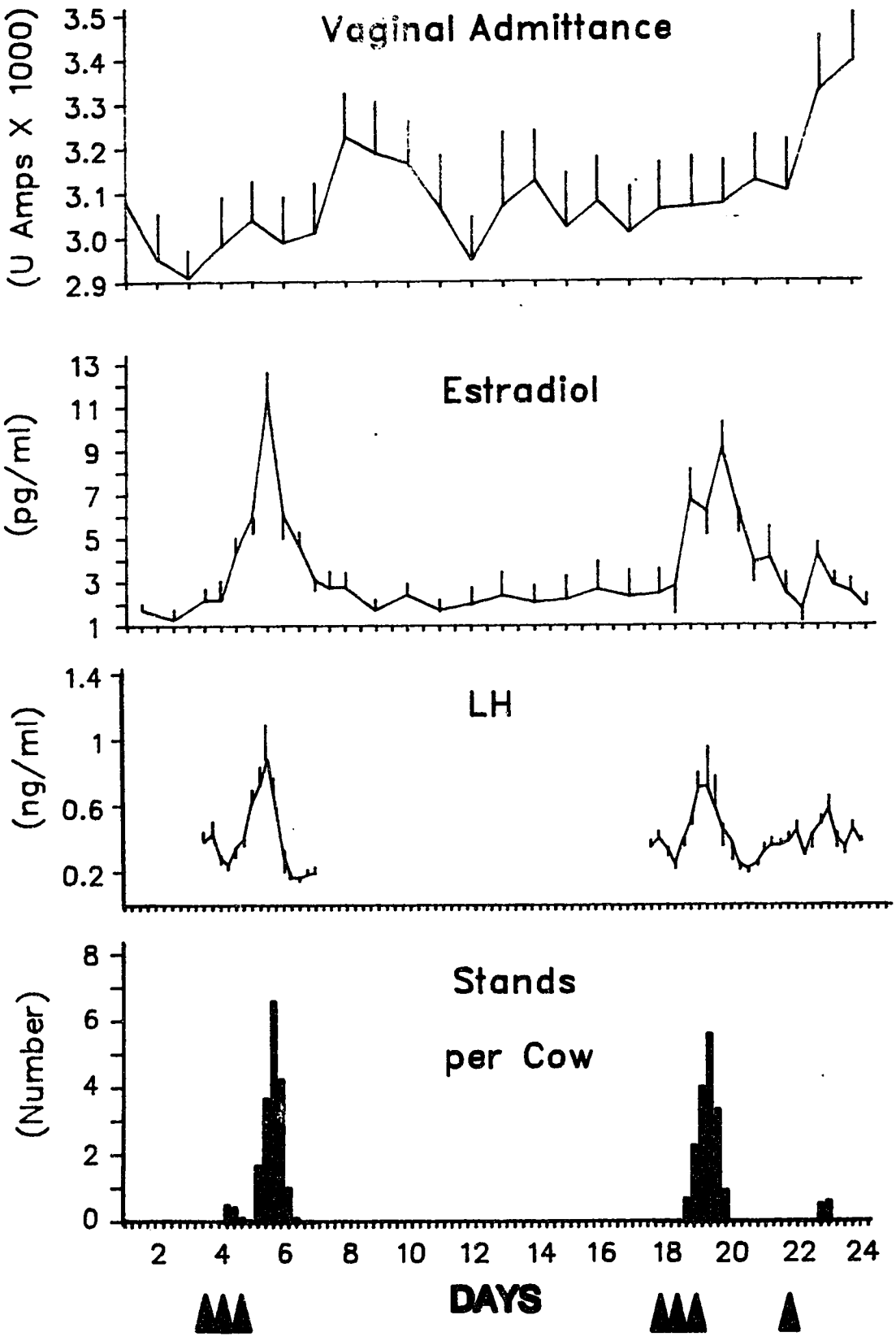
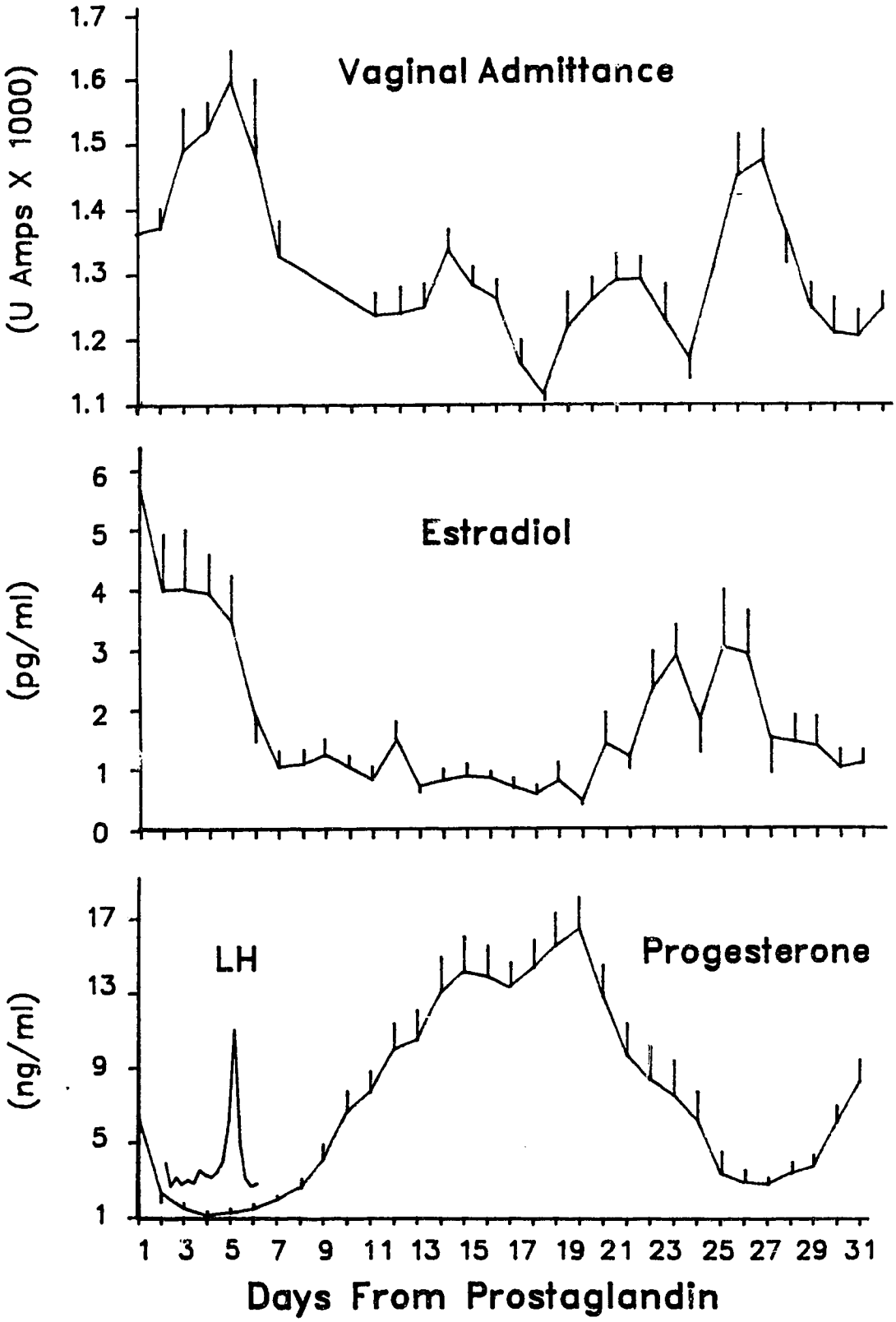

Vaginal Admittance
(U Amps X 1000)
3.5
3.4
3.3
3.2
3.1
3.0
2.9
Estradiol
(pg/ml)
13
11
9
7
5
3
1
LH
(ng/ml)
1.4
1
0.6
0.2
Stands
per Cow
(Number)
8
6
4
2
0
2
4
6
8
10
12
14
16
18
20
22
24
DAYS

Figure III-6. Daily mean vaginal probe admittance values (u Amps, 200 mV, 50 KHz) of 20 heifers plotted with mean plasma estradiol (pg/ml, daily) and progesterone (ng/ml, daily with SEM bars) concentrations, and LH (ng/ml, every 4 h for 4 d) surge for 6 heifers. Time is days from synchronizing prostaglandin injection. Bars indicate SEM.


Vaginal Admittance
(U Amps X 1000)
1.7
1.6
1.5
1.4
1.3
1.2
1.1
Estradiol
(pg/ml)
6
5
4
3
2
1
0
LH
Progesterone
(ng/ml)
17
13
9
5
1
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31
Days From Prostaglandin

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IV. GENERAL DISCUSSION

The limited information on the effect of growth hormone (GH) on the ovary and thus reproductive performance and the need for an estrous detection aid that doesn't require animal interaction has led to the two experiments reported in this thesis. Milk production increases in response to exogenous GH treatment between 16 and 40% have been reported (Eppard et al, 1987). GH-treated cows require enhanced management in order to fulfil their elevated nutritional requirements. A number of studies have quantified the effects of GH treatment on reproductive performance. However, most of these studies have dealt with limited cow numbers and have only studied [illegible] lactation effects. The results range from slightly antagonistic to no substantial impact of GH treatment on standard measures of reproductive fitness.

There is a growing body of evidence linking insulin-like growth factor-I (IGF-I) to ovarian activity and fertility (Miller et al., 1986; Holland et al., 1988; Rutter et al., 1989; Echternkamp et al., 1990; Spicer et al., 1990). Low fertility is a major management concern on dairy farms (Pelissier, 1971; Spalding et al., 1975). Culling for reproductive failure averages between 20 and 30% of all herd disposals (Burnside et al., 1971). A calving interval of 12 to 13 months is an optimal goal for maximum profit (Adamowicz et al., 1986). To achieve this, cows must be pregnant within 100 days of calving. Unfortunately, this critical 100 d breeding period coincides with the period of highest milk production and negative to neutral energy balance. High milk production and the accompanying negative energy balance have been linked

to suboptimal reproductive performance (Butler et al., 1981; Laben et al., 1982; Staples et al., 1990). Thus, genetic selection for superior milk production potential, may have a negative impact on reproductive performance.

After parturition many high producing cows fail to show signs of estrous behavior prior to their first ovulation. Many of the conventional estrous detection aids (eg. heat mount detectors, teaser animals, and video cameras) are rendered useless during this period of postpartum lactational anestrus as no standing behavior occurs. It has been postulated that the lack of estrous behavior response at the first postpartum ovulation is due to a lack of progesterone priming of the neural centres responsible for initiating estrous activity (Carrick and Shelton, 1969).

Our results from experiment 1 confirm that exogenous GH increases IGF-I both in circulation and in follicular fluid. The identification of mRNA for IGF-I, GH receptor and IGF Type-I receptor in bovine granulosa cells suggests a potential autocrine and(or) paracrine mode of action for IGF-I in granulosa cells. One key regulatory step that was not investigated is that of the IGF-I binding proteins (Adashi, 1989; Blum et al., 1989).

The following is a possible model for GH action in the early postpartum period. Exogenous GH increases circulatory and possibly follicular fluid IGF-I concentrations. Increased concentrations of both GH and IGF-I stimulates granulosa cells of developing follicles. IGF-I could act in an endocrine, paracrine, or autocrine manner to enhance steroidogenesis leading to increased production of estrogen at the first

and second postpartum ovulations.

Detailed studies of the IGF-I binding protein's role in modulating IGF-I actions on the ovary are required. These could include simultaneous determinations of IGF-I and IGF-I binding protein concentrations in follicular fluid and circulation at various stages of follicular development. As more information becomes available about IGF-I binding proteins, [illegible] studies of their synthesis in granulosa and thecal cells relative to GH treatment could be initiated.

The probe we studied appeared to respond positively to elevated estrogen concentrations. In the second experiment using ovariectomized cows, the probe admittance values increased following the second estradiol challenge which was at least 18 days following progesterone implantation. Control cows also showed standing estrous behavior and had increasing vaginal admittance values following this challenge suggesting that progesterone is not required for the admittance values to respond to estrogen. The value of the probe studied to predict impending estrus or ovulation is limited because of the latent response to the preovulatory estrogen surge. However, these readings could be used to monitor estrogen fluctuations after calving which could be interpreted to approximate the second postpartum estrus. Future studies into the roles of estrogen and progesterone in altering vulval admittance values with a redesigned vulval probe are necessary. It is hoped that these studies and those on the role of GH and IGF-I in reproduction will eventually lead to a better understanding of ovarian function in the dairy cow.

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