


# Follicle stimulating hormone isoforms and plasma concentrations of estradiol and inhibin A in dairy cows with ovulatory and non-ovulatory follicles during the first postpartum follicle wave

Short Title: FSH Isoforms and Ovulation

Stephen T. Butler[1, 3, 4], Susanne H. Pelton[1], Phillip G. Knight[2] and W.R. Butler[1]

[1]Department of Animal Science, Cornell University, Ithaca, NY 14853, [2]School of Animal and Microbial Sciences, University of Reading, Whiteknights, Reading RG6 6AJ, UK.

[3]Present address: Teagasc, Moorepark Dairy Production Research Centre, Fermoy, Co. Cork, Ireland.

[4]Corresponding author: S.T. Butler, Teagasc, Moorepark Dairy Production Research Centre, Fermoy, Co. Cork, Ireland.; Tel.: +353-25-42252; Fax: +353-25-42340; Email: stephen.butler@teagasc.ie

(Key words: FSH isoforms, ovulation, estradiol, inhibin A, dairy cow)

## ABSTRACT

Following parturition, all cows display a wave of ovarian follicular growth, but a large proportion fail to generate a preovulatory rise in estradiol, and hence fail to ovulate. Follicle stimulating hormone (FSH) exists as multiple isoforms in the circulation depending on the type and extent of glycosylation, and this has pronounced effects on its biological properties. This study examined differences in plasma FSH, estradiol, and inhibin A concentrations, and the distribution of FSH isoforms in cows with ovulatory or atretic dominant follicles during the first postpartum follicle wave. Plasma FSH isoform distribution was examined in both groups during the period of final development of the dominant follicle by liquid phase isoelectric focusing. Cows with an ovulatory follicle had higher circulating estradiol and inhibin A concentrations, and lower plasma FSH concentrations. The distribution of FSH isoforms displayed a marked shift toward the less acidic isoforms in cows with ovulatory follicles. A higher proportion of the FSH isoforms had a pI >5.0 in cows with ovulatory follicles compared to those with atretic follicles. In addition, cows with ovulatory follicles had greater dry matter intake, superior energy balance, elevated circulating concentrations of insulin and insulin-like growth factor-I, and lower plasma nonesterified fatty acids. The shift in FSH isoforms toward a greater abundance of the less acidic isoforms appears to be a key component in determining the capability for producing a preovulatory rise in estradiol, and this shift in FSH isoforms was associated with more favorable bioenergetic and metabolic status.

## INTRODUCTION

The expulsion of the foetal-placental unit at parturition in dairy cows is associated with clearance of gestational hormones from the circulation, facilitating an increase in pituitary follicle-stimulating hormone (FSH) release, which in turn orchestrates the synchronous resumption of ovarian follicular development during the first week postpartum (1). The first postpartum dominant follicle ovulates, becomes atretic, or develops into a cyst, with approximate frequencies of 40, 40 and 20%, respectively (2). This has a major impact on the duration of postpartum anestrus, as atretic and cystic follicles are likely to be replaced by follicles with similar fates (2,3). A prompt resumption of ovulatory activity following parturition is desirable; cows that have an early commencement of luteal activity have an improved likelihood of conception after breeding (4).

Timing of the first postpartum ovulation is correlated with timing of the onset of recovery from the negative energy balance (NEB) nadir (5,6). Infusion of exogenous pulses of LH during the first postpartum follicle wave in dairy (7) and beef (8) cows increased the likelihood of ovulation. However, some cows completely failed to exhibit an increase in circulating estradiol in response to frequent exogenous LH pulses (8), indicating that other factors in addition to frequent LH pulses are necessary. Multiple isoforms of FSH are present in the circulation due to variation in the extent and type of glycosylation on $\alpha$ and $\beta$-subunits; in cattle, terminally sialylated oligosaccharide structures are most prevalent (9). The isoelectric point (pI) of FSH has a wide range depending on the abundance of sialic acid residues; heavily sialylated isoforms have an acid pH, and isoforms with little or no sialic acid have a less acidic or basic pH. In cattle, most (~70%) FSH isoforms have a pI less than 5.0 (10). The heterogeneity in glycosylation has significant effects on the biological properties of FSH. Less acidic isoforms have greater in vitro bioactivity than more acidic isoforms, but less acidic isoforms are more rapidly cleared from circulation and thus have lower in vivo bioactivity than more acidic isoforms (11,12). In human beings, a shift toward greater pituitary release of less acidic serum FSH isoforms in response to GnRH is observed during the preovulatory period (13). It is

currently thought that frequent transient stimulation with more bioactive, faster clearing isoforms represents a key mode of FSH action during the preovulatory period (14).

This study was carried out to examine differences in circulating estradiol, inhibin A and FSH concentrations, and to compare the distribution of FSH isoforms during the final stages of follicle development in dairy cows with ovulatory and non-ovulatory follicles during the first postpartum follicle wave.

## MATERIALS AND METHODS

All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee. As part of a large study examining the effects of treatment with propylene glycol (a glucogenic precursor) during the interval from late pregnancy through early lactation, multiparous Holstein cows (n = 56) were monitored from day –21 to day 30 relative to parturition (15). Nine cows with ovulatory and nine with non-ovulatory (atretic) first postpartum follicle waves were randomly selected for further analysis. The only provisions placed on cows selected were that none of the animals selected suffered any clinical illness or metabolic disorder during the experimental period, and that the cows with non-ovulatory follicles did not exhibit a preovulatory-style rise in circulating estradiol concentrations. The restriction on using only cows that did not exhibit a preovulatory-style rise in circulating estradiol was imposed because most cows with atretic follicles during the first postpartum follicle wave have low circulating estradiol concentrations throughout the period of dominant follicle development (15). All Cows were housed in individual tie stalls starting on day 21 prior to expected parturition, moved to individual box stalls for a 2-4 day period around the time of parturition, and then returned to individual tie stalls until day 30 postpartum. The management of the animals, energy balance (EB) determination, blood sampling routine, and the methods used to measure circulating concentrations of estradiol, insulin, insulin-like growth factor-I (IGF-I), glucose and nonesterified fatty acids (NEFA) were previously reported (15).

Ovarian follicular activity of all cows was examined by linear array ultrasonography with a 7.5-MHz transrectal transducer (Aloka 210; Corometrics Medical Systems Inc., Wallingford, CT) three times per week (Monday, Wednesday and Friday) beginning on days 8-10 postpartum and continuing until ovulation, or until day 30 for cows failing to ovulate. The diameter of dominant follicles between ultrasound examinations was calculated by linear interpolation. Additionally, if the final measurement of follicle diameter in cows with ovulatory follicles was recorded prior to or on the day of the preovulatory rise in estradiol, a follicle diameter was calculated by linear interpolation such that the largest follicle diameter was recorded for the day following the preovulatory rise in estradiol (ovulation occurs approximately 28 h after the onset of the LH surge). Ovulation was verified by ultrasonography. Follicles were considered to be dominant when a diameter of >10 mm was reached in the absence of other large growing follicles (16).

Plasma FSH and inhibin A concentrations were determined from day of parturition until day of ovulation in ovulatory cows, and from day of parturition until day of maximum follicle diameter in non-ovulatory cows. Plasma FSH was determined using an RIA (1) and plasma inhibin A concentrations were determined by ELISA using highly purified 32 kDa bovine inhibin-A as standard (17). The detection limit of the assays were 0.17 ng/ml and 40 pg/ml, and the inter- and intra-assay coefficients of variation were 13% and 8%, and 12% and 9% for FSH and inhibin A, respectively.

**Plasma FSH isolation and isoelectric focusing**

An affinity column to isolate FSH from plasma was generated by covalently coupling guinea pig anti-bFSHβ antiserum (AFP859691, US National Hormone and Peptide Program) to immobilized Protein A using the ImmunoPure rProtein A IgG Plus Orientation Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Plasma was pooled from the day preceding and the day of maximum estradiol concentrations for cows with ovulatory follicles, and the day preceding and the day of maximum follicle diameter for cows with non-ovulatory

follicles to generate a sample volume of 5.5 ml. The plasma sample was combined with an equal volume of PBS (0.1 M phosphate, 0.15 M NaCl, pH 7.2), and slowly passed over the FSH affinity column (~ 4 ml/h). The column was washed with 10 ml of PBS, and FSH was eluted with 10 ml of elution buffer (0.1 M glycine·HCl, pH 2.8). The 10 ml eluate was neutralized with 500 µl of 1 M Tris·HCl, pH 9.5, and dialyzed overnight against 4 L dd $H_2O$. Following dialysis, 550 µl of Bio-Lyte 3/10 ampholytes 40% w/v (Bio-Rad Laboratories, Hercules, CA) were added, and the sample was diluted to a volume of 55 ml with dd $H_2O$. Isoelectric focusing of the diluted sample by liquid phase electrophoresis was carried out with the Rotofer preparative isoelectric focusing (PIEF) cell, which was assembled and used according to the manufacturers instructions (Bio-Rad Laboratories, Hercules, CA). Focusing was carried out at 15 W constant power for 3 h 45 min, and 20 fractions were simultaneously collected. The pH of each fraction was measured and the fractions were dried using a vacuum dryer. The fractions were reconstituted in 200 µl dd $H_2O$, the pH adjusted to neutral (7.2–7.4), and the volume of each tube brought to 300 µl with dd $H_2O$. 200 µl of PBS was added to each tube, and then assayed for FSH by RIA [1]. The assay detection limit for the Rotofer fractions was 0.05 ng FSH. The percentage of total FSH recovered from the sample was calculated for each fraction. The first and last fractions collected from the Rotofer apparatus were extremely acidic or basic, respectively, and were not included in the analysis. A blank sample containing only water and ampholytes was also run through the Rotofer PIEF cell and included in the FSH assay. Average binding from the most acidic fraction through the basic fraction of pH 8 was 99.4%, indicating absence of interference of ampholytes with FSH assay values. Fractions with an original pH above 8 were excluded because a decrease in the percent binding of tracer was observed indicating interference in the assay. A solution of 5.5 ml PBS containing 10 ng of purified pituitary FSH was prepared, passed over the FSH affinity column, and eluted FSH was subjected to isoelectric focusing as described above. The focused fractions were then neutralized and assayed for FSH by RIA. The total amount of FSH recovered was 7.4 ng.

**Statistical Analysis**

Data were analyzed using SAS (SAS Institute, Cary, NC). Hormone, metabolite, and production data were analyzed as repeated measures using the MIXED procedure. Fixed effects included follicle type, time and their interaction. Cow within follicle type was used as a random effect, with day as the repeated statement, and a first-order autoregressive plus random effect covariance structure was used (18). When the interaction between follicle type and time was significant ($P<0.05$), pair-wise comparisons of individual means were carried out using the Tukey-Kramer test.

The estradiol, FSH, inhibin and follicle diameter data were all normalized to day of maximum follicle diameter (day 0). To minimize the effect of substantial differences among cows in baseline concentrations of estradiol, inhibin A and FSH, the plasma concentration of each hormone measured on day -7 (earliest measurement available for all cows) was used as a covariate in the statistical analysis. The proportion of FSH isoforms with a pI >5 was calculated for each cow, and differences between ovulatory and non-ovulatory cows were analyzed using a one-way ANOVA test.

## RESULTS

Circulating concentrations of estradiol are illustrated in Figure 1. Cows with an ovulatory follicle had a distinct preovulatory rise in circulating estradiol during the final stages of follicular growth, whereas cows with non-ovulatory follicles had low concentrations of estradiol throughout follicle development, and all non-ovulatory follicles subsequently underwent atresia. Circulating estradiol began to deviate on day -4, and were significantly higher in cows with ovulatory follicles compared with cows with non-ovulatory follicles by day -3. Peak concentrations of circulating estradiol were observed on the day prior to maximum follicle diameter in the cows with a follicle that would ovulate [it is presumed that ovulation occurred on average 28 h after the peak of the preovulatory rise in estradiol]. Circulating inhibin A was elevated ($P<0.001$) in cows with ovulatory follicles compared to those with non-ovulatory

follicles as illustrated in Figure 1. The deviation between groups was evident as early as day -5, but mean concentrations were not significantly higher in ovulatory cows until day -3. Circulating inhibin A concentrations returned to baseline concentrations on day +1.

Plasma concentrations of FSH were lower ($P<0.01$) in cows with ovulatory follicles compared with cows not ovulating the dominant follicle (Figure 1). The cows with a non-ovulatory follicle displayed relatively constant circulating FSH, whereas pre- and peri-ovulatory increases in plasma FSH were evident (d -1 to +1) in cows with an ovulatory follicle. Follicle diameter was larger ($P=0.05$) in cows with an ovulatory follicle (Figure 2).

The distribution of FSH isoforms during the final stages of follicle development in three representative cows with an ovulatory follicle and three representative cows with a non-ovulatory follicle are depicted in Figures 3 and 4. A shift in FSH isoform profile toward a greater abundance of less acidic isoforms is apparent in the cows with an ovulatory follicle compared to cows with a non-ovulatory follicle. The proportion of FSH isoforms in fractions with a pH > 5.0 was calculated for each cow. Cows with an ovulatory follicle had a significantly higher percentage of FSH isoforms with a pI >5 ($32.5 \pm 0.6\%$ vs. $43.2 \pm 1.2\%$; $P<0.001$).

Dry matter intake (DMI), EB, milk production, and circulating levels of metabolic hormones and metabolites are summarized in Table 1. Prepartum DMI, EB and plasma insulin were higher, and circulating NEFA was lower, in cows with future ovulatory follicles. Postpartum DMI, EB, and plasma insulin and IGF-I concentrations were higher, while circulating NEFA concentrations were lower in cows with ovulatory follicles.

## DISCUSSION

This study has highlighted marked differences in endocrine status between cows with ovulatory and non-ovulatory follicles during the first postpartum follicle wave. As expected, all cows with an ovulatory follicle displayed a preovulatory rise in estradiol. In addition, cows with ovulatory follicles had greater circulating inhibin A concentrations, and reduced plasma FSH, reflecting the combined negative feedback effects of estradiol and inhibin A on pituitary FSH

release (19). During the final stages of follicle development, a pronounced shift toward an increase in less acidic FSH isoforms was observed in cows with an ovulatory follicle.

The inhibins are dimers composed of a unique α-subunit with either a βA or βB subunit, resulting in inhibin A (α-βA) or inhibin B (α-βB) (20). Granulosa cell secretion of dimeric inhibin A is regulated by FSH (21,22), and increases as the dominant follicle approaches preovulatory status (17,23). In the current study, cows with ovulatory follicles had higher circulating inhibin A compared to cows with non-ovulatory follicles, despite having lower circulating FSH concentrations. Thus, cows with non-ovulatory follicles have reduced inhibin A production in addition to lower estradiol output. Inhibin A acts at the level of the pituitary to reduce FSH release (19), and locally at the level of the ovary to stimulate thecal androgen production (23), but appears to inhibit granulosa cell estradiol synthesis (24). More acidic FSH isoforms were better at stimulating inhibin-α subunit gene expression than less acidic isoforms in vitro (25). Our in vivo observations provide little support for this effect, as the more acidic isoforms were more abundant in the non-ovulatory cows, yet they had lower circulating inhibin A concentrations.

It is widely held that an increase in LH pulse frequency during the preovulatory period stimulates greater follicular androgen synthesis, which in turn serves as a substrate for aromatase to synthesize estradiol (26,27). Administration of frequent exogenous LH pulses proved to be an important stimulus for ovulation in some early postpartum anestrous cows, but failed to increase circulating estradiol—and hence failed to induce ovulation—in approximately half of the cows (8). Thus, in addition to critical LH pulses, the signal that specifically increases granulosa cell aromatase activity may also have been lacking in anovulatory cows. It has previously been demonstrated that a shift in the FSH isoform distribution occurs prior to ovulation in human beings (13,28) and cattle (10). Though less acidic isoforms have a short half-life in circulation (12), they have potent biological activities at the level of the ovary (11,29), and display a greater capacity to induce aromatase expression than the more acidic isoforms (25). In the current study, we observed an increase in less acidic FSH isoforms during the final stages of follicle

development in cows with an ovulatory follicle, associated with significantly greater circulating inhibin A and estradiol concentrations. Increased output of inhibin by the selected dominant follicle may upregulate LH-induced androgen secretion that is required to sustain a high level of estradiol secretion from the preovulatory follicle (30).

Each pulse of hypothalamic GnRH stimulates pituitary release of a pulse of both LH and FSH, but additional non-GnRH associated pulses of FSH also exist (31,32). As it is difficult to identify FSH pulses in the peripheral circulation, it is thought that the FSH released during episodic FSH pulses is comprised of the faster clearing isoforms (32). Administration of frequent exogenous pulses of GnRH results in increased pituitary (33) and circulating (13) concentrations of less acidic isoforms. Moreover, high circulating estradiol concentrations result in increased pituitary synthesis of less acidic isoforms via reduced expression and activity of enzymes involved in posttranslational processing of FSH (34). Thus, increased hypothalamic GnRH pulse frequency stimulates pituitary release of LH and less acidic FSH isoforms, increasing follicular estradiol synthesis, which in turn has feedback effects on the pituitary to sustain the synthesis and release of less acidic isoforms. The complexity of this system is increased by the enhanced negative feedback effects of estradiol on hypothalamic GnRH release during negative energy balance (35,36). The positive and negative feedback effects of estradiol are mediated by specific regions of the hypothalamus (37), and estrogen receptor immunoreactivity in these specific regions is sensitive to nutritional status (38).

We propose that a sensor of energy balance plays a vital role in determining estrogen receptor expression in particular hypothalamic nuclei. During negative energy balance, the sensitivity of hypothalamic estradiol negative feedback centers is enhanced, and GnRH pulsatility is reduced. Consequently, pituitary release of LH and the less acidic FSH isoforms is also reduced, resulting in diminished follicular estradiol synthesis. However, because of the heightened sensitivity to the negative feedback effects of estradiol, the estradiol produced maintains low frequency hypothalamic GnRH pulse release, ultimately resulting in follicular atresia. The situation is reversed for animals in less negative energy balance, with estradiol

negative feedback centers being less sensitive to estradiol, allowing frequent GnRH pulses to stimulate release of LH and less acidic FSH isoforms, and thus generating an estrogen-active follicle. As the sensitivity of the negative feedback effects of estradiol are reduced, frequent release of hypothalamic GnRH pulses is maintained. Together with the effects of estradiol to increase pituitary synthesis of less acidic isoforms, frequent stimulation of the ovary with LH and less acidic FSH isoforms is continued, resulting in a robust preovulatory rise in estradiol, ultimately leading to ovulation following the LH surge. LH pulse frequency was not measured in this study, but it is presumed that LH pulses were more frequent in cows with ovulatory follicles. Though all the cows reported in this study were in NEB during the postpartum period, the calculated EB status, DMI, and hormone and metabolite profiles were indicative of better energetic status in cows with an ovulatory follicle (Table 1). These variables are reported in greater detail and discussed in a separate publication (15). The importance of circulating IGF-I during bovine follicular development is established (39).

In conclusion, this study illustrated that cows with ovulatory follicles had less negative energy balance and greater dry matter intake throughout the transition period, and this was associated with an improved metabolic hormone profile. Information regarding energetic status is conveyed to hypothalamic nuclei responsible for gonadotropin release. It is likely that the combination of frequent LH pulses and accompanying pituitary release of less acidic FSH isoforms provides a stimulatory environment for development of estrogen-active dominant follicles and ovulation.

## ACKNOWLEDGEMENTS

We are grateful to B. Berggren-Thomas, T. Janda, T. Muscato, and M. Partridge for technical assistance, to the farm staff at the Cornell University Dairy Teaching and Research Center for care of the cows, and to D.E. Bauman and A.N. Pell for generously allowing use of laboratory equipment. Advice and assistance from G.G. Ignotz and W. B. Currie is also gratefully acknowledged. Inhibin antiserum was generously provided by N.P. Groome (Oxford Brookes University, UK), and antisera to human IGF-1 and bovine FSH-β was obtained through NHPP, NIDDK, and Dr. A.F. Parlow. This research was supported by funds allocated to USDA regional project NE-161, and a Walsh Fellowship from Teagasc (The Irish Agriculture and Food Development Authority) to STB.

## REFERENCES


1 Beam SW, Butler WR. Energy balance and ovarian follicle development prior to the first ovulation postpartum in dairy cows receiving three levels of dietary fat. Biol Reprod 1997;56:133-142.

2 Beam SW, Butler WR. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. J Reprod Fertil Suppl 1999;54:411-424.

3 Garverick HA. Ovarian follicular cysts in dairy cows. J Dairy Sci 1997;80:995-1004.

4 Darwash AO, Lamming GE, Woolliams JA. The phenotypic association between the interval to post-partum ovulation and traditional measures of fertility in dairy cattle. Anim Sci 1997;65:9-16.

5 Butler WR, Smith RD. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. J Dairy Sci 1989;72:767-783.

6 Canfield RW, Butler WR. Energy balance and pulsatile LH secretion in early postpartum dairy cattle. Domest Anim Endocrinol 1990;7:323-330.

7 Hampton JH, Salfen BE, Bader JF, Keisler DH, Garverick HA. Ovarian follicular responses to high doses of pulsatile luteinizing hormone in lactating dairy cattle. J Dairy Sci 2003;86:1963-1969.

8 Duffy P, Crowe MA, Boland MP, Roche JF. Effect of exogenous LH pulses on the fate of the first dominant follicle in postpartum beef cows nursing calves. J Reprod Fertil 2000;118:9-17.

9 Green ED, Baenziger JU. Asparagine-linked oligosaccharides on lutropin, follitropin, and thyrotropin. II. Distributions of sulfated and sialylated oligosaccharides on bovine, ovine, and human pituitary glycoprotein hormones. J Biol Chem 1988;263:36-44.

10 Cooke DJ, Crowe MA, Roche JF. Circulating FSH isoform patterns during recurrent increases in fsh throughout the oestrous cycle of heifers. J Reprod Fertil 1997;110:339-345.

11 Ulloa-Aguirre A, Midgley AR, Jr., Beitins IZ, Padmanabhan V. Follicle-stimulating isohormones: Characterization and physiological relevance. Endocr Rev 1995;16:765-787.

12 West CR, Carlson NE, Lee JS, McNeilly AS, Sharma TP, Ye W, Padmanabhan V. Acidic mix of FSH isoforms are better facilitators of ovarian follicular maturation and E2 production than the less acidic. Endocrinology 2002;143:107-116.

13 Zambrano E, Olivares A, Mendez JP, Guerrero L, Diaz-Cueto L, Veldhuis JD, Ulloa-Aguirre A. Dynamics of basal and gonadotropin-releasing hormone-releasable serum follicle-stimulating hormone charge isoform distribution throughout the human menstrual cycle. J Clin Endocr Metab 1995;80:1647-1656.

14 Ulloa-Aguirre A, Timossi C, Barrios-de-Tomasi J, Maldonado A, Nayudu P. Impact of carbohydrate heterogeneity in function of follicle-stimulating hormone: Studies derived from in vitro and in vivo models. Biol Reprod 2003;69:379-389.

15 Butler ST, Pelton SH, Butler WR. Energy balance, metabolic status, and the first postpartum ovarian follicle wave in cows administered propylene glycol. J Dairy Sci 2006;89:2938-2951.

16 Savio JD, Boland MP, Roche JF. Development of dominant follicles and length of ovarian cycles in post-partum dairy cows. J Reprod Fertil 1990;88:581-591.

17 Bleach EC, Glencross RG, Feist SA, Groome NP, Knight PG. Plasma inhibin A in heifers: Relationship with follicle dynamics, gonadotropins, and steroids during the estrous cycle and after treatment with bovine follicular fluid. Biol Reprod 2001;64:743-752.

18 Littell RC, Henry PR, Ammerman CB. Statistical analysis of repeated measures data using SAS procedures. J Anim Sci 1998;76:1216-1231.
19 Findlay JK, Clarke IJ. Regulation of the secretion of FSH in domestic ruminants. J Reprod Fertil Suppl 1987;34:27-37.
20 Lin SY, Morrison JR, Phillips DJ, de Kretser DM. Regulation of ovarian function by the TGF-β superfamily and follistatin. Reproduction 2003;126:133-148.
21 Boudjemaa ML, Rouillier P, Bhatia B, Silva JM, Guilbault LA, Price CA. Effect of FSH and cell localization on dimeric inhibin-A secretion from bovine granulosa cells in culture. J Endocrinol 2000;165:207-215.
22 Glister C, Tannetta DS, Groome NP, Knight PG. Interactions between follicle-stimulating hormone and growth factors in modulating secretion of steroids and inhibin-related peptides by nonluteinized bovine granulosa cells. Biol Reprod 2001;65:1020-1028.
23 Knight PG, Glister C. Potential local regulatory functions of inhibins, activins and follistatin in the ovary. Reproduction 2001;121:503-512.
24 Jimenez-Krassel F, Winn ME, Burns D, Ireland JL, Ireland JJ. Evidence for a negative intrafollicular role for inhibin in regulation of estradiol production by granulosa cells. Endocrinology 2003;144:1876-1886.
25 Timossi CM, Barrios-de-Tomasi J, Gonzalez-Suarez R, Arranz MC, Padmanabhan V, Conn PM, Ulloa-Aguirre A. Differential effects of the charge variants of human follicle-stimulating hormone. J Endocrinol 2000;165:193-205.
26 Baird DT. Pulsatile secretion of LH and ovarian estradiol during the follicular phase of the sheep estrous cycle. Biol Reprod 1978;18:359-364.
27 Diskin MG, Mackey DR, Roche JF, Sreenan JM. Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. Anim Reprod Sci 2003;78:345-370.
28 Padmanabhan V, Lang LL, Sonstein J, Kelch RP, Beitins IZ. Modulation of serum follicle-stimulating hormone bioactivity and isoform distribution by estrogenic steroids in normal women and in gonadal dysgenesis. J Clin Endocr Metab 1988;67:465-473.
29 Padmanabhan V, Lee JS, Beitins IZ. Follicle-stimulating isohormones: Regulation and biological significance. J Reprod Fertil Suppl 1999;54:87-99.
30 Knight PG, Glister C. TGF-β superfamily members and ovarian follicle development. Reproduction 2006;132:191-206.
31 Padmanabhan V, McFadden K, Mauger DT, Karsch FJ, Midgley AR, Jr. Neuroendocrine control of follicle-stimulating hormone (FSH) secretion. I. Direct evidence for separate episodic and basal components of FSH secretion. Endocrinology 1997;138:424-432.
32 Padmanabhan V, Brown MB, Dahl GE, Evans NP, Karsch FJ, Mauger DT, Neill JD, Van Cleeff J. Neuroendocrine control of follicle-stimulating hormone (FSH) secretion: III. Is there a gonadotropin-releasing hormone-independent component of episodic FSH secretion in ovariectomized and luteal phase ewes? Endocrinology 2003;144:1380-1392.
33 Galle PC, Ulloa-Aguirre A, Chappel SC. Effects of oestradiol, phenobarbitone and luteinizing hormone releasing hormone upon the isoelectric profile of pituitary follicle-stimulating hormone in ovariectomized hamsters. J Endocrinol 1983;99:31-39.
34 Ulloa-Aguirre A, Maldonado A, Damian-Matsumura P, Timossi C. Endocrine regulation of gonadotropin glycosylation. Arch Med Res 2001;32:520-532.

35 Imakawa K, Day ML, Zalesky DD, Clutter A, Kittok RJ, Kinder JE. Effects of 17 beta-estradiol and diets varying in energy on secretion of luteinizing hormone in beef heifers. J Anim Sci 1987;64:805-815.
36 Ichimaru T, Mori Y, Okamura H. A possible role of neuropeptide Y as a mediator of undernutrition to the hypothalamic gonadotropin-releasing hormone pulse generator in goats. Endocrinology 2001;142:2489-2498.
37 Caraty A, Fabre-Nys C, Delaleu B, Locatelli A, Bruneau G, Karsch FJ, Herbison A. Evidence that the mediobasal hypothalamus is the primary site of action of estradiol in inducing the preovulatory gonadotropin releasing hormone surge in the ewe. Endocrinology 1998;139:1752-1760.
38 Hileman SM, Lubbers LS, Jansen HT, Lehman MN. Changes in hypothalamic estrogen receptor-containing cell numbers in response to feed restriction in the female lamb. Neuroendocrinology 1999;69:430-437.
39 Sudo N, Shimizu T, Kawashima C, Kaneko E, Tetsuka M, Miyamoto A. Insulin-like growth factor-I (IGF-I) system during follicle development in the bovine ovary: Relationship among IGF-I, type 1 IGF receptor (IGFR-1) and pregnancy-associated plasma protein-a (PAPP-A). Mol Cell Endocrinol 2007;264:197-203.

FIGURE LEGENDS

Figure 1. Circulating plasma concentrations of estradiol, inhibin A, and FSH during the final stages of follicular development in cows with ovulatory (Ov) and non-ovulatory (Non-Ov) follicles during the first postpartum follicle wave (n=9/group). Upper panel: a pre-ovulatory rise in circulating estradiol commenced in ovulatory cows 4 days prior to maximum follicle diameter, and plasma concentrations were first significantly higher on day -3. Peak concentrations were observed on the day prior to maximum follicle diameter. Cows with non-ovulatory follicles had low and stable circulating estradiol concentrations throughout the final stages of follicle development. A significant effect of follicle type was observed ($P<0.001$; pooled SEM = 0.22 pg/ml). * denotes that the follicle types differ at the indicated time point ($P<0.001$). Middle panel: Circulating concentrations of inhibin-A were higher in cows with an ovulatory follicle (follicle type, $P<0.001$; pooled SEM = 15.7 pg/ml). Differences first became significant on day -3 and inhibin A concentrations started to return to basal concentrations on day 0. * denotes that the follicle types differ at the indicated time point ($P<0.05$). Lower panel: Circulating concentrations of FSH were lower in cows with an ovulatory follicle (follicle type, $P<0.01$; pooled SEM = 0.04 ng/ml). Cows with ovulatory follicles exhibited pre-ovulatory (day -1 vs. day -2; $P<0.01$) and peri-ovulatory (day 1 vs. day 0; $P=0.08$) increases in circulating FSH.

Figure 2. Follicle growth pattern in cows with ovulatory (Ov) and non-ovulatory (Non-Ov) first postpartum follicle waves. Follicle diameter was measured by transrectal ultrasound 3 days per week, and the diameter between measurements was calculated by linear interpolation. Follicle diameter was larger in ovulatory cows (follicle type, $P = 0.051$; pooled SEM = 0.9 mm). Statistical analysis was conducted from day -6 to day 0; days 1 to 6 are included for illustrative purposes only.

Figure 3. Distribution of FSH isoforms in plasma collected from 3 representative cows with ovulatory follicles during the preovulatory peak in circulating estradiol. FSH was isolated from plasma using a specific affinity column and isoelectric focusing was carried out using liquid phase electrophoresis. All fractions were assayed for FSH by RIA and fraction number is shown on the horizontal axis. The total amount of FSH recovered in fractions within the pH gradient 3 to 8 for all cows with ovulatory follicles was $1.23 \pm 0.16$ ng (mean ± SEM). The FSH profile is indicated by the open circles (○), and the pH gradient is illustrated by the solid line.

Figure 4. Distribution of FSH isoforms in plasma collected from 3 representative cows with non-ovulatory follicles during the period of maximum follicle diameter. FSH was isolated from plasma using a specific affinity column and isoelectric focusing was carried out using liquid phase electrophoresis. All fractions were assayed for FSH by RIA and fraction number is shown on the horizontal axis. The total amount of FSH recovered in fractions within the pH gradient 3 to 8 for all cows with non-ovulatory follicles was $1.15 \pm 0.04$ ng (mean ± SEM).The FSH profile is indicated by the open circles (○), and the pH gradient is illustrated by the solid line.

Table 1. Pre- and postpartum means for EB, DMI, energy corrected milk (ECM) and hormone and metabolite concentrations in dairy cows (n = 9/group) with non-ovulatory (Non-Ov) and ovulatory (Ov) dominant follicles.

| | Prepartum | | | | Postpartum | | | |
|---|---|---|---|---|---|---|---|---|
| | Non-Ov | Ov | SEM | P-value | Non-Ov | Ov | SEM | P-value |
| EB (Mcal/day)[1] | 9.0 | 13.6 | 0.66 | <0.001 | -11.4 | -6.9 | 0.90 | <0.001 |
| DMI (kg/day)[1] | 12.9 | 15.6 | 0.46 | <0.001 | 18.9 | 20.9 | 0.55 | 0.018 |
| ECM (kg/day)[1] | | | | | 50.4 | 48.1 | 1.5 | 0.3 |
| Insulin (ng/ml)[2] | 0.64 | 1.10 | 0.12 | 0.014 | 0.31 | 0.45 | 0.02 | <0.001 |
| Glucose (mg/dl)[2] | 64.9 | 65.1 | 1.1 | 0.9 | 52.7 | 56.9 | 1.6 | 0.080 |
| NEFA (mmol/l)[3] | 255 | 112 | 48 | 0.050 | 494 | 319 | 34 | <0.001 |
| IGF-I (ng/ml)[4] | 116 | 127 | 8.8 | 0.4 | 41.6 | 68.7 | 6.4 | 0.009 |

[1] Daily prepartum EB and DMI measurements commenced on day -21 and continued until day -1. Daily postpartum EB, DMI, and ECM measurements commenced on day 0 and continued until day 30.

[2] Insulin and glucose were measured on prepartum days -3, -6 and -9, and then every other day until day 24 postpartum.

[3] NEFA was measured on prepartum days -3, -6 and -9, daily from parturition until day 3 postpartum, and every third day thereafter until day 24.

[4] IGF-I was measured on day -7 prepartum, the day of parturition, and days 5, 15 and 25 postpartum.

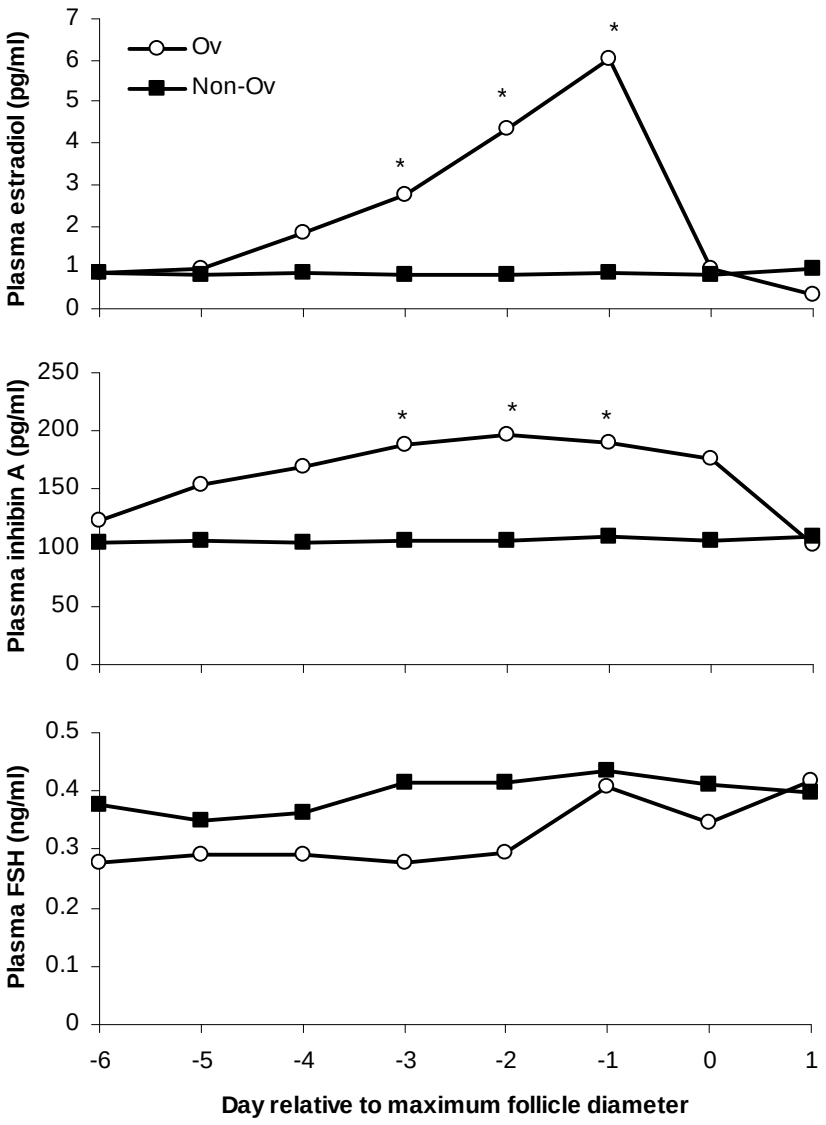


Figure 1 (Butler)

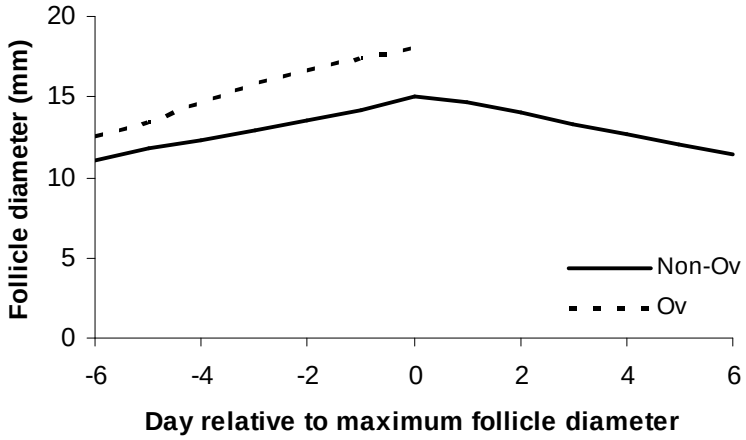


Figure 2 (Butler)

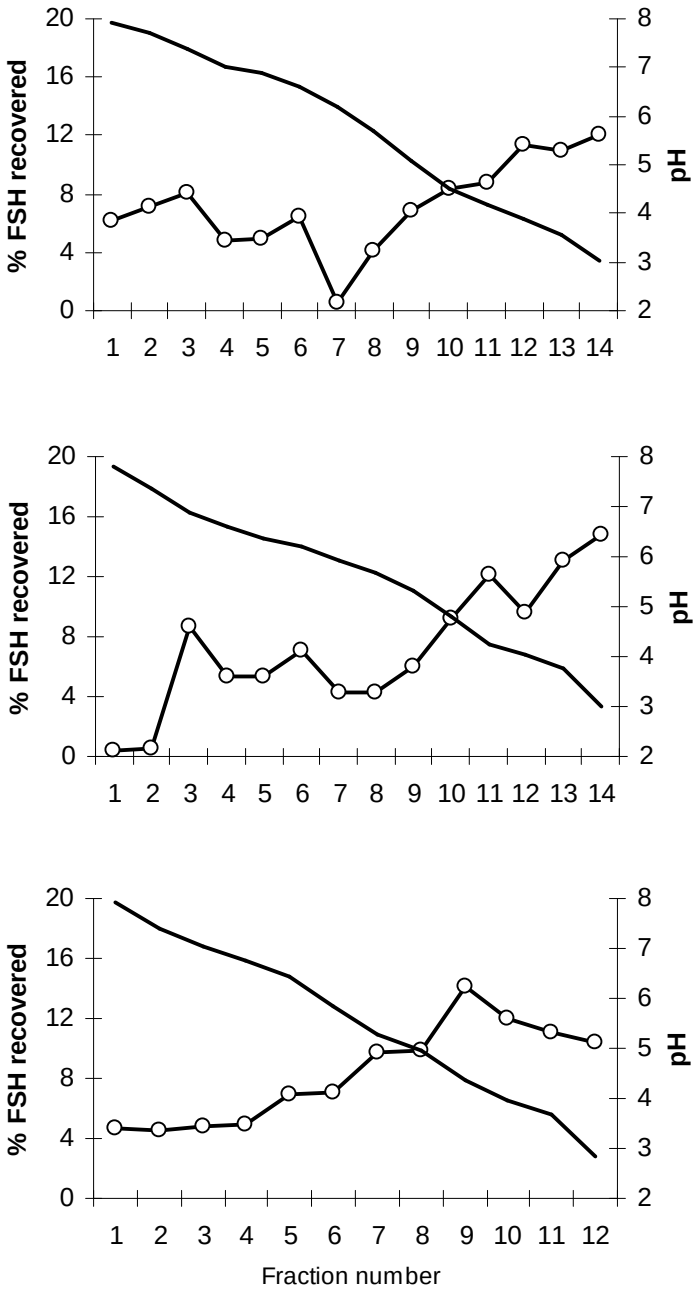


Figure 3 (Butler)

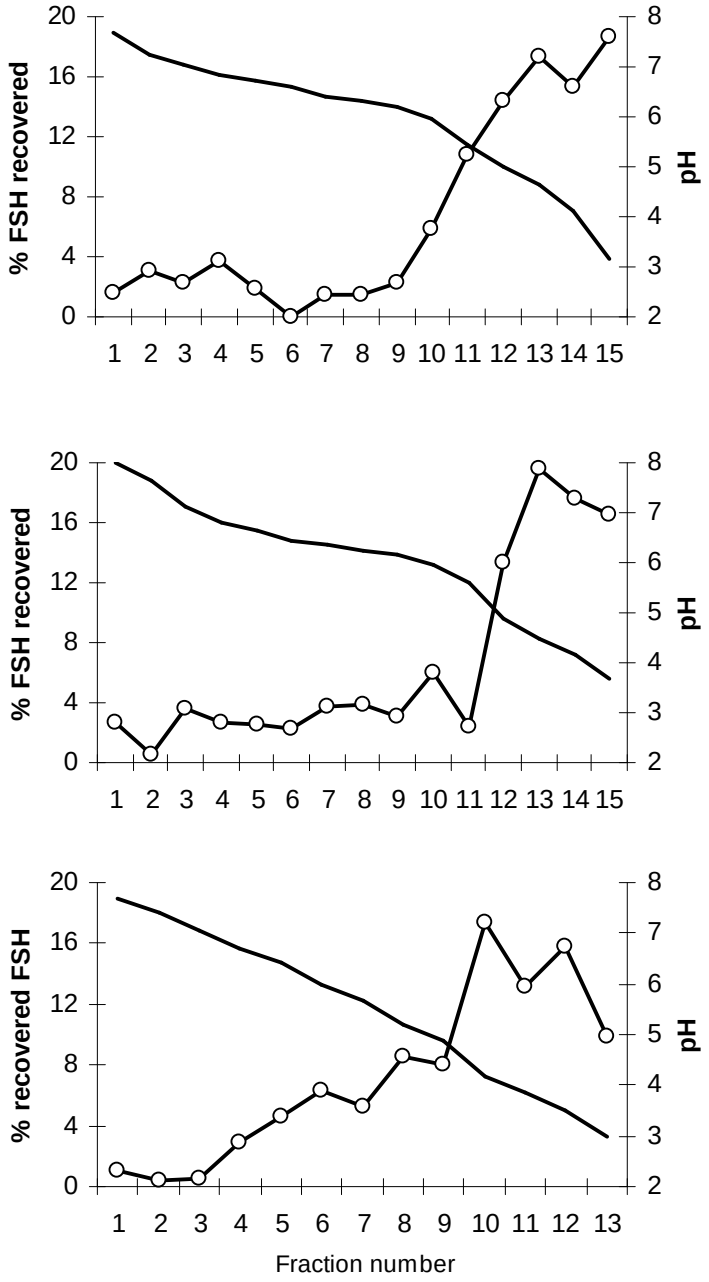


Figure 4 (Butler)