MILLER PEREIRA PALHÃO

INDUCED CODOMINANCE AND DOUBLE OVULATION AND NEW APPROACHES ON LUTEOLYSIS IN CATTLE

Dissertation submitted in partial fulfillment of the requirements for the degree of *Doctor Scientiae* in Animal Science.

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APPROVED on February 26th, 2010.

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Prof. Marcelo Teixeira Rodrigues
(Advisor)

DEDICATION

For this dedication I would like to paraphrase the professor Phillip L. Senger. “There are two lasting benefits we can give our students, children and other people we associate with. The first are **roots** and the other **wings**.”

This thesis is dedicated to my grandmothers - Dinorah Ribeiro and Maria do Carmo Ribeiro (*in memoriam*) - to my grandfathers - Joacir Ribeiro and Pedro Palhão (my **roots**) - and to a little angel that inspires me every day - Eloa A. Palhão (my **wings**).

A better world would come from benefits of science, discovering the closest balance between man and environment (**Miller Pereira Palhão**, 2010).

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BIOGRAPHY

Miller Pereira Palhão is son of Milton Pereira Palhão and Tânia Maria Ribeiro Palhão, was born on January 10t^{h} (1978), in the city of Paraguaçu, state of Minas Gerais.

Had finished (July 2003) the School of Veterinary Science at the Federal University of Lavras (UFLA), Lavras, MG.

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Had joined (August 2006) the Doctoral Scientiae program at the Department of Animal Science, Federal University of Viçosa (UFV). He had finished (February 2009) a trainee program in Physiology of Animal Reproduction at the Pathobilogical Science of the University of Wisconsin, Madison, US. Submitting this dissertation on February 2010.

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PALHÃO, Miller Pereira, D.Sc., Universidade Federal de Viçosa, fevereiro de 2010. **Indução de codominância e dupla ovulação e novas abordagens em luteólise de bovinos**. Orientador: Marcelo Teixeira Rodrigues. Co-orientadores: João Henrique Moreira Viana e Álan Maia Borges.

O modelo de punção e aspiração folicular no quarto dia após a ovulação foi utilizado nos capítulos 2 e 3, com a finalidade de aumentar a incidência de ovulações duplas em novilhas Holandesas. Deste modo, os folículos ovarianos (≥ 5 mm) foram destruídos 4 dias após a ovulação, induzindo um proeminente pico de FSH e a emergência de uma nova onda folicular. Dois dias mais tarde (6º dia), duas injeções de prostaglandina F2α (intervaladas de 12 h) foram administradas, favorecendo a ovulação. O objetivo do primeiro estudo (Capítulo 2) foi comparar o crescimento folicular e as concentrações plasmáticas dos hormônios reprodutivos em novilhas apresentando simples versus dupla ovulação. Do total de 31 novilhas, 16 (52%) ou 15 (48%) desenvolveram, respectivamente, um único ou mais de um folículo dominante (≥ 10 mm) na nova onda emergida após a punção e aspiração folicular. Do grupo de novilhas com dois folículos dominantes, o segundo maior folículo ovulou em 9 (60%), e a taxa geral de ovulações múltiplas foi de 29% (9/31). Posteriormente, o crescimento folicular e as concentrações hormonais plasmáticas foram comparados entre os grupos de novilhas com ovulações simples (n=12) e duplas (n=8). Quando os dados foram normalizados para o pico de LH, as concentrações médias de FSH - antes e após o pico -, o intervalo (horas) da divergência folicular ao pico pré-ovulatório de LH e o diâmetro do folículo pré-ovulatório (F1) foram menores em animais com dupla ovulação. Ao passo que as concentrações de estradiol (E2), 6 h antes e no momento do pico de LH (Hora 0), foram maiores quando comparadas ao grupo de animais com ovulações simples. Nestes termos, o diâmetro pré-ovulatório de F1 foi 1,5 mm menor em novilhas

com ovulações duplas, quando o pico de LH foi induzido aproximadamente 14 h antes do observado para aquelas com ovulação simples. O objetivo dos experimentos realizados no capítulo 3 foi determinar o papel do estradiol-17β (E2) nas diferenças entre animais com ovulações simples e duplas. Foram administradas oito (0,15 mg/injeção) ou 4 injeções de E2 (0,07 ou 0,09 mg/injeção), a cada intervalo de 6 h, em dois experimentos. Os experimentos iniciaram próximo ao momento esperado da divergência folicular (n = 6 a 8 novilhas/grupo). Em cada experimento, os tratamentos com E2 induziram os picos preovulatórios de FSH e LH, concomitantemente, em média de 24 a 34 h após a primeira injeção, comparando com 58 h no grupo veículo. Ao momento do pico de LH, o diâmetro médio do folículo preovulatório foi de 13 mm no grupo controle e 10 mm em todos os grupos tratados com E2. Desta maneira, os tratamentos com E2 induziram um pico de LH significantemente precoce com ovulação de folículos significantemente menores. O tratamento com E2 não reduziu as concentrações de FSH antes do pico preovulatório de FSH como foi demonstrado para novilhas com ovulações duplas. As doses de 0,15 mg de E2 foram associadas com uma maior concentração de FSH ao pico preovulatório de FSH, no entanto, as doses de 0,07 ou 0,09 mg não tiveram efeito similar. Estes resultados não suportaram a hipótese de que o E2 foi responsável pelas reduzidas concentrações de FSH antes do pico preovulatório de FSH reportados em novilhas com ovulações duplas. Porém, as hipóteses de que as concentrações de E2, aumentadas em novilhas com dois folículos preovulatórios, foram responsáveis pela ocorrência de um pico preovulatório de LH precoce e pelo menor diâmetro dos folículos preovulatórios em novilhas com dupla ovulação. Os efeitos luteolíticos da prostaglandina F2alpha (PGF) exógena foram também estudados durante o diestro em 42 novilhas Holandesas. As concentrações plasmáticas de PGF foram acessadas através da análise de seu metabólito (PGFM). No experimento 1, uma única injeção de 4,0 mg de PGF administrada dentro do corno

uterino ipsilateral ao corpo lúteo resultou em um declínio rápido na concentração de progesterona, enquanto que injeções sequenciais de 0,25 ou 1,0 mg a cada 12 h resultou em uma queda gradual da progesterona ($P<0,05$) seguindo cada injeção. Ocorreu um aumento na concentração de progesterona durante os primeiros 5 minutos após a injeção de 4,0 mg, seguindo de decréscimo e luteólise. Com os resultados do experimento 2, a infusão intrauterina de 2 h com um total de 0,5 mg de PGF foi considerada a que melhor simulou um pulso natural de PGFM. No experimento 3, a simulação de pulsos sequenciais a cada 12 h resultou em uma queda continua na concentração de progesterona, atingindo níveis <1 ng/ml no início do quarto pulso simulado. Em contraste, a simulação de um único pulso implicou em um decréscimo na concentração de progesterona por 6 h, permanecendo constante por 3 dias, seguindo o retorno as concentrações semelhantes às do grupo controle. Os resultados indicaram que uma dose excessiva de PGF pode estimular uma resposta não fisiológica da progesterona e suportam a hipótese de que pulsos sequenciais de PGF são necessários para estimular a luteólise natural em bovinos.

ABSTRACT

PALHÃO, Miller Pereira, D.Sc., Universidade Federal de Viçosa, February, 2010. **Induced codominance and double ovulation and new approaches on luteolysis in cattle**. Advisor: Marcelo Teixeira Rodrigues. Co-advisors: João Henrique Moreira Viana and Álan Maia Borges.

The day-4 ablation model for increasing the incidence of double ovulations in heifers was used for the chapters 2 and 3. In this regard, follicles ≥ 5 mm were ablated at 4 d post-ovulation to induce a prominent FSH surge and a new follicular wave, and two injections of prostaglandin F2α (12 h apart) were given two days later (6 d) to favor ovulation. The objective of the first study (Chapter 2) was to compare follicle growth and plasma hormone concentrations associated with single versus double ovulations. From a total of 31 heifers, 16 (52%) or 15 (48%) developed, respectively, a single or more than one dominant follicle (≥ 10 mm) in the follicular wave after ablation. For heifers with two dominant follicles, second-largest follicle ovulated in 9 (60%) heifers and the overall double ovulation rate was 29% (9/31). Follicle diameters and plasma hormone concentrations were compared between single (n = 12) and double ovulators (n = 8). When the data were normalized to the peak of LH surge, a lower concentration of FSH averaged over hours and greater concentrations E2 before and at peak were observed in double ovulators. Consequently, the interval from follicle deviation to the preovulatory peak of LH surge was shorter and the diameter of the largest preovulatory follicle was smaller in double than single ovulators. In double-ovulating heifers when the peak of LH/FSH surge was observed approximately 14 h before than single-ovulating, the ovulatory F1 was 1.5 mm smaller. The objective of the chapter 3 was to determine the role of the oestratiol-17β (E2) in reported follicle and hormone differences between single and double ovulations. The E2 was given to heifers for eight treatment (1.2 mg/treatment) or four treatments (0.07 or 0.09 mg/treatment) at 6-h

intervals in two experiments beginning at the time off the expected deviation (n = 6 to 8 heifers/treatment group). In each experiment, the E2 treatments induced concomitant preovulatory surges in LH and FSH at mean of 24 to 34 h after first treatment, compared to 58 h in the vehicle groups. At the time of the LH peak, the diameter of the preovulatory follicle was a mean of 13 mm in the vehicle groups and 10 mm in all E2-treated groups. Thus, E2 treatments induced a significantly earlier LH surge with ovulation of significantly smaller follicles. The E2 treatment did not lower the FSH concentrations before the preovulatory FSH surge as has been reported for double ovulations. The 0.15 mg doses of E2 were associated with greater FSH concentration at peak of the preovulatory FSH surge, but the 0.07 or 0.09 mg doses did not have a similar effect. Results did not support the hypothesis that E2 is responsible for the reported reduced FSH concentrations before the preovulatory FSH surge in double ovulators. Results supported the hypotheses that increased E2 concentrations in heifers with double preovulatory follicles accounts for the reported earlier occurrence of the preovulatory LH surge and smaller preovulatory follicles in double ovulations. The luteolytic effects of exogenous prostaglandin F2alpha (PGF) were also studied during mid-diestrus in 42 Holstein heifers. Plasma concentrations of PGF were assessed by assay of PGFM. In experiment 1, a single intrauterine injection of 4.0 mg of PGF into the uterine horn ipsilateral to the corpus luteum resulted in a precipitous progesterone decline, whereas sequential injections of 0.25 or 1.0 mg every 12 h resulted in a stepwise decrease ($P<0.05$) following each injection. A progesterone increase occurred during the first 5 min before the luteolytic decrease but only for the 4.0-mg dose. From the results of experiment 2, a 2-h intrauterine infusion of a total of 0.5 mg of PGF was judged to best simulate a natural PGFM pulse. In experiment 3, simulation of sequential pulses at 12-h intervals resulted in a continuous precipitous decrease in progesterone to <1 ng/ml by the beginning of the fourth simulated pulse. In contrast, a single simulated

pulse resulted in a 6-h progesterone decrease to a constant concentration for 3 days after treatment, followed by a return to control concentrations. Results indicated that excessive PGF doses may stimulate nonphysiologic progesterone responses and supported the hypothesis that sequential PGF pulses are required to stimulate natural luteolysis in cattle.

THESIS ORGANIZATION

CHAPTER 1 – Literature review focused on mainly aspects of the folliculogesis, follicle deviation, follicle codominance, double ovulation and on effects of the estradiol over follicle development and hormonal profile. Additional information on vascularity of the corpus luteum and luteolysis were also provided. The end of this chapter brings the introduction and objectives for this dissertation.

CHAPTER 2 - There apparently have been no published reports in cattle on the pre-ovulatory temporal relationships between follicle development and plasma gonadotropin concentrations in association with single versus double ovulations. The experiment was designed to compare follicle growth and plasma hormone concentrations associated with single versus double ovulations.

CHAPTER 3 - These experiments tested the hypothesis that the increased concentrations of E2 from double preovulatory follicles (CHAPTER 2) accounts for the reduced concentrations of FSH, smaller preovulatory follicles, and earlier occurrence of the LH surge in double-ovulating heifers.

CHAPTER 4 – This last chapter is an additional study developed during the trainee period at the PBS department of the University of Wisconsin, Madison US. It was designed to: 1) determine if low IU doses of PGF require sequential treatment for the completion of luteolysis, 2) develop a dose and convenient method of delivery of exogenous PGF that would result in an approximation of a natural PGFM pulse, 3) simulate PGFM pulses and requirement for the stimulation of complete luteolysis.

CHAPTER 1

1.0. LITERATURE REVIEW

The literature review presented in this chapter will focus primarily on folliculogenesis, the physiologic mechanisms involved in the regulation of follicle deviation, codominance and double ovulation during the cattle estrous cycle. The enrollment of the estradiol on follicle development will be included. Extra information about angiogenesis, regulation of the luteal blood flow and luteolysis will be given. Most of the general aspects can cover different species, but the main focus will be the bovine and specifically Holstein heifers.

1.1. ESTROUS CYCLE AND FOLLICULAR WAVE

After puberty the female enters a period of reproductive cyclicity which continues throughout most of her productive life. Estrous cycles consist of a series of predictable reproductive events beginning at estrus (heat) and ending at the subsequent estrus (Senger 1999). In cattle, the reported average length of the estrous cycle varies between 18-24 days (Wishart 1972, Sirois & Fortune 1988, Ginther *et al.* 1989). There are four stages encompassing the estrus interval: proestrus, estrus, metestrus and diestrus. Each of these stages is a subdivision of luteal and follicular phases of the cycle. The luteal phase includes metestrus and diestrus, and the follicular phase embraces the proestrus and estrus (Senger, 1999; Hafez & Hafez 2004).

The estrous cycle and its phases in cattle were first described about 80 years ago (McNutt 1927). About nineteen years later, the mystery behind the estrous cycle events started to be uncovered. The relationship between the development of ovarian follicle and hormonal profile was described using a mouse model (Bullough 1946). However, until 1960, the available studies had concluded that there was no cyclic variation in

follicle number. Thus, in 1960, Rajakoski (1960) first proposed the theory of follicular growth pattern in two waves during the estrous cycle in cattle. This study was severely criticized because the methodology was based on qualitative assessment of data and not on current knowledge of the hormones profiles. In this regard, until the 1980's many studies have shown contradictory results regarding the theory of follicular waves and follicle development [reviewed in (Fortune 1993, Adams 1999)]. Only in the late 1980's, the introduction of ultrasonography allowed an understanding of follicular dynamics and their relationship to hormonal changes during the estrous cycle (Ginther *et al.* 1989). It was clearly demonstrated that follicular growth in cattle occurs in a wavelike pattern and the majority of the estrous cycles (>95%) are composed of either two or three follicular waves [reviewed in (Adams 1998)].

The predominance of two versus three waves of follicle development has been reported to vary in different laboratories (Ginther *et al.* 1989, Fortune 1993, Kulick *et al.* 1999, Noseir 2003, Sartori *et al.* 2004). However, the emergence of the first follicular wave occurs consistently on the day of ovulation (Day 0) in two- and three-waves estrous cycles. The emergence of the second wave occurs on Day 9 or 10 in two-wave cycles, and on Day 8 or 9 in three-wave cycles. A third wave emerges on Day 15 or 16 in three-wave cycles (Ginther *et al.* 1989, Kulick *et al.* 1999, Sartori *et al.* 2004). During the diestrus, with a progesterone (P4) environment, the dominant follicles of the successive waves undergo atresia. Conversely, the dominant follicle present at the onset of luteolysis becomes the ovulatory follicle.

In a two-waves estrous cycles, the corpus luteum (CL) begins to regress earlier (Day 16) than in three-waves [Day 19; (Ginther *et al.* 1989)], resulting in a shorter interovulatory interval (19-20 days versus 22-23 days, respectively). Thus, the so-called 21-day cycle exists only as an average between two- and three-wave cycles (Adams *et al.* 2008). The reason why some cattle have two waves of follicular development while

others have three is unclear (Salfen *et al.* 1999). The influence of the genetic, environmental and nutritional factors, in addition to factors related to age and parity on the number of the follicular waves per cycle have been intensely studied, but the results are not conclusive (Fortune 1993, Noseir 2003).

1.2. FOLLICLE DYNAMIC

1.2.1 EMERGENCE AND COMMON-GROWTH PHASE

At specific times during the luteal phase of the estrous cycle and at intervals of 7-10 days in most other reproductive status in cows (including the pre-pubertal, post-partum and early pregnant), a sudden (within 2-3 days) growth of 8-41 small follicles is initially detected by ultrasonography at a diameter of 3-4 mm (Roche *et al.* 1998, Ireland *et al.* 2000, Ginther *et al.* 2003b, Bo *et al.* 2008). Gonadotropins are probably not involved in the initiation of follicle growth, although previous studies investigating gene expression in pré-antral follicle have demonstrated that FSH receptor (FSHr) mRNA can be detected in follicles with only one or two layers of cells (Webb, 2003). However, the final stages of follicle growth are definitely dependent on gonadotropins profile (Webb *et al.* 2007). Thus, the development of the antral follicular beyond 3–4 mm is an FSH-dependent process (Austin *et al.* 2001). The emergence of the follicular wave is stimulated by a rise in plasma concentration of FSH (follicle stimulating hormone), that reaches the peak when the largest follicle is about 5 mm in diameter. The cohort of follicles develops at similar growth rate for approximately 2-3 days. Within 12 h of the peak in FSH, a group of three to five growing follicles of 5–6 mm in diameter is detectable, and subsequent follicular selection proceeds during declining FSH concentrations (Ginther *et al.* 2003b, Beg & Ginther 2006).

1.2.2. FOLLICLE DEVIATION

The FSH concentrations decline during the remaining common-growth phase and the nadir is reached 10 to 24 h after follicular selection (Ginther *et al.* 1999, Bergfelt *et al.* 2000, Ginther *et al.* 2000). The essence of selection of a dominant follicle has been postulated (Ginther *et al.* 2000) to be a close, within 8 h (Ginther *et al.* 1999), two-way functional coupling between the changing concentrations of FSH and the changing diameters of follicles. The growing follicles cause the FSH decline from the peak of the surge until the selection of the dominant follicle, even though the follicles continue to require FSH. Thus, only the more developed largest follicle (F1) is able to utilize the low FSH. The remaining follicles have not reached a similar stage of development, and because of their continued dependency on FSH, they become susceptible to the low concentration.

In this regard, at the end of the common-growth phase, usually one follicle continues to grow and becomes the developing dominant follicle and the remaining follicles (subordinates) regress. The end of the common-growth phase and the beginning of a distinctive difference in growth rates between the two largest follicles has been termed follicle deviation (Ginther *et al.* 1997) and, in Holstein heifes, occurs when the largest follicle reaches a mean diameter of 8.5 mm (Ginther *et al.* 1996). Even though the F1 is the most likely to reach a critical development stages first and become dominant, there is no evidence that dominance is exerted by the F1 over its cohort during the common-growth phase (Ginther *et al.* 2004, Beg & Ginther 2006). In this regard, the F1 at first detection of a follicular wave became the dominant follicle in about 60% of cattle (Ginther *et al.* 1996).

The phenomenon of follicle deviation has been attributable to enhanced sensitivity or responsiveness of the future dominant follicle to FSH and Luteinizing Hormone (LH) [reviewed by (Ginther *et al.* 1996)]. On average, a transient elevation in

LH begins before deviation and decreases after deviation (Kulick *et al.* 1999, Bergfelt *et al.* 2000, Kulick *et al.* 2001). The LH requirements for the development of F1 after deviation were indicated by the smaller diameter when LH is experimentally reduced (Ginther *et al.* 2001), and suppression of LH prevents follicular growth beyond 9 mm (Gong *et al.* 1995). In addition, when the dominant follicle reaches the diameter of about 10 mm, it acquires LH receptors on its granulosa cells and the ovulatory capacity in response to the LH surge (Sartori *et al.* 2001).

The selection of a dominant follicle during the oestrous cycle in cattle occurs during each of one or two anovulatory follicular waves and during the ovulatory wave [reviewed in (Fortune 2001, Ginther *et al.* 2003a, Beg & Ginther 2006, Webb *et al.* 2007, Adams *et al.* 2008, Mihm & Evans 2008, Aerts & Bols 2009)]. Deviation in individual waves leads to the dominance of one follicle that may become the ovulatory follicle depending of the stage of luteal phase. After follicle deviation the subordinate follicles cease growth and eventually decrease in size. This phenomenon is associated with loss of gonadotropin binding sites in granulosa and thecal cells, limited capacity to produce estradiol (Ireland & Roche 1983), and onset of granulosa cell apoptosis, coinciding with follicle atresia (Austin *et al.* 2001).

1.2.3. FOLLICLE DEVELOPMENT AND ESTRADIOL PRODUCTION

Plasma concentrations of estrogens depend mostly on the balance between the ovarian production (Nelson & Bulun 2001) and the rate of its metabolism (Sangsritavong *et al.* 2002). Follicular fluid concentrations of estradiol began to increase differentially in F1 than second largest follicle (Ginther *et al.* 1997, Austin *et al.* 2001, Beg *et al.* 2001) or shortly before the expected beginning of deviation (Mihm *et al.* 2000, Beg *et al.* 2002, Ginther *et al.* 2003b). In a follicular wave, as the dominant follicle(s) grow(s) the concentrations of estradiol in follicular fluid increase. In cattle, during the early part of

the follicular wave the plasma concentrations of estradiol are about 0.2 pg/ml but increase to about 2 pg/ml just after the time of deviation or up to 10 pg/ml one day before ovulation (Kulick *et al.* 1999). According to the deviation mechanism, the continued follicular growth during and after deviation and subsequent estradiol secretion seem to be dependent on LH action (Ginther *et al.* 2003b). Additionally, once an increase in estradiol has occurred, it has the capacity to increase its own synthesis by up-regulating thecal synthesis of androgens (Wrathal & Knight 1995) and increasing pregnenolone synthesis in the granulosa cells, preventing its metabolism to progesterone in both granulosa and theca cells. Therefore, in bovine follicles, estradiol promotes the use of the delta 5 pathway for synthesis of androgens by theca cells and inhibits progesterone production (Fortune & Quirk 1988).

The exam of follicle population on days 3, 5, 7, 11 and 13 of the estrous cycle had revealed the development of three large estrogen-active follicles over the course of the cycle at about 7 days intervals, with only the last follicle successfully ovulating (Ireland & Roche 1983). The granulosa cells of large follicles have elongated mitochondria that become more rounded near ovulation. Lipid bodies appeared in granulosa cells of healthy follicles near the time of ovulation, as well as, rough endoplasmic reticulum (ER) and free ribosomes. Theca internal cells hypertrophied and their mitochondrias were generally oval in shape. The theca cells had more lipid bodies than granulosa cells (Fortune & Quirk 1988). The increasing secretion of estradiol by the dominant follicle has been associated with luteolysis and has a positive effect on the hypothalamus triggering GnRH release and consequently the LH surge that is responsible for ovulation (Voss & Fortune 1993, Niswender *et al.* 2000).

1.3. RELATIONSHIP BETWEEN ESTRADIOL, FOLLICLE DEVELOPMENT AND GONADOTROPIN PROFILES

The factors that regulate the recurrent increase/decrease in concentrations of FSH during the oestrous cycle are mainly of ovarian origin, particularly from the growing follicles ≥ 5 mm(Gibbons *et al.* 1997) and from the dominant follicles (Ginther *et al.* 1996). Thus, the cauterization of the dominant follicle(s) 3 or 5 d after ovulation resulted in a surge of the FSH concentration beginning one day later and an early emergence of the subsequent follicular wave (Adams *et al.* 1992).

After follicle emergence, the initial decline in FSH concentrations after the peak occurs when the future dominant follicle and its largest companions are approximately 6 mm (Ginther *et al.* 1996). Aromatase, an enzyme in the estradiol synthetic pathway, is present in 4 mm follicles (McNatty *et al.* 1984), but low levels of estradiol are present in 5 to 7 mm follicles (Echternkamp *et al.* 1994). It is not known whether estradiol enters the circulation in concentrations that would have a negative-feedback effect on FSH during the initial decline after the peak of the FSH surge (Ginther *et al.* 1996). However, shortly before or at the beginning of deviation in heifers, the follicular fluid concentrations of E2 begin to increase faster in the future dominant than in the future largest subordinate follicle (Ginther *et al.* 1997, Beg *et al.* 2001, Beg *et al.* 2002).

During the ovulatory wave, the maximum concentrations of the E2 surge has been reported to occur synchronous with the peak of the LH/FSH surge in single (Sirois *et al.* 1990, Bergfelt *et al.* 1997, Haughian *et al.* 2004), but there are no information for double ovulating heifers.

It has been suggested that steroidal and non-steroidal products of the dominant follicle suppress FSH and/or LH secretions, preventing the growth of smaller subordinate follicles (Beg *et al.* 2002). The effect of E2 on follicle cell proliferation and differentiation has been studied (Richards 1980). The normal levels of E2 observed at

follicular phase of the oestrous cycle promotes granulosa cells proliferation, follicular growth in rats and cattle, enhances aromatase activity and increase the sensitivity of granulose cells to FSH and LH. However, as observed for subordinate follicles after the beginning of deviation, the developing dominant follicle is also sensitive to changes in peripheral gonadotropins (Turzillo & Fortune 1993).

The mechanism by which exogenous E2 terminates follicular growth has been assumed to be mediated through FSH inhibition (Barnes *et al.* 1981, Kesner *et al.* 1982, Price & Webb 1988, Bolt *et al.* 1990), LH release (Macmillan & Thatcher 1991) or a direct effect of estradiol within the ovary (Engelhardt *et al.* 1989, Hutz *et al.* 1989, Rajamahendran & Walton 1990).

The oestradiol transiently suppresses FSH concentrations in ovariectomised heifers, and the magnitude of the suppression is dose-dependent. The FSH concentrations begin to increase 1–2 days after oestradiol benzoate (ODB) administration while concentrations of E2 were declining but still high (O'Rourke *et al.* 2000). One study reported that 10 mg of oestradiol 17β administered intramuscularly (IM), either alone or in combination with norgestomet, suppressed plasma FSH in ovariectomised heifers for at least 64 h (Bolt *et al.* 1990). The suppressive effect of 1 mg of estradiol 17β on FSH was also reported when the E2 was injected IM (Kesner *et al.* 1982, Price & Webb 1988) or administrated in intramuscular implant to intact heifers (Barnes *et al.* 1981). However, other authors (Schoenemann *et al.* 1985) have found little or no effect of 1 mg of estradiol 17β on FSH in ovariectomised animals. The consequences of lack in FSH concentrations upon follicle development after deviation have not been reported. However, treated cattle with ear implant of high estradiol 17β have shown reduced ovulation rate, number of follicles larger than 6 mm, number of estrogen-active follicles and the corpus luteum weight (Price & Webb 1988).

As part of the negative feedback complex in ovariectomised ewe, E2 is reported to suppress LH pulse amplitude (Goodman & Karsch 1980, Rawlings *et al.* 1984). The removal of the progesterone causes an increase in LH pulse frequency, but the static concentration of E2 (implant releasing) did not induce the peak of LH surge. The rise in E2 concentrations, observed during the follicular phase of the estrous cycle, may be necessary to induce an LH peak (Price & Webb 1988). In addition, the GnRH (gonadotropin releasing hormone) -induced LH release or hCG (human chorionic gonadotropin) has been reported to induce follicle luteinization and atresia in the cow (Rajamahendran & Walton 1990, Macmillan & Thatcher 1991).

1.4. CODOMINANCE AND DOUBLE OVULATION

The deviation mechanism in monovulatory species including cattle allows one follicle to become dominant and ovulate. But occasionally, two or more follicles within the same wave become dominant, resulting in a phenomenon that has been termed codominance (Kulick *et al.* 2001, Beg *et al.* 2003, Acosta *et al.* 2005, Lopez *et al.* 2005). The induced-waves with codominant follicles have more follicles ≥ 4 mm at follicle emergence. However, the interval from emergence to deviation and the diameter of two largest follicles at the beginning of deviation did not differ between heifers with single- and double-dominant follicles (Acosta *et al.* 2005). Codominant follicles may result in multiple ovulations or single ovulation by undergoing two deviations (Kulick *et al.* 2001, Beg *et al.* 2003, Acosta *et al.* 2005). The first deviation occurs when the largest follicle is about 8.5 mm and is indicated by reduced growth of the third-largest follicle. The second deviation between the codominant follicles occurs 36–50 h after the first deviation and is associated temporally with a more precipitous decrease in FSH after the beginning of the first deviation. The incidence of spontaneous codominance in Holstein heifers during the first wave of the oestrous cycle (wave 1) has been reported

as 28% (Beg *et al.* 2003) and 35% (Kulick *et al.* 2001) and in Holstein cows as 40% (Lopez *et al.* 2005). Spontaneous codominant follicles were associated with greater FSH and LH concentrations before deviation and a greater reduction in FSH after the beginning of deviation in heifers (Kulick *et al.* 2001) and cows (Lopez *et al.* 2005). The estradiol concentration was higher after deviation for double-dominant versus single-dominant heifers (Kulick *et al.* 2001) and cows (Lopez *et al.* 2005).

The incidence of spontaneous multiple ovulations in Holstein heifers has been reported as 1–4% (Wiltbank *et al.* 2000), and in cows as 15–28% (Lopez-Gatius *et al.* 2005, Mann *et al.* 2007) or up to 40% in high producing dairy cows (Wiltbank *et al.* 2000). However, the temporal relationships between the gonadotropin concentrations and spontaneous multiple ovulations in dairy cattle are not complete understanding. In one study of single versus multiple ovulations (Mann *et al.* 2007), no significant difference in plasma concentrations of E2 around the time of ovulation was found. In mares, it was concluded that a preovulatory difference in hormone concentrations between single and double ovulations was an effect rather than a cause of double ovulations (Ginther *et al.* 2008a, Ginther & Almamun 2009).

1.5. REPROGRAMMING OF FOLLICULAR CELLS

The transformation of granulosa cells into luteal cells occurs within few hours (Richards *et al.* 2002). Structural and genomics changes lead the terminal differentiation of follicular cells into nondividing progesterone-producing luteal cells (Stocco *et al.* 2007). The process of luteinization is followed by an expression of a new set of molecules that will allow luteal cells to survive in a differential environment. In this regard, the final luteal cell phenotype depends on a specific combination of genes encoding for regulatory proteins such as receptors, transcription factors, and signaling proteins, which ensures the expression of only those genes necessary for luteal cell

function (Stocco *et al.* 2007). Genes that are rapidly and transiently induced by the LH surge have been thought to be involved in ovulation and induction of luteinization (Park & Mayo 1991, Natraj & Richards 1993, Lim *et al.* 1997, Sterneck *et al.* 1997, Espey *et al.* 2000, Park *et al.* 2001). Among the key molecules involved in this process are: progesterone receptor (PR), cycloxygenase-2 (COX-2), CATT/enhancer binding protein β (C/EBP β), early growth response protein-1 (Egr-1), and Nurr77. During luteinization, changes in the expression of receptors also occur. One of the most important changes during luteinization is the alteration in the cellular responsiveness to external signals allowing luteal cells to respond to a new set of hormones. According to Stocco, (2007), the most studied receptors are those for FSH, LH, prolactin, estrogen, and P4. As a response to the LH surge, a silencing of the FSH receptor, a transient decline in LH receptors and a sustained stimulation of the prolactin receptors occur. Additionally, a rapid yet short-lived increase in PR expression and a shift in the expression of the estrogen receptor from ERβ to ERα are induced in the CL.

1.6. LUTEAL PHASE

After ovulation, and through LH action, the follicular cells luteinize, forming the corpus luteum of the subsequent cycle (Fields & Fields , Milvae *et al.* 1996). The CL is a transient endocrine gland with the major physiological function of producing P4 (Ginther 1998, Stocco *et al.* 2007). Circulating P4 blocks estrous behavior and maintains the animal in a non-receptive state. Morphologic changes in the CL structure occur progressively in the establishment of the functional gland. The luteogenesis (luteinizing process of the CL) involves the transformation of the pre-ovulatory follicle into a structure with higher vascularity and able to secrete greater amounts of P4 (Smith *et al.* 1994, Niswender *et al.* 2000, Schams & Berisha 2004). Progesterone also prepares the tubular genitalia for the potential reception of an embryo. If the cow does not

become pregnant, the CL regresses (luteolysis). The cow then returns to estrus and is afforded another opportunity to become pregnant. If the cow becomes pregnant, the CL is maintained and continues to produce progesterone, which is essential for the continuation of pregnancy (Ginther 1998) in this species. The normal development of the CL and its capacity to produce P4, growth factors, angiogenic factors, and vasoatives substances are dependent on vascularity, i.e. blood supply (Acosta & Miyamoto 2004). The regression of the CL occurs around 15-19 days after ovulation in non-pregnant animals and is attributed to the pulsatile secretion of Prostaglandin (PG) F2α from the uterus in many species including cows (reviewed (Arosh *et al.* 2004, Weems *et al.* 2006). The transfer of PGF2α from the uterus to the ovary involves special vasculature relationships that have been studied and reported previously (reviewed in (Ginther 1974). This regression is characterized by loss of functionality (P4 production) and the breakdown of the cellular structure.

1.7. CORPUS LUTEUM AND ANGIOGENESIS

It has been suggested that a critical part of CL development is the intense angiogenic activity and development of an adequate blood supply (Niswender *et al.* 1994, Fraser & Wulff 2003). Blood vessels invade the antral space of the preovulatory follicle and an extensive and dense capillary network allows the hormone producing cells to obtain oxygen, nutrients, and hormone precursors and provides a mechanism for speedy and efficient output of progesterone from the CL. This rapid formation of the vascular supply of the cells layers from the granulosa cells might be related to the existence of angiogenic activity of the follicular fluid (Schams & Berisha 2004), which, apparently, is induced by the lysis of the follicular basal membrane that separates the theca and granulosa cells during the periovulatory period (Thibault *et al.* 1993). Hyperplasia of the thecal cells, downregulation of granulosa cells, and proliferation of the fibroblastic

and endothelial cells also occur during the luteinizing (Niswender *et al.* 1994, Luck & Zhao 1995, Irving-Rodgers *et al.* 2006). The molecular regulation of angiogenesis in the CL is complex, with a growing list of regulators including VEGF that are essential for CL angiogenesis (Ferrara *et al.* 1998), basic fibroblast growth factor (bFGF), endocrine gland-derived VEGF (EG-VEGF; (LeCouter *et al.* 2002), and angiopoietins (Ang). A more in depth review of the molecular regulation of angiogenesis and the role of vascular endothelial growth factors is described (Acosta & Miyamoto 2004, Kaczmarek *et al.* 2005, Stocco *et al.* 2007). Macroscopically, the luteinization process results in progressive increase in size and changes in shape, consistency, and echogenic status of the CL (Tom *et al.* 1998). The development of high luteal vascularity due to substantial luteal angiogenesis seems to be responsible for the hypo-echogenic status during ultrasonographic exams. Each cell is in direct contact with several capillaries, giving the CL one of the highest rates of blood flow in the organism. It has been reported that the blood supply to the CL is higher than to any other corporal tissue (Wiltbank 1994) and during diestrus from 65 to 95% of the blood supply to the ovary goes to the luteal gland.

1.8. LUTEAL BLOOD FLOW AND COLOR-DOPPLER ULTRASONOGRAPHY

The recently introduction of Color-Doppler ultrasonography in large animal reproduction has also provided additional information about folliculogenesis and CL vascularity using a non-invasive approach (Acosta *et al.* 2002, Acosta *et al.* 2003, Acosta & Miyamoto 2004, Ginther & Utt 2004, Miyamoto *et al.* 2006, Ginther 2007, Herzog & Bollwein 2007, Araujo *et al.* 2008). Several groups have reported that there are cyclic changes in blood flow (BF) to the ovaries in cattle, sheep, and pigs (reviewed by (Fortune & Quirk 1988)). Ovary BF is greatest during the luteal phase and lowest

around the time of estrus. The increased BF during the luteal phase appears to be due to BF to the CL itself and BF to the luteal ovary declines sharply with luteolysis. Since changes in BF accompany the differentiation of the CL, it makes sense to ask whether changes in the microcirculation of ovarian follicles occur as a follicle is selected and attains dominance. Pre-antral follicles have no special vascular supply, but the theca internal and external begin to acquire a vascular sheath or network around the time of antrum formation. Capillaries do not penetrate through the basement membrane to the granulosa cell layer until the beginning of luteinization. Blood flow to the follicular compartiment increased significantly in the 12-18 h before ovulation, while the stromal blood flow remained constant (Fortune & Quirk 1988).

Studies have demonstrated the relationship of vascularity of the follicle to capacity for dominance before deviation, and the increasing vascularity of the follicle before ovulation (Acosta 2007, Gastal *et al.* 2007). Color-Doppler ultrasonography also provides information regarding functionality of the CL structure (Ginther 2007). It is worth noting that earlier experimental approaches have not clarified whether the decreased luteal blood flow at the end of the diestrus is a cause or consequence of luteolysis (Knickerbocker *et al.* 1988). Luteal blood flow in cows (using Color-Doppler) has been found to increase before the decrease in P4 during both induced (Acosta *et al.* 2002) and spontaneous (Miyamoto *et al.* 2005, Ginther *et al.* 2007c) luteolysis. In horses, there was no indication that either an acute increase or decrease in blood flow occurred prior to the decrease in P4 during either spontaneous (Ginther *et al.* 2007b, Ginther *et al.* 2008b) or induced (Ginther *et al.* 2007a) luteolysis. In cattle, the relationship among CL echotexture, vascularity and its functional status during luteolysis using B-mode/Color- Doppler ultrasonography has been reported (Araujo *et al.* 2008). Luteal blood flow (r=0.80) and mean pixel value (r=0.41) were correlated ($P<0.0001$) to plasma progesterone concentrations. It was concluded that evaluation of

CL by B-mode (mean pixel value) and blood flow by Color-Doppler ultrasound reflected morphologic and functional changes in CL. However, CL blood flow was a better indicator of continuing luteolysis in cattle. Thus, it is evident that there are many ways to determine/measure the length of luteal phase, functionality of the gland and timing of luteolysis. Using ultrasonography, for instance, characteristics of the CL can be compared with circulating P4, or with luteal P4, etc.

1.9. LUTEOLYSIS

Prostaglandin F2α is secreted by the uterus and released in pulses that terminate the luteal phase in many animal species (Arosh *et al.* 2004, Weems *et al.* 2006). The release of PGF2α occurs in several pulses (4-7 large pulses) of short duration (3-4 hours length) for 2-3 days during and after the decline in circulating progesterone (Kindhal *et al.* 1981, Mann & Lamming 2006, Ginther *et al.* 2007c). This phenomenon is known as luteolysis and is one of the most important events regulating fertility and the reproductive process (McCracken *et al.* 1999). Regression of the CL is essential for normal cyclicity as it allows the development of a new ovulatory follicle, whereas prevention of luteolysis is necessary to establish and maintain pregnancy (Okuda *et al.* 2002). It is well known that P4, E2, and oxytocin regulate the uterine secretion of PGF2α that causes luteolysis (reviewed in (Weems *et al.* 2006) by binding to their respective endometrial receptors. During early and mid-luteal phases, E2 receptor expression is suppressed in the endometrium of ewes presumably by an inhibitory action of increasing P4 concentrations (Wathes & Hamon 1993). There is also an inhibitory action of P4 on oxytocin receptor (OTR) gene expression in cows during the early and mid-luteal phase of the cycle (McCracken *et al.* 1999, Ivell *et al.* 2000). The effect of P4 on uterine sensitivity to oxytocin involves a nongenomic action of progesterone on the uterine OTR. P4 binds to the OTR with high affinity, inhibiting

oxytocin binding to OTR, and consequently suppresses the induction of inositol triphosphate production and calcium mobilization by oxytocin (Zingg *et al.* 1998, Bogacki *et al.* 2000). During the late luteal phase, E2 receptors in the uterus increase after a decrease in uterine P4 receptor and/or decreased uterine P4 sensitivity (Meyer *et al.* 1988). Subsequently, stimulation of E2 receptor by circulating E2 stimulates the synthesis of OTR in the endometrium with subsequent oxytocin-induced release of PGF2α from the uterus (Flint & Sheldrick 1985, Silvia *et al.* 1991, Mann *et al.* 2001). Prolonged exposure to P4 promotes the accumulation of arachidonic acid and cyclooxygenase (COX) necessary for synthesis of PGF2α. In cattle, the initial rise in OTR preceding luteolysis occurs on Day 15-16 after estrus (Mann & Lamming 1994, Robinson *et al.* 1999). In this regard, uterine venous PGF first increases on day 15-16 after estrus in cows (Thatcher *et al.* 2001) with increases in circulating concentrations of PGF metabolite (PGFM) generally used as a monitor for uterine PGF secretion (Ginther *et al.* 2007c). Many studies have demonstrated that PGF2α pulses physiologically increases during luteolysis in the uterine venous blood during the follicular phase of the estrous cycle by endometrial cells. However, one recent paper has proposed a new concept of local endocrine regulation that questions this idea. It has been proposed (Krzymowski & Stefanczyk-Krzymowska 2004) that the uterine PGF2α pulses occur due to remodeling of the endometrium (apoptosis, necrosis and phagocytosis) and pulsatile excretion of PGF2α occurs due to rhythmic uterine contractions caused by oxytocin. Thus, there is still substantial controversy on the physiologic mechanisms involved in luteolysis.

1.10. INTRODUCTION

For the first part of this dissertation, the relationship between follicle development and hormone profiles in Holstein heifers with double ovulation has been studied.

In this regard, the selection of a dominant follicle during the oestrous cycle in cattle occurs during each of one or two anovulatory follicular waves and during the ovulatory wave (reviewed in (Aerts & Bols 2009)). The deviation mechanism in monovulatory species including cattle allows one follicle to become dominant and ovulate. But occasionally, two or more follicles within the same wave become dominant, resulting in a phenomenon that has been termed codominance (Acosta *et al.* 2005, Lopez *et al.* 2005). The incidence of spontaneous codominance in Holstein heifers during the first wave of the oestrous cycle (wave 1) has been reported as 28% (Beg *et al.* 2003) and 35% (Kulick *et al.* 2001) and in Holstein cows as 40% (Lopez *et al.* 2005). Spontaneous codominant follicles were associated with greater FSH and LH concentrations before deviation and a greater reduction in FSH after the beginning of deviation in heifers (Kulick *et al.* 2001) and cows (Lopez *et al.* 2005). The oestradiol (E2) concentration was higher after deviation for double-dominant versus single-dominant heifers (Kulick *et al.* 2001) and cows (Lopez *et al.* 2005).

The incidence of spontaneous multiple ovulations in Holstein heifers has been reported as 1–4% (Wiltbank *et al.* 2000) and in cows as 15–28% (Lopez-Gatius *et al.* 2005, Mann *et al.* 2007) and up to 40% in high producing dairy cows (Wiltbank *et al.* 2000). Temporal relationships between the gonadotropin concentrations and spontaneous multiple ovulations in dairy cattle have not been reported. In one study of single versus multiple ovulations (Mann *et al.* 2007), no significant difference in plasma concentrations of E2 around the time of ovulation was found. In mares, double ovulations are associated with greater preovulatory E2 concentrations, lower FSH concentrations, and smaller preovulatory follicles and it was concluded that a preovulatory difference in hormone concentrations between single and double ovulations was an effect rather than a cause of double ovulations (Ginther *et al.* 2008a, Ginther & Almamun 2009).

Only a few studies have been done in monovular species on the preovulatory hormonal changes associated with double ovulation, owing to the low natural incidence. Approaches that have been used include selecting cattle with a high twinning rate (Echternkamp *et al.* 2004), selecting mares with multiple follicles of preovulatory diameter (Ginther & Almamun 2009), or increasing the incidence of double ovulation without the use of exogenous hormones and mares (Ginther & Almamun 2009). Follicle ablation by aspiration of the contents has been used in both species to increase the prominence of the resulting FSH surge and the incidence of codominance (Acosta *et al*. 2005; Ginther *et al*. 2008). Additionally, the administration of PGF2α 1.5 days after ablation resulted in 54% of double ovulation in beef cows (Mussard *et al.* 2007).

The second part of this dissertation gives a new insight mechanisms associated with the luteolytic process in cattle, involving the administration of exogenous PGF delivery from uterus to CL.

Secretion of prostaglandin F2alpha (PGF) by the uterus, augmented by intraluteal PGF production, terminates the luteal phase in many species, including cattle (McCracken *et al.* 1999, Arosh *et al.* 2004, Schams & Berisha 2004, Weems *et al.* 2006, Skarzynski *et al.* 2008). The minimal effective intrauterine (IU) dose of PGF (1–2 mg (Louis *et al.* 1974)) when given into the uterine horn ipsilateral to the corpus luteum (CL) in cattle is about one tenth of the minimal systemic dose (15 mg (Lauderdale & Fokolowsky 1979)). The IU effectiveness in cattle results from a unilateral utero-ovarian pathway (Ginther *et al.* 1966, Ginther *et al.* 1967). The transfer of the PGF involves passage from the uterine vein to the closely adherent ovarian artery (Miller *et al.* 1981).

Based on the PGFM concentrations, PGF is released from the uterus in pulses occurring approximately every 12 h in association with luteolysis in cattle (Kindahl *et al.* 1976a, Kindahl *et al.* 1976b, Mann & Lamming 2006, Ginther *et al.* 2007c). The

pulsatile release of PGF has been assumed to be an important aspect of luteolysis (Schams & Berisha 2004, Mann & Lamming 2006). However, the necessity for natural pulsatile delivery of PGF has not been demonstrated. Treatment of cattle with sequential pulses of PGF to simulate the natural pulse-delivery system has not been reported, to our knowledge. Reported studies of the mechanisms associated with the luteolytic process in cattle that involved administration of exogenous PGF apparently did not account for the potential natural necessity of pulsatile delivery from uterus to CL.

1.11. OBJECTIVES

The follicle ablation on day 4 and PGF2α administration on day 6 for increasing the incidence of double ovulations in heifers were used for chapters 2 and 3. The objective of the chapter 2 was to compare follicle growth and plasma hormone concentrations associated with single versus double ovulations.

Preliminary results from chapter 2 indicated that the preovulatory follicles in double-ovulating heifers are associated with an earlier and greater increase in circulating oestradiol-17β (E2) concentrations, lower FSH concentrations, an earlier LH surge, and ovulation at a smaller follicle diameter.

The experiments of the chapter 3 was designed to test the hypothesis that increased E2 concentrations from double preovulatory follicles account for the reduced concentrations of FSH, smaller preovulatory follicles, and earlier occurrence of the LH surge in double-ovulating heifers.

The objectives of the chapter 4 were: 1) to determine if low IU doses of PGF require sequential treatment for the completion of luteolysis (experiment 1), 2) to develop a dose and convenient method of delivery of exogenous PGF that would result in an approximation of a natural PGFM pulse (experiment 2), and 3) to simulate PGFM

pulses to test the hypothesis that sequential PGF pulses are required for the stimulation of complete luteolysis (experiment 3). Apparent nonphysiologic responses to various doses of PGF also were considered.

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CHAPTER 2

FOLLICLE AND HORMONE DYNAMICS IN SINGLE VERSUS DOUBLE OVULATING HEIFERS ***(Reproduction 138 561–570, 2009).***

[DINÂMICA FOLICULAR E HORMONAL EM NOVILHAS COM SIMPLES VERSUS DUPLA OVULAÇÃO].

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2.1. ABSTRACT

The present experiment used the day-4 ablation model for increasing the incidence of double ovulations in heifers. The objective was to compare follicle growth and plasma hormone concentrations associated with single versus double ovulations. Follicles ≥ 5 mm were ablated at 4 d post-ovulation to induce a prominent FSH surge and a new follicular wave, and two injections of prostaglandin F2α (12 h apart) were given two days later (6 d) to favor ovulation. Beginning on day 5, the three largest follicles of the induced wave were scanned twice a day until 36 h after the next ovulation. Blood samples were taken at 6-h intervals starting when the largest follicle reached ≥ 8.0 mm (expected deviation at 8.5 mm, Ginther *et al.* 1996) and continued until 36 h after the next ovulation. Concentrations of LH and FSH were measured by validated RIAs for cattle (LH, Ginther *et al*.1999, -FSH, Adams *et al.* 1992) and concentrations of oestradiol by a commercially available RIA kit (Siddiqui *et al*. 2009). From a total of 31 heifers, 16 (52%) or 15 (48%) developed a single or more than one dominant follicle (≥ 10 mm) in the follicular wave after ablation, respectively. For heifers with two dominant follicles, second-largest follicle ovulated in 9 (60%) heifers and the overall double ovulation rate was 29% (9/31). Follicle diameters and plasma hormone concentrations were compared between single ovulators (n = 12) and double ovulators

(n = 8). Diameter of the preovulatory follicles did not increase between the LH peak and ovulation in either the single or double ovulations. In double ovulators, the interval from follicle deviation to the peak of the preovulatory LH surge was shorter (1.9 ± 0.2 d vs 2.5 ± 0.2 d; $P<0.02$) and the diameter of the largest preovulatory follicle was smaller (12.2 ± 0.5 mm vs 13.3 ± 0.3 mm; $P<0.02$) than single ovulators, respectively. The LH concentrations of the preovulatory surge did not differ between single and double ovulators for 24 h on each side of the peak (main effect of hour, only; $P<0.0001$). When data were normalized to the LH peak, the peak of the preovulatory FSH and estradiol surges occurred in synchrony with the peak of LH surge for both groups. A group effect ($P<0.0001$) for FSH resulted from a lower concentration averaged over hours in double ovulators. Estradiol showed a group by-hour interaction ($P<0.008$), reflecting greater concentrations in the double ovulators before and at peak. In conclusion, an increased incidence of double dominant follicles and double ovulations was induced by ablation of follicles ≥ 5 mm and administration of PGF2a during the nadir in FSH concentrations between FSH surges. Probably, the presence of two preovulatory follicles resulted in an earlier and greater estradiol concentration which led to lower FSH concentration, an earlier appearance of the LH surge, and ovulation of a smaller follicle. The differences in hormone concentrations during the preovulatory period were a result rather than a cause of double ovulations.

2.2. RESUMO

O presente experimento usou o modelo de punção e aspiração (ablação) folicular no quarto dia do ciclo estral para aumentar a incidência de duplas ovulações em novilhas da raça Holandesa. O objetivo foi comparar o crescimento folicular ovariano e as concentrações hormonais de animais que apresentaram simples e dupla ovulação. Quarto dias após a ovulação todos os folículos ≥ 5 mm foram aspirados para induzir

um proeminente pico nas concentrações plasmáticas de FSH e uma nova onda de crescimento folicular. Duas injeções de prostaglandina F2α (intervaladas de 12 h) foram administradas dois dias mais tarde (6^{o} dia), promovendo a luteólise e permitindo a ovulação. A partir do dia 5, os três maiores folículos da nova onda foram monitorados duas vezes ao dia até 36 h após a ovulação. Amostras de sangue foram coletadas a cada 6 h, iniciando quando o maior folículo alcançou um diâmetro ≥ 8,0 mm (o diâmetro esperado para a divergência folicular é 8,5 mm, Ginther *et al.* 1996), e continuando até 36 h após a ovulação. As concentrações plasmáticas de LH e FSH foram mensuradas pela técnica de RIA, validada para bovinos (LH, Ginther *et al.*1999, -FSH, Adams *et al.* 1992), e as concentrações de estradiol por um Kit de RIA disponível comercialmente (Siddiqui *et al*. 2009). Do total de 31 novilhas submetidas ao protocolo, 16 (52%) ou 15 (48%) desenvolveram, respectivamente, um ou mais de um folículo dominante (≥ 10 mm) na nova onda folicular, emergida após a ablação folicular. Das novilhas que apresentaram dois folículos dominantes, o segundo maior folículo ovulou em 60% (9/15) animais e a incidência geral de dupla ovulação foi de 29% (9/31). Os diâmetros foliculares e as concentrações hormonais foram comparados entre os grupos de animais com simples (n = 12) e dupla ovulações (n = 8). O diâmetro dos folículos preovulatórios não aumentou, em nenhum dos grupos, durante o intervalo que vai do pico de LH a ovulação. Nos animais com dupla ovulação, o intervalo da divergência folicular ao pico preovulatório de LH foi mais curto (1,9 ± 0,2 d vs 2,5 ± 0,2 d; P<0,02) e o diâmetro preovulatório do maior folículo foi menor (12,2 ± 0,5 mm vs 13,3 ± 0,3 mm; P< 0,02). As concentrações plasmáticas de LH não diferiram entre os grupos durante o intervalo que vai de 24 h antes até 24 h após o pico preovulatório (somente o efeito de hora foi significante, P<0,0001). Quando os dados foram normalizados para o pico de LH, o pico preovulatório de FSH e as concentrações mais elevadas de estradiol ocorreram em sincronia com o pico de LH, em ambos os grupos. Um efeito de grupo (P<0,0001) foi

observado no perfil de FSH e foi resultado de uma concentração de FSH média menor no grupo com dupla ovulação. O efeito da interação, Grupo x Hora (P<0,008), para as concentrações de estradiol foi reflexo de uma maior concentração antes e ao pico no grupo com dupla ovulação. Pode-se concluir que uma maior incidência de folículos codominantes e duplas ovulações foi induzida pela aspiração dos folículos ≥ 5 mm e pela administração de PGF2α quando a concentração de FSH atingiu os níveis mais baixos durante a primeira onda folicular. Provavelmente, a presença de dois folículos preovulatórios resultou em uma maior concentração de estradiol, que levou a menores níveis plasmáticos de FSH, a um precoce pico preovulatório de LH e a ovulação de um folículo menor. As diferenças nas concentrações hormonais, durante o período preovulatório, foram um resultado e não uma causa das ovulações duplas.

2.3. INTRODUCTION

The selection of a dominant follicle during the oestrous cycle in cattle occurs during each of one or two anovulatory follicular waves and during the ovulatory wave (reviewed in (Fortune *et al.* 2001, Ginther *et al.* 2003, Beg & Ginther 2006, Webb *et al.* 2007, Adams *et al.* 2008, Mihm & Evans 2008, Aerts & Bols 2009)). Follicular growth in a wave is characterized by a 2 or 3 day common-growth phase after follicle emergence at 4.0 mm. At the end of the common-growth phase, usually one follicle continues to grow and becomes the developing dominant follicle and the remaining follicles (subordinates) regress. The end of the common-growth phase and the beginning of a distinctive difference in growth rates between the two largest follicles have been termed follicle deviation (Ginther *et al.* 1997) and, in cattle, occurs when the largest follicle reaches a mean diameter of 8.5 mm (Ginther *et al.* 1996).

On average, a transient elevation in LH begins before deviation and decreases after deviation (Kulick *et al.* 1999, Bergfelt *et al.* 2000, Ginther *et al.* 2001). The

functional requirements of F1 (largest follicle) for LH after deviation is indicated by a smaller diameter when LH is experimentally reduced (Ginther *et al.* 2001) and by cessation of growth beyond 9 mm when LH is suppressed (Gong *et al.* 1995). The dominant follicle acquires ovulatory capacity when it reaches about 10 mm in Holstein heifers (Sartori *et al.* 2001).

The deviation mechanism in monovulatory species including cattle allows one follicle to become dominant and ovulate. But occasionally, two or more follicles within the same wave become dominant, resulting in a phenomenon that has been termed codominance (Kulick *et al.* 2001, Beg *et al.* 2003, Acosta *et al.* 2005, Lopez *et al.* 2005). Induced follicle waves that developed codominant follicles have more follicles ≥ 4 mm but a similar interval from emergence to deviation and a similar diameter of two largest follicles at the beginning of deviation (Acosta *et al.* 2005). Codominant follicles may result in multiple ovulations but may also result in a single ovulation by undergoing two deviations (Kulick *et al.* 2001, Beg *et al.* 2003, Acosta *et al.* 2005). The first deviation occurs when the largest follicle is about 8.5 mm and is indicated by reduced growth of the third-largest follicle. The second deviation between the codominant follicles occurs 36–50 h after the first deviation. The second deviation is associated temporally with a more precipitous decrease in FSH after the beginning of the first deviation. The incidence of spontaneous codominance in Holstein heifers during the first wave of the oestrous cycle (wave 1) has been reported as 28% (Beg *et al.* 2003) and 35% (Kulick *et al.* 2001) and in Holstein cows as 40% (Lopez *et al.* 2005). Spontaneous codominant follicles were associated with greater FSH and LH concentrations before deviation and a greater reduction in FSH after the beginning of deviation in heifers (Kulick *et al.* 2001) and cows (Lopez *et al.* 2005). The oestradiol (E2) concentration was higher after deviation for double-dominant versus single-dominant heifers (Kulick *et al.* 2001) and cows (Lopez *et al.* 2005).

The incidence of spontaneous multiple ovulations in Holstein heifers has been reported as 1–4% (Wiltbank *et al.* 2000) and in cows as 15–28% (Lopez-Gatius *et al.* 2005, Mann *et al.* 2007) and up to 40% in high producing dairy cows (Wiltbank *et al.* 2000). Temporal relationships between the gonadotropin concentrations and spontaneous multiple ovulations in dairy cattle have not been reported. In one study of single versus multiple ovulations (Mann *et al.* 2007), no significant difference in plasma concentrations of E2 around the time of ovulation was found. In another series of studies (Echternkamp *et al.* 1990, Echternkamp *et al.* 2004), cows were genetically selected for double ovulations and twin births (defined as twinners) and were compared with controls at 0, 1, 2, and 3 days after induction of luteolysis with prostaglandin F2a (PGF2α). Cows were slaughtered on the indicated days, precluding determination of ovulation rate or normalizing to ovulation or to the LH surge. No differences were found after PGF2α treatment between the twinner and control cows in follicular-fluid concentration of ovarian steroids or in plasma concentration of LH and FSH. In mares, it was concluded that a preovulatory difference in hormone concentrations between single and double ovulations was an effect rather than a cause of double ovulations (Ginther *et al.* 2008, Ginther & Almamun 2009).

A 75% incidence of codominance occurred in Holstein heifers after ablation of all follicles ≥ 5 mm 4 days after ovulation, compared with 25% in controls and in groups with follicle ablation on other days (Acosta *et al.* 2005). The greater rate of codominance was associated with a more prominent FSH surge after follicle ablation on day 4 when FSH concentrations were low. The experiment was done during the anovulatory wave 1, and the codominant follicles did not result in double ovulations. The luteal phase was terminated by PGF2α treatment 4 days after expected deviation, which was apparently too late for ovulation by the dominant follicles. In this regard, aspiration of all follicles ≥ 5 mm 6.4 days after ovulation followed by administration of

PGF2α 1.5 days later resulted in 54% double-ovulation rate in beef cows (Mussard *et al.* 2007). There apparently have been no published reports in cattle on the pre-ovulatory temporal relationships between follicle development and plasma gonadotropin concentrations in association with single versus double ovulations. The present experiment used follicle ablation on day 4 and PGF2α administration on day 6 for increasing the incidence of double ovulations in heifers. The objective was to compare follicle growth and plasma hormone concentrations associated with single versus double ovulations.

2.4. MATERIAL AND METHODS

2.4.1. Animals and ultrasonography

A total of 31 Holstein heifers aged 12–18 months and weighting 330–575 kg were used. All heifers had a single ovulation during the previous oestrous cycle with at least two cycles of 16–24 days. The heifers did not have apparent abnormalities of the reproductive tract, based on transrectal ultrasound examinations (Ginther 1998) and were acclimated to the handling procedures for at least 2 weeks prior to the experiment. Heifers remained healthy and had good body condition throughout the experiment. The feeding program consisted of access to a mixture of grass and alfalfa hay, water and minerals with grain supplementation ad libitum. Animals were handled in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research.

A duplex B-mode (grey scale) and pulsed-wave color-Doppler ultrasound instrument (Aloka SSD 3500; Aloka American, Wallingford, CT, USA) equipped with a lineararray 7.5-MHz transducer was used. Transrectal scanning of the ovaries for measurement of follicles, detecting ovulation and determining the cross-sectional area of the corpus luteum was done as described (Ginther 1998). Heifers were checked once

daily for ovulation beginning when a pre-ovulatory follicle was present. The day of ovulation was designated day 0. Follicle ablations were done by ultrasound-guided transvaginal aspiration of follicle contents, using a 17-ga needle connected to a vacuum pump as described (Bergfelt *et al.* 1994, Bodensteiner *et al.* 1996). Any follicle site that refilled with a fluid collection of ≥ 5 mm 24 h later was aspirated again. If needed for generation of optimal ultrasound images, heifers were sedated during scanning with xylazine hydrochloride (0.05 mg/kg, i.m.; Xila-ject, Phoenix Pharmaceutical, Inc., St Joseph, MO, USA) as described previously (Araujo & Ginther 2009).

2.4.2. Experimental design

On day 4, all follicles ≥ 5 mmin both ovaries were ablated to induce a surge of FSH that was expected to stimulate an increased incidence of codominant follicles (Acosta *et al.* 2005). Beginning on day 5, the three largest follicles of the induced wave were scanned twice a day, and follicle identity was maintained from examination-to-examination as described (Pierson & Ginther 1988, Sirois & Fortune 1988). Scanning every 12 h continued until 36 h after the next ovulation. The heifers received two injections of PGF2α (25 mg; Lutalyse; Pfizer Animal Health, New York City, NY, USA) 12 h apart on day 6 to terminate the luteal phase. Blood samples were taken at 6-h intervals starting when the largest follicle reached ≥ 8.0 mm. This was just before expected deviation at 8.5 mm (Ginther *et al.* 1996). Blood sampling every 6 h continued until 36 h after the next ovulation.

2.4.3. Definitions and experimental groups

The three largest follicles at the ultrasound examination just before a follicle ovulated were designated F1, F2 and F3, according to decreasing diameter. Follicular wave emergence was defined retrospectively as occurring when F1 was ≥ 4.0 mm. A

dominant follicle was defined as one that reached ≥ 10 mm (Kulick *et al.* 2001, Lopez *et al.* 2005). In heifers that developed one dominant follicle, the beginning of deviation was based on inspection of the data profiles of follicle diameters for each heifer and was defined as the beginning of a greater diameter difference between the two largest follicles between successive ultrasound examinations as described (Ginther *et al.* 1997). In heifers that developed two dominant follicles, the beginning of deviation was clarified when indicated by comparing the diameter changes between F1 and F3 (Kulick *et al.* 2001, Acosta *et al.* 2005). When the beginning of deviation was not apparent in a heifer, the mean diameter of F1 at deviation within the group was used to represent deviation in that heifer. This occurred in 4 out of 12 single and 3 out of 8 double ovulators. All end points involving ovulation were related to the first ovulation in the double ovulators. The examination 12 h before ovulation was used to determine diameter of the preovulatory follicle. Discrete end points were length of the interovulatory interval and intervals from follicular emergence to follicular deviation, peak of the LH surge to ovulation, beginning of deviation to peak of the LH surge, and largest diameter of F1 to ovulation. Follicular growth rate was calculated for F1 and F2 from the total and daily diameter increases between discrete events.

The pre-ovulatory and periovulatory FSH surges were defined as the surges temporally related to the LH surge and to ovulation respectively, as described (Haughian *et al.* 2004). The first nadir for an FSH surge was the lowest value preceding the progressive increase in concentrations. The second nadir for the pre-ovulatory surge was the same as the first nadir of the periovulatory surge and was the lowest value between the two FSH surges. Concentrations of FSH and time of occurrence of nadirs and peaks were compared among groups.

2.4.4. Blood samples and hormone assays

Blood samples from the coccygeal vein were collected into heparinised tubes and centrifuged (1500 g for 10 min) and plasma was separated and stored at -20^{o}C until assayed. All plasma samples were assayed for LH and FSH. However, E2 determinations were done on samples collected every 12 h from 48–12 h before the peak of the LH surge and then on samples collected every 6 h until 18 h after the peak of LH. Concentrations of LH and FSH were measured by validated RIAs for cattle (Bolt & Rollins 1983, Bolt *et al.* 1990) with modifications as reported for LH (Ginther *et al.* 1999) and FSH (Adams *et al.* 1992) in our laboratory. For LH assay, USDA-bLH-B-6 was used for iodination and the reference standard, and USDA-309-684P was used as the primary antiserum. For FSH assay, USDA-bFSH-I-2 (AFP-5318C) was used for iodination and the reference standard, and NIDDK-anti-oFSH-1 was used as the primary antiserum. The standards and antibodies were purchased from A F Parlow (National Hormone and Pituitary Program, Torrance, CA, USA). Plasma concentrations of E2 were measured as described and validated in our laboratory (Siddiqui *et al.* 2009), using a commercially available RIA kit (Double Antibody Estradiol: Diagnostics Products Corporation, Los Angeles, CA, USA). The intra-and interassay coefficients of variation and mean sensitivity respectively, were 9.1%, 5.2% and 0.1 ng/ml for LH; 6.1%, 8.0%, and 0.02 ng/ml for FSH; and 14.1%, 7.7% and 0.1 pg/ml for E2.

2.4.5. Statistical analysis

Follicle diameter and hormone concentrations were divided into two data sets, one normalized (hour 0) to the beginning of deviation and encompassing -24 or -12 to 24 h and the second normalized (hour 0) to the peak of LH and encompassing -24 to 24 h. For hormone data, suspected outliers were challenged using Dixon's outlier test (Kanji, 1993). Statistical outliers were found for 4 out of 307 observations (1.3%) for LH and 5

out of 307 observations (1.6%) for FSH and were excluded from further analysis. In order to verify the normality distribution, the data for all analyses were examined using the Shapiro–Wilk test and transformed to natural logarithms or ranks when indicated. Data for follicle diameter and hormone concentration were analyzed by SAS MIXED procedure (version 9.1.3, SAS Institute Inc., Cary, NC, USA) for main effects of group and time (hour) and their interaction, using a REPEATED statement to account for sequential measurements. Calculated end points for time intervals and follicular growth rates were analyzed by SAS GLM procedure for main effect of group. Differences between two means were evaluated by Student's t-test. Differences among more than two means (F1, F2, and F3 within a group) were further analyzed by Duncan's multiple-range test. The significant probability level adopted was 5%, and values between > 5% to ≤ 10% were considered as approaching significance. Data are presented as the mean ± S.E. unless otherwise indicated.

2.5. RESULTS

The number of heifers with single and multiple dominant follicles and the number with multiple dominant follicles that developed multiple ovulations are shown (Fig. 2.1). In the 22 single ovulators, data were not available for seven heifers and three heifers were not used, owing to three dominant follicles, non-response to PGF2α, or delayed emergence of the induced follicular wave. One heifer with three ovulations was excluded from the nine heifers with multiple ovulations. Thus, 12 singles ovulators and 8 double ovulators were compared in the statistical analyses. Ovulations in double ovulators were synchronized within 6 h in 6 out of 8 heifers, whereas in the remaining two the second ovulation occurred from F2 at 12 or 36 h after ovulation from F1. Double ovulations occurred in the same ovary in 4 out of 8 heifers and in each ovary in 4 out of 8 heifers.

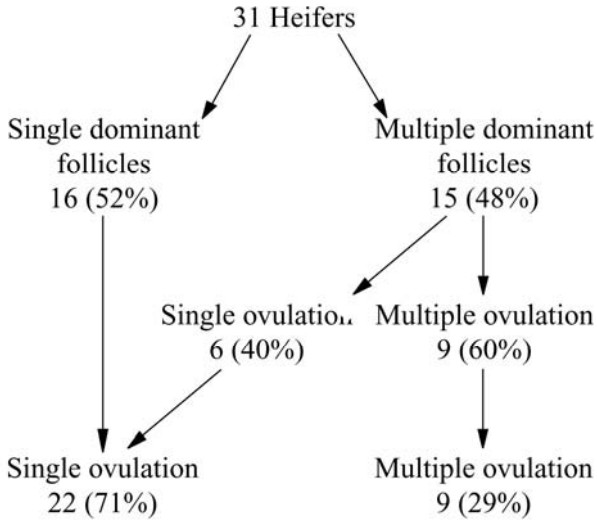

Figure 2.1. Diagrammatic outcome for single and multiple dominant follicles and single and multiple ovulations after ablating all follicles (≥ 5 mm) and treatment with PGF2α.

2.5.1. Single versus double ovulators

There was no difference ($P<0.05$) between groups in the number of follicles ablated on day 4. The interval from ablation to emergence and emergence to deviation did not differ between single and double ovulators (Table 2.1). In addition, the day of emergence and deviation, diameter at emergence and deviation, and follicle growth rate between emergence and deviation did not differ between groups for either F1 or F2 (not shown). The interval from deviation to maximum diameter of F1, to the peak of the LH surge, and to ovulation was significantly shorter in the double ovulators than in the single ovulators. A reduction in the interval from the pretreatment to post-treatment ovulation in double ovulators approached significance. The diameter of F1 and F2 at deviation was not different between the single and double ovulators (Table 2.2). The diameter of F1 (largest ovulatory follicle) was greater and the diameter of F2 was less in the single ovulators than in the double ovulators at maximum diameter, at the peak of the LH surge, and at preovulation of F1. The growth rate of F1 between deviation and the LH peak (combined for groups, 1.8 ± 0.1 mm/day) and between the LH peak and preovulation (0.1 ± 0.1 mm/day) were not different between single and double ovulators.

Table 2.1. Mean (± S.E.) for intervals between follicle events in single and double ovulators.*

Interval	Single ovulators (n = 12)	Double ovulators (n = 8)	Probability
Ablation to emergence (d)	1.2 ± 0.1	1.3 ± 0.2	NS
Emergence to deviation (d)	2.4 ± 0.1	2.4 ± 0.1	NS
Deviation to max. diam. F1 (d)	3.1 ± 0.2	2.4 ± 0.3	$P<0.03$
Deviation to LH peak (d)	2.5 ± 0.2	1.9 ± 0.2	$P<0.02$
Deviation to ovulation (d)	3.9 ± 0.2	3.3 ± 0.1	$P<0.01$
LH peak to ovulation (h)	33.5 ± 0.9	32.2 ± 1.9	NS
Max. diam. F1 to ovulation (h)	19.5 ± 2.6	19.5 ± 2.2	NS
Ovulation to ovulation (d)	12.2 ± 0.2	11.7 ± 0.2	$P<0.06$

*Ovulatory wave induced by ablating all follicles ≥ 5 mm 4 d after ovulation and treating with PGF2α 6 d after ovulation.

Table 2.2. Mean (± S.E.) diameters of F1 and F2 in single and double ovulators.*

Diameter (mm)	Double ovulators (n = 8)	Single ovulators (n = 12)	Probability
At deviation			
F1	8.9 ± 0.2	8.8 ± 0.2	NS
F2	8.3 ± 0.2	8.3 ± 0.3	NS
At maximum			
F1	13.9 ± 0.3	12.3 ± 0.5	$P<0.004$
F2	9.5 ± 0.2	11.4 ± 0.3	$P<0.0001$
At peak of LH surge			
F1	13.3 ± 0.3	12.2 ± 0.5	$P<0.02$
F2	8.9 ± 0.3	11.1 ± 0.4	$P<0.0001$
At preovulation F1			
F1	13.5 ± 0.3	12.0 ± 0.6	$P<0.01$
F2	8.6 ± 0.4	10.8 ± 0.4	$P<0.0005$

*Ovulatory wave induced by ablating all follicles ≥ 5 mm 4 d after ovulation and treating with PGF2α 6 d after ovulation.

Data and results of the analyses for diameters of F1, F2, and F3 normalized to deviation and to the LH peak are shown (Fig. 2.2). When normalized to deviation, only the main effect of hour, representing a gradual diameter increase, was significant for each follicle. For F1 normalized to the LH peak, the hour effect was significant, representing increasing diameter averaged over groups. The group effect was also

significant, owing to a greater diameter in the single ovulators, averaged over hours. For F2 normalized to the LH peak, the main effects of group and hour and their interaction were significant. These effects were attributable to a greater average diameter with a greater increase in diameter before than after the LH peak in the double ovulators. There were no significant differences for F3 normalized to the LH peak.

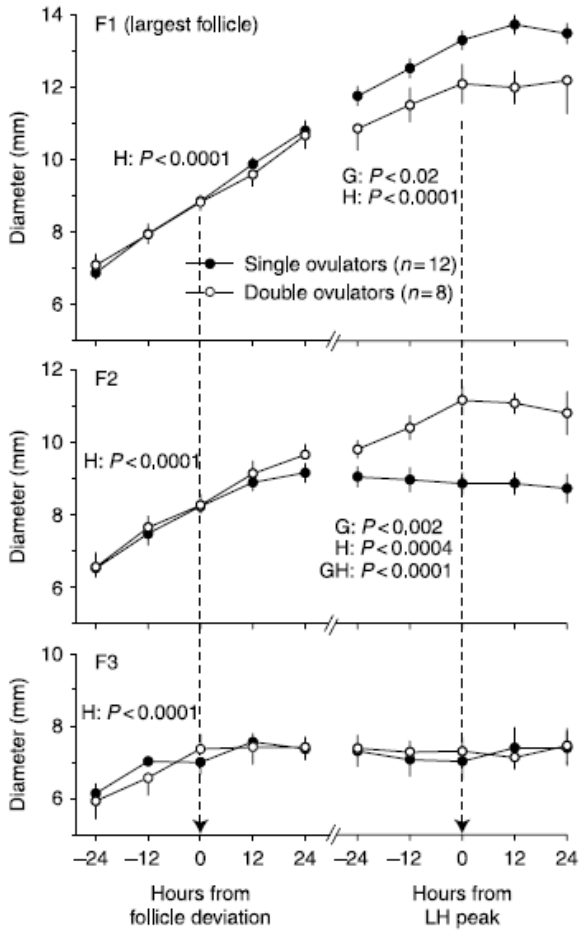

Figure 2.2. Mean (± S.E.) for diameters of the three largest follicles (F1, F2, and F3) in single and double ovulators. Significant main effects of group (G) and hour (H) and an interaction (GH) are shown for 24 h before to 24 h after the beginning of follicle deviation and for 24 h before to 24 h after the peak of the preovulatory LH surge

Normalized to the beginning of deviation, there were no significant effects for LH, but a main effect of hour for FSH represented declining concentrations (Fig. 2.3). Although the interaction for FSH was not significant, a decrease during 12–24 h after deviation was significant ($P<0.05$) only for the double ovulators. A lower concentration in the double than in the single ovulators at 24 h approached significance ($P<0.06$). For

LH, FSH, and E2 concentrations normalized to the peak of LH, there was a main effect of hour for each hormone. The means for the pre-ovulatory peaks of LH, FSH, and E2 occurred synchronously among hormones for both single and double ovulators when data were normalized to the LH peak. Both the first and second nadirs were synchronous between concentrations of LH and FSH. In addition to the hour effect for each hormone, a group effect was detected for FSH and an interaction for E2. Differences between FSH and LH resulted from an FSH increase beginning at the second nadir and represented the beginning of the periovulatory surge. In addition, a group effect for FSH resulted from a greater concentration averaged over hours combined for both surges in single than in double ovulators (0.23 ± 0.01 vs 0.19 ± 0.02 ng/ml). The interaction for E2 represented a more prominent increase in the double than in single ovulators, with greater concentration in the double ovulators at -12 h ($P<0.09$) and -6 h ($P<0.03$) before the peak and at the peak ($P<0.01$).

In addition, the hour of the periovulatory peak in FSH concentration was not significantly different between single and double ovulators (35.0 ± 3.3 and 32.3 ± 2.8 h after peak LH respectively; not shown). The concentration at the periovulatory peak also was similar between single and double ovulators (0.49 ± 0.03 vs 0.44 ± 0.04 ng/ml).

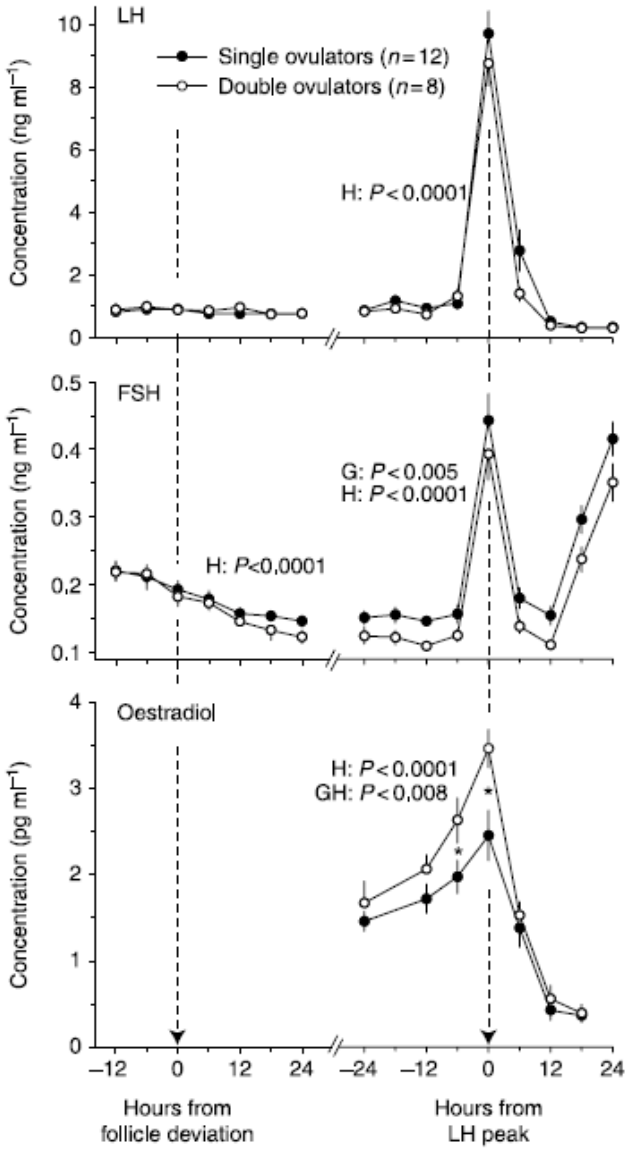

Figure 2.3. Mean (± S.E.) for concentrations of LH, FSH, and oestradiol in single and double ovulations. Significant main effects of group (G) and hour (H) and an interaction (GH) are shown for 12 h before to 24 h after the beginning of follicle deviation and for 24 h before to 24 h after the peak of the preovulatory LH surge for LH and FSH and 24 h before to 20 h after peak of preovulatory LH surge for oestradiol. An asterisk indicates a difference ($P<0.05$) between groups when a significant interaction was obtained.

2.5.2. Single versus double dominant follicles in single ovulators

In the single-ovulating group, the length of the interovulatory period and the growth of F1 were not different between subgroups with one versus two dominant follicles (not shown). Concentration of LH did not differ between subgroups when data were normalized either to deviation or to the LH peak (not shown). For F2, a significant hour effect normalized to deviation reflected growth of the follicle averaged over subgroups (Fig. 2.4). A significant interaction of subgroup and hour for F2 reflected a constant

diameter increase for 12 h before and 24 h after deviation in the double-dominant subgroup and a reduced diameter increase after deviation in the single-dominant subgroup. As a result, F2 was smaller (P<0.002) in the single-dominant subgroup (8.8 ± 0.2 mm) than in the double-dominant subgroup (10.3 ± 0.4 mm) 24 h after deviation. The only significant difference between the single- and double-dominant subgroups normalized to the LH peak was a greater diameter of F2 in the double-dominant subgroup throughout the period, as indicated by a significant group effect without an interaction. The main effect of hour for FSH normalized to deviation was significant, resulting from a continuing decrease averaged over subgroups (Fig. 2.4). However, the interaction was also significant, reflecting greater concentrations before deviation and 6 h after deviation in the double-dominant subgroup, with no difference thereafter. Concentrations of FSH were not different between single and double ovulators normalized to the LH peak.

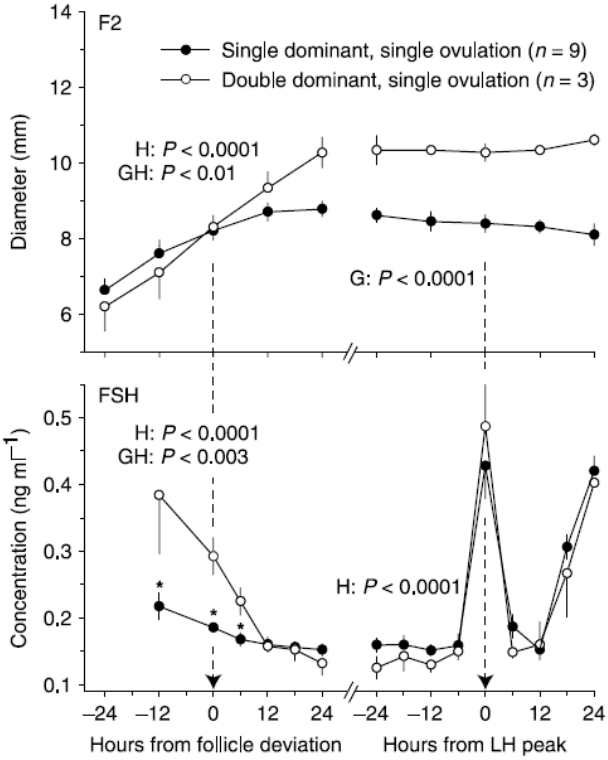

Figure 2.4. Mean (± S.E.) for diameter of F2 and concentration of FSH in single ovulators with single and codominant follicles. Significant main effects of group (G) and hour (H) and an interaction (GH) are shown for 12 h before to 24 h after the beginning of follicle deviation and for 24 h before and 24 h after

the peak of the pre-ovulatory LH surge. An asterisk indicates a difference ($P<0.05$) between groups when a significant interaction was obtained.

2.6. DISCUSSION

It is not practical to use natural ovulations in Holstein heifers to study the temporal relationships between follicles and hormones associated with single versus multiple ovulations, owing to the low natural incidence of multiple ovulations. In the contemporary heifers that served as the source of the heifers for the present study, the natural incidence was 1.5% (1 out of 67 oestrous cycles). Follicle ablation during the nadir in FSH concentrations between the surges that stimulate waves 1 and 2 (Kulick *et al.* 2001) was used to increase the incidence of multiple ovulations without the use of exogenous gonadotropins. An increased incidence of multiple dominant follicles and multiple ovulations in the present day-4 ablation model agrees with the results of ablating on days 4, 5 or 6 (Gibbons *et al.* 1997, Acosta *et al.* 2005, Mussard *et al.* 2007). Days 4–6 approximate the reported period of low FSH concentrations between FSH surges (Kulick *et al.* 2001).

The concentrations of FSH were not determined immediately post-ablation in the present study. However, a previous study indicated that ablation of the follicles of wave 1 during the FSH nadir removed the FSH suppression and was followed by an FSH surge more prominent than a natural surge (Acosta *et al.* 2005). An increased number of follicles during the common-growth phase of the induced wave and an increased incidence of multiple dominant follicles were attributed to the prominent FSH surge. Greater pre-deviation concentrations of LH and FSH and lesser post-deviation concentrations of FSH occurred during codominance in the ablation model (Acosta *et al.* 2005) and in naturally occurring codominance (Kulick *et al.* 2001, Lopez *et al.* 2005). Greater concentrations of E2 were associated with the LH surge of the double ovulators in the present study and ~2 days after the beginning of deviation in natural

codominance (Kulick *et al.* 2001). Apparently, follicle and hormone dynamics during the ovulatory period were similar between double ovulations in the follicle-ablation model and in natural double ovulations.

The frequency of conversion of natural double dominants to double ovulations apparently has not been studied in cattle in the same experiment. The lack of information is attributable to use of anovulatory wave 1 in most wave characterization studies, without an associated induction of luteolysis. The natural double-ovulation rate is low (about 2%) in both heifers (Wiltbank *et al.* 2000) and ponies (Adams *et al.* 1992). In Holstein heifers, the incidence of double dominants during natural ovulatory waves has been reported as 10% (Kulick *et al.* 2001). In pony mares, two dominant follicles developed in 34% of natural ovulatory waves, but conversion to double ovulatory follicles occurred in only 9% of mares with double dominants (Jacob *et al.* 2009). These results indicate that the incidence of conversion of natural double dominants to double ovulations is low in both species. A high conversion rate (60%) of double dominant follicles to double ovulations was found in the present study in heifers for ovulatory waves that were induced by ablation and PGF2α administration. In ponies, both dominant follicles ovulated more frequently in the induced waves than in the natural waves (Ginther *et al.* 2008). These results of both species indicate that waves induced by a combination of ablation and PGF2α treatment have more dominant follicles and a higher conversion rate of the double dominant follicles to double ovulations than for natural waves.

The role of the induction of luteolysis 2 days after follicle ablation on the increased double-ovulation rate was not studied. The decrease in progesterone from the PGF2α treatment may have allowed an LH increase while the follicles were still in their common-growth phase, owing to the loss of the negative effect of progesterone on LH (Ginther *et al.* 2001). However, concentrations of LH during the common-growth phase

were not adequately considered in the present study, owing to the availability of blood samples only for 12 h before the beginning of deviation. In mares, an increase in LH concentrations during the common-growth phase is associated with an increase in double ovulations (Ginther *et al.* 2009) and treatment with PGF2α during diestrous increases the incidence of double ovulations (Ginther & Almamun 2009). These results in mares indicate a need for similar studies in heifers, i.e. the increased double-ovulation rate in the present study in heifers may not be entirely a result of a more prominent post-ablation FSH surge.

In horses (Ginther *et al.* 2008, Ginther *et al.* 2009) and sheep (McNatty *et al.* 1984), the pre-ovulatory diameter of each ovulatory follicle in double ovulators is less than for single ovulators. Similar information apparently has not been available for cattle, but it has been reported that each corpus luteum is smaller for two corpora lutea than for one corpus luteum (Mann *et al.* 2007). In the present study, diameter of F1 was about 1.5 mm smaller in double ovulators than in single ovulators and in double ovulators F2 was about 1 mm smaller than F1 both at maximum diameter and 12 h before ovulation (preovulatory). The greater diameter of F1 in single versus double ovulators began within 1 day before the LH peak or about 2.5 days before ovulation. The corresponding diameter differences in mares also became apparent within 2.5 days before ovulation (Ginther *et al.* 2009). The interval from wave emergence to deviation was not different between single and double ovulators in heifers (present study) and mares (Ginther *et al.* 2008), but the interval from deviation to ovulation was shorter in double ovulators in heifers (significant) and in mares (approached significance). In the heifers, the shorter interval from deviation to ovulation in the double ovulators accounted for the smaller F1 in that the growth rate of F1 did not differ between groups. These results indicate that in dairy heifers (present study) and in mares (Ginther *et al.* 2008) the relationships of follicle diameter to double ovulation are evident only after

deviation. Furthermore, the interval from deviation to the LH peak was shorter in the double-ovulating heifers, indicating that the smaller pre-ovulatory diameter was at least partially a function of an earlier LH surge. The earlier LH surge in double ovulators may reflect the earlier and greater increase in E2 by the double follicles. In this regard, exogenous E2 induces a LH surge in ovariectomised and ovarian-intact cattle (Hobson & Hansel 1972, Engelhardt *et al.* 1989). The diameter of F2 in the double ovulators ranged from 9.1 to 12.0 mm at the peak of the LH surge; two follicles (25%) were <11 mm. This result seems consistent with the conclusion that ovulatory capacity is acquired at about 10 mm, based on inducing ovulation by exogenous LH at various diameters in Holstein heifers (Sartori *et al.* 2001).

The maximum concentrations of E2 and FSH in the pre-ovulatory surges occurred at the peak of the LH surge for both single and double ovulators. The close temporal relationship between the three hormones has been previously reported for single ovulators (Bergfelt *et al.* 1997, Haughian *et al.* 2004). An apparently unreported observation in cattle was a reduction or cessation in the diameter increase for F1 in both single and double ovulators and for F2 in double ovulators beginning at the synchronized LH, FSH and E2 peaks. On a temporal basis, the decreases in LH, FSH or E2 after the peak concentrations could have been related to or involved in the reduction in follicle growth beginning about 33 h before ovulation. A similar cessation or reduction in follicle growth occurs in mares 2 days before ovulation in synchrony with the peak of the pre-ovulatory E2 surge, but both LH and FSH continue to increase until after ovulation (Ginther *et al.* 2009). Therefore, on a species comparative basis, the reduction in follicle growth and the beginning of an E2 decrease apparently represent the same mechanism. The reduction in follicle growth in mares has been attributed to the attainment of a critical level of LH, based on an immediate cessation of growth and an E2 reduction when an ovulatory dose of hCG is given (Gastal *et al.* 2006). In heifers,

a similar critical level of LH may be reached during the LH surge. The molecular basis for an apparent negative effect of a critical level of LH in blocking E2 production by down-regulation of aromatase and other steroidogenic enzymes in horses and cattle has been discussed (Gastal *et al.* 2006).

The LH concentrations were similar between single and double ovulators at the LH surge. Also in mares, the preovulatory concentrations of LH were not different between single and double ovulators (Ginther *et al.* 2008, Ginther *et al.* 2009). Lower FSH concentrations in double-ovulating heifers seemed to begin 24 h after deviation. The lower FSH in double ovulators continued throughout the 24 h before and 24 h after the peaks of the preovulatory LH/FSH surges. This conclusion relies statistically on the main effect of group without a detected group-by-hour interaction. The lower FSH was temporally associated with greater E2 concentrations before and at the peak of the FSH surge. A similar FSH/E2 relationship occurs in double-ovulating mares during the pre-ovulatory period (Ginther *et al.* 2008, Ginther *et al.* 2009). The greater plasma E2 in the two species is attributable to the presence of two pre-ovulatory follicles in the double ovulators. A negative effect of exogenous E2 on FSH has been shown in heifers (Miller *et al.* 1981). In the present study, E2 concentration was not different between single and double ovulators after the peak of the pre-ovulatory surge, but FSH continued to be lower after the peak of the pre-ovulatory FSH surge and during the ascending portion of the periovulatory surge. The reason for the apparent continuation of the lower concentrations of FSH in the double ovulators after the pre-ovulatory peak is not known, but may be a consequence of the earlier establishment of reduced concentrations.

The greater LH and FSH concentrations that have been reported before and at deviation in cattle that developed double dominant follicles (Kulick *et al.* 2001, Acosta *et al.* 2005, Lopez *et al.* 2005) were not detected during the 12 h before deviation in the

present study. A major difference between the reported studies and the present study was that the outcome (ovulation or atresia) of each of the double dominant follicles was not determined in the reported studies and presumably would have included follicles that would and would not have ovulated. The high levels of FSH before deviation in cattle that later develop multiple dominant follicles seems consistent with reports in women. Women with a history of giving birth to spontaneous twins have higher FSH concentrations than in controls during the portion of the menstrual cycle when follicles are recruited (Lambalk *et al.* 1998). By contrast, mares with multiple dominant follicles had lower concentrations of FSH before deviation (Ginther *et al.* 2008); the enhanced FSH suppressing effect of multiple follicles was attributed to greater pre-deviation inhibin output. The role of inhibin in the FSH reduction at deviation and at ovulation was not determined in the present study, owing to the unavailability of inhibin antigen. In this regard, both inhibin and E2 increase during days 3–9 of the bovine oestrous cycle (Martin *et al.* 1991), which is expected to encompass deviation. In Holstein cows, double and triple dominant follicles were associated with greater FSH and lower immunoreactive-inhibin concentrations 24–12 h before the beginning of deviation than for single dominant follicles (Lopez *et al.* 2005). Inhibin-A concentrations were low on the day of ovulation (Bleach *et al.* 2001).

Comparisons were also made between subgroups with single and double dominant follicles within the single-ovulating group. This has not been done previously. No significant effects were found on diameter of F1 and concentration of LH either by normalization to deviation or to the LH peak. The diameter of F2 was greater in the single ovulators with two dominant follicles than with one dominant follicle by 24 h after the beginning of deviation and then remained at approximately the same diameter until 24 h after the LH/FSH preovulatory peak. Thus, the presence of the large anovulatory F2 during the pre-ovulatory period did not alter the gonadotropin

concentrations and morphologic regression of F2 did not occur despite great fluctuations in concentrations of each gonadotropin. The most pronounced difference between subgroups was the greater FSH concentrations before and at the beginning of deviation in the double-dominant subgroup. This is consistent with the results of previous comparisons between heifers with single and double dominant follicles when the outcome of the double dominant follicles was unknown (Kulick *et al.* 2001, Acosta *et al.* 2005). In the present study, the greater FSH concentrations near the beginning of deviation occurred when only one follicle of double dominant follicles ovulated, but not when both follicles ovulated. Although interesting, reservation on this difference between double dominants with single versus double ovulations is recommended because of the small number (n = 3) of double dominants with a single ovulation.

In conclusion, an increased incidence of double dominant follicles and double ovulations was induced by ablation of follicles ≥ 5 mm during the nadir in FSH concentrations between FSH surges, followed in 2 days by administration of PGF2α. The interval from follicle deviation to the peak of the pre-ovulatory LH surge was shorter and the diameter of the pre-ovulatory follicles at the peak of the surge was smaller in double ovulators. Diameter of the pre-ovulatory follicles did not increase between the LH peak and ovulation. When data were normalized to the LH peak, the peak of the preovulatory FSH and E2 surges occurred in synchrony with the LH peak in both single and double ovulators. Concentration of LH from 24 h before to 24 h after the peak did not differ between single and double ovulators, but the concentration of E2 was greater and concentration of FSH was lower in the double ovulators. The interpretation was that the presence of two preovulatory follicles resulted in an earlier and greater E2 concentration which led to lower FSH concentration, an earlier appearance of the LH surge, and ovulation of a smaller follicle.

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CHAPTER 3

ROLE OF INCREASED OESTRADIOL ON AN EARLIER LH SURGE, DECREASED DIAMETER OF THE PREOVULATORY FOLLICLE, AND DECREASED FSH CONCENTRATION PRECEDING DOUBLE OVULATION IN HEIFERS ***(to be submitted to Reproduction).***

[EFEITOS DO AUMENTO NAS CONCENTRAÇÕES DE ESTRADIOL SOBRE A OCORRÊNCIA DE UM PICO DE LH PRECOCE, REDUÇÃO DO DIÂMETRO DO FOLÍCULO PREOVULATÓRIO E DIMINUIÇÃO DO FSH PLASMÁTICO EM NOVILHAS COM OVULAÇÃO DUPLA]

Authors: Miller P. Palhão; Mohamed A Beg; Marcelo T. Rodrigues and Oliver J. Ginther.

3.1. ABSTRACT

The day-4 ablation model for increasing the incidence of double ovulations in heifers was also used for this study. The objective was to determine the role of the oestratiol-17β (E2) in reported follicle and hormone differences between single and double ovulations. The E2 was given to heifers for eight treatment (1.2 mg/treatment) or four treatments (0.07 or 0.09 mg/treatment) at 6-h intervals in two experiments beginning at the time off the expected deviation (n = 6 to 8 heifers/treatment group). In each experiment, the E2 treatments induced concomitant preovulatory surges in LH and FSH at mean of 24 to 34 h after first treatment, compared to 58 h in the vehicle groups. At the time of the LH peak, the diameter of the preovulatory follicle was a mean of 13 mm in the vehicle groups and 10 mm in all E2-treated groups. Thus, E2 treatments induced a significantly earlier LH surge with ovulation of significantly smaller follicles. The E2 treatment did not lower the FSH concentrations before the preovulatory FSH surge as has been reported for double ovulations. The 0.15 mg doses of E2 were associated with greater FSH concentration at peak of the preovulatory FSH surge, but the 0.07 or 0.09

mg doses did not have a similar effect. Results did not support the hypothesis that E2 is responsible for the reported reduced FSH concentrations before the preovulatory FSH surge in double ovulators. Results supported the hypotheses that increased E2 concentrations in heifers with double preovulatory follicles accounts for the reported earlier occurrence of the preovulatory LH surge and smaller preovulatory follicles in double ovulations.

3.2. RESUMO

O presente experimento também usou o modelo de punção e aspiração (ablação) folicular no quarto dia do ciclo estral para aumentar a incidência de duplas ovulações em novilhas da raça Holandesa. O objetivo foi determinar o papel do estradiol-17β (E2) nas diferenças entre animais com ovulações simples e duplas. Foram administradas oito (0,15 mg/injeção) ou 4 injeções de E2 (0,07 ou 0,09 mg/injeção), a cada intervalo de 6 h, em dois experimentos. Os experimentos iniciaram próximo ao momento esperado da divergência folicular (n = 6 a 8 novilhas/grupo). Em cada experimento, os tratamentos com E2 induziram os picos preovulatórios de FSH e LH, concomitantemente, em média de 24 a 34 h após a primeira injeção, comparando com 58 h no grupo veículo. Ao momento do pico de LH, o diâmetro médio do folículo preovulatório foi de 13 mm no grupo controle e 10 mm em todos os grupos tratados com E2. Desta maneira, os tratamentos com E2 induziram um pico de LH significantemente precoce com ovulação de folículos significantemente menores. O tratamento com E2 não reduziu as concentrações de FSH antes do pico preovulatório de FSH como foi demonstrado para novilhas com ovulações duplas. As doses de 0,15 mg de E2 foram associadas com uma maior concentração de FSH ao pico preovulatório de FSH, no entanto, as doses de 0,07 ou 0,09 mg não tiveram efeito similar. Estes resultados não suportaram a hipótese de que o E2 foi responsável pelas reduzidas concentrações de FSH antes do pico

preovulatório de FSH reportados em novilhas com ovulações duplas. Porém, as hipóteses de que as concentrações de E2, aumentadas em novilhas com dois folículos preovulatórios, foram responsáveis pela ocorrência de um pico preovulatório de LH precoce e pelo menor diâmetro dos folículos preovulatórios em novilhas com dupla ovulação.

3.3. INTRODUCTION

Only a few studies have been done in monovular species on the preovulatory hormonal changes associated with double ovulation, owing to the low natural incidence. Approaches that have been used include selecting cattle with a high twinning rate (Echternkamp *et al.* 2004), selecting mares with multiple follicles of preovulatory diameter (Ginther *et al.* 2008), and increasing the incidence of double ovulation without the use of exogenous hormones in heifers (Palhao *et al.* 2009) and mares (Ginther *et al.* 2009). Follicle ablation by aspiration of contents has been used in both species to increase the prominence of the resulting FSH surge and the incidence of double ovulation. In cattle, follicles emerge in waves, and the dominant follicle of the first postovulatory wave attains the diameter of a preovulatory follicle but does not ovulate, unless luteal regression occurs (Mussard *et al.* 2007, Palhao *et al.* 2009). A bovine model for increasing the double-ovulation rate involves aspirating the contents of all follicles ≥ 5 mm 4 to 6 days postovulation (Acosta *et al.* 2005, Palhao *et al.* 2009); FSH of the surge that stimulated emergence of the wave is at low concentration at 4 to 6 days (Kulick *et al.* 2001). The resulting FSH surge rebounds above normal prominence and induces an increased incidence of codominant follicles (≥ 10 mm) and, if prostaglandin F2α (PGF) is given, an increased incidence of double ovulation (e.g., 29% vs 2%; Palhao *et al.* 2009).

The emerging follicles of a wave grow in a common-growth phase until the beginning of diameter deviation when the largest follicle is 8.0 to 8.9 mm (Beg *et al.* 2002) or a mean of 8.5 mm (Ginther *et al.* 1996). Deviation is characterized by a continued growth rate of the future dominant follicle and a reduced growth rate and regression of the subordinate follicles (Ginther *et al.* 1996). Intrafollicular oestradiol begins to increase differentially in the future dominant follicle when the follicle is 7.0 to 7.9 mm or before the beginning of diameter deviation (Beg *et al.* 2002). Circulating oestradiol begins to increase at the beginning of diameter deviation (Ginther *et al.* 1999, 2000). Experimentally increasing or decreasing the circulating oestradiol concentration at the expected beginning of deviation results in a decrease or increase, respectively, in FSH concentration (Ginther *et al.* 2000), indicating a functional relationship between the two hormones. In addition, oestradiol has a local functional role in deviation, independent of the systemic negative effect on FSH (Beg *et al.* 2003).

Spontaneous codominant follicles compared to a single dominant follicle in heifers are associated with greater FSH and LH concentrations before deviation, a greater reduction in FSH after the beginning of deviation, and greater concentrations of oestradiol-17β (E2) after deviation (Kulick *et al.* 2001) and cows (Lopez *et al.* 2005). About 60% of codominant follicles result in double ovulations in cattle (Palhao *et al.* 2009). Double ovulation is temporally associated with an earlier and greater increase in circulating E2 concentrations, an earlier LH surge, ovulation at a smaller follicle diameter, and lower FSH concentrations in heifers (Palhao *et al.* 2009) and mares (Ginther *et al.* 2008, 2009). Preovulatory circulating LH concentrations were not altered in either species. These results have indicated that differences in hormone concentrations between single and double ovulators during the preovulatory period in monovular species can be considered a result rather than a cause of double ovulation. The interpretation (Palhao *et al.* 2009) that increased E2 from the double follicles

hastens the LH surge and lowers the concentrations of FSH and diameter of follicles was based on temporal rather than functional research results.

The present experiments tested three hypotheses that the increased E2 concentrations from double follicles accounts for the following reported characteristics of double ovulation in heifers: (1) earlier occurrence of the preovulatory LH surge, (2) smaller preovulatory follicles, and (3) lower concentrations of FSH.

3.4. MATERIAL AND METHODS

3.4.1. Animals and ultrasonography

Thirty-five Holstein heifers aged 12–18 mon and weighting 330–580 kg were used. All heifers had a single ovulation during the previous oestrous cycle with at least two consecutive oestrous cycles of 16 to 24 days. The heifers were acclimated to the handling procedures for at least 2 wk and did not have apparent abnormalities of the reproductive tract based on transrectal ultrasound examinations (Ginther 1998). Heifers remained healthy and in good body condition throughout the experiments. Feeding consisted of a mixture of grass and alfalfa hay and free access to water and a mineral supplement. Animals were handled in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research.

A duplex B-mode (grey-scale) and pulsed-wave colour-Doppler ultrasound instrument (Aloka SSD 3500; Aloka American, Wallingford, CT, USA) equipped with a linear-array 7.5-MHz transducer was used. Transrectal ultrasonography of the ovaries for detecting ovulation, measuring follicles, and ablating follicles were done as described (Ginther 1998). Heifers were checked once daily for ovulation beginning when a preovulatory follicle was present. Four days after ovulation, all follicles ≥ 5 mm in both ovaries were ablated to induce a surge of FSH and an associated follicular wave (Fig. 1; Gibbons *et al.* 1997, Acosta *et al.* 2005, Palhao *et al.* 2009). The induced wave

was considered the experimental follicular wave. Follicle ablations were done by ultrasound-guided transvaginal aspiration of follicle contents, using a 17-ga needle connected to a vacuum pump (Bergfelt *et al.* 1994, Bodensteiner *et al.* 1996). Follicle sites with a refilled fluid collection of ≥ 5 mm in diameter 5 days postovulation were aspirated again, and the three largest follicles of the induced wave were scanned twice a day until the next ovulation or ovulations. Follicle identity was maintained from examination-to-examination as described (Pierson & Ginther 1988, Ginther 1998). The three largest follicles (F1, F2, F3; F1 = largest) were identified according to diameter of F1 on the day preceding ovulation or had reached 108 h without ovulating. The identities were used retrospectively to identify follicles at the expected beginning of the experiments (Hour 0; beginning of follicle deviation).

Six days after ovulation (2 d after follicle ablation), heifers received two injections of 25 mg PGF2α (Dinoprost tromethamine; Lutalyze; Pfizer Animal Health, New York City, NY, USA) 12 h apart to induce luteolysis. When the largest follicle of the induced follicular wave reached ≥ 8.2 mm (expected beginning of deviation), the heifers were randomized into experimental groups. The randomization distributed the means and ranges for body weights, so that there were no significant differences in body weight among experimental groups. A diameter of 8.2 mm was selected to represent the expected beginning of deviation so that the actual diameter would be approximately 8.5 mm, owing to the 12-h interval between examinations. Beginning at Hour 0, heifers were given an intramuscular injection of a designated dose of oestradiol benzoate or vehicle every 6 h for eight (experiment 1) or four (experiment 2) treatments. Blood samples were taken from the tail vein every 6 h until 11 days after the pretreatment ovulation, and measurement of follicles continued every 12 h until ovulation or Hour 108 (Fig. 3.1). The oestradiol benzoate was dissolved in benzyl

alcohol and diluted in sesame oil, and 2 ml of the solution or the sesame vehicle was used for each designated dose.

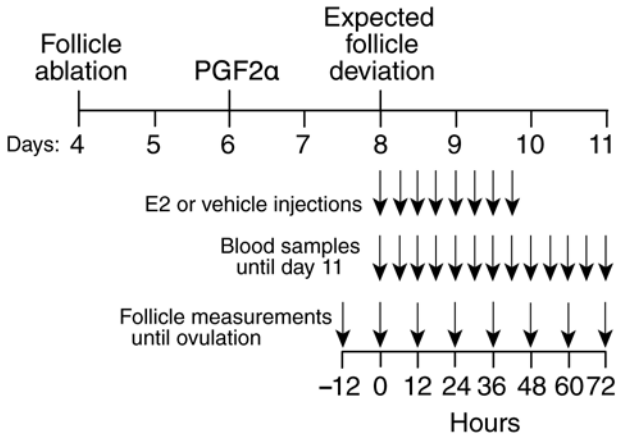

Figure 3.1. Diagram of protocol for Experiment 1. Days = number of days after ovulation. Hours = number of hours after the beginning of deviation. Protocol for Experiment 2 was similar, except that four injections instead of eight injections of E2 or vehicle were given at Hours 0, 6, 12, and 18. The dose/injection of E2 was 0.15 mg in experiment 1 and 0.07 or 0.09 mg in experiment 2.

A dominant follicle of the experimental follicular wave was defined as one that attained 10 mm (Kulick *et al.* 2001, Sartori *et al.* 2001). The preovulatory diameter used the measurements at the last examination before ovulation. Discrete end points during the experimental follicular wave were number of dominant follicles and ovulations per heifer; diameters of F1 at maximum, at the LH peak, and at preovulation; diameter of F2 at maximum; intervals from Hour 0 to the LH peak and from the LH peak to ovulation; and concentrations at the LH peak for LH, FSH, and E2. The preovulatory and periovulatory FSH surges were defined as the surge occurring in synchrony with the LH surge and the surge encompassing ovulation, respectively (Haughian *et al.* 2004).

3.4.2. Experimental design

3.4.2.1. Experiment 1

This initial experiment used a known (Ginther *et al.* 2000) pharmacological dose of E2. At the expected beginning of deviation (Hour 0), heifers were randomized into two

groups (n = 8 heifers/group) to receive either vehicle or a total dose of 1.2 mg E2 in eight treatments (0.15 mg/treatment) at 6-h intervals. The eight treatments were used to approximate the reported 48-h interval from follicle deviation to the preovulatory peak of E2 concentrations in double ovulators (Palhao *et al.* 2009). The total dose of E2 (1.2 mg) was greater than the 1.0 mg single dose that resulted an E2 concentration that was about 5-fold greater than for the maximum mean concentration of the follicular phase (Araujo *et al.* 2009).

3.4.2.2. Experiment 2

Prior to experiment 2, a dose-titration study was done to compare the circulating E2 concentrations for dose/treatment of 0.00, 0.05, 0.07, 0.09 and 0.11 mg (n = 3 heifers/dose) given in four treatments at 6-h intervals. The heifers were randomized within replicates according to the day of the estrous cycle. Blood samples were taken at 0 (just before first treatment), 6, 12, 18, and 24 h (6 h after the last treatment). The resulting E2 concentrations were compared with concentrations that were previously observed for the preovulatory surge of single and double ovulators (Palhao *et al.* 2009). The doses that most closely resulted in the E2 concentrations for single and double ovulators were 0.07 and 0.09 mg/treatment, respectively, and were used in experiment 2 to represent maximal physiological doses. Based on the titration study, the heifers of experiment 2 were randomized into three groups to receive vehicle (n = 6) or a total E2 dose of 0.28 mg (n = 7) or 0.36 mg (n = 6) in four treatments at 6-h intervals (0.07 or 0.09 mg/treatment). Four treatments at 6-h intervals were used, owing to the occurrence of the peak of the induced LH surge after four or five E2 treatments in experiment 1.

3.4.3. Blood samples and hormone assays

Blood samples were collected into heparinized tubes and centrifuged (1500 x g for 10 min), and the plasma was separated and stored at –20 ^{o}C until assayed. Concentrations of FSH and LH were assayed for all samples. Assay of E2 was done 24, 12, 6, and 0 h before the peak of the LH surge and 6, 12, and 24 h after the peak. An additional sample 48 h after the peak was assayed in experiment 1. Concentrations of FSH, LH, and E2 were measured by RIAs as previously described for bovine plasma in our laboratory (Palhao *et al.* 2009). The intra- and interassay coefficients of variation and mean sensitivity, respectively, were 9.1%, 5.2%, and 0.1 ng/ml for LH; 6.1%, 8.0%, and 0.02 ng/ml for FSH; and 14.1%, 7.7%, and 0.1 pg/ml for E2.

3.4.4. Statistical analyses

Data for follicle and hormone end points that were not normally distributed, according to Shapiro-Wilk tests, were transformed to natural logarithms or ranks. Sequential diameters of follicles and hormone concentrations were normalized to Hour 0 (expected beginning of deviation and hour of first treatment) and also to the peak of the LH surge. Data were analyzed for the main effects of group (E2 versus vehicle) and hour and their interaction. The SAS MIXED procedure with a REPEATED statement was used to account for the autocorrelation between sequential measurements (9.2 Version; SAS Institute Inc., Cary, NC, USA). If a significant main effect of hour was obtained, differences between Least Square Means for hormone concentrations or follicle diameter among hours within each group were compared using the Least Significant Difference test. When a significant effect of group or interaction was detected, the difference between two groups within an hour were assessed by a Student's *t*-test, and Duncan's multiple range tests was used to assess differences among three groups. The single-point or discrete data were analyzed by one-way ANOVA. Frequency data were

evaluated by Fisher's Exact test. Data are given as the mean ± S.E., unless otherwise stated. A probability of $P \leq 0.05$ indicated that a difference was significant, and probabilities between $P > 0.05$ and $P \leq 0.1$ indicated that significance was approached.

3.5. RESULTS

Probabilities for main effects (group or hour) and the interaction that were significant or approached significance are shown in the indicated figures or legends, and probabilities for differences in discrete end points are shown in the tables.

3.5.1. Experiment 1

After inspection of the follicle data, the E2-treated heifers were subdivided into an E2/ovulatory group (n = 4) and an E2/anovulatory group (n = 4). Ovulation occurred at Hours 60 or 72 in the E2/ovulatory group, whereas in the E2/anovulatory group, only one heifer spontaneously ovulated and the ovulation occurred after Hour 108. The frequency of codominant follicles was greater in the vehicle group than in the E2/ovulatory group (Table 3.1). Codominant follicles did not develop in any heifer in the E2/ovulatory group. In the E2/anovulatory group, 4 of 4 heifers developed a second dominant follicle in the experimental follicular wave but not until Hours 72 to 120. Heifers in the E2/anovulatory group were treated after Hour 108 with GnRH (not part of experiment). An induced ovulation occurred from F2 in the heifer that spontaneously ovulated from F1 and from both F1 and F2 in the remaining three heifers. The diameter of F1 reached 17.5 to 21.2 mm before the induced ovulation. In comparison, the largest preovulatory follicle among heifers in the E2/ovulatory group and vehicle group was 11.3 mm and 14.6 mm, respectively.

Table 3.1. Mean (± S.E.) for discrete follicle and hormone characteristics for heifers treated with 1.2 mg of oestradiol every 6 h for eight treatments beginning at Hour 0[a]. Experiment 1.

End points	Group			Probability
	Vehicle	E2/ ovulatory	E2/ Anovulatory[b]	
Number of heifers	8	4	4	- - -
Number/heifer				
Dominant follicles	2.0 ± 0.2[c]	1.0 ± 0.0[d]	1.8 ± 0.3[c]	$P<0.0001$
Ovulations	1.5 ± 0.3	1.0 ± 0.0	(none)	$P<0.1$
Diameters (mm)				
F1 at maximum	13.8 ± 0.3[c]	11.0 ± 0.4[d]	15.3 ± 1.6[c]	$P<0.01$
F1 at LH peak	13.1 ± 0.3[c]	10.4 ± 0.3[d]	9.4 ± 0.2[e]	$P<0.0001$
F1 preovulatory	13.5 ± 0.3	10.6 ± 0.4	(none)	$P<0.0002$
F2 at maximum	11.0 ± 0.3[c]	8.2 ± 0.6[d]	10.7 ± 0.6[c]	$P<0.003$
Intervals (h)				
Hour 0 to 1st dominance	21.0 ± 3.0[c]	21.0 ± 5.7[c]	57.0 ± 17.9[d]	$P<0.02$
Hour 0 to 2nd dominance	41.1 ± 3.6	(none)	96.0 ± 6.9	$P<0.0001$
Hour 0 to LH peak	57.8 ± 4.1[c]	27.0 ± 1.7[d]	24.0 ± 0.0[d]	$P<0.0001$
LH peak to ovulation	33.8 ± 3.0	39.0 ± 3.9	(none)	NS
Concentrations at LH peak				
LH (ng/ml)	7.6 ± 0.6	8.0 ± 0.7	8.5 ± 0.4	NS
FSH (ng/ml)	0.42 ± 0.0	0.55 ± 0.0	0.54 ± 0.1	NS
Oestradiol (pg/ml)	2.6 ± 0.3[c]	6.0 ± 0.7[d]	7.7 ± 1.3[d]	$P<0.0003$

[a] Hour 0 = Expected beginning of follicle deviation when the largest follicle of the induced follicular wave was ≥ 8.2 mm. The follicular wave was induced by ablation of follicles ≥ 5 mm 4 days after ovulation and treatment with PGF2α 2 days later.
[b] No ovulations before termination of experiment at Hour 108.
[cde] Means within a row with a different superscript letter are different ($P<0.05$).

An overview is shown of the temporal relationships among groups, treatments, diameters of F1, mean hours of the peak of the LH/FSH surge, and mean hours of ovulation (Fig. 3.2). The earliest ovulation occurred at Hour 60 in three heifers, and therefore diameters of F1, F2, and F3 for the three groups were analysed for Hours 0 to 48. Diameter of F1 for Hours 0 to 48 involved significant main effects (group and hour) and a group-by-hour interaction (Fig. 3.3). The interaction represented a greater diameter in the vehicle and E2/ovulatory groups than in the E2/anovulatory group at Hours 12 and 24 and a greater diameter in the vehicle group than in the other two

groups at Hours 36 and 48. Diameter increased (P<0.05) between Hours 36 and 48 in the vehicle group but not in the other two groups. Within the E2/anovulatory group, the only significant increase in F1 diameter before Hour 48 occurred between Hours 24 and 36. Diameter of F2 also showed significant main effects and an interaction. The interaction represented greater diameter in the vehicle group than in the E2-treated groups at Hours 24 and 36 and differences among all groups at Hour 48. Diameters increased continuously in the vehicle group, but the only increase (P<0.05) before Hour 48 in the two E2-treated groups occurred between Hours 24 and 36. An apparent decrease (P<0.05) in diameter occurred in the E2/ovulatory group between Hours 36 and 48 but not in the E2/anovulatory group. Diameter of F3 had only a significant hour effect (not shown). Mean F3 diameter averaged over the three groups increased (P<0.05) between Hours 0 (7.0 ± 0.2 mm) and 24 (7.7 ± 0.2 mm) with no further changes.

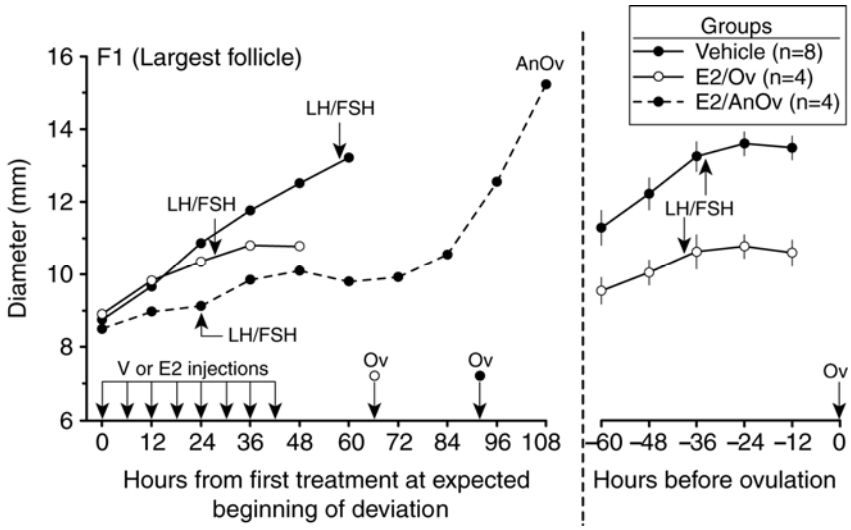

Figure 3.2. Overview showing temporal relationships among treatments for E2 or vehicle (V); means for diameters of F1; and mean hours of the peak of the LH/FSH surges, ovulation (Ov), and anovulation (AnOv) in the three experimental groups (vehicle, E2 ovulatory [E2/Ov], and E2 anovulatory [E2/Anov]). Also included are the means (± S.E.) for preovulatory diameters of F1 for the vehicle and E2/ovulatory groups; the main effects of group (P<0.05) and hour (P<0.0001) were significant. Experiment 1.

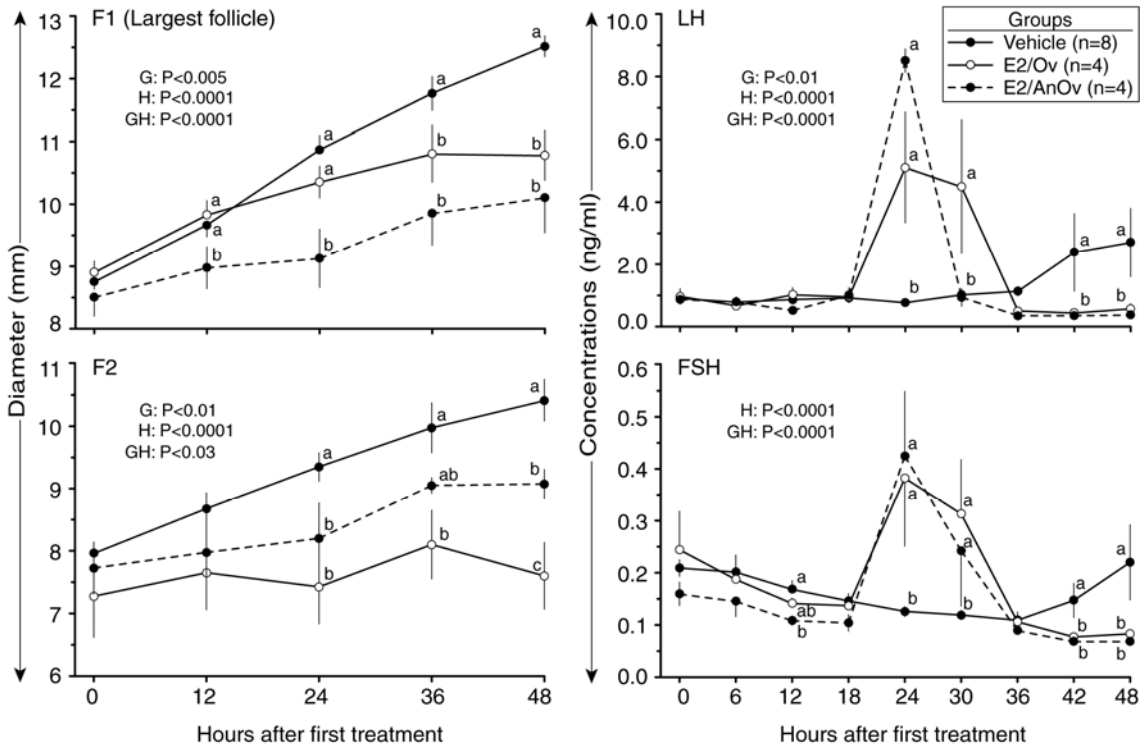

Figure 3.3. Mean (± S.E.) diameter of F1 and F2 and concentrations of LH and FSH in vehicle, E2/ovulatory (E2/Ov), and E2/anovulatory (E2/Anov) groups. Significant main effects of group (G) and hour (H) and an interaction (GH) are shown for Hour 0 to 48 for each end point. Means among groups within an hour with a different letter (abc) are different (P<0.05). Experiment 1.

The diameter of F1 for –60 to –12 h preceding ovulation showed main effects of group (vehicle and E2/ovulatory groups) and hour (Fig. 3.2). The hour effect was further considered by analysing growth rates, using the four 12-h intervals before ovulation. The main effects of group (P<0.05) and hour (P<0.0001) were significant, but the interaction was not. The group effect was from a more rapid growth from –60 to –12 h in the vehicle group (2.2 ± 0.3 mm) than in the E2/ovulatory group (1.1 ± 0.4 mm). The hour effect represented primarily a greater growth rate (P<0.05) between –60 and –36 h (1.7 ± 0.2 mm) than between –36 and –12 h (0.2 ± 0.2 mm).

The interval from Hour 0 to the peak of the preovulatory LH surge was greatest for the vehicle group, with no differences between the two E2 groups (Table 3.1). The interval from the LH peak to ovulation was not different between the vehicle and E2/ovulatory groups. The interval from Hour 0 to development of the first dominant follicle (10 mm) was greatest in the E2/anovulatory group. The mean diameter of F1 at

the LH peak was progressively less among the vehicle, E2/ovulatory, and E2/anovulatory groups. Diameter of F1 at maximum and at preovulation and diameter of F2 at maximum were greater in the vehicle group than in the E2/ovulatory group. Multiple ovulations occurred in 3 of 8 (38%) heifers in the vehicle group and in 0 of 4 heifers by Hour 108 in the E2/anovulatory group. One heifer (vehicle group) had three codominant follicles and three ovulations. For the three heifers with multiple ovulations in the vehicle group, the preovulatory diameter was greater (approached significance, $P<0.07$) for F1 (13.4 ± 0.8 mm) than for F2 (11.6 ± 0.5 mm).

Concentrations of LH and FSH for Hours 0 to 48 each showed significant main effects and an interaction (Fig. 3.3). The interaction for each gonadotropin was attributable primarily to greater concentrations at Hour 24 in each of the two E2-treated groups than in the vehicle group and greater concentrations at Hours 42 and 48 in the vehicle group. The LH peak was elicited at 25.5 ± 1.0 h (range, 24 to 30 h) at about the time of the fifth treatment (Fig. 3.2); the plasma concentration of E2 was 6.8 ± 0.8 pg/ml at the time of the peak of LH in the combined E2-treated groups. The peaks of the LH and FSH preovulatory surges occurred in the E2-treated groups at the same 6-h examination in all heifers, except for a 1-h difference in one heifer.

Concentration of LH normalized to the peak of the LH surge showed a significant hour effect, but the group effect and interaction were not significant (Fig. 3.4). There were no differences among groups in LH concentrations at the LH peak (Table 3.1). Concentration of FSH normalized to the preovulatory LH peak (0 h) for –18 to 12 h had a significant hour effect and an interaction (Fig. 3.4). The area under the curve (ng/6 h per ml) for –6 to 6 h was less ($P<0.05$) in the vehicle group (1.64 ± 0.17) than in each of the E2/ovulatory (2.04 ± 0.16) and E2/anovulatory (1.98 ± 0.18) groups. The concentration of FSH at the peak was not different among groups (Table 3.1) but was greater ($P<0.05$) in the combined E2-treated groups than in the vehicle group. The

FSH concentration for the periovulatory surge began to increase 12 h after the preovulatory peak in the vehicle group and gradually in the other groups beginning at 24 h (Fig. 3.4). As a consequence, concentrations were greater in the vehicle group at 12 to 36 h. The increasing concentrations during 12 to 60 h did not differ between the two E2-treated groups.

Concentrations of endogenous E2 in the vehicle group were at a peak at the same hour as the LH peak in 6 of 8 heifers. Endogenous and exogenous E2 in the E2-treated groups increased gradually after –24 h to the greatest concentrations at 6 to 24 h and then decreased (Fig. 3.4). Concentrations were lower in the vehicle group at all hours after –24 h. Concentrations were greater in the E2/anovulatory group than in the E2/ovulatory group at –12, –6, and 12 h. When the two E2-treated groups were analysed separately, only the hour effect was significant.

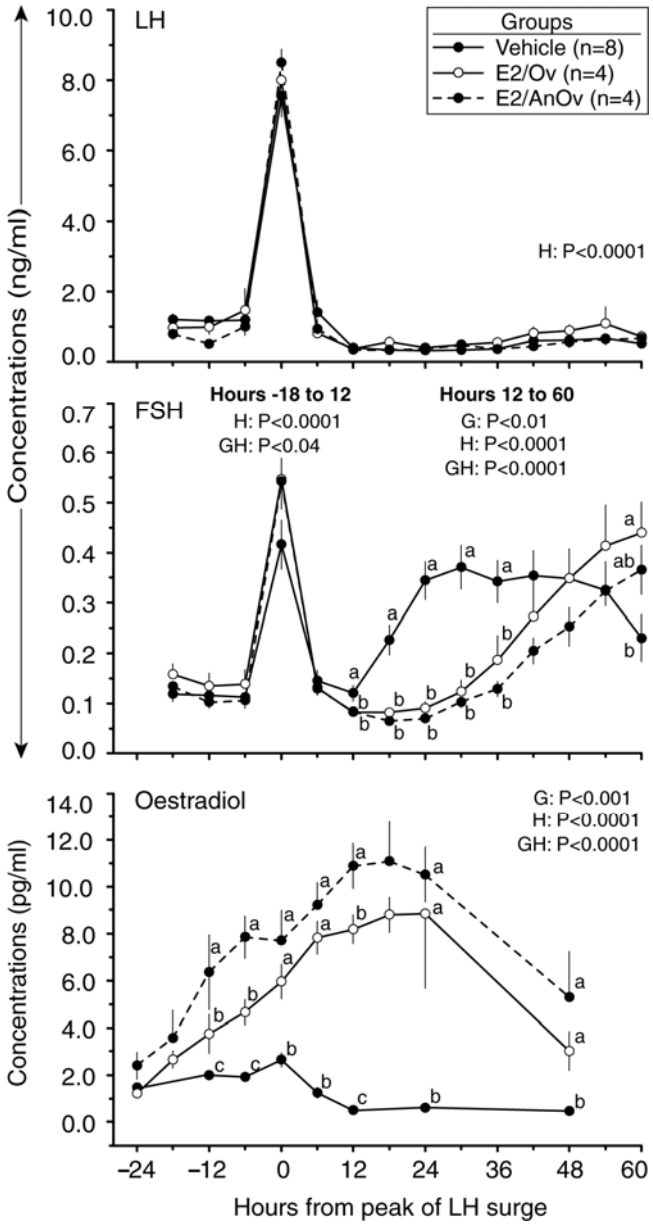

Figure 3.4. Mean (± S.E.) concentrations of LH, FSH and E2 in vehicle, E2/ovulatory (E2/Ov) and E2/anovulatory (E2/Anov) groups. Significant main effects of group (G) and hour (H) and an interaction (GH) are shown for 18 h before to 60 h after the peak of preovulatory LH surge for LH, for –18 to 12 h and 12 to 60 h for FSH, and 24 h before to 48 h after peak of preovulatory LH surge for E2. The interaction was significant for FSH and E2, and means among groups within an hour with a different letter (abc) are different (P<0.05). Peak concentrations of FSH was not different among groups but was greater (P<0.05) for the combined E2-treated groups than for the vehicle group. Experiment 1.

3.5.2. Experiment 2

Three heifers, one in each group, did not ovulate by Hour 108. Two of these had an apparent preovulatory LH surge at the expected hour, according to the LH concentrations in other heifers in their group and were included in the statistical analyses, except that normalization to ovulation was precluded. The other heifer did not

have an LH surge by Hour 108 and was excluded. In all three heifers, F1 grew to ≥ 15.1 mm, whereas the largest preovulatory follicle was 11.6 mm among heifers that ovulated before Hour 108. The discrete variables (Table 3.2) and sequential follicle diameters and LH concentrations for all analyses and for FSH in the analyses for Hours 0 to 48 were not significantly different between the two E2-treated groups (0.07 and 0.09 mg of E2) and were combined into a single E2 group for further data analyses. As a result of these adjustments, the number of heifers in the analyses of the vehicle versus the combined E2 groups was 6 and 12, respectively.

Table 3.2. Mean (± S.E.) for discrete follicle and hormone characteristics for heifers treated with 0.07 or 0.09 mg oestradiol (E2) or vehicle every 6 h for four treatments beginning at Hour 0[a]. Experiment 2

End points	Group		Probability
	Vehicle	E2[b]	
Number of heifers	6	12	- - -
Number/heifer			
Dominant follicles	1.3 ± 0.2	1.1 ± 0.1	NS
Ovulations	1.0 ± 0.3	0.9 ± 0.1	NS
Diameters (mm)			
F1 at maximum	13.5 ± 0.5[c]	10.7 ± 0.2[d]	$P<0.0001$
F1 at LH peak	13.1 ± 0.6[c]	10.2 ± 0.2[d]	$P<0.0001$
F1 preovulatory	13.3 ± 0.5[c]	10.5 ± 0.3[d]	$P<0.0001$
F2 at maximum	9.2 ± 0.6	9.1 ± 0.2	NS
Intervals (h)			
Hour 0 to first dominance	20.0 ± 2.5	27.3 ± 2.8	NS
Hour 0 to LH peak	57.6 ± 4.5[c]	34.0 ± 2.6[d]	$P<0.0002$
Concentrations at LH peak			
LH (ng/ml)	11.1 ± 1.9	11.6 ± 1.6	NS
FSH (ng/ml)	0.45 ± 0.1	0.45 ± 0.0	NS
Oestradiol (pg/ml)	3.2 ± 0.4[c]	4.6 ± 0.5[d]	$P<0.05$

[a] Hour 0 = Expected beginning of follicle deviation when the largest follicle of the induced follicular wave was ≥ 8.2 mm. The follicular wave was induced by ablation of follicles ≥ 5 mm 4 days after ovulation and treatment with PGF2α 2 days later.
[b] End points were not different between E2 doses of 0.07 and 0.09 mg, and data were combined.
[cd] Means within a row with a different superscript letter are different ($P<0.05$).

The numbers of codominant follicles and ovulations were not different between the vehicle and combined E2 groups (Table 3.2). Two heifers in each of the vehicle and

E2 groups had two dominant follicles and one heifer (vehicle group) double ovulated. Diameter of F1 during Hours 0 to 48 showed significant main effects (group and hour) and a significant interaction of group by hour (Fig. 3.5). The interaction represented similar diameters between groups for Hours 0 to 24, followed by greater diameters in the vehicle group at Hours 36 and 48. Diameter of F2 showed a significant hour effect and an interaction (Fig. 3.5). The interaction represented a progressive increase for F2 between Hours 0 and 36 in the vehicle group. The increase in F2 for Hours 0 to 12 was similar between the vehicle and E2 groups, but thereafter F2 remained at an approximately constant diameter in the E2 groups. As a result F2 tended to be larger (P<0.06) in the vehicle group than in the E2 groups at Hour 36. Diameter of F3 was not different between groups or among hours (not shown). The interval from Hour 0 to the LH peak and diameters of F1 at maximum, at the LH peak, and at preovulation were greater in the vehicle group than in the combined E2 group (Table 3.2).

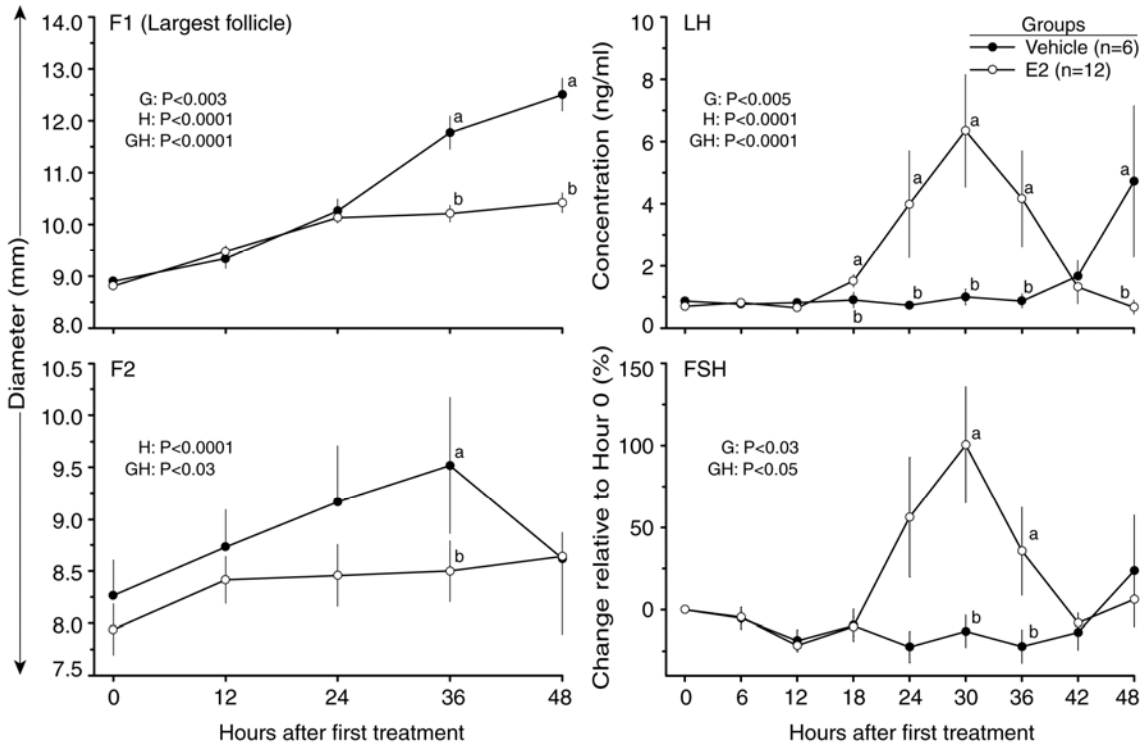

Figure 3.5. Mean (± S.E.) diameter of F1 and F2, concentration of LH, and percentage change in FSH in vehicle and combined E2-treated groups. Significant main effects of group (G) and hour (H) and an interaction (GH) are shown for Hours 0 to 48 after the first treatment. Means between groups within an hour with a different letter (ab) are different (P<0.05). Experiment 2.

Concentrations of LH for Hours 0 to 48 showed a significant main effect of hour and a group-by-hour interaction (Fig. 3.5). The interaction was attributable primarily to greater concentrations between Hours 18 and 36 in the combined E2 groups than in the vehicle group and greater concentrations (approached significance, $P<0.08$) at Hour 48 in the vehicle group. The LH peak was elicited at Hour 34.0 ± 2.6 or 16 h after the last E2 treatment. Concentration of FSH was greater ($P<0.02$) at Hour 0 in the E2 group than in the vehicle group, and the concentrations were converted to percentage change from Hour 0. The percentage change showed a group effect and an interaction. The percentage change in concentrations by Hours 30 ($P<0.01$) and Hour 36 ($P<0.05$) were greater in the combined E2 groups than in the vehicle group.

When the data were normalized to ovulation, the diameter of F1 had significant effects of group and hour similar to experiment 1 (data not shown). Concentration of LH and FSH were not different between groups at the hour of the LH peak (Table 3.2). Only the hour effect for –24 to 60 h relative to the LH peak was significant (Fig. 3.6). For FSH, the hour effect and the interaction from –24 h to 60 h relative to the peaks of the FSH surge were significant (Fig. 3.6). The interaction was represented primarily by lower concentrations in the E2 group at 12 to 24 h.

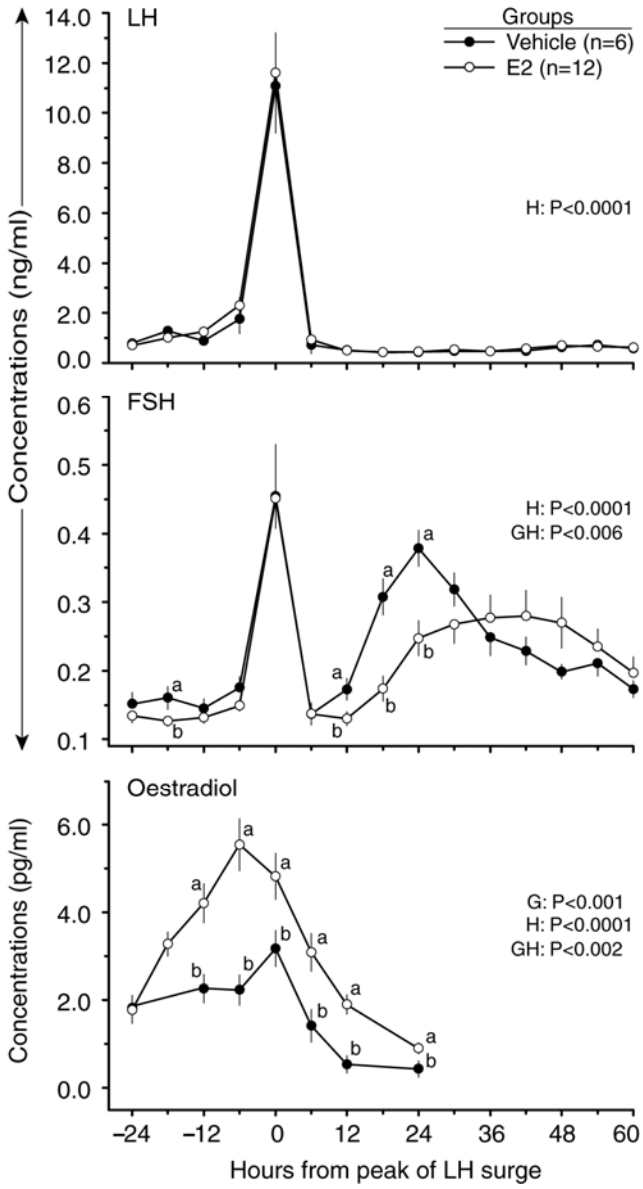

Figure 3.6. Mean (± S.E.) concentrations of LH, FSH and oestradiol in vehicle and combined E2-treated groups. Significant main effects of group (G) and hour (H) and an interaction (GH) are shown for 24 h before to 60 h after the peak of preovulatory LH surge for LH and FSH and 24 h before to 24 h after peak of preovulatory LH surge for E2. Means between groups within an hour with a different letter (ab) are different (P<0.05). Experiment 2.

When E2 concentrations were normalized to the LH peak, significant effects of group, hour, and their interaction were found (Fig. 3.6). In the combined E2-treated groups, concentrations of oestradiol increased until –6 h before the LH peak, whereas in the vehicle group, the only significant increase occurred between –24 and 0 h. The concentration of E2 at the LH peak was greater in the E2 group than in the vehicle group (Table 3.2), and the maximum occurred earlier in relation to the peak of the LH

surge (Fig. 3.6). In the vehicle group, the peak concentration occurred at the LH peak (Fig. 3.6, Table 3.2). Concentration of E2 decreased until 12 h and 24 h after the LH peak in the vehicle and E2 groups, respectively.

3.6. DISCUSSION

The previously reported (Acosta *et al.* 2005) follicle-ablation model for increasing the double ovulation rate in Holstein heifers (e.g., 29% vs 2%; Palhao *et al.* 2009) was effective as shown in the combined vehicle groups of the present experiments (4/14, 29%). Follicular codominance was observed in 75% (6 of 8) of the heifers in the vehicle group of experiment 1 and the conversion to multiple ovulations was 50% (3 of 6). These results agree with the reported 60% frequency of conversion with the follicle-ablation model (Palhao *et al.* 2009). In both experiments, development of double dominant follicles was reduced and double ovulation did not occur in the E2 groups. This is attributable to the early induction of the LH surge by E2 so that F2 did not have ovulatory capacity at the time of the surge. The interval from expected deviation or the beginning of E2 treatments (Hour 0) to the LH surge was 24 to 30 h shorter in the E2-treated groups than in the vehicle groups, and the diameter of F2 (7 to 8 mm) was well below the diameter (about 10 mm; Sartori *et al.* 2001) associated with acquisition of ovulatory capacity. Thus, double ovulation was precluded in the E2 groups.

Our previous study (Palhao *et al.* 2009) indicated that differences between single and double ovulators in follicle diameters and systemic concentrations of LH, FSH, and E2 were a result rather than a cause of double dominant follicles and double ovulations in heifers. The interpretation on a temporal basis was that greater E2 concentrations from two dominant follicles resulted in an earlier occurrence of concomitant LH and FSH preovulatory surges, and the smaller preovulatory follicles in turn were attributed to the earlier occurrence of the LH surge and lower concentrations

of FSH. The present two experiments examined these relationships on a functional rather a temporal basis. In experiment 1, the E2 protocol (0.15 mg/treatment every 6 h for eight treatments) was expected to exceed physiological concentrations according to previous studies (O'Rourke *et al.* 2000, Araujo *et al.* 2009). In experiment 2, the E2 protocol was adjusted by reducing the number of 6-h treatments to four and lowering the doses to 0.07 or 0.09 mg/treatment. The number of treatments was reduced so that the last treatment at Hour 18 would precede the peak of the induced LH surge; in experiment 1, the peak occurred at 24 to 36 h after first treatment. Reducing the dose per treatment to more closely mimic endogenous concentrations in double ovulators was only partly successful in that the concentration at the LH peak was 30% greater than in the vehicle group. However, the range of E2 concentrations in 7 of 12 individuals heifers in the E2-treated group overlapped with the range of concentrations in the vehicle group. The disparity between the E2 and vehicle groups was greater 6 and 12 h before than at the LH peak.

Synchronous occurrence of the preovulatory LH and FSH surges in the E2- and vehicle-treated groups is consistent with the previous study (Palhao *et al.* 2009) and with synchronous occurrence in GnRH-treated and control heifers (Haughian *et al.* 2004). The similarity in the time of occurrence and pattern between the preovulatory LH and FSH surges indicated a common stimulatory mechanism, and the commonality was not disturbed by various doses of E2. The reduction in the interval from follicle deviation to the gonadotropin surges in all E2-treated groups than in the vehicle groups supports the hypothesis that the increased circulatory E2 from two dominant follicles in double ovulators accounts for the reported (Palhao *et al.* 2009) earlier occurrence of the LH surge in double ovulators. The E2 treatments induced an earlier LH surge but did not alter the concentrations of LH during the surge. In this regard, a pharmaceutical

dose of E2 induces an LH surge in ovarian-intact and ovariectomized cattle (Hansel *et al.* 1975).

Smaller diameter of preovulatory follicles (Palhao *et al.* 2009) and smaller corpora lutea (Mann *et al.* 2007) have been reported in cattle and smaller follicles have also been reported in horses (Ginther et al. 2008, 2009) and sheep (McNatty et al. 1984) in double ovulators than in single ovulators. The present results supported the hypothesis that increased E2 accounts for ovulation of smaller follicles in double ovulators. The smaller follicle diameter is considered to be an indirect effect of E2 from stimulation of an LH surge when the preovulatory follicles were about 21% smaller in the E2 groups than in the vehicle groups.

The continuing growth of the preovulatory follicle in both the vehicle and E2-treated groups in both experiments ceased and began an average plateau extending from the peak of the LH surge to ovulation (approximately 36 h). This phenomenon was only recently reported in cattle (Palhao *et al.* 2009) and occurred in spontaneous ovulators while both LH and E2 were decreasing. A similar phenomenon occurs in horses beginning 2 days before ovulation, except that LH continues to increase until after ovulation, but E2 decreases during the plateau in follicle diameter (Gastal *et al.* 2006, Ginther *et al.* 2009). Considering the two species, the trigger for the growth cessation apparently is attainment of a critical concentration of LH. Results of the present studies are novel in that they indicate that a critical diameter of the preovulatory follicle and decreasing concentrations of E2 are not components of mechanism for inducing the cessation in growth of the preovulatory follicle. The molecular basis for an apparent negative effect of a critical level of LH in terminating growth of the follicle and blocking E2 production by down-regulation of aromatase and other steroidogenic enzymes in cattle and horses has been discussed (Gastal *et al.* 2006).

The high doses and prolonged treatment with E2 (eight treatments of 0.15 mg/treatment at 6-h intervals) did not significantly alter the FSH concentrations in the E2/ovulatory group before the beginning of the preovulatory FSH surge. The low doses (0.07 and 0.09 mg/treatment for four treatments) appeared to result in lower FSH concentrations in the combined E2-treated groups at 18 h before the peak of the FSH surge. However, this apparent difference was confounded by the lower concentrations at Hour 0; there were no differences between groups after conversion of FSH to percentage change from Hour 0 in the analyses for Hours 0 to 48. However, differences in FSH concentrations were found during the preovulatory FSH surge. In experiment 1, high E2 doses had a novel positive rather than a negative effect on the FSH surge. This was shown by the greater concentrations at the peak and in area under the curve in the E2-treated groups than in the vehicle group. However, the lower E2 doses and fewer treatments in experiment 2 did not alter the FSH concentrations at the peak of the surge. Although statistical comparisons between the two separate experiments are not appropriate, the apparent differences between experiments suggested that the effect of E2 on the preovulatory FSH surge was dose dependent with the high dose being positive to FSH during the surge and the low doses being ineffective. Thus, the hypothesis that E2 had a negative effect on FSH was not supported before the FSH preovulatory surge.

The most dramatic negative effect of E2 on FSH concentrations involved the periovulatory FSH surge in both experiments. In the vehicle groups, the FSH concentrations at the nadirs 6 h before and after the peak of the preovulatory surge were similar. That is, the division between the preovulatory and periovulatory surges was complete and distinct, confirming a previous report (Haughian *et al.* 2004). In both experiments, E2 treatment was associated with a delay in the onset of the periovulatory surge. With the eight high doses of E2 (experiment 1), the periovulatory surge began

about 12 h later than with the four lower doses (experiment 2). Thus, during the preovulatory period from the ending nadirs of the LH and FSH preovulatory surges to ovulation (approximately 30 h), FSH concentrations were considerably lower in the E2-treated groups. This was attributable to the continuation of E2 treatments and therefore the continuing high E2 concentrations for at least 24 h after the LH surge in experiment 1, compared to the beginning of an E2 decrease at 6 h before the LH peak in experiment 2. An extensive study of the negative effects of various doses and routes of administration of E2 on FSH in heifers and including a review of the literature has been reported (O'Rourke *et al.* 2000). Assay of inhibin was not done for these studies, owing to the unavailability of a purified inhibin. This was unfortunate because inhibin from the follicles is a suppressor of FSH in addition to E2 (Bleach *et al.* 2001**).**

An unexpected observation was the anovulatory response to the induced LH surge in 50% of the E2-treated heifers in experiment 1. The F1 diameters at Hour 24 in individuals were 9.6, 10.5, 10.6, and 10.7 mm in the ovulatory heifers and 7.8, 9.2, 9.5, and 10.0 mm in the anovulatory heifers. Ovulatory capacity in heifers does not usually occur if the largest follicle is <10.0 mm at the time of LH treatment (Sartori *et al*. 2001) and the smaller diameter in the E2/anovulatory group compared to the E2/ovulatory group likely accounts for the anovulation. The increase in diameter of F1 and F2 between Hours 24 and 36 in the anovulatory heifers can be attributed to the FSH preovulatory surge at that time. Regrowth of F1 in the four heifers in the E2/anovulatory group began after the E2 treatments were completed. The growth occurred during the inclining FSH concentrations of the delayed secretion of FSH during the equivalent of a periovulatory surge. The anovulatory follicles can be considered cystic in that they attained diameters at Hours 108 to 228 that were approximately 4 to 6 mm greater than the largest preovulatory follicle in the vehicle group. The anovulatory follicles also apparently produced increased E2, as indicated by

the sporadically greater concentrations relative to the peak of the LH/FSH preovulatory surges than in the E2/ovulatory group. Although the experiment terminated at Hour 108, it was observed that the apparently cystic follicles responded to treatment with GnRH. The observation that follicles that failed to ovulate after the LH surge continued to expand as apparent cystic follicles during the subsequent FSH surge (comparable to the periovulatory surge) may be useful in proposals on a components of the mechanism associated with the natural occurrence of follicular cysts. In this regard, follicular cysts occur when heifers are treated with high doses of E2 or are exposed for a long time to E2 or when E2 treatment is given at a time when the LH surge occurs in the absence of a potentially ovulatory follicle (reviewed in Gumen and Wiltbank 2005).

In conclusion, a high dose of E2 (0.15 mg/treatment every 6 h for eight treatments) and a low dose (0.07 or 0.09 mg/treatment every 6 h for four treatments) beginning at expected follicle deviation were used in separate experiments. Both doses induced concomitant preovulatory surges in LH and FSH within 24 h at a time when the largest follicle was only about 10 mm. These results supported the hypothesis that the reported greater E2 concentrations in heifers with double dominant follicles and double ovulations accounts for the earlier development of the preovulatory LH surge and ovulation of smaller follicles. Treatment with E2 also delayed the periovulatory FSH surge resulting in reduced FSH concentrations during the approximately 24 h before ovulation. The results provided functional indications that the reported temporal differences between single and double ovulators in E2 and FSH concentration, time of the LH surge, and diameter of the ovulatory follicles can be attributable to greater circulating E2 concentrations from double follicles in double ovulators.

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CHAPTER 4

NECESSITY OF SEQUENTIAL PULSES OF PROSTAGLANDIN (DINOPROST, LUTALYSE) FOR COMPLETE PHYSIOLOGIC LUTEOLYSIS IN CATTLE ***(Biology of Reproduction 80, 641–648, 2009).***

[NECESSIDADE DE PULSOS SEQUENCIAIS DE PROSTAGLANDINA (DINOPROST, LUTALYSE) PARA A COMPLETA LUTEÓLISE EM VACAS]

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4.1. ABSTRACT

The luteolytic effects of exogenous prostaglandin (PGF - Dinoprost tromethamine [Lutalyse]; Pfiser Animal Health, New York, NY) that did and did not simulate natural 13,14-dihydro-15-keto-PGF (PGFM) pulses were studied during mid-diestrus in 42 Holstein heifers. Plasma concentrations of PGF were assessed by assay of PGFM. In experiment 1, a single intrauterine injection of 4.0 mg of PGF into the uterine horn ipsilateral to the corpus luteum resulted in a precipitous progesterone decline, whereas sequential injections of 0.25 or 1.0 mg every 12 h resulted in a stepwise decrease ($P<0.05$) following each injection. A progesterone increase occurred during the first 5 min before the luteolytic decrease but only for the 4.0-mg dose. From the results of experiment 2, a 2-h intrauterine infusion of a total of 0.5 mg of PGF was judged to best simulate a natural PGFM pulse. In experiment 3, simulation of sequential pulses at 12-h intervals resulted in a continuous precipitous decrease in progesterone to <1 ng/ml by the beginning of the fourth simulated pulse. In contrast, a single simulated pulse resulted in a 6-h progesterone decrease to a constant concentration for 3 days after treatment, followed by a return to control concentrations. The mean (± S.E.) interval between the pretreatment and post-treatment ovulations was shorter ($P<0.05$) in the

group with sequential simulated pulses (14 ± 1 day) than in the group with a single pulse (21 ± 1 day). Results indicated that excessive PGF doses may stimulate nonphysiologic progesterone responses and supported the hypothesis that sequential PGF pulses are required to stimulate natural luteolysis in cattle.

4.2. RESUMO

Os efeitos luteolíticos da prostaglandina (PGF - Dinoprost tromethamine [Lutalyse]; Pfiser Animal Health, New York, NY) exógena, que simulam ou não os pulsos naturais de 13,14-dihydro-15-keto-PGF (PGFM), foram estudados durante o diestro em 42 novilhas da raça Holandesa. As concentrações plasmáticas de PGF foram acessadas por meio da análise de seu metabólito (PGFM). No experimento 1, uma única injeção de 4,0 mg de PGF administrada dentro do corno uterino ipsilateral ao corpo lúteo resultou em um declínio rápido na concentração de progesterona, enquanto que injeções sequenciais de 0,25 ou 1,0 mg a cada 12 h resultaram em uma queda gradual (P<0,05) seguindo cada injeção. Ocorreu um aumento na concentração de progesterona durante os primeiros 5 minutos após a injeção de 4,0 mg, seguindo de decréscimo e luteólise. Este aumento inicial não foi observado com as demais concentrações de PGF. Com os resultados do experimento 2, a infusão intrauterina de 2 h com um total de 0,5 mg de PGF foi considerada a que melhor simula um pulso natural de PGFM. No experimento 3, a simulação de pulsos sequenciais a cada 12 h resultou em uma queda continua na concentração de progesterona, atingindo concentrações <1 ng/ml no início do quarto pulso simulado. Em contraste, a simulação de um único pulso implicou em um decréscimo na concentração de progesterona por 6 h, permanecendo constante por 3 dias, seguindo o retorno as concentrações semelhantes às do grupo controle. O intervalo médio da ovulação pré-tratamento à ovulação pós-tratamento foi mais curto (P<0,05) no grupo que simulou pulsos sequenciais (14,0 ± 1,0 dias), quando comparado aquele

que simulou somente um pulso (21,0 ± 1,0 dias). Os resultados indicaram que uma dose excessiva de PGF pode estimular uma resposta não fisiológica da progesterona e suportam a hipótese de que pulsos sequenciais de PGF são necessários para estimular a luteólise natural em bovinos.

4.3. INTRODUCTION

Secretion of prostaglandin F2alpha (PGF) by the uterus, augmented by intraluteal PGF production, terminates the luteal phase in many species, including cattle (McCracken *et al.* 1999, Arosh *et al.* 2004, Schams & Berisha 2004, Weems *et al.* 2006, Skarzynski *et al.* 2008). The minimal effective intrauterine (IU) dose of PGF (1–2 mg (Louis *et al.* 1974)) when given into the uterine horn ipsilateral to the corpus luteum (CL) in cattle is about one tenth of the minimal systemic dose (15 mg (Lauderdale & Fokolowsky 1979)). The IU effectiveness in cattle results from a unilateral utero-ovarian pathway (Ginther *et al.* 1966, Ginther *et al.* 1967). Surgical anastomoses of the uterine vein or the ovarian artery ipsilateral of an intact uterine horn to the corresponding vessel on the unilaterally hysterectomized side have demonstrated that a venoarterial unilateral pathway adequately accounts for the local luteolytic effect of a uterine horn on the CL in the adjacent ovary (Mapletoft *et al.* 1976). The transfer of the PGF involves passage from the uterine vein to the closely adherent ovarian artery (Miller *et al.* 1981). In this regard, injection of a large dose (6 mg) of PGF into a uterine horn is followed by greater PGF concentrations in the ipsilateral ovarian artery than in the carotid artery (Hixon & Hansel 1974). Thus, the natural utero-ovarian route of delivery should be considered in studies of the mechanism of luteolysis that use exogenous PGF. A complexity in using the jugular vein for the PGF treatment and sampling is that 65% of PGF is metabolized during one passage through the lungs in cattle (Davis *et al.* 1985). The main plasma metabolite of PGF is 13,14-dihydro-15-keto-PGF (PGFM) (Kindahl

et al. 1976a), and assay of PGFM has been used as an indicator of PGF release into the circulation (Kindahl *et al.* 1976b).

Based on the PGFM concentrations, PGF is released from the uterus in pulses occurring approximately every 12 h in association with luteolysis in cattle (Kindahl *et al.* 1976a, Kindahl *et al.* 1976b, Mann & Lamming 2006, Ginther *et al.* 2007). The pulsatile release of PGF has been assumed to be an important aspect of luteolysis (Schams & Berisha 2004, Mann & Lamming 2006). However, the necessity for natural pulsatile delivery of PGF has not been demonstrated. Treatment of cattle with sequential pulses of PGF to simulate the natural pulse-delivery system has not been reported, to our knowledge. Sequential hour-long infusions of PGF into the ovarian artery of an autotransplanted ovary have been performed in ewes (Schramm *et al.* 1983, McCracken *et al.* 1984). Four sequential hour-long infusions in 19 h caused complete CL regression in one of four ewes, whereas five infusions in 25 h caused permanent regression in four of four ewes. Sequential hour-long infusions required considerably less PGF for luteolysis than continuous infusion. Results suggested that sequential pulses of PGF may be a component of the luteolytic process in ewes. However, the concentrations of PGF or PGFM and the extent to which each hour-long infusion simulated a natural PGF pulse were not given. Reported studies of the mechanisms associated with the luteolytic process in cattle that involved administration of exogenous PGF apparently did not account for the potential natural necessity of pulsatile delivery from uterus to CL.

The objectives of the present experiments were the following: 1) to determine if low IU doses of PGF require sequential treatment for the completion of luteolysis (experiment 1), 2) to develop a dose and convenient method of delivery of exogenous PGF that would result in an approximation of a natural PGFM pulse (experiment 2), and 3) to simulate PGFM pulses to test the hypothesis that sequential PGF pulses are

required for the stimulation of complete luteolysis (experiment 3). Apparent nonphysiologic responses to various doses of PGF also were considered.

4.4. MATERIAL AND METHODS

4.4.1. Animals

Animals were handled in accord with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research. Holstein heifers, aged 17–20 mo, were used in three experiments. Heifers remained healthy and in good body condition throughout each experiment. Animals were selected with docile temperament and no apparent abnormalities of the reproductive tract, as determined by ultrasound examinations (Ginther 1998). If more than one CL was present, the heifer was not used. The animals were acclimated to the handling procedures for at least 2 wk before experimentation. When indicated, heifers were sedated with a low dose of xylazine hydrochloride (14 mg/heifer i.m. of Xila-Ject; Phoenix Pharmaceutical Inc., St. Joseph, MO). Xylazine sedation reportedly (Araujo & Ginther 2009) produces hemodynamic effects when assessed in a major artery (internal iliac) but does not affect local vascular perfusion in the ovaries, based on the vascular resistance index at the ovarian pedicle and percentage of CL area with color Doppler blood flow signals.

4.4.2. Catheterizations

Injection or infusion of PGF (dinoprost tromethamine [Lutalyse]; Pfizer Animal Health, New York, NY) into the uterine horn ipsilateral to the CL (IU) was performed during mid-diestrus in all experiments. The time of injection or the beginning of infusion was designated as Hour 0. Infusion of PGF was performed at a constant rate using a variable-flow peristaltic minipump (catalog No. 13-876-4; Fisher Scientific, Pittsburgh, PA). The pump was calibrated to deliver the specified dose of PGF in 3 h in 9 ml of

vehicle (experiment 2) or in 2 h in 6 ml (experiment 3). The intravenous (IV) infusion for one group in experiment 3 was made through an indwelling surgical tubing (Tygon tubing [inner diameter, 0.040 inches]; Norton, Akron, OH) placed and secured into a jugular vein. After the IV tubing was inserted, the heifer's head was no longer restrained or approached during the hours of infusion. The IU injections (experiment 1) and IU infusions (experiments 2 and 3) were made into the lower horizontal portion of the spiraled uterine horn (segment 3 (Ginther 1998)) ipsilateral to the CL. For IU injection and infusion of PGF, the tubing (polytetrafluoroethylene AWG Tubing-TFT 15 NT [inner diameter, 0.059 inches and outer diameter, 0.083 inches]; Parker Hannifin Corporation, Fort Worth, TX) was initially inserted through an artificial insemination pipette into segment 1, the horn was straightened transrectally, and the tubing was pushed into segment 3. The tubing was secured by digital compression of the horn while the pipette was withdrawn. Maintenance of the IU location was confirmed before and at the end of infusion by transrectal ultrasonography. A session was defined as a 5-h or 6-h period after the injection or the beginning of infusion.

4.4.3. Ultrasonography

A duplex B-mode (gray scale) and pulsed wave color Doppler ultrasound instrument (Aloka SSD 3550; Aloka American, Wallingford, CT) equipped with a linear array 7.5-MHz transducer was used for transrectal scanning of the ovaries to determine the day of ovulation and cross-sectional area of the CL. The scanner's tracing function in B mode was used to determine cross-sectional area (in centimeters squared) of the CL, as previously described (Ginther 2007). A decrease in progesterone to <1 ng/ml was used as an indicator of complete luteolysis (Mann & Lamming 2006). The length of the interval from ovulation to progesterone level <1 ng/ml and the experimental interovulatory interval were determined in experiments 1 and 3.

4.4.4. Experimental Design

4.4.4.1. Experiment 1: IU Injection

This experiment was performed to determine if a dose of PGF that was ineffective as a luteolytic treatment when given in a single IU injection 9 or 10 days after ovulation would be effective when given sequentially. Simulation of a natural PGFM pulse was not expected, based on a previous study (Lamond *et al.* 1973) of circulating PGF concentration following IU injection. Doses of PGF were chosen that were expected to be ineffective, borderline, and effective for inducing luteolysis when given as a single IU treatment ipsilateral to the CL (Inskeep 1973). The four experimental groups were controls (0.0 mg of PGF, vehicle only) and the PGF doses of 0.25, 1.0, and 4.0 mg (n = 4) given in 1 ml of vehicle. Sequential treatment sessions were begun every 12 h for sessions 1 to 4, except that only session 1 was used for the 4.0-mg group, owing to expected immediate completion of luteolysis. Collection of blood samples was performed hourly at Hours 0–6 for each treatment session through an indwelling cannula in a jugular vein. In addition, a sample was collected at Minutes 0, 2, 5, 10, 15, 30, 45, and 60 after treatment with PGF but only for session 1. Concentrations of PGFM were determined for the frequent blood samples collected during the first hour of session 1 and hourly at Hours 0–6 for sessions 1–4. Progesterone was assayed for the samples collected at Minutes 0, 5, 15, 30, and 60 for the first hour of session 1 to determine if the reported immediate post treatment increase in progesterone (Lamond *et al.* 1973) occurred in each PGF treated group. Progesterone was also assayed for samples collected at 6-h intervals during 0–48 h after the beginning of session 1, thereby encompassing all of the four sessions. Thereafter, progesterone was assayed every 12 h until 24 h after morphologic regression seemed complete, based on ultrasonographic assessment of CL area (in centimeters squared) and CL color Doppler blood flow signals that involved <20% of the CL area (Ginther 2007).

Natural PGFM pulses were characterized for comparison with the experimental PGFM concentrations from the IU injections of PGF. Hourly samples were taken during a 12-h window, beginning 15 days after ovulation in four heifers. The 12-h window was used each day until CL regression was apparent by color Doppler ultrasonography. Six pulses of PGFM were identified during luteolysis (rapidly decreasing progesterone) by statistical comparison of the values for a suspected pulse against the intraassay coefficient of variation (CV), as previously described (Ginther 2007). The mean ± SD for the peak of the pulses was determined as an indicator of the variation among natural pulses.

4.4.4.2. Experiment 2: dose titration for IU Infusion

This was a preliminary dose titration study for guidance on the dose of PGF to be used for IU infusion in experiment 3. Doses of 0.125, 0.25, 0.5, 1.0, and 2.0 mg/3 h were infused (n = 2) on Day 8. Blood samples for PGFM assay were collected every hour at Hours 0–6 and for progesterone at Hours 0, 6, 24, and 48. Data were assessed by inspection. However, for guidance in the selection of a dose of PGF that best simulated a spontaneous PGFM pulse, the observations (n = 18) comprising the three highest values for the six natural pulses from experiment 1 were compared with the mean PGFM concentrations for the IU infusion doses comprising Hours 1, 2, and 3 (n = 6/dose). The dose that seemed to best simulate the PGFM concentrations and the shape of the data profile of the natural pulses and the doses that caused an apparent transient decrease in progesterone were considered in selecting a dose for experiment 3.

4.4.4.3. Experiment 3: Sequential IU

The primary objective of this experiment was to determine if sequential simulated pulses begun 9 or 10 days after ovulation would induce complete luteolysis, whereas a

single simulated pulse would produce only a transient or no decrease in progesterone. A total 0.5-mg dose of PGF (0.25 mg/h for 2 h) was used, based on the results of experiment 2. A 2-h infusion was selected for delivery of the PGF dose, rather than the 3 h used in experiment 2, based on the judgment that the 2-h infusion would adequately simulate a natural pulse. An IV group was included to compare IV vs. IU infusion. The experimental groups involved vehicle IU for a single session (session 1; 0.0-mg IU1) and a dose of PGF of 0.5-mg IV for a single session (0.5-mg IV1), 0.5-mg IU for a single session (0.5-mg IU1), and 0.5-mg IU for four sequential sessions (sessions 1–4) at 12-h intervals (0.5-mg IU4; n = 4/group). For analyses that were limited to session 1, the 0.5-mg IU1 and 0.5-mg IU4 groups were combined (n = 8). Blood samples for PGFM and progesterone assay were collected at Minutes 0, 2, 10, 15, 30, 45, and 60 for the 0.5-mg IV1 and 0.5-mg IU1 groups and were used to compare the IU rate of absorption into the circulation with increases from direct IV infusion. Samples for PGFM and progesterone were collected from all groups at Hours 0, 1, 2, 3, 4, and 5. Additional samples for progesterone only were collected at Hours 12, 24, 36, and 48 and daily thereafter until ovulation. Cross-sectional area (in centimeters squared) of the CL was determined at Hours 0, 2, 12, 24, 36, and 48. Data for CL area were converted to percentage change from Hour 0, owing to apparent differences among groups before infusion began. Concentrations of PGFM for the six natural pulses from experiment 1 were used to judge the success in simulating a natural pulse by 2-h IU infusion of PGF.

4.4.5. Blood samples and hormone assays

Blood samples were collected into heparinized tubes and centrifuged (2000 X g for 10 min), and plasma was decanted and stored (-20^{o}C) until assay. Plasma progesterone concentrations were measured using a solid-phase radioimmunoassay kit containing antibody-coated tubes and 125I-labeled progesterone (Coat-A-Count Progesterone;

Diagnostic Products Corporation, Los Angeles, CA), as previously described (Ginther 2007). The intraassay CV and sensitivity ranged from 5.8% to 11.6% and 0.02 to 0.03 ng/ml, respectively, for all experiments. Blood samples for PGFM assay were collected and immediately placed in ice-cold water for 10 min before centrifuging and storing at -20^{o}C. The plasma samples were assayed for PGFM by a modification of a radioimmunoassay procedure (Meyer *et al.* 1995, Mattos *et al.* 2004) that was adapted and validated in our laboratory for bovine plasma, and has been described in detail (Ginther 2007). The cross-reactivity of PGFM antibody with arachidonic acid, PGF2α, PGF1a, and PGE2 was <1.0% (Meyer *et al.* 1995). The intraassay CV and sensitivity ranged from 7.2% to 12.0% and 33.3 to 69.4 pg/ml, respectively, for all experiments.

4.4.6. Statistical analysis

Data were examined for normality using Shapiro-Wilk test. Data that were not normally distributed were transformed to natural logarithms or ranks. Individual end points were analyzed for time effects (minute, hour, or day), and comparisons involving groups were analyzed for main effects (group and time) and the interaction. The data were analyzed by SAS MIXED procedure (version 9.1.3; SAS Institute, Inc., Cary, NC) using a REPEATED statement with autoregressive structure to account for autocorrelation between sequential measurements and spatial power to account for uneven intervals between samples. Student paired t-tests were used to locate differences between two times within a group when a significant time effect was found. When a group-3-time interaction was significant or approached significance, the interaction was further examined by study of the time effect in each group or combinations of groups. Tukey multiple-range test was used when comparisons were made within a time among more than two means. $P<0.05$ indicated that a difference was significant, and $P<0.05$ to

$P<0.1$ indicated that significance was approached. Data are presented as the mean ± SEM unless otherwise indicated.

4.5. RESULTS

Probabilities for the main effects and interactions that were significant or approached significance and differences between selected means are given in the figures.

4.5.1. Experiment 1: IU Injection

Circulating concentrations of PGFM after an IU injection for each PGF group (doses of 0.25, 1.0, and 4.0 mg) increased in 2 min, reached maximum in 10 min, and then decreased to near the base concentration in 1 h (Fig. 4.1). The maximum concentration at 10 min for the 0.25-mg dose (1799 ± 137 pg/ ml) was greater ($P<0.0001$) than that at the peak of the six natural pulses (1065 ± 77 pg/ml; Fig. 4.2). The mean ± SD for the peak of the natural PGFM pulses was 1065 ± 188 pg/ml (range, 879-1339 pg/ml). The PGFM concentrations at Hours 0–6 in each of the four sessions within each of the 0.0-, 0.25-, and 1.0-mg groups did not differ among sessions (data not shown).

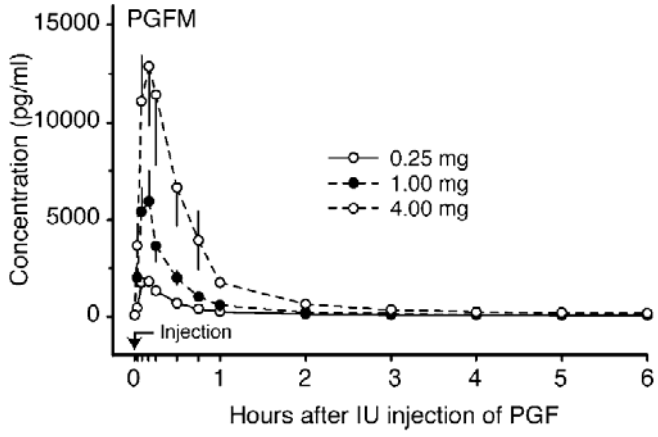

Figure 4.1. Mean (± S.E.) concentrations of PGFM after injection of the indicated doses of PGF into the uterus (n = 4). The maximum mean concentration occurred 10 min after injection. Experiment 1.

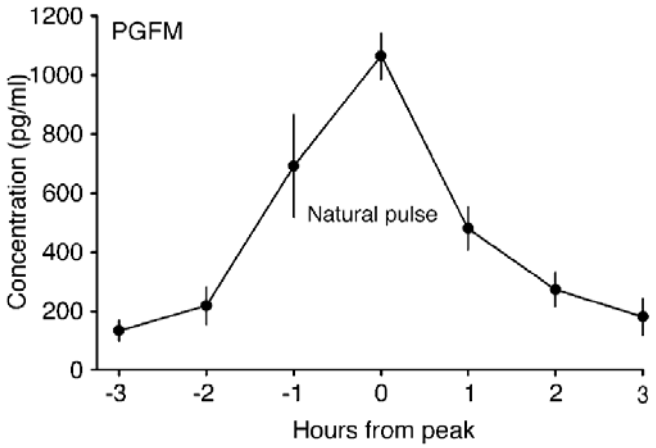

Figure 4.2. Mean (± S.E.) concentrations of PGFM pulses during the luteolytic period (n = 6). Experiment 1.

Concentrations of progesterone showed significant main effects of group (dose) and time (minutes or hours) and a group-3-time interaction for the first 60 min, for the 6 h of session 1, and for every 6 h for 48-h posttreatment, except that the interaction only approached significance ($P<0.1$) for the 6 h of session 1 (Fig. 4.3). When each group was analyzed separately for each time period (60 min, 6 h, and 48 h), the time effect was not significant for the 0.0-mg group, and only the main effect of time (minutes or hours) was significant ($P<0.001$) for the three groups that received PGF. The differences ($P<0.05$) among minutes or hours averaged over the three PGF groups are shown (Fig. 4.3). Although the interaction was not significant, an increase ($P<0.05$) between Minutes 0 and 5 occurred exclusively for the 4.0-mg group. Averaged over the three PGF groups, progesterone decreased progressively between Minutes 15 and 60. The decrease in progesterone between Hours 0 and 1 was significant ($P<0.0005$) for each PGF group, but the increase between Hours 1 and 2 was significant ($P<0.03$) only for the 0.25- and 1.0-mg groups. When the 0.25-mg and 1.0-mg groups for every 6 h for 48 h were analyzed after removing the 0.0-mg and 4.0-mg groups, only the hour effect was significant ($P<0.001$). The hour effect represented significant decreases combined for the 0.25- and 1.0-mg groups between Hours 0 and 6 ($P<0.03$), Hours 12 and 18

(P<0.0004), and Hours 24 and 30 (P<0.002) but not between Hours 6 and 12 and Hours 18 and 24. Owing to the mean decrease in progesterone to <1 ng/ml within 48 h after PGF treatment in the three PGF groups, the subsequent progesterone concentrations are not shown. However, the intervals from the pretreatment ovulation to a progesterone decrease to <1 ng/ml and to the post-treatment ovulation were shorter (P<0.05) for each of the groups that received PGF than for the 0.0-mg group (Table 4.1).

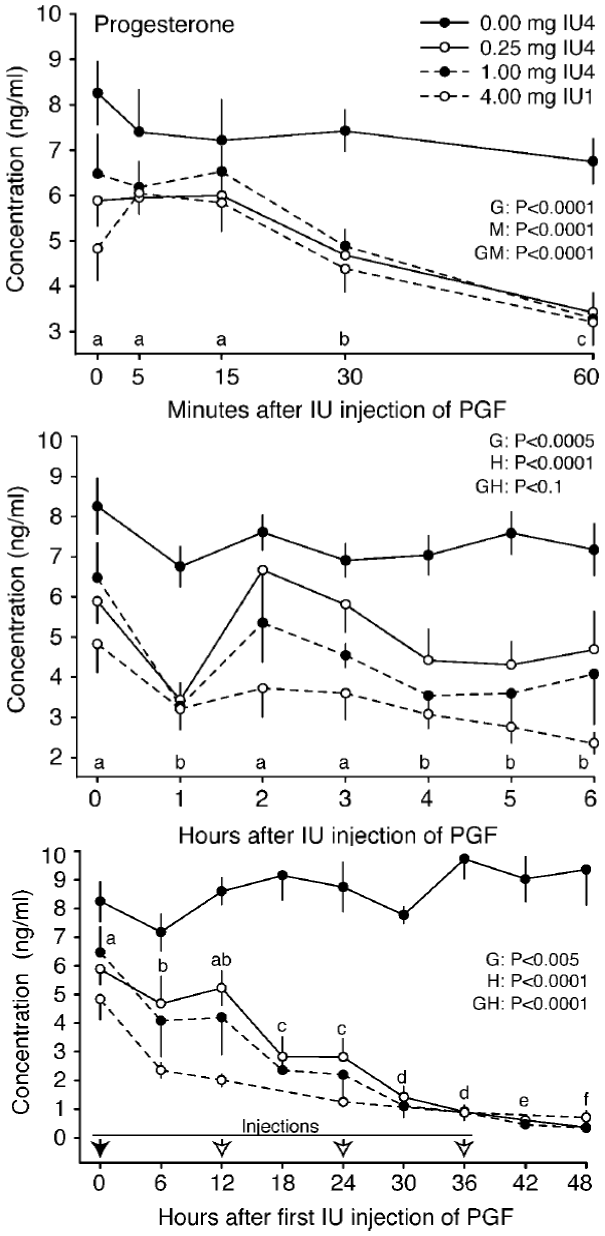

Figure 4.3. Mean (± S.E.) concentrations of progesterone during the first hour, 6 h, and 48 h after IU injection of PGF (n = 4). The 4.0-mg IU1 group was injected only at the beginning of session 1 (solid arrow on x-axis), and the remaining groups were injected at the beginning of each of sessions 1–4 at 12-h intervals (solid and open arrows). Main effects (group [G] and minute [M] or hour [H]) and interactions

(GM or GH) that were significant or approached significance are shown. Means that are different ($P<0.05$) among minutes and hours averaged for the three PGF-treated groups (two top panels) or for the two groups with lowest doses of PGF (bottom panel) are indicated by a–f. An increase ($P<0.05$) between Minutes 0 and 5 was found only in the 4.0-mg group, and an increase ($P<0.05$) between Hours 1 and 2 was found only in the 0.25-mg and 1.0-mg groups. Experiment 1.

Table 4.1. Mean (± S.E.) effects of PGF treatment at mid-diestrus on the intervals from pretreatment ovulation until progesterone decrease to <1 ng/ml, and from pretreatment ovulation until post-treatment ovulation.

Experiment (route)[a]	Dose	No. of sessions[b]	No. of days from pre-treatment ovulation until P4 is <1 ng/ml	No. of days from pre-treatment until post-treatment ovulation
1 (Injected IU)	0.0 mg	1	18.9 ± 1.4[c]	21.2 ± 1.5[c]
	0.25g	4	12.0 ± 0.0[d]	13.3 ± 0.9[d]
	1.0 mg	4	11.3 ± 0.4[d]	13.2 ± 0.8[d]
	4.0 mg	1	10.5 ± 0.3[d]	15.2 ± 0.5[d]
3 (Infused IU or IV)[e]	0.0 mg (IU)	1	18.8 ± 1.2[c]	21.7 ± 1.7[c]
	0.5 mg (IV)	1	16.3 ± 1.8[c]	19.5 ± 1.5[c]
	0.5 mg (IU)	1	17.5 ± 0.5[c]	20.8 ± 1.1[c]
	0.5 mg (IU)	4	10.2 ± 0.6[d]	13.5 ± 0.6[d]

[a] n = 4 heifers/group.

[b] No. of sequential treatment periods at 12-h intervals.

[c,d] Within each experiment and interval, means without common superscripts are significantly different ($P<0.05$).

[e] Infusion dose was given during 2-h interval.

4.5.2. Experiment 2: Dose Titration for IU Infusion

The mean concentrations of PGFM for Hours 0–6 and of progesterone for Hours 0, 6, 24, and 48 from IU infusion of PGF for Hours 0–3 are shown (n = 2; Fig. 4.4). Compared with the observations comprising the mean of the three highest values for the six natural pulses (746 ± 86 pg/ml), the concentration was greater for the 2.0-mg (2018 ± 136 pg/ml; $P<0.0008$) and 1.0-mg (1180 ± 68 pg/ml; $P<0.006$) groups, was not significantly different for the 0.5-mg group (806 ± 49 pg/ml), and was lesser for the 0.25-mg (413 ± 23 pg/ml; $P<0.02$) and 0.125-mg (250 ± 41 pg/ml; $P<0.002$) groups. Progesterone concentrations decreased to <1 ng/ml by Hour 24 in the 2.0-mg group, decreased transiently to approximately 3 ng/ml in the 1.0-mg and 0.5-mg groups, and decreased slightly or did not seem to decrease in the 0.25-mg and 0.125-mg groups. The decrease and then increase in progesterone concentration seemed to be most prominent

for the 0.5-mg group. The 0.5-mg dose was selected for experiment 3 after considering both the progesterone responses and the differences in PGFM concentrations between experimental and natural pulses.

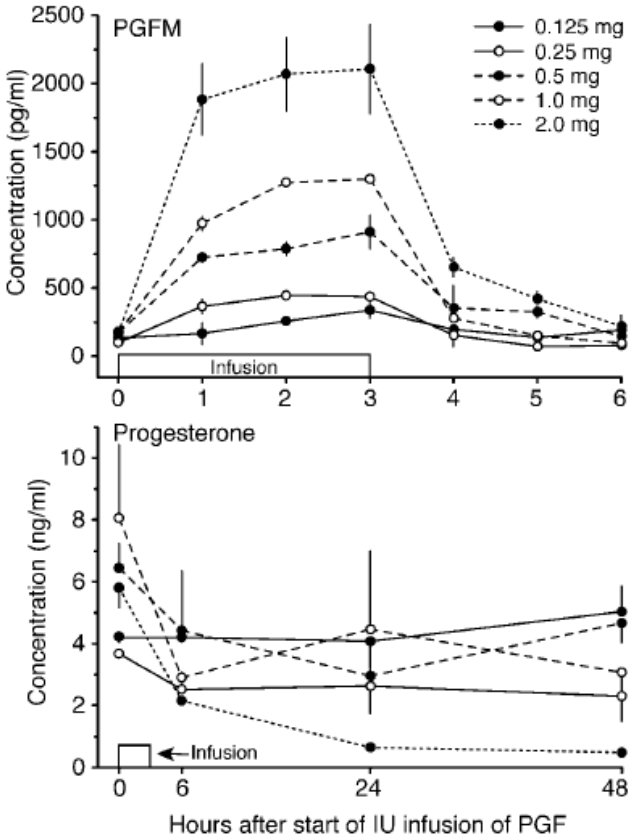

Figure 4.4. Mean (± S.E.) PGFM and progesterone concentrations associated with a 3-h IU infusion of PGF (n = 2). The 0.5-mg dose was chosen for use in experiment 3, owing to the production of a simulated PGFM pulse that was similar to a natural pulse from experiment 1 and progesterone concentrations that seemed to decrease and then increase. Experiment 2.

4.5.3. Experiment 3: Sequential IU Infusions

The IV infusion of PGF during session 1 (0.5-mg IV1) resulted in an immediate (≤2 min) increase in PGFM to a concentration that was maintained until the end of infusion at Hour 2 and then decreased rapidly (Fig. 4.5). The IU infusion during session 1 (0.5-mg IU) was associated with a gradual increase in circulating PGFM between Minutes 2 and 45 compared with the immediate and greater increase in the IV group. The concentration in the two combined 0.5-mg IU groups at Hour 1 of session 1 was maintained until Hour 2 and decreased thereafter. However, the concentration was

lower (P<0.02) at Hours 1 and 2 but was greater (P<0.009) at Hour 3 than that for IV infusion.

Progesterone concentrations during the first hour of infusion in session 1 showed a similar reduction in concentrations between Minutes 0 and 60 for the IV and IU infusion routes (main effect of minute; Fig. 4.5). For progesterone concentrations during Hours 0–5 of session 1, the hour effect and the interaction of group (0.0-mg IU, 0.5-mg IV, and 0.5-mg IU) and hour were significant. When groups were analyzed separately, only the combined 0.5-mg IU groups showed an effect of hour (P<0.0002). The interaction was attributable primarily to a decrease in progesterone between Hours 0 and 1 in the 0.5-mg IU groups (P<0.006).

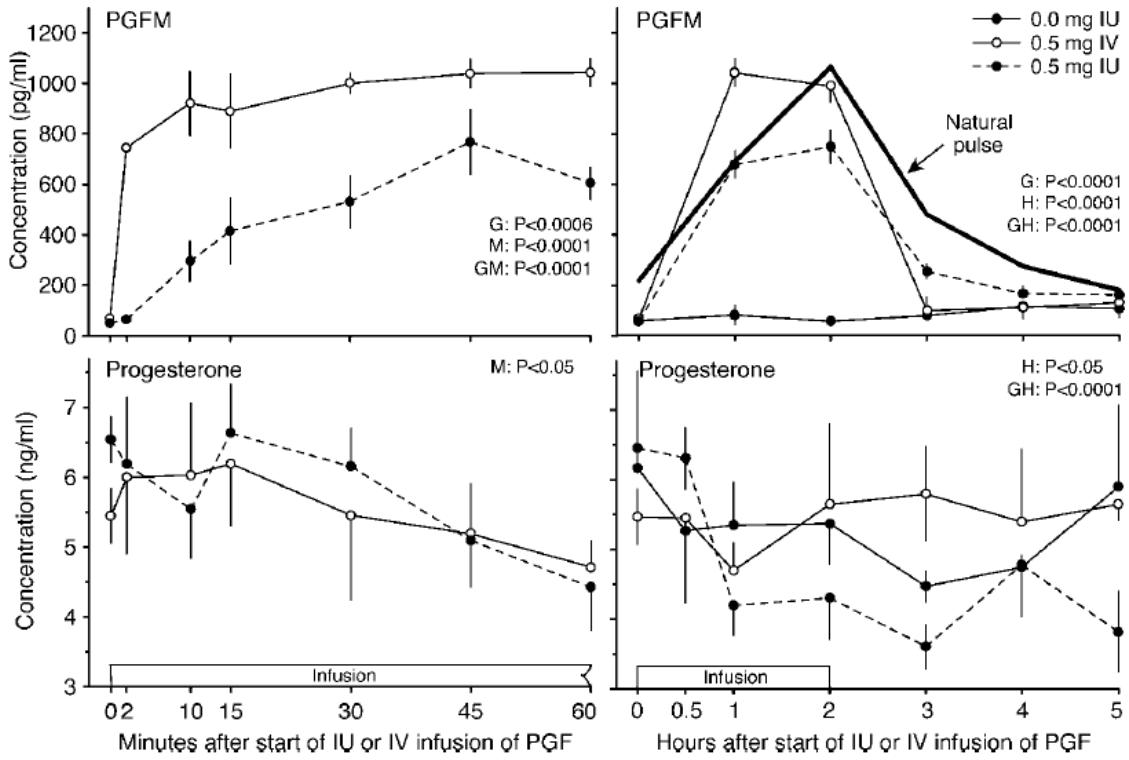

Figure 4.5. Mean (± S.E.) PGFM and progesterone concentrations for the first hour (left) and 5 h (right) associated with a 2-h IU or IV infusion of PGF (n = 4). During the first hour, only the 0.5-mg IV and 0.5-mg IU groups were considered. During the 5 h, the two groups with 0.5-mg IU infusion (see Fig.6) were combined (n = 8). The concentration profile for a natural pulse of PGFM from experiment 1 is superimposed on the upper right panel for comparative inspection. Main effects (group [G] and minute [M] or hour [H]) and the interactions (GM or GH) that were significant are shown. The interaction for progesterone during the 5 h was primarily the result of a precipitous decrease between Hours 0.5 and 1.0 in the 0.5-mg IU group. Experiment 3.

The two main effects (group and day) and the interaction (group-3-day) for progesterone concentrations during 0.5-day and 1-day intervals were significant (Fig. 4.6). The interaction represented primarily lower concentrations in the 0.5-mg IU4 group (infused during sessions 1–4) on Days 1–5 than in the other groups and an intermediate and transient reduction on Days 0.5–3 in the 0.5-mg IU1 group (infused only for session 1). The apparent reduced concentrations in the IV1 group on Days 6–9, compared with the 0.0-mg IU1 and 0.5-mg IU1 groups, were not significant. As expected, the concentrations decreased similarly between 0 and 0.5 days after the session 1 treatment in the 0.5-mg IU1 and 0.5-mg IU4 groups (single vs. four sequential sessions). Concentrations continued to decrease after 0.5 days in the sequential (0.5-mg IU4) group but not in the single (0.5-mg IU1) group. When the 0.0-mg IU1 group and the 0.5-mg IV1 group were analyzed separately from the other two groups, only the main effect of day was significant ($P<0.0001$). The apparent increases and decreases during Days 0, 0.5, and 1 in these two groups were not significant. Concentrations were lower ($P<0.05$) in the 0.5-mg IU1 group than in the 0.0-mg IU1 and 0.5-mg IV1 groups on Days 0.5–3 but not thereafter. The intervals from the pretreatment ovulation to the day when progesterone level was <1 ng/ml and to the post-treatment ovulation were shortest for the sequential treatments (IU4 group), with no differences among the other three groups (Table 4.1).

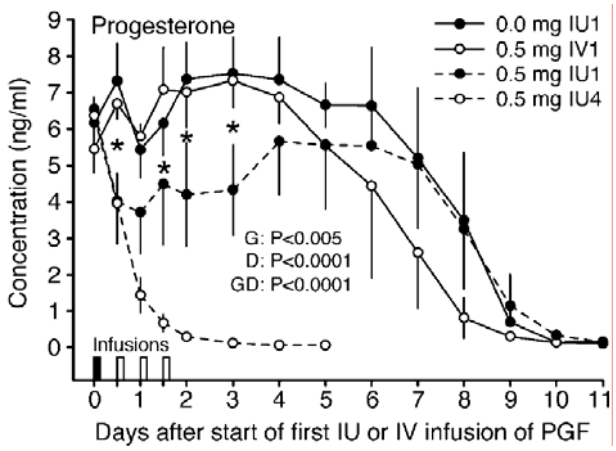

Figure 4.6. Mean (± S.E.) concentrations of progesterone associated with 2-h IU or IV infusion of PGF (n = 4). The 0.0-mg IU1, 0.5-mg IV1, and 0.5-mg IU1 groups were infused only during session 1 (solid box on x-axis), and the 0.5-mg IU4 group was infused during each of sessions 1–4 at 12-h intervals (solid and open boxes). Significant main effects (group [G] and day [D]) and interaction (GD) are shown. The interaction resulted primarily from lower concentrations on Days 1–5 in the 0.5-mg IU4 group than in the other groups and from lower concentrations on Days 0.5–3 in the 0.5-mg IU1 group than in the 0-mg and 0.5-mg IV1 groups. Asterisks indicate a day when the concentration in the 0.5-mg IU1 group was different ($P<0.05$) from that of the 0.0-mg IU1 and 0.5-mg IV1 groups. Experiment 3.

For percentage change in CL area (in centimeters squared), both main effects and the interaction were significant (Fig. 4.7). When groups were analyzed separately, a significant hour effect occurred in both the single (0.5-mg IU1; $P<0.004$) and sequential (0.5-mg IU4; $P<0.0001$) IU infusion groups. The hour effect represented a decrease ($P<0.05$) between Hours 0 and 12 for each group and subsequent decreases only for the sequential 0.5-mg IU4 group, as shown. At Hours 24, 36, and 48, percentages were lesser ($P<0.05$) in the sequential group than in the single group and were lesser ($P<0.05$) in the single group than in the IV and 0.0-mg groups.

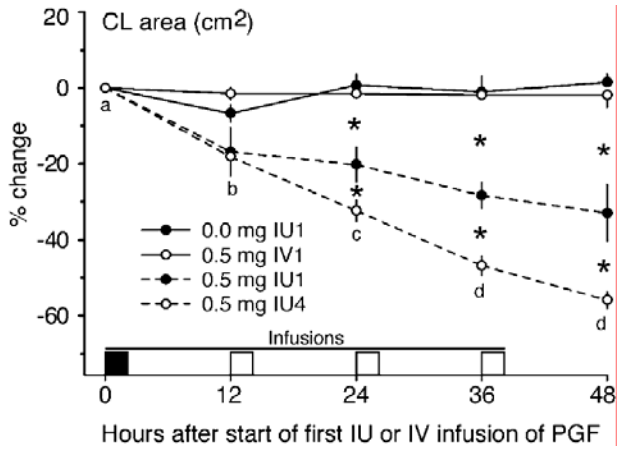

Figure 4.7. Mean (± S.E.) for percentage change in CL area (in centimeters squared) from the area at Hour 0 associated with 2-h IU or IV infusion of PGF (n = 4). The 0.0-mg IU1, 0.5-mg IV1, and 0.5-mg IU1 groups were infused only during session 1 (solid box on x-axis), and the 0.5-mg IU4 group was infused during each of sessions 1–4 at 12-h intervals (solid and open boxes). Main effects of group and hour and the interaction were significant ($P<0.0001$). Means that are different ($P<0.05$) among hours for the 0.5-mg IU4 group are indicated by a–d. Asterisks indicate an hour when the group below an asterisk was different ($P<0.05$) from the groups above the asterisk. Experiment 3.

4.6. DISCUSSION

The increase to maximum in circulating PGFM concentrations within 10 min after the IU injection of each dose of PGF (0.25, 1.0, and 4.0 mg) is consistent with previous studies (Lamond *et al.* 1973, Stellflug *et al.* 1975). The maximum concentrations greatly exceeded the peak of a natural PGFM pulse, even for the smallest dose. Although the rapid absorption rate from the uterus resulted in PGFM concentrations that did not simulate a natural pulse, some information was obtained on the role of exposure of the CL to sequential bursts of PGF. The highest dose (4.0 mg) induced luteolysis (mean progesterone level, <1 ng/ml) within 36 h, as expected (Inskeep 1973). However, the 0.25- and 1.0-mg doses induced stepwise CL regression, in keeping with the sequential treatment session every 12 h. The stepwise regression was shown by the progesterone decrease during the 6 h of each of sessions 1, 2, and 3 and the absence of a decrease in the 6-h periods between sessions. The decrease in progesterone to <1 ng/ml occurred after three sessions of PGF injection, suggesting that only three consecutive bursts of PGF were needed for the completion of luteolysis. Similarly, progesterone

decreased to <1 ng/ml after three simulated PGFM pulses in experiment 3. However, a study with different numbers of sessions would be needed for a firmer conclusion on the number of required exposures to PGF. The stepwise regression of the lower doses was as effective as the precipitous progesterone decrease of the high dose, as indicated by the similar decrease to a mean of <1 ng/ml within 36 h for the two lower doses and the high dose. The results suggested that natural sequential delivery of PGF is important for luteolysis, but this conclusion is tentative in that the sequential bursts did not resemble natural pulses.

Examination of the post-treatment progesterone profile after the injected low IU doses of PGF indicated that a progesterone decrease did not begin until 15 min after treatment, followed by a continuous rapid decrease until 1 h. However, concentrations increased during the next hour, followed by an apparent slower gradual decrease until 6 h. These are novel observations that require confirmation but suggest that the initial response to a burst of PGF is a transient rapid decrease in progesterone, followed by a slower rate of decrease until another PGF burst occurs. In response to simulating a PGFM pulse, the progesterone decline occurred only between 0.5 and 1.0 h after initiation of the simulated pulse, even though the PGF infusion continued for another hour. In mares (Ginther *et al.* 2008), plasma progesterone concentrations decreased after the peak of each individual natural PGFM pulse. However, in a limited study (Ginther 2007) in heifers, progesterone levels did not differ significantly during the 7 h associated with individual PGFM pulses. These observations indicate a need for further study of the progesterone response to each natural PGF pulse in cattle.

Inspection of published characteristics and profiles of bovine PGFM pulses (Kindahl *et al.* 1976b, Mann & Lamming 2006, Ginther *et al.* 2007) suggested that a simple constant infusion rate of PGF for 2 or 3 h would be adequate for simulating the function of a single natural PGF pulse, based on the 2- or 3-h period of major

concentrations in natural PGFM pulses. A second assumption was that the mean characteristics of a natural PGFM pulse need not be closely duplicated, as indicated by the wide variation among peaks of PGFM pulses. A third assumption was that the elevated nadir between PGFM pulses during the luteolytic period (Ginther 2007) did not represent an essential component of the luteolytic mechanism and is supported by an increase in CL blood flow during each pulse and not between pulses (Ginther 2007). Improved simulation of the mean shape of natural PGFM pulses and the nadirs between pulses would require minipumps with programmable changes in flow rates. In addition, pumps that are attachable to the animal may minimize the adverse effects of prolonged animal restraint. Such a refined approach was beyond the goal of the present investigations, but the results may encourage more refinement in future studies.

The simulated PGFM pulses from IU infusion of PGF approached the characteristics of a natural pulse, especially after considering the leeway indicated by the variation in the peak of natural pulses. In addition, the IU infusion (in contrast to the IV route) more closely simulated the gradual increase and decrease that occurs on each side of a natural PGFM peak. The precipitous progesterone decrease from a single 2-h IU infusion of 0.5 mg of PGF and the return to a concentration similar to the control concentrations by 3 days contrast with the continuous decrease from sequential IU sessions at 12-h intervals. The rapid 24-h decline in progesterone from the sequential PGF infusions and the slower rate of decline during the next 12 h are consistent with the reduction rate during spontaneous luteolysis when data are normalized retrospectively to a decrease to <1.0 ng/ml of progesterone (Ginther 2007). The comparatively slower rate of the mean decline in the controls than in the sequentially treated group (experiment 3) is attributable to the beginning of luteolysis at different times among control individuals.

The relationships in progesterone concentrations among groups were consistent with the reduced area (in centimeters squared) of a cross section of the CL during Hours 24, 36, and 48 in the sequential IU group and an intermediate area reduction in the single IU group. No reduction in CL area was detected in the controls or the IV group. In addition, the progesterone and CL results were consistent with a reduced interval from ovulation to a progesterone concentration of <1 ng/ml and to the post-treatment ovulation in the group with sequential simulation of pulses. These intervals in the other two PGF-treated groups were similar to the intervals in the control group. Therefore, the results well supported the hypothesis that sequential PGF pulses are required for the natural completion of luteolysis in cattle.

The transient increase in progesterone concentrations that occurred within 5 min after IU injection of a dose of PGF that caused luteolysis with a single treatment (4.0 mg) agrees with findings that an initial increase in progesterone occurred within 5 or 10 min after systemic, IU, or intraluteal PGF administration of a single luteolytic dose (Lamond *et al.* 1973, Hixon & Hansel 1974, Miyamoto *et al.* 1997, Skarzynski *et al.* 2003). However, in the present investigations, an initial increase in progesterone before the decrease associated with luteolysis was not detected with doses of PGF that required sequential IU treatment for complete luteolysis (0.25 or 1.0 mg; experiment 1) or in association with simulated PGFM pulses. This observed dose-sensitive phenomenon apparently represented a nonphysiologic response to unnatural doses and delivery of PGF. Although further study is needed, it seems that many of the reported studies during the past few decades on the nature of the luteolytic process in cattle may have resulted in dubious interpretations, owing to potential artifactual or pharmacologic responses to unnatural doses or unnatural delivery of PGF to the CL. A dose and method of delivery that approximated the endogenous system were included in the

present investigations. Nevertheless, some reservation is required, owing to the unnatural time of treatment during mid-diestrus.

In conclusion, sequential injections of 0.25 and 1.0 mg of PGF every 12 h and a single injection of 4.0 mg were made into the uterine horn ipsilateral to the CL during mid-diestrus. Each dose induced luteolysis in a mean of 36 h. However, the high dose induced an initial precipitous decline in progesterone, followed by a more gradual but continuous decline. In contrast, the sequential lower doses induced stepwise CL regression, in keeping with the sequential treatments. An approximate simulation of natural PGFM pulses was performed by constant IU infusion of a total of 0.5 mg of PGF during 2 h beginning every 12 h. Simulation of a single pulse induced a transient decrease in progesterone that returned to pretreatment concentrations in 4 days, with no effect on the interval to post-treatment ovulation. Sequential infusion of PGF to simulate PGFM pulses induced a continuing decrease in progesterone and shortened the interval to ovulation by approximately 7 days. Results supported the hypothesis that sequential PGF pulses are required for completion of luteolysis in cattle. In addition, it was noted that an unnatural dose or route of delivery of exogenous PGF may produce a progesterone response that may not occur naturally such as the frequently reported immediate and transient increase in circulating progesterone before the luteolytic decrease.

4.7. REFERENCES

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CONCLUSION

In conclusion for the first part of this dissertation an increased incidence of double dominant follicles and double ovulations was induced by ablation of follicles ≥ 5 mm during the nadir in FSH concentrations between FSH surges, followed in 2 days by administration of PGF2α. The interval from follicle deviation to the peak of the pre-ovulatory LH surge was shorter and the diameter of the pre-ovulatory follicles at the peak of the surge was smaller in double ovulators. Concentration of E2 was greater and concentration of FSH was lower in the double ovulators. The sequential injections of a high dose of E2 (0.15 mg/treatment every 6 h for eight treatments) and a low dose (0.07 or 0.09 mg/treatment every 6 h for four treatments) beginning at expected follicle deviation induced concomitant preovulatory surges in LH and FSH within 24 h at a time when the largest follicle was only about 10 mm. These results supported the hypothesis that the greater E2 concentrations in heifers with double dominant follicles and double ovulations accounts for the earlier development of the preovulatory LH surge and ovulation of smaller follicles. Treatment with E2 also delayed the periovulatory FSH surge resulting in reduced FSH concentrations during the approximately 24 h before ovulation. The results provided functional indications that the reported temporal differences between single and double ovulators in E2 and FSH concentration, time of the LH surge, and diameter of the ovulatory follicles can be

attributable to greater circulating E2 concentrations from double follicles in double ovulators.

On the other hand, the second part of this dissertation had shown the sequential injections of 0.25 and 1.0 mg of PGF every 12 h and a single injection of 4.0 mg made into the uterine horn ipsilateral to the CL during mid-diestrus. Each dose induced luteolysis in a mean of 36 h. However, the high dose induced an initial precipitous decline in progesterone, followed by a more gradual but continuous decline. In contrast, the sequential lower doses induced stepwise CL regression, in keeping with the sequential treatments. An approximate simulation of natural PGFM pulses was performed by constant IU infusion of a total of 0.5 mg of PGF during 2 h beginning every 12 h. Results supported the hypothesis that sequential PGF pulses are required for completion of luteolysis in cattle.

5.0. A.I. APPENDICES – DATA AND STATISTICAL ANALYSIS FROM CHAPTER 2

5.1. Data of the Table 2.1.

The SAS System

Heifer	Group	End point	Y	Heifer	Group	End point	Y	Heifer	Group	End point	Y
94.0	**1**	1	1	**94.0**	**1**	3	4.25	**94.0**	**1**	5	5
99.0	**1**	1	1	**99.0**	**1**	3	3	**99.0**	**1**	5	3.5
302.0	**1**	1	2	**302.0**	**1**	3	1.75	**302.0**	**1**	5	3
520.0	**1**	1	1	**520.0**	**1**	3	3	**520.0**	**1**	5	3.25
524.0	**1**	1	2	**524.0**	**1**	3	3.5	**524.0**	**1**	5	4
99.1	**1**	1	2	**99.1**	**1**	3	2.5	**99.1**	**1**	5	3.5
38.1	**1**	1	1	**38.1**	**1**	3	2.5	**38.1**	**1**	5	4
43.0	**1**	1	1	**43.0**	**1**	3	3.5	**43.0**	**1**	5	4
44.1	**1**	1	1	**44.1**	**1**	3	3.5	**44.1**	**1**	5	4
99.2	**1**	1	1	**99.2**	**1**	3	2.75	**99.2**	**1**	5	3.75
26.0	**1**	1	1	**26.0**	**1**	3	3	**26.0**	**1**	5	4
6280.0	**1**	1	1	**6280.0**	**1**	3	3.5	**6280.0**	**1**	5	4.5
10.0	**2**	1	1	**10.0**	**2**	3	2.25	**10.0**	**2**	5	3.25
10.1	**2**	1	1	**10.1**	**2**	3	1.75	**10.1**	**2**	5	2.75
16.0	**2**	1	1	**16.0**	**2**	3	2.5	**16.0**	**2**	5	3
16.1	**2**	1	2.75	**16.1**	**2**	3	1.5	**16.1**	**2**	5	2.5
522.0	**2**	1	2	**522.0**	**2**	3	2.5	**522.0**	**2**	5	3.5
525.0	**2**	1	1	**525.0**	**2**	3	3	**525.0**	**2**	5	3.5
6264.0	**2**	1	1	**6264.0**	**2**	3	4	**6264.0**	**2**	5	4.5
88.0	**2**	1	1	**88.0**	**2**	3	2	**88.0**	**2**	5	3
94.0	**1**	2	3.25	**94.0**	**1**	4	3.75	**94.0**	**1**	6	30
99.0	**1**	2	2.75	**99.0**	**1**	4	2	**99.0**	**1**	6	36
302.0	**1**	2	2.25	**302.0**	**1**	4	1.75	**302.0**	**1**	6	30
520.0	**1**	2	3	**520.0**	**1**	4	2	**520.0**	**1**	6	30
524.0	**1**	2	1.75	**524.0**	**1**	4	2.75	**524.0**	**1**	6	30
99.1	**1**	2	2.5	**99.1**	**1**	4	2	**99.1**	**1**	6	36
38.1	**1**	2	2.5	**38.1**	**1**	4	2.5	**38.1**	**1**	6	36
43.0	**1**	2	2.5	**43.0**	**1**	4	2.5	**43.0**	**1**	6	36
44.1	**1**	2	2.5	**44.1**	**1**	4	2.5	**44.1**	**1**	6	36
99.2	**1**	2	2.25	**99.2**	**1**	4	2.5	**99.2**	**1**	6	30
26.0	**1**	2	2.5	**26.0**	**1**	4	2.5	**26.0**	**1**	6	36
6280.0	**1**	2	1.5	**6280.0**	**1**	4	3	**6280.0**	**1**	6	36
10.0	**2**	2	2.5	**10.0**	**2**	4	1.5	**10.0**	**2**	6	42
10.1	**2**	2	2.75	**10.1**	**2**	4	1.5	**10.1**	**2**	6	30
16.0	**2**	2	3.25	**16.0**	**2**	4	1.75	**16.0**	**2**	6	30
16.1	**2**	2	2.5	**16.1**	**2**	4	1.25	**16.1**	**2**	6	30
522.0	**2**	2	2	**522.0**	**2**	4	2.25	**522.0**	**2**	6	30
525.0	**2**	2	2.5	**525.0**	**2**	4	2	**525.0**	**2**	6	36
6264.0	**2**	2	1.5	**6264.0**	**2**	4	3	**6264.0**	**2**	6	36
88.0	**2**	2	2.5	**88.0**	**2**	4	2	**88.0**	**2**	6	24

Heifer	Group	End point	Y	Heifer	Group	End point	Y	Heifer	Group	End point	Y
94.0	**1**	7	18	**16.0**	**2**	7	12	**43.0**	**1**	8	11.75
99.0	**1**	7	12	**16.1**	**2**	7	24	**44.1**	**1**	8	11.75
302.0	**1**	7	30	**522.0**	**2**	7	24	**99.2**	**1**	8	11.75
520.0	**1**	7	6	**525.0**	**2**	7	12	**26.0**	**1**	8	12.25
524.0	**1**	7	12	**6264.0**	**2**	7	12	**6280.0**	**1**	8	11.75
99.1	**1**	7	24	**88.0**	**2**	7	24	**10.0**	**2**	8	11.5
38.1	**1**	7	36	**94.0**	**1**	8	14	**10.1**	**2**	8	11.25
43.0	**1**	7	12	**99.0**	**1**	8	12	**16.0**	**2**	8	12
44.1	**1**	7	12	**302.0**	**1**	8	12	**16.1**	**2**	8	12.5
99.2	**1**	7	24	**520.0**	**1**	8	12	**522.0**	**2**	8	12.25
26.0	**1**	7	24	**524.0**	**1**	8	12.5	**525.0**	**2**	8	11.75
6280.0	**1**	7	24	**99.1**	**1**	8	12.75	**6264.0**	**2**	8	11.75
10.0	**2**	7	24	**38.1**	**1**	8	11.75	**88.0**	**2**	8	10.75
10.1	**2**	7	24								

5.2. Statistical Analysis of data from Table 2.1.


```
---------------------------------- endpoint=1 ----------------------------------

                           The UNIVARIATE Procedure
                              Variable:  resid_y

                             Tests for Normality

          Test                  --Statistic---    -----p Value------
          Shapiro-Wilk          W     0.650783    Pr < W     <0.0001
          Kolmogorov-Smirnov    D     0.430956    Pr > D     <0.0100
          Cramer-von Mises      W-Sq  0.676161    Pr > W-Sq  <0.0050
          Anderson-Darling      A-Sq  3.365819    Pr > A-Sq  <0.0050

---------------------------------- endpoint=2 ----------------------------------

                           The UNIVARIATE Procedure
                              Variable:  resid_y

                             Tests for Normality

          Test                  --Statistic---    -----p Value------
          Shapiro-Wilk          W     0.903817    Pr < W      0.0487
          Kolmogorov-Smirnov    D     0.251907    Pr > D     <0.0100
          Cramer-von Mises      W-Sq  0.195081    Pr > W-Sq   0.0052
          Anderson-Darling      A-Sq  0.949356    Pr > A-Sq   0.0141

---------------------------------- endpoint=3 ----------------------------------

                           The UNIVARIATE Procedure
                              Variable:  resid_y

                             Tests for Normality

          Test                  --Statistic---    -----p Value------
          Shapiro-Wilk          W       0.9734    Pr < W      0.8244
          Kolmogorov-Smirnov    D      0.11379    Pr > D     >0.1500
          Cramer-von Mises      W-Sq  0.043058    Pr > W-Sq  >0.2500
          Anderson-Darling      A-Sq   0.27579    Pr > A-Sq  >0.2500

---------------------------------- endpoint=4 ----------------------------------

                           The UNIVARIATE Procedure
                              Variable:  resid_y


                             Tests for Normality

          Test                  --Statistic---    -----p Value------
```

```
                Shapiro-Wilk          W     0.907905    Pr < W      0.0581
                Kolmogorov-Smirnov    D     0.179553    Pr > D      0.0893
                Cramer-von Mises      W-Sq  0.105718    Pr > W-Sq   0.0901
                Anderson-Darling      A-Sq  0.677201    Pr > A-Sq   0.0688

        ---------------------------------- endpoint=5 ----------------------------------

                                The UNIVARIATE Procedure
                                   Variable:  resid_y

                                   Tests for Normality

                Test                  --Statistic---    -----p Value------
                Shapiro-Wilk          W     0.937449    Pr < W      0.2145
                Kolmogorov-Smirnov    D     0.175636    Pr > D      0.1022
                Cramer-von Mises      W-Sq  0.077205    Pr > W-Sq   0.2198
                Anderson-Darling      A-Sq  0.487091    Pr > A-Sq   0.2081

        ---------------------------------- endpoint=6 ----------------------------------

                                The UNIVARIATE Procedure
                                   Variable:  resid_ly

                                   Tests for Normality
                Test                  --Statistic---    -----p Value------
                Shapiro-Wilk          W     0.912153    Pr < W      0.0701
                Kolmogorov-Smirnov    D     0.227977    Pr > D     <0.0100
                Cramer-von Mises      W-Sq   0.17142    Pr > W-Sq   0.0111
                Anderson-Darling      A-Sq  0.939631    Pr > A-Sq   0.0151

        ---------------------------------- endpoint=7 ----------------------------------

                                The UNIVARIATE Procedure
                                   Variable:  resid_y

                                   Tests for Normality
                Test                  --Statistic---    -----p Value------
                Shapiro-Wilk          W      0.86887    Pr < W      0.0112
                Kolmogorov-Smirnov    D     0.269105    Pr > D     <0.0100
                Cramer-von Mises      W-Sq  0.265893    Pr > W-Sq  <0.0050
                Anderson-Darling      A-Sq  1.433567    Pr > A-Sq  <0.0050

        ---------------------------------- endpoint=8 ----------------------------------

                                The UNIVARIATE Procedure
                                   Variable:  resid_ly

                                   Tests for Normality
                Test                  --Statistic---    -----p Value------
                Shapiro-Wilk          W     0.907471    Pr < W      0.0571
                Kolmogorov-Smirnov    D     0.164546    Pr > D     >0.1500
                Cramer-von Mises      W-Sq  0.111824    Pr > W-Sq   0.0759
                Anderson-Darling      A-Sq  0.690163    Pr > A-Sq   0.0633


                                  The GLM Procedure
                               Class Level Information
                              Class       Levels  Values

heifer          20  10 10.1 16 16.1 26 38.1 43 44.1 88 94 99 99.1 99.2 302 520
                    522 524 525 6264 6280
group            2  1 2

                    Number of Observations Read          20
                    Number of Observations Used          20

---------------------------------- endpoint=1 ----------------------------------

                               The GLM Procedure
Dependent Variable: y
                                      Sum of
 Source                     DF        Squares    Mean Square   F Value   Pr > F
 Model                       1     0.04218750     0.04218750      0.14   0.7112
 Error                      18     5.36718750     0.29817708
 Corrected Total            19     5.40937500
```

```
              R-Square     Coeff Var      Root MSE        y Mean
              0.007799      42.41211      0.546056      1.287500

                          Least Squares Means
                                                 H0:LSMean1=LSMean2
                     group        y LSMEAN       Pr > |t|
                     1          1.25000000         0.7112
                     2          1.34375000

--------------------------------- endpoint=2 ---------------------------------

                              The GLM Procedure
Dependent Variable: y
                                       Sum of
 Source                      DF        Squares    Mean Square   F Value   Pr > F
 Model                        1     0.00000000     0.00000000      0.00   1.0000
 Error                       18     4.35937500     0.24218750
 Corrected Total             19     4.35937500

              R-Square     Coeff Var      Root MSE        y Mean
              0.000000      20.18976      0.492125      2.437500

                           Least Squares Means
                                                 H0:LSMean1=LSMean2
                     group        y LSMEAN       Pr > |t|
                     1          2.43750000         1.0000
                     2          2.43750000

--------------------------------- endpoint=3 ---------------------------------

                              The GLM Procedure
Dependent Variable: y
                                       Sum of
 Source                      DF        Squares    Mean Square   F Value   Pr > F
 Model                        1     1.87500000     1.87500000      3.76   0.0684
 Error                       18     8.98437500     0.49913194
 Corrected Total             19    10.85937500

              R-Square     Coeff Var      Root MSE        y Mean
              0.172662      25.11974      0.706493      2.812500



                           Least Squares Means
                                                 H0:LSMean1=LSMean2
                     group        y LSMEAN       Pr > |t|
                     1          3.06250000         0.0684
                     2          2.43750000

--------------------------------- endpoint=4 ---------------------------------
                              The GLM Procedure
Dependent Variable: y
                                       Sum of
 Source                      DF        Squares    Mean Square   F Value   Pr > F
 Model                        1     1.57552083     1.57552083      5.35   0.0327
 Error                       18     5.29947917     0.29441551
 Corrected Total             19     6.87500000

              R-Square     Coeff Var      Root MSE        y Mean
              0.229167      24.11559      0.542601      2.250000

                           Least Squares Means
                                                 H0:LSMean1=LSMean2
                     group        y LSMEAN       Pr > |t|
                     1          2.47916667         0.0327
                     2          1.90625000

--------------------------------- endpoint=5 ---------------------------------
                              The GLM Procedure
Dependent Variable: y
                                       Sum of
 Source                      DF        Squares    Mean Square   F Value   Pr > F
 Model                        1     1.87500000     1.87500000      5.81   0.0269
 Error                       18     5.81250000     0.32291667
 Corrected Total             19     7.68750000
```

```
              R-Square     Coeff Var      Root MSE        y Mean
              0.243902      15.67607      0.568258      3.625000

                          Least Squares Means
                                                 H0:LSMean1=LSMean2
                     group        y LSMEAN       Pr > |t|
                     1          3.87500000         0.0269
                     2          3.25000000

---------------------------------- endpoint=6 ----------------------------------

                            The GLM Procedure
Dependent Variable: ly
                                      Sum of
 Source                     DF        Squares    Mean Square   F Value   Pr > F
 Model                       1     0.01042696     0.01042696      0.63   0.4377
 Error                      18     0.29788513     0.01654917
 Corrected Total            19     0.30831210

              R-Square     Coeff Var      Root MSE       ly Mean
              0.033820      3.687216      0.128644      3.488909

                                      Least Squares Means
                                                 H0:LSMean1=LSMean2
                     group       ly LSMEAN       Pr > |t|
                     1          3.50755162         0.4377
                     2          3.46094386

---------------------------------- endpoint=7 ----------------------------------
                            The GLM Procedure
Dependent Variable: y
                                      Sum of
 Source                     DF        Squares    Mean Square   F Value   Pr > F
 Model                       1       0.000000       0.000000      0.00   1.0000
 Error                      18    1143.000000      63.500000
 Corrected Total            19    1143.000000
              R-Square     Coeff Var      Root MSE        y Mean
              0.000000      40.86507      7.968689      19.50000

                            Least Squares Means
                                                 H0:LSMean1=LSMean2
                     group        y LSMEAN       Pr > |t|
                     1          19.5000000         1.0000
                     2          19.5000000

---------------------------------- endpoint=8 ----------------------------------
                            The GLM Procedure
Dependent Variable: ly
                                      Sum of
 Source                     DF        Squares    Mean Square   F Value   Pr > F
 Model                       1     0.00728821     0.00728821      2.88   0.1066
 Error                      18     0.04547625     0.00252646
 Corrected Total            19     0.05276447
              R-Square     Coeff Var      Root MSE       ly Mean
              0.138127      2.023860      0.050264      2.483566

                            Least Squares Means
                                                 H0:LSMean1=LSMean2
                     group       ly LSMEAN       Pr > |t|
                     1          2.49915244         0.1066
                     2          2.46018608
```

5.3. Data of the Table 2.2.

Heifer	Group	End point	Y	Heifer	Group	End point	Y	Heifer	Group	End point	Y

94.0	**1**	1	9.3	**94.0**	**1**	3	14.7	**94.0**	**1**	5	14.3
99.0	**1**	1	9.7	**99.0**	**1**	3	12.7	**99.0**	**1**	5	12.2
302.0	**1**	1	9.4	**302.0**	**1**	3	13.5	**302.0**	**1**	5	13.5
520.0	**1**	1	8.7	**520.0**	**1**	3	13.5	**520.0**	**1**	5	12.9
524.0	**1**	1	8.8	**524.0**	**1**	3	15.9	**524.0**	**1**	5	14
99.1	**1**	1	8.8	**99.1**	**1**	3	13.3	**99.1**	**1**	5	13
38.1	**1**	1	8.6	**38.1**	**1**	3	13.4	**38.1**	**1**	5	13.4
43.0	**1**	1	7.9	**43.0**	**1**	3	13.2	**43.0**	**1**	5	11.5
44.1	**1**	1	9	**44.1**	**1**	3	14.4	**44.1**	**1**	5	14.3
99.2	**1**	1	8.7	**99.2**	**1**	3	14.1	**99.2**	**1**	5	13.1
26.0	**1**	1	9.3	**26.0**	**1**	3	15.2	**26.0**	**1**	5	14.3
6280.0	**1**	1	8	**6280.0**	**1**	3	13.1	**6280.0**	**1**	5	13
10.0	**2**	1	.	**10.0**	**2**	3	11.4	**10.0**	**2**	5	.
10.1	**2**	1	9.1	**10.1**	**2**	3	12.6	**10.1**	**2**	5	12
16.0	**2**	1	9.5	**16.0**	**2**	3	12.9	**16.0**	**2**	5	12.4
16.1	**2**	1	8	**16.1**	**2**	3	9.7	**16.1**	**2**	5	9.6
522.0	**2**	1	.	**522.0**	**2**	3	11.9	**522.0**	**2**	5	11.5
525.0	**2**	1	9.1	**525.0**	**2**	3	13.5	**525.0**	**2**	5	12.9
6264.0	**2**	1	8.3	**6264.0**	**2**	3	14.6	**6264.0**	**2**	5	14.2
88.0	**2**	1	8.9	**88.0**	**2**	3	11.9	**88.0**	**2**	5	11.9
94.0	**1**	2	9	**94.0**	**1**	4	9.6	**94.0**	**1**	6	8.2
99.0	**1**	2	9	**99.0**	**1**	4	9.8	**99.0**	**1**	6	9.8
302.0	**1**	2	9.3	**302.0**	**1**	4	9.4	**302.0**	**1**	6	9
520.0	**1**	2	7.7	**520.0**	**1**	4	8.6	**520.0**	**1**	6	7.9
524.0	**1**	2	8.2	**524.0**	**1**	4	9.2	**524.0**	**1**	6	8.6
99.1	**1**	2	8	**99.1**	**1**	4	9.3	**99.1**	**1**	6	8.6
38.1	**1**	2	7.6	**38.1**	**1**	4	8.8	**38.1**	**1**	6	7.6
43.0	**1**	2	7.6	**43.0**	**1**	4	8.5	**43.0**	**1**	6	7.8
44.1	**1**	2	7.7	**44.1**	**1**	4	8.6	**44.1**	**1**	6	8
99.2	**1**	2	8.7	**99.2**	**1**	4	10.5	**99.2**	**1**	6	9.8
26.0	**1**	2	8.5	**26.0**	**1**	4	11	**26.0**	**1**	6	10.5
6280.0	**1**	2	7.7	**6280.0**	**1**	4	10.7	**6280.0**	**1**	6	10.5
10.0	**2**	2	.	**10.0**	**2**	4	10.3	**10.0**	**2**	6	.
10.1	**2**	2	8.5	**10.1**	**2**	4	11.6	**10.1**	**2**	6	11.6
16.0	**2**	2	9.2	**16.0**	**2**	4	12	**16.0**	**2**	6	12
16.1	**2**	2	7.8	**16.1**	**2**	4	10.4	**16.1**	**2**	6	9.1
522.0	**2**	2	.	**522.0**	**2**	4	10.6	**522.0**	**2**	6	10.6
525.0	**2**	2	8	**525.0**	**2**	4	12.5	**525.0**	**2**	6	11.9
6264.0	**2**	2	7.3	**6264.0**	**2**	4	11.9	**6264.0**	**2**	6	11.3
88.0	**2**	2	8.8	**88.0**	**2**	4	11.5	**88.0**	**2**	6	11.5

Heifer	**Group**	**End point**	**Y**	**Heifer**	**Group**	**End point**	**Y**	**Heifer**	**Group**	**End point**	**Y**
94.0	**1**	7	14	**16.0**	**2**	7	12.1	**43.0**	**1**	8	7.6
99.0	**1**	7	13	**16.1**	**2**	7	9.2	**44.1**	**1**	8	7.8
302.0	**1**	7	12	**522.0**	**2**	7	11.5	**99.2**	**1**	8	10.3
520.0	**1**	7	14	**525.0**	**2**	7	13.5	**26.0**	**1**	8	10.8
524.0	**1**	7	16	**6264.0**	**2**	7	14.6	**6280.0**	**1**	8	10.7
99.1	**1**	7	13	**88.0**	**2**	7	11.5	**10.0**	**2**	8	10.3

38.1	**1**	7	13	**94.0**	**1**	8	6.7	**10.1**	**2**	8	11.4
43.0	**1**	7	13	**99.0**	**1**	8	9.2	**16.0**	**2**	8	11.3
44.1	**1**	7	14	**302.0**	**1**	8	8.8	**16.1**	**2**	8	9.5
99.2	**1**	7	13	**520.0**	**1**	8	6.9	**522.0**	**2**	8	9.4
26.0	**1**	7	15	**524.0**	**1**	8	8.5	**525.0**	**2**	8	12.5
6280.0	**1**	7	13	**99.1**	**1**	8	8	**6264.0**	**2**	8	11.3
10.0	**2**	7	11	**38.1**	**1**	8	8.4	**88.0**	**2**	8	11
10.1	**2**	7	12								

5.4. Statistical analysis of data from Table 2.2.

The SAS System

```
---------------------------------- endpoint=1 ----------------------------------

                          The UNIVARIATE Procedure
                             Variable:  resid_y
                             Tests for Normality
          Test                  --Statistic---    -----p Value------
          Shapiro-Wilk            W     0.957216    Pr < W      0.5489
          Kolmogorov-Smirnov      D      0.11061    Pr > D     >0.1500
          Cramer-von Mises        W-Sq  0.037034    Pr > W-Sq  >0.2500
          Anderson-Darling        A-Sq  0.278156    Pr > A-Sq  >0.2500

---------------------------------- endpoint=2 ----------------------------------

                          The UNIVARIATE Procedure
                             Variable:  resid_y
                             Tests for Normality
          Test                  --Statistic---    -----p Value------
          Shapiro-Wilk            W     0.927522    Pr < W      0.1756
          Kolmogorov-Smirnov      D      0.15953    Pr > D     >0.1500
          Cramer-von Mises        W-Sq   0.08885    Pr > W-Sq   0.1488
          Anderson-Darling        A-Sq  0.534493    Pr > A-Sq   0.1506

---------------------------------- endpoint=3 ----------------------------------

                          The UNIVARIATE Procedure
                             Variable:  resid_y
                             Tests for Normality
          Test                  --Statistic---    -----p Value------
          Shapiro-Wilk            W      0.96067    Pr < W      0.5573
          Kolmogorov-Smirnov      D     0.189934    Pr > D      0.0575
          Cramer-von Mises        W-Sq  0.072688    Pr > W-Sq   0.2470
          Anderson-Darling        A-Sq  0.417319    Pr > A-Sq  >0.2500

---------------------------------- endpoint=4 ----------------------------------

                          The UNIVARIATE Procedure
                             Variable:  resid_y
                             Tests for Normality
          Test                  --Statistic---    -----p Value------
          Shapiro-Wilk            W      0.92936    Pr < W      0.1501
          Kolmogorov-Smirnov      D     0.153683    Pr > D     >0.1500
          Cramer-von Mises        W-Sq  0.056023    Pr > W-Sq  >0.2500
          Anderson-Darling        A-Sq  0.441124    Pr > A-Sq  >0.2500

---------------------------------- endpoint=5 ----------------------------------

                          The UNIVARIATE Procedure
                             Variable:  resid_y
                             Tests for Normality
          Test                  --Statistic---    -----p Value------
          Shapiro-Wilk            W     0.959137    Pr < W      0.5555
          Kolmogorov-Smirnov      D     0.145143    Pr > D     >0.1500
          Cramer-von Mises        W-Sq  0.061379    Pr > W-Sq  >0.2500
          Anderson-Darling        A-Sq  0.387387    Pr > A-Sq  >0.2500

---------------------------------- endpoint=6 ----------------------------------

                          The UNIVARIATE Procedure
                             Variable:  resid_y
                             Tests for Normality
```

```
                Test                 --Statistic---    -----p Value------
                Shapiro-Wilk         W     0.974748    Pr < W      0.8657
                Kolmogorov-Smirnov   D     0.089768    Pr > D     >0.1500
                Cramer-von Mises     W-Sq  0.026381    Pr > W-Sq  >0.2500
                Anderson-Darling     A-Sq  0.187558    Pr > A-Sq  >0.2500

--------------------------------- endpoint=7 ---------------------------------

                            The UNIVARIATE Procedure
                               Variable:  resid_y
                              Tests for Normality
                Test                 --Statistic---    -----p Value------
                Shapiro-Wilk         W      0.95015    Pr < W      0.3694
                Kolmogorov-Smirnov   D     0.139391    Pr > D     >0.1500
                Cramer-von Mises     W-Sq  0.085693    Pr > W-Sq   0.1686
                Anderson-Darling     A-Sq  0.503705    Pr > A-Sq   0.1891

--------------------------------- endpoint=8 ---------------------------------

                            The UNIVARIATE Procedure
                               Variable:  resid_y
                              Tests for Normality
                Test                 --Statistic---    -----p Value------
                Shapiro-Wilk         W     0.955921    Pr < W      0.4659
                Kolmogorov-Smirnov   D      0.12373    Pr > D     >0.1500
                Cramer-von Mises     W-Sq  0.035486    Pr > W-Sq  >0.2500
                Anderson-Darling     A-Sq  0.279762    Pr > A-Sq  >0.2500

                          The GLM Procedure
                       Class Level Information

Class       Levels  Values
heifer          20  10 10.1 16 16.1 26 38.1 43 44.1 88 94 99 99.1 99.2 302 520
                    522 524 525 6264 6280
group            2  1 2
                    Number of Observations Read          20
                    Number of Observations Used          18

--------------------------------- endpoint=1 ---------------------------------

                            The GLM Procedure
Dependent Variable: y
                                    Sum of
 Source                     DF        Squares    Mean Square   F Value   Pr > F
 Model                       1     0.00444444     0.00444444      0.01   0.9042
 Error                      16     4.75833333     0.29739583
 Corrected Total            17     4.76277778

              R-Square     Coeff Var      Root MSE        y Mean
              0.000933      6.169781      0.545340      8.838889

                          Least Squares Means
                                          H0:LSMean1=LSMean2
                    group        y LSMEAN       Pr > |t|
                    1          8.85000000         0.9042
                    2          8.81666667




--------------------------------- endpoint=2 ---------------------------------

                            The GLM Procedure
Dependent Variable: y
                                    Sum of
 Source                     DF        Squares    Mean Square   F Value   Pr > F
 Model                       1     0.00111111     0.00111111      0.00   0.9597
 Error                      16     6.74333333     0.42145833
 Corrected Total            17     6.74444444
              R-Square     Coeff Var      Root MSE        y Mean
              0.000165      7.863774      0.649198      8.255556

                          Least Squares Means
                                          H0:LSMean1=LSMean2
                    group        y LSMEAN       Pr > |t|
                    1          8.25000000         0.9597
```

```
                    2          8.26666667

---------------------------------- endpoint=3 ----------------------------------

                              The GLM Procedure
Dependent Variable: y
                                       Sum of
 Source                     DF        Squares    Mean Square   F Value   Pr > F
 Model                       1    12.35208333    12.35208333      8.83   0.0082
 Error                      18    25.18541667     1.39918981
 Corrected Total            19    37.53750000

               R-Square     Coeff Var      Root MSE        y Mean
               0.329060      8.910535      1.182874      13.27500

                            Least Squares Means
                                                 H0:LSMean1=LSMean2
                     group        y LSMEAN       Pr > |t|
                     1          13.9166667         0.0082
                     2          12.3125000

---------------------------------- endpoint=4 ----------------------------------

                              The GLM Procedure
Dependent Variable: y
                                       Sum of
 Source                     DF        Squares    Mean Square   F Value   Pr > F
 Model                       1    16.42800000    16.42800000     23.21   0.0001
 Error                      18    12.74000000     0.70777778
 Corrected Total            19    29.16800000

               R-Square     Coeff Var      Root MSE        y Mean
               0.563220      8.215774      0.841295      10.24000

                            Least Squares Means
                                                 H0:LSMean1=LSMean2
                     group        y LSMEAN       Pr > |t|
                     1           9.5000000         0.0001
                     2          11.3500000

---------------------------------- endpoint=5 ----------------------------------

                              The GLM Procedure
Dependent Variable: y
                                       Sum of
 Source                     DF        Squares    Mean Square   F Value   Pr > F
 Model                       1     6.58286341     6.58286341      5.55   0.0307
 Error                      17    20.16345238     1.18608543
 Corrected Total            18    26.74631579

               R-Square     Coeff Var      Root MSE        y Mean
               0.246122      8.480506      1.089075      12.84211

                            Least Squares Means
                                                 H0:LSMean1=LSMean2
                     group        y LSMEAN       Pr > |t|
                     1          13.2916667         0.0307
                     2          12.0714286

---------------------------------- endpoint=6 ----------------------------------
                              The GLM Procedure
Dependent Variable: y
                                       Sum of
 Source                     DF        Squares    Mean Square   F Value   Pr > F
 Model                       1    23.07369048    23.07369048     21.50   0.0002
 Error                      17    18.24630952     1.07331232
 Corrected Total            18    41.32000000

               R-Square     Coeff Var      Root MSE        y Mean
               0.558415      10.68049      1.036008      9.700000

                            Least Squares Means
                                                 H0:LSMean1=LSMean2
                     group        y LSMEAN       Pr > |t|
                     1           8.8583333         0.0002
                     2          11.1428571
```

```
---------------------------------- endpoint=7 ----------------------------------

                            The GLM Procedure
Dependent Variable: y
                                    Sum of
 Source                     DF        Squares    Mean Square   F Value   Pr > F
 Model                       1    10.09200000    10.09200000      6.46   0.0205
 Error                      18    28.13750000     1.56319444
 Corrected Total            19    38.22950000

               R-Square     Coeff Var      Root MSE        y Mean
               0.263985      9.695834      1.250278      12.89500

                          Least Squares Means

                                              H0:LSMean1=LSMean2
                     group        y LSMEAN       Pr > |t|
                     1          13.4750000         0.0205
                     2          12.0250000

---------------------------------- endpoint=8 ----------------------------------

                            The GLM Procedure
Dependent Variable: y
                                    Sum of
 Source                     DF        Squares    Mean Square   F Value   Pr > F
 Model                       1    23.14408333    23.14408333     14.49   0.0013
 Error                      18    28.74791667     1.59710648
 Corrected Total            19    51.89200000

               R-Square     Coeff Var      Root MSE        y Mean
               0.446005      13.27486      1.263767      9.520000

                          Least Squares Means
                                              H0:LSMean1=
                                                LSMean2
                     group        y LSMEAN       Pr > |t|
                     1          8.6416667         0.0013
                     2         10.8375000
```

5.5. Data of the Figure 2.2.

The SAS System

Heifer	Group	Foll	-24	-12	0	12	24
94.0	**1**	**1**	13.40	14.00	14.30	14.70	14.20
99.0	**1**	**1**	10.90	.	12.20	.	12.70
302.0	**1**	**1**	11.50	12.10	13.50	13.00	12.40
520.0	**1**	**1**	11.60	12.80	12.90	13.20	13.50
524.0	**1**	**1**	12.10	12.50	14.00	14.80	15.50
99.1	**1**	**1**	11.20	12.10	13.00	13.30	12.80
38.1	**1**	**1**	12.30	13.20	13.40	12.90	13.20
43.0	**1**	**1**	10.20	10.70	11.50	12.70	13.20
44.1	**1**	**1**	12.90	13.40	14.30	14.00	14.40
99.2	**1**	**1**	12.00	12.60	13.10	14.00	12.60
26.0	**1**	**1**	12.10	12.50	14.30	15.20	14.50
6280.0	**1**	**1**	10.80	11.80	13.00	13.10	12.70
10.0	**2**	**1**	.	.	.	11.40	.
10.1	**2**	**1**	10.50	11.50	12.00	12.20	.
16.0	**2**	**1**	11.20	11.90	12.40	12.50	12.10
16.1	**2**	**1**	8.70	9.30	9.60	9.70	9.20
522.0	**2**	**1**	.	11.00	11.50	11.70	11.50
525.0	**2**	**1**	12.00	12.30	13.00	12.90	13.50
6264.0	**2**	**1**	12.70	13.30	14.20	14.00	14.60
88.0	**2**	**1**	10.00	11.20	11.90	11.50	.
94.0	**1**	**2**	8.60	8.90	8.20	7.80	6.70
99.0	**1**	**2**	9.60	.	9.80	.	9.20
302.0	**1**	**2**	9.00	8.90	9.00	8.70	8.80
520.0	**1**	**2**	7.60	7.10	7.90	7.70	6.90
524.0	**1**	**2**	9.00	9.00	8.60	8.90	8.50
99.1	**1**	**2**	8.80	9.30	8.60	8.80	8.00

38.1	**1**	**2**	8.50	7.80	7.60	8.60	8.80
43.0	**1**	**2**	8.20	8.50	7.80	8.00	7.80
44.1	**1**	**2**	8.20	8.10	8.00	8.00	8.20
99.2	**1**	**2**	10.50	10.40	9.80	10.50	10.30
26.0	**1**	**2**	10.90	10.30	10.50	10.40	10.80
6280.0	**1**	**2**	9.60	10.30	10.50	10.10	10.70
10.0	**2**	**2**	.	.	.	10.30	.
10.1	**2**	**2**	9.60	10.10	11.60	11.50	.
16.0	**2**	**2**	10.20	11.40	12.00	11.70	11.30
16.1	**2**	**2**	8.70	9.00	9.10	9.80	9.50
522.0	**2**	**2**	.	9.70	10.60	10.50	9.40
525.0	**2**	**2**	10.10	11.00	12.00	11.90	12.50
6264.0	**2**	**2**	10.30	10.40	11.30	11.90	11.30
88.0	**2**	**2**	9.90	11.20	11.50	11.00	.
94.0	**1**	**3**	6.30	6.80	5.20	.	.
99.0	**1**	**3**	7.70	.	7.30	.	7.10
302.0	**1**	**3**	6.70	6.30	.	.	.
520.0	**1**	**3**	7.40	6.90	6.40	6.50	5.70
524.0	**1**	**3**	4.10	4.10	4.40	4.60	.
99.1	**1**	**3**	8.20	8.10	8.90	8.10	9.00
38.1	**1**	**3**	8.20	8.30	7.20	8.00	7.70
43.0	**1**	**3**	7.80	7.50	7.70	7.60	7.00
44.1	**1**	**3**	7.60	7.60	7.90	7.50	7.00
99.2	**1**	**3**	5.70	5.00	4.80	.	.
26.0	**1**	**3**	8.20	7.80	7.90	6.80	6.10
6280.0	**1**	**3**	9.80	9.40	9.60	10.10	9.50
10.0	**2**	**3**	.	.	.	7.20	.
10.1	**2**	**3**	8.30	7.00	7.50	7.50	.
16.0	**2**	**3**	8.10	8.30	8.90	8.30	8.00
16.1	**2**	**3**	8.10	7.80	8.10	8.60	8.90
522.0	**2**	**3**	.	7.70	7.30	6.60	6.50
525.0	**2**	**3**	6.70	6.40	5.70	6.20	6.50
6264.0	**2**	**3**	6.90	7.60	6.80	6.10	7.40
88.0	**2**	**3**	6.20	6.20	6.80	6.60	.

5.6. Statistical Analysis of data from Figure 2.2.

The SAS System

```
------------------------------------ fol=1 ------------------------------------

                          The UNIVARIATE Procedure
                       Variable:  Resid e (Residual)
                            Tests for Normality
              Test                 --Statistic---    -----p Value------
              Shapiro-Wilk         W     0.990366    Pr < W      0.7512
              Kolmogorov-Smirnov   D     0.050803    Pr > D     >0.1500
              Cramer-von Mises     W-Sq  0.034403    Pr > W-Sq  >0.2500
              Anderson-Darling     A-Sq  0.250244    Pr > A-Sq  >0.2500

------------------------------------ fol=2 ------------------------------------

                          The UNIVARIATE Procedure
                       Variable:  Resid e (Residual)
                            Tests for Normality
              Test                 --Statistic---    -----p Value------
              Shapiro-Wilk         W     0.985335    Pr < W      0.4017
              Kolmogorov-Smirnov   D     0.072262    Pr > D     >0.1500
              Cramer-von Mises     W-Sq  0.062012    Pr > W-Sq  >0.2500
              Anderson-Darling     A-Sq  0.405601    Pr > A-Sq  >0.2500

------------------------------------ fol=3 ------------------------------------

                          The UNIVARIATE Procedure
                       Variable:  Resid e (Residual)
                            Tests for Normality
              Test                 --Statistic---    -----p Value------
              Shapiro-Wilk         W     0.986965    Pr < W      0.5706
              Kolmogorov-Smirnov   D     0.048774    Pr > D     >0.1500
              Cramer-von Mises     W-Sq   0.03098    Pr > W-Sq  >0.2500
```

```
Anderson-Darling      A-Sq  0.255873    Pr > A-Sq  >0.2500

------------------------------------ fol=1 ------------------------------------

                          The Mixed Procedure

                    Number of Observations
                Number of Observations Read            100
                Number of Observations Used             91
                Number of Observations Not Used          9
                Covariance Parameter Estimates

                Cov Parm          Subject          Estimate
                heifer(group)                        0.8893
                AR(1)             heifer(group)      0.7120
                Residual                             0.3862

                      Type 3 Tests of Fixed Effects

                                Num     Den
                Effect          DF      DF    F Value    Pr > F
                group            1      18       6.96    0.0167
                time             4      63      25.33    <.0001
                group*time       4      63       1.24    0.3029

------------------------------------ fol=2 ------------------------------------

                          The Mixed Procedure

                    Number of Observations
                Number of Observations Read            100
                Number of Observations Used             91
                Number of Observations Not Used          9

                      Covariance Parameter Estimates

                Cov Parm          Subject          Estimate
                heifer(group)                        0.8431
                AR(1)             heifer(group)      0.4893
                Residual                             0.2363

                      Type 3 Tests of Fixed Effects

                                Num     Den
                Effect          DF      DF    F Value    Pr > F
                group            1      18      13.43    0.0018
                time             4      63       5.88    0.0004
                group*time       4      63       8.94    <.0001

------------------------------------ fol=3 ------------------------------------

                          The Mixed Procedure

                    Number of Observations
                Number of Observations Read            100
                Number of Observations Used             83
                Number of Observations Not Used         17

                      Covariance Parameter Estimates

                Cov Parm          Subject          Estimate
                heifer(group)                        1.4555
                AR(1)             heifer(group)      0.4222
                Residual                             0.2979


                      Type 3 Tests of Fixed Effects

                                Num     Den
                Effect          DF      DF    F Value    Pr > F
                group            1      18       0.30    0.5913
                time             4      55       0.81    0.5265
                group*time       4      55       1.09    0.3688

------------------------------------ fol=1 ------------------------------------
```

The MEANS Procedure
Analysis Variable : e

fol	group	N Obs	N	Mean	Std Error
1	1	60	58	12.9448276	0.1490679
	2	40	33	11.7272727	0.2518971

---------- fol=1 ----------

The TTEST Procedure
Statistics

Variable	group	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev
e	1	58	12.646	12.945	13.243	0.9598	1.1353
e	2	33	11.214	11.727	12.24	1.1637	1.447
e	Diff (1-2)		0.6733	1.2176	1.7619	1.0958	1.2563

Statistics

Variable	group	Upper CL Std Dev	Std Err	Minimum	Maximum
e	1	1.3899	0.1491	10.2	15.5
e	2	1.914	0.2519	8.7	14.6
e	Diff (1-2)	1.4724	0.2739		

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
e	Pooled	Equal	89	4.44	<.0001
e	Satterthwaite	Unequal	54.6	4.16	0.0001

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
e	Folded F	32	57	1.62	0.1091

---------- fol=2 time=-24 ----------

The MEANS Procedure
Analysis Variable : e

fol	group	N Obs	N	Mean	Std Error
2	1	12	12	9.0416667	0.2786489
	2	8	6	9.8000000	0.2422120

---------- fol=2 time=-12 ----------

fol	group	N Obs	N	Mean	Std Error
2	1	12	11	8.9636364	0.3239733
	2	8	7	10.4000000	0.3287784

---------- fol=2 time=0 ----------

fol	group	N Obs	N	Mean	Std Error
2	1	12	12	8.8583333	0.3028797
	2	8	7	11.1571429	0.3872105

---------- fol=2 time=12 ----------

fol	group	N Obs	N	Mean	Std Error
2	1	12	11	8.8636364	0.3110898
	2	8	8	11.0750000	0.2833158

---------- fol=2 time=24 ----------

fol	group	N Obs	N	Mean	Std Error
2	1	12	12	8.7250000	0.3898572
	2	8	5	10.8000000	0.5932959

---------- fol=2 time=-24 ----------

The TTEST Procedure
Statistics

Variable	group	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev
e	1	12	8.4284	9.0417	9.655	0.6838	0.9653
e	2	6	9.1774	9.8	10.423	0.3703	0.5933
e	Diff (1-2)		-1.677	-0.758	0.16	0.6452	0.8664

Statistics

Variable	group		Upper CL Std Dev	Std Err	Minimum	Maximum
e		1	1.6389	0.2786	7.6	10.9
e		2	1.4551	0.2422	8.7	10.3
e	Diff (1-2)		1.3185	0.4332		

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
e	Pooled	Equal	16	-1.75	0.0992
e	Satterthwaite	Unequal	15	-2.05	0.0578

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
e	Folded F	11	5	2.65	0.2925

---------- fol=2 time=-12 ----------

Statistics

Variable	group	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev
e		1	11	8.2418	8.9636	9.6855	0.7508	1.0745
e		2	7	9.5955	10.4	11.204	0.5605	0.8699
e	Diff (1-2)			-2.464	-1.436	-0.409	0.7468	1.0027

Statistics

Variable	group		Upper CL Std Dev	Std Err	Minimum	Maximum
e		1	1.8857	0.324	7.1	10.4
e		2	1.9155	0.3288	9	11.4
e	Diff (1-2)		1.526	0.4848		

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
e	Pooled	Equal	16	-2.96	0.0092
e	Satterthwaite	Unequal	14.9	-3.11	0.0072

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
e	Folded F	10	6	1.53	0.6261

---------- fol=2 time=0 ----------

Statistics

Variable	group	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev
e		1	12	8.1917	8.8583	9.525	0.7433	1.0492
e		2	7	10.21	11.157	12.105	0.6602	1.0245
e	Diff (1-2)			-3.343	-2.299	-1.255	0.7808	1.0405

Statistics

Variable	group		Upper CL Std Dev	Std Err	Minimum	Maximum
e		1	1.7814	0.3029	7.6	10.5
e		2	2.2559	0.3872	9.1	12
e	Diff (1-2)		1.5599	0.4949		

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
e	Pooled	Equal	17	-4.65	0.0002
e	Satterthwaite	Unequal	12.9	-4.68	0.0004

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
e	Folded F	11	6	1.05	1.0000

---------- fol=2 time=12 ----------

Statistics

Variable	group	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev
e		1	11	8.1705	8.8636	9.5568	0.7209	1.0318
e		2	8	10.405	11.075	11.745	0.5298	0.8013
e	Diff (1-2)			-3.137	-2.211	-1.286	0.7082	0.9437

Statistics

Variable	group		Upper CL Std Dev	Std Err	Minimum	Maximum

```
      e                   1    1.8107     0.3111        7.7       10.5
      e                   2    1.6309     0.2833        9.8       11.9
      e         Diff (1-2)     1.4148     0.4385

                                T-Tests
    Variable    Method          Variances      DF    t Value    Pr > |t|
    e           Pooled          Equal          17      -5.04      0.0001
    e           Satterthwaite   Unequal      16.9      -5.26      <.0001

                          Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F        10         7       1.66    0.5163

-------------------------------- fol=2 time=24 --------------------------------

                                Statistics
                                Lower CL           Upper CL  Lower CL
  Variable  group            N      Mean       Mean      Mean   Std Dev  Std Dev
  e                  1      12    7.8669     8.725    9.5831    0.9567   1.3505
  e                  2       5    9.1527      10.8    12.447    0.7948   1.3266
  e         Diff (1-2)               -3.6    -2.075     -0.55     0.993   1.3442

                              Statistics
                             Upper CL
      Variable  group           Std Dev    Std Err    Minimum    Maximum
      e                   1     2.293     0.3899        6.7       10.8
      e                   2    3.8122     0.5933        9.4       12.5
      e         Diff (1-2)     2.0804     0.7155

                                T-Tests
    Variable    Method          Variances      DF    t Value    Pr > |t|
    e           Pooled          Equal          15      -2.90      0.0110
    e           Satterthwaite   Unequal      7.68      -2.92      0.0201

                          Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F        11         4       1.04    1.0000
```

5.7. bLH data from of Figure 2.3.

The SAS System

Heifer	Group	-24	-18	-12	-6	0	6	12	18	24
94.0	**1**	0.48	0.37	0.52	0.47	8.79	6.45	0.53	0.19	0.24
99.0	**1**	0.78	1.45	0.82	1.20	7.89	5.32	0.62	0.28	0.26
302.0	**1**	0.83	0.63	0.69	1.75	8.33	0.94	0.37	0.31	0.33
520.0	**1**	1.16	.	1.27	0.72	9.00	1.42	0.38	0.17	0.11
524.0	**1**	0.91	0.54	0.56	0.61	9.83	1.46	0.34	0.35	0.31
99.1	**1**	1.01	1.53	1.66	1.02	8.40	3.35	0.50	0.19	0.35
38.1	**1**	1.06	1.58	0.73	0.10	15.20	0.10	0.73	0.44	0.47
43.0	**1**	0.91	1.81	0.56	1.66	12.60	3.56	0.64	0.57	0.43
44.1	**1**	.	0.98	1.58	1.36	12.80	1.78	0.58	0.46	0.43
99.2	**1**	0.42	1.08	0.66	0.58	8.87	6.63	0.74	0.33	0.35
26.0	**1**	1.04	0.87	1.00	1.47	7.30	1.73	0.42	0.35	0.31
6280.0	**1**	0.94	2.07	1.20	2.11	7.50	0.59	0.29	0.31	0.31
10.0	**2**	.	.	.	1.51	9.07	0.66	0.34	0.30	0.42
10.1	**2**	1.01	0.85	0.51	1.08	8.23	2.24	0.31	0.46	.
16.0	**2**	0.57	0.93	0.76	1.14	9.09	1.04	0.34	0.29	0.15
16.1	**2**	1.02	1.22	0.54	1.42	10.69	1.61	0.47	0.20	0.19
522.0	**2**	.	.	0.56	2.43	7.83	1.60	0.44	0.42	0.42
525.0	**2**	0.61	0.95	1.04	0.98	6.99	1.30	0.33	0.23	0.41
6264.0	**2**	1.21	0.62	0.99	0.72	5.93	2.24	0.47	0.19	0.19
88.0	**2**	0.58	0.99	0.74	.	12.20	0.50	0.35	0.32	0.36

5.8. Statistical Analysis of the bLH Data from Figure 2.3.

The SAS System

The UNIVARIATE Procedure
Variable: Resid re (Residual)

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.933167	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.068202	Pr > D	0.0497
Cramer-von Mises	W-Sq	0.156367	Pr > W-Sq	0.0206
Anderson-Darling	A-Sq	1.295311	Pr > A-Sq	<0.0050

The Mixed Procedure

Number of Observations

Number of Observations Read	180
Number of Observations Used	171
Number of Observations Not Used	9

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
heifer(group)		2.2463
AR(1)	heifer(group)	0.1269

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
group	1	18	0.79	0.3865
time	8	135	50.80	<.0001
group*time	8	135	0.78	0.6246

5.9. bFSH Data of Figure 2.3.

Heifer	Group	-24	-18	-12	-6	0	6	12	18	24
94.0	**1**	0.16	0.14	0.15	0.13	0.32	0.23	0.12	0.29	0.42
99.0	**1**	0.17	0.20	0.16	0.18	0.38	0.19	0.14	0.30	0.53
302.0	**1**	0.16	0.16	0.16	0.18	0.29	0.14	0.19	0.37	0.48
520.0	**1**	0.17	.	0.13	0.15	0.34	0.13	0.10	0.23	0.34
524.0	**1**	0.09	0.09	0.11	0.06	0.42	0.14	0.10	0.39	0.36
99.1	**1**	0.14	0.16	0.16	0.12	0.52	0.27	0.15	0.28	0.49
38.1	**1**	0.15	0.16	0.13	0.16	0.43	0.15	0.18	0.34	0.43
43.0	**1**	0.19	0.22	0.19	0.24	0.37	0.26	0.14	0.23	0.37
44.1	**1**	0.21	0.15	0.18	0.20	0.78	0.18	0.24	0.32	0.37
99.2	**1**	0.16	0.19	0.15	0.17	0.45	.	0.17	0.22	0.50
26.0	**1**	0.12	0.11	0.11	0.12	0.40	0.16	0.10	0.18	0.23
6280.0	**1**	0.10	0.13	0.13	0.16	0.61	0.14	0.21	0.40	0.47
10.0	**2**	.	.	.	0.12	0.34	0.09	0.10	0.22	0.36
10.1	**2**	0.13	0.11	0.12	0.10	0.32	0.17	0.09	0.30	.
16.0	**2**	0.12	0.13	0.11	0.12	0.37	0.11	0.12	0.30	0.49
16.1	**2**	0.17	0.17	0.13	0.16	0.51	0.16	0.11	0.20	0.39
522.0	**2**	.	.	0.11	0.14	0.32	0.13	0.10	0.17	0.28
525.0	**2**	0.08	0.09	0.09	0.08	0.58	0.17	0.12	0.24	0.31
6264.0	**2**	0.12	0.12	0.11	0.11	0.25	0.14	0.11	0.18	0.33
88.0	**2**	0.11	0.12	0.10	0.16	0.46	0.12	0.14	0.28	0.30

5.10. Statistical Analysis of the bFSH Data from Figure 2.3.

The UNIVARIATE Procedure
Variable: Resid Le (Residual)

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.993412	Pr < W	0.6324
Kolmogorov-Smirnov	D	0.045809	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.036686	Pr > W-Sq	>0.2500

```
Anderson-Darling      A-Sq  0.208148    Pr > A-Sq  >0.2500

                  The Mixed Procedure

            Number of Observations
      Number of Observations Read             180
      Number of Observations Used             172
      Number of Observations Not Used           8
        Covariance Parameter Estimates

      Cov Parm          Subject          Estimate
      heifer(group)                       0.01298
      AR(1)             heifer(group)      0.1655
      Residual                            0.04833

         Type 3 Tests of Fixed Effects

                    Num     Den
Effect               DF      DF    F Value    Pr > F
group                 1      18      11.58    0.0032
time                  8     136      90.66    <.0001
group*time            8     136       0.37    0.9371

                The MEANS Procedure
               Analysis Variable : e
               N
 group    Obs      N            Mean       Std Error
     1    108    106       0.2343396       0.0128542
     2     72     66       0.1925758       0.0144685
                The TTEST Procedure

                          Statistics
                        Lower CL          Upper CL  Lower CL
Variable  group            N      Mean    Mean      Mean   Std Dev  Std Dev
e                  1     106    0.2089  0.2343    0.2598    0.1166   0.1323
e                  2      66    0.1637  0.1926    0.2215    0.1004   0.1175
e         Diff (1-2)             0.0025  0.0418     0.081    0.1147   0.1269
                          Statistics
                       Upper CL
     Variable  group     Std Dev    Std Err    Minimum    Maximum
     e                1     0.153     0.0129       0.06       0.78
     e                2    0.1419     0.0145       0.08       0.58
     e         Diff (1-2)    0.142     0.0199

                          T-Tests
   Variable    Method          Variances      DF    t Value    Pr > |t|
   e           Pooled          Equal         170       2.10      0.0373
   e           Satterthwaite   Unequal       150       2.16      0.0325

                  Equality of Variances
      Variable    Method      Num DF    Den DF    F Value    Pr > F
      e           Folded F       105        65       1.27    0.3026
```

5.11. Estradiol data of Figure 2.3.

Heifer	Group	-24	-12	-6	0	6	12	18
94.0	**1**	1.40	1.20	2.15	2.51	1.60	0.34	0.18
99.0	**1**	1.23	0.92	1.52	1.94	1.41	0.26	0.34
302.0	**1**	1.50	1.78	1.64	1.83	0.45	0.21	0.18
99.1	**1**	1.94	2.01	3.03	3.52	2.05	1.38	0.71
38.1	**1**	2.10	2.23	3.05	3.58	2.94	0.55	.
43.0	**1**	0.88	1.58	1.63	1.50	1.37	0.61	0.81
44.1	**1**	1.31	2.75	1.94	3.96	1.22	0.21	0.13
99.2	**1**	1.34	1.25	1.78	2.23	1.03	0.18	0.18

26.0	**1**	1.28	1.64	1.44	1.42	0.57	0.29	0.40
6280.0	**1**	1.60	1.82	1.56	2.03	1.17	0.23	0.34
10.0	**2**	.	.	2.20	3.31	1.09	0.43	0.41
10.1	**2**	1.48	2.32	3.35	4.05	1.72	0.90	0.24
16.0	**2**	2.23	2.61	3.43	4.29	1.34	0.36	0.18
16.1	**2**	1.84	1.93	3.24	3.50	1.15	0.21	0.18
522.0	**2**	.	1.56	1.96	2.85	1.38	0.18	0.18
525.0	**2**	0.83	2.01	2.26	2.55	1.30	0.64	0.68
6264.0	**2**	1.20	1.46	1.51	4.03	2.38	0.26	0.31
88.0	**2**	2.44	2.54	3.12	3.14	1.86	1.46	0.94

5.12. Statistical Analysis of the estradiol Data from Figure 2.3.


```
                          The UNIVARIATE Procedure
                        Variable:  Resid e (Residual)

                             Tests for Normality
          Test                  --Statistic---    -----p Value------
          Shapiro-Wilk          W     0.981637    Pr < W      0.0955
          Kolmogorov-Smirnov    D     0.076404    Pr > D      0.0802
          Cramer-von Mises      W-Sq  0.110921    Pr > W-Sq   0.0832
          Anderson-Darling      A-Sq   0.70119    Pr > A-Sq   0.0692

                          The Mixed Procedure

                       Number of Observations
                  Number of Observations Read             126
                  Number of Observations Used             122
                  Number of Observations Not Used           4

                Covariance Parameter Estimates

                  Cov Parm          Subject          Estimate
                  heifer(group)                       0.08037
                  SP(POW)           heifer(group)      0.8337
                  Residual                             0.2184

                       Type 3 Tests of Fixed Effects
                             Num     Den
                Effect        DF      DF    F Value    Pr > F
                group          1      16       3.86    0.0670
                time           6      92      58.52    <.0001
                group*time     6      92       2.76    0.0163

---------------------------------- time=-24 -------------

                           The MEANS Procedure
                           Analysis Variable : e
                           N
            group    Obs      N            Mean       Std Error
                1     10     10       1.4580000       0.1117219
                2      8      6       1.6700000       0.2515287
---------------------------------- time=-12 -----------------------------------

                           Analysis Variable : e
                           N
            group    Obs      N            Mean       Std Error
                1     10     10       1.7180000       0.1691797
                2      8      7       2.0614286       0.1711227

---------------------------------- time=-6 ------------------------------------

                           Analysis Variable : e
                           N
            group    Obs      N            Mean       Std Error
                1     10     10       1.9740000       0.1895972
                2      8      8       2.6337500       0.2603428

---------------------------------- time=0 -------------------------------------
```

```
                          Analysis Variable : e
                          N
                group    Obs      N            Mean       Std Error
                    1     10     10       2.4520000       0.2894086
                    2      8      8       3.4650000       0.2191053

---------------------------------- time=6 ----------------------------------

                          Analysis Variable : e
                          N
                group    Obs      N            Mean       Std Error
                    1     10     10       1.3810000       0.2271830
                    2      8      8       1.5275000       0.1533524

---------------------------------- time=12 ----------------------------------

                          Analysis Variable : e
                          N
                group    Obs      N            Mean       Std Error
                    1     10     10       0.4260000       0.1157123
                    2      8      8       0.5550000       0.1550115

---------------------------------- time=18 ----------------------------------

                          Analysis Variable : e
                          N
                group    Obs      N            Mean       Std Error
                    1     10      9       0.3633333       0.0814282
                    2      8      8       0.3900000       0.0989408

---------------------------------- time=-24 ----------------------------------

                           The TTEST Procedure
                                Statistics
                              Lower CL           Upper CL  Lower CL
 Variable  group             N      Mean    Mean      Mean   Std Dev  Std Dev
 e                   1      10    1.2053   1.458    1.7107     0.243   0.3533
 e                   2       6    1.0234    1.67    2.3166    0.3846   0.6161
 e         Diff (1-2)              -0.727  -0.212    0.3025    0.3401   0.4646

                               Statistics
                             Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                   1     0.645     0.1117       0.88        2.1
      e                   2    1.5111     0.2515       0.83       2.44
      e         Diff (1-2)     0.7326     0.2399

                                 T-Tests
    Variable    Method           Variances      DF    t Value    Pr > |t|
    e           Pooled           Equal          14      -0.88      0.3918
    e           Satterthwaite    Unequal      7.02      -0.77      0.4663

                           Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F         5         9       3.04    0.1403

---------------------------------- time=-12 ----------------------------------
                           The TTEST Procedure
                                Statistics
                              Lower CL           Upper CL  Lower CL
 Variable  group             N      Mean    Mean      Mean   Std Dev  Std Dev
 e                   1      10    1.3353   1.718    2.1007     0.368    0.535
 e                   2       7    1.6427  2.0614    2.4802    0.2917   0.4527
 e         Diff (1-2)              -0.873  -0.343    0.1857    0.3721   0.5037

                               Statistics
                             Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                   1    0.9767     0.1692       0.92       2.75
      e                   2     0.997     0.1711       1.46       2.61
      e         Diff (1-2)     0.7796     0.2482

                                 T-Tests
    Variable    Method           Variances      DF    t Value    Pr > |t|
    e           Pooled           Equal          15      -1.38      0.0948
```

```
    e           Satterthwaite    Unequal      14.3      -1.43      0.1750
                          Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F         9         6       1.40    0.7062

---------------------------------- time=-6 ----------------------------------

                           The TTEST Procedure
                                Statistics
                              Lower CL          Upper CL  Lower CL
  Variable  group             N      Mean    Mean      Mean   Std Dev  Std Dev
  e                   1      10    1.5451   1.974    2.4029    0.4124   0.5996
  e                   2       8    2.0181  2.6338    3.2494    0.4869   0.7364
  e         Diff (1-2)              -1.326   -0.66    0.0068    0.4937   0.6629

                               Statistics
                             Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                   1    1.0946     0.1896       1.44       3.05
      e                   2    1.4987     0.2603       1.51       3.43
      e         Diff (1-2)     1.0089     0.3144

                                T-Tests
    Variable    Method           Variances      DF    t Value    Pr > |t|
    e           Pooled           Equal          16      -2.10      0.0321
    e           Satterthwaite    Unequal      13.5      -2.05      0.0605

                           Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F         7         9       1.51    0.5539

---------------------------------- time=0 -----------------------------------

                           The TTEST Procedure
                                Statistics
                              Lower CL          Upper CL  Lower CL
  Variable  group             N      Mean    Mean      Mean   Std Dev  Std Dev
  e                   1      10    1.7973   2.452    3.1067    0.6295   0.9152
  e                   2       8    2.9469   3.465    3.9831    0.4097   0.6197
  e         Diff (1-2)              -1.817  -1.013    -0.209    0.5954   0.7995

                               Statistics
                             Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                   1    1.6708     0.2894       1.42       3.96
      e                   2    1.2613     0.2191       2.55       4.29
      e         Diff (1-2)     1.2167     0.3792

                                T-Tests
    Variable    Method           Variances      DF    t Value    Pr > |t|
    e           Pooled           Equal          16      -2.67      0.0127
    e           Satterthwaite    Unequal      15.7      -2.79      0.0133
                           Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F         9         7       2.18    0.3165


---------------------------------- time=6 -----------------------------------

                           The TTEST Procedure
                                Statistics
                              Lower CL          Upper CL  Lower CL
  Variable  group             N      Mean    Mean      Mean   Std Dev  Std Dev
  e                   1      10    0.8671   1.381    1.8949    0.4942   0.7184
  e                   2       8    1.1649  1.5275    1.8901    0.2868   0.4337
  e         Diff (1-2)             -0.76  -0.147    0.4673    0.4546   0.6104
                               Statistics
                             Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                   1    1.3115     0.2272       0.45       2.94
      e                   2    0.8828     0.1534       1.09       2.38
      e         Diff (1-2)     0.929      0.2896
                                T-Tests

    Variable    Method           Variances      DF    t Value    Pr > |t|
    e           Pooled           Equal          16      -0.51      0.6198
```

```
    e           Satterthwaite    Unequal      15.1      -0.53      0.6008

                          Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F         9         7       2.74    0.1970

---------------------------------- time=12 ------------------------------------

                           The TTEST Procedure
                                Statistics
                              Lower CL          Upper CL  Lower CL
 Variable  group            N      Mean    Mean      Mean   Std Dev  Std Dev
 e                   1     10    0.1642   0.426    0.6878    0.2517   0.3659
 e                   2      8    0.1885   0.555    0.9215    0.2899   0.4384
 e         Diff (1-2)             -0.53  -0.129    0.2725    0.2974   0.3993

                                Statistics
                               Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                   1     0.668     0.1157       0.18       1.38
      e                   2    0.8923      0.155       0.18       1.46
      e         Diff (1-2)     0.6077     0.1894

                                  T-Tests
    Variable    Method           Variances      DF    t Value    Pr > |t|
    e           Pooled           Equal          16      -0.68      0.5055
    e           Satterthwaite    Unequal      13.7      -0.67      0.5159

                          Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F         7         9       1.44    0.6001

---------------------------------- time=18 ------------------------------------

                           The TTEST Procedure
                                Statistics
                              Lower CL          Upper CL  Lower CL
 Variable  group            N      Mean    Mean      Mean   Std Dev  Std Dev
 e                   1      9    0.1756  0.3633    0.5511     0.165   0.2443
 e                   2      8     0.156    0.39     0.624     0.185   0.2798
 e         Diff (1-2)             -0.297  -0.027    0.2442    0.1932   0.2615

                                Statistics
                               Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                   1     0.468     0.0814       0.13       0.81
      e                   2    0.5696     0.0989       0.18       0.94
      e         Diff (1-2)     0.4047     0.1271
                                  T-Tests
    Variable    Method           Variances      DF    t Value    Pr > |t|
    e           Pooled           Equal          15      -0.21      0.8366
    e           Satterthwaite    Unequal      14.1      -0.21      0.8381

                          Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F         7         8       1.31    0.7067
```

5.13. Follicle (F2) data from -24 to 24 h after follicle deviation – Figure 2.4.

Heifer	Group	Foll	-24	-12	0	12	24
94.0	**1**	**2**	6.20	8.40	9.00	9.50	9.20
99.0	**1**	**2**	7.70	.	9.00	.	9.60
302.0	**1**	**2**	7.50	8.90	9.30	9.40	9.00
520.0	**1**	**2**	6.60	.	7.70	8.60	7.60
524.0	**1**	**2**	6.20	.	8.20	.	9.20
99.1	**1**	**2**	5.40	6.80	8.00	9.00	8.80
38.1	**1**	**2**	5.90	6.60	7.30	8.00	8.80
43.0	**1**	**2**	7.70	7.60	7.60	7.90	8.20
44.1	**1**	**2**	6.50	7.30	7.70	8.50	8.60
99.2	**3**	**2**	5.40	.	8.70	9.90	10.20

26.0	**3**	**2**	7.50	7.80	8.50	9.60	11.00
6280.0	**3**	**2**	5.70	6.40	7.70	8.50	9.60

5.14. Statistical analysis of the follicle (F2) data from -24 to 24 h after follicle deviation – Figure 2.4.


```
                          The UNIVARIATE Procedure
                        Variable:  Resid e (Residual)

                             Tests for Normality
          Test                  --Statistic---    -----p Value------
          Shapiro-Wilk          W     0.962264    Pr < W      0.0870
          Kolmogorov-Smirnov    D     0.094093    Pr > D     >0.1500
          Cramer-von Mises      W-Sq  0.095611    Pr > W-Sq   0.1289
          Anderson-Darling      A-Sq  0.613666    Pr > A-Sq   0.1060

---------------------------------- fol=2 ----------------------------------

                           The Mixed Procedure
                         Number of Observations
                    Number of Observations Read              60
                    Number of Observations Used              54
                    Number of Observations Not Used           6

                        Covariance Parameter Estimates
                    Cov Parm          Subject          Estimate
                    heifer(group)                      7.73E-19
                    AR(1)             heifer(group)      0.7642
                    Residual                             0.5706

                          Type 3 Tests of Fixed Effects
                                Num     Den
                    Effect       DF      DF    F Value    Pr > F
                    group         1      10       0.22    0.6496
                    time          4      34      30.22    <.0001
                    group*time    4      34       4.10    0.0081

------------------------------ fol=2 time=-24 ------------------------------

                           The MEANS Procedure
                          Analysis Variable : e
                          N
                    group    Obs     N            Mean       Std Error
                        1      9     9       6.6333333       0.2758824
                        2      3     3       6.2000000       0.6557439

------------------------------ fol=2 time=-12 ------------------------------
                          Analysis Variable : e
                          N
                    group    Obs     N            Mean       Std Error
                        1      9     6       7.6000000       0.3678768
                        2      3     2       7.1000000       0.7000000
-------------------------------- fol=2 time=0 --------------------------------

                          Analysis Variable : e
                          N
                    group    Obs     N            Mean       Std Error
                        1      9     9       8.2000000       0.2415229
                        2      3     3       8.3000000       0.3055050

------------------------------ fol=2 time=12 -------------------------------

                          Analysis Variable : e
                          N
                    group    Obs     N            Mean       Std Error
                        1      9     7       8.7000000       0.2390457
                        2      3     3       9.3333333       0.4255715

------------------------------ fol=2 time=24 -------------------------------

                          Analysis Variable : e
                          N
                    group    Obs     N            Mean       Std Error
                        1      9     9       8.7777778       0.1984508
```

```
                    2      3     3      10.2666667       0.4055175

---------------------------------- time=-24 ----------------------------------

                            The TTEST Procedure
                                 Statistics
                               Lower CL          Upper CL  Lower CL
Variable  group             N      Mean    Mean      Mean   Std Dev  Std Dev
e                   1       9    5.9971  6.6333    7.2695     0.559   0.8276
e                   2       3    3.3786     6.2    9.0214    0.5914   1.1358
e         Diff (1-2)              -0.9  0.4333    1.7669    0.6273   0.8978

                                Statistics

                              Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                   1    1.5856     0.2759        5.4        7.7
      e                   2    7.1381     0.6557        5.4        7.5
      e         Diff (1-2)     1.5755     0.5985

                                T-Tests
   Variable    Method           Variances      DF    t Value    Pr > |t|
   e           Pooled           Equal          10       0.72      0.4857
   e           Satterthwaite    Unequal      2.75       0.61      0.5891

                           Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F         2         8       1.88    0.4274

---------------------------------- time=-12 ----------------------------------

                            The TTEST Procedure
                                 Statistics
                               Lower CL          Upper CL  Lower CL
Variable  group             N      Mean    Mean      Mean   Std Dev  Std Dev
e                   1       6    6.6543     7.6    8.5457    0.5625   0.9011
e                   2       2    -1.794     7.1    15.994    0.4417   0.9899
e         Diff (1-2)              -1.331     0.5    2.3311    0.5906   0.9165

                                Statistics
                              Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                   1    2.2101     0.3679        6.6        8.9
      e                   2    31.589        0.7        6.4        7.8
      e         Diff (1-2)     2.0182     0.7483
                                T-Tests
   Variable    Method           Variances      DF    t Value    Pr > |t|
   e           Pooled           Equal           6       0.67      0.5289
   e           Satterthwaite    Unequal       1.6       0.63      0.6053
                           Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F         1         5       1.21    0.6440

---------------------------------- time=0 ----------------------------------

                            The TTEST Procedure
                                 Statistics
                               Lower CL          Upper CL  Lower CL
Variable  group             N      Mean    Mean      Mean   Std Dev  Std Dev
e                   1       9     7.643     8.2     8.757    0.4894   0.7246
e                   2       3    6.9855     8.3    9.6145    0.2755   0.5292
e         Diff (1-2)              -1.125    -0.1    0.9248    0.4821   0.6899

                                Statistics
                              Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                   1    1.3881     0.2415        7.3        9.3
      e                   2    3.3256     0.3055        7.7        8.7
      e         Diff (1-2)     1.2108       0.46
                                T-Tests
   Variable    Method           Variances      DF    t Value    Pr > |t|
   e           Pooled           Equal          10      -0.22      0.8323
   e           Satterthwaite    Unequal      4.81      -0.26      0.8080
                           Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F         8         2       1.88    0.7877
```

```
---------------------------------- time=12 ----------------------------------

                          The TTEST Procedure
                               Statistics
                             Lower CL          Upper CL  Lower CL
Variable  group            N      Mean    Mean      Mean   Std Dev  Std Dev
e                  1       7    8.1151     8.7    9.2849    0.4076   0.6325
e                  2       3    7.5022  9.3333    11.164    0.3838   0.7371
e         Diff (1-2)             -1.684  -0.633    0.4172    0.4459   0.6602

                               Statistics
                              Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                   1    1.3927      0.239        7.9        9.5
      e                   2    4.6326     0.4256        8.5        9.9
      e         Diff (1-2)     1.2647     0.4556
                                 T-Tests
    Variable    Method           Variances      DF    t Value    Pr > |t|
    e           Pooled           Equal           8      -1.39      0.2019
    e           Satterthwaite    Unequal      3.35      -1.30      0.2766

                           Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F         2         6       1.36    0.6523

---------------------------------- time=24 ----------------------------------
                          The TTEST Procedure
                               Statistics
                             Lower CL          Upper CL  Lower CL
Variable  group            N      Mean    Mean      Mean   Std Dev  Std Dev
e                  1       9    8.3201  8.7778    9.2354    0.4021   0.5954
e                  2       3    8.5219  10.267    12.011    0.3657   0.7024
e         Diff (1-2)             -2.407  -1.489    -0.571     0.432   0.6182
                               Statistics
                              Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                   1    1.1406     0.1985        7.6        9.6
      e                   2    4.4143     0.4055        9.6         11
      e         Diff (1-2)      1.085     0.4122
                                 T-Tests
    Variable    Method           Variances      DF    t Value    Pr > |t|
    e           Pooled           Equal          10      -3.61      0.0047
    e           Satterthwaite    Unequal      3.03      -3.30      0.0452

                           Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F         2         8       1.39    0.6058
```

5.15. Follicle (F2) data from -24 to 24 h after peak of LH surge – Figure 2.4.

Heifer	Group	Foll	-24	-12	0	12	24
94.0	**1**	**2**	8.60	8.90	8.20	7.80	6.70
99.0	**1**	**2**	9.60	.	9.80	.	9.20
302.0	**1**	**2**	9.00	8.90	9.00	8.70	8.80
520.0	**1**	**2**	7.60	7.10	7.90	7.70	6.90
524.0	**1**	**2**	9.00	9.00	8.60	8.90	8.50
99.1	**1**	**2**	8.80	9.30	8.60	8.80	8.00
38.1	**1**	**2**	8.50	7.80	7.60	8.60	8.80
43.0	**1**	**2**	8.20	8.50	7.80	8.00	7.80
44.1	**1**	**2**	8.20	8.10	8.00	8.00	8.20
99.2	**2**	**2**	10.50	10.40	9.80	10.50	10.30
26.0	**2**	**2**	10.90	10.30	10.50	10.40	10.80
6280.0	**2**	**2**	9.60	10.30	10.50	10.10	10.70

5.16. Statistical analysis of the follicle (F2) data from -24 to 24 h after peak of LH surge – Figure 2.4.


```
---------------------------------- fol=2 ----------------------------------

                          The UNIVARIATE Procedure
                        Variable:  Resid e (Residual)

                            Tests for Normality
          Test                  --Statistic---    -----p Value------
          Shapiro-Wilk          W     0.970705    Pr < W      0.1730
          Kolmogorov-Smirnov    D      0.06952    Pr > D     >0.1500
          Cramer-von Mises      W-Sq  0.079709    Pr > W-Sq   0.2124
          Anderson-Darling      A-Sq  0.549978    Pr > A-Sq   0.1539

                           The Mixed Procedure
                         Number of Observations
                Number of Observations Read              60
                Number of Observations Used              58
                Number of Observations Not Used           2
                      Covariance Parameter Estimates

                Cov Parm          Subject          Estimate
                heifer(group)                        0.2388
                AR(1)             heifer(group)      0.4599
                Residual                             0.2165
                       Type 3 Tests of Fixed Effects

                              Num     Den
                Effect         DF      DF    F Value    Pr > F
                group           1      10      25.82    <0.0001
                time            4      38       0.23    0.9173
                group*time      4      38       1.28    0.2954
---------------------------------- fol=2 ----------------------------------

                           The MEANS Procedure
                          Analysis Variable : e
                          N
                group    Obs     N            Mean       Std Error
                    1     45    43       8.3720930       0.1028565
                    2     15    15      10.3733333       0.0886226

---------------------------------- fol=2 ----------------------------------
                           The TTEST Procedure
                                Statistics
                              Lower CL          Upper CL  Lower CL
 Variable  group             N      Mean    Mean      Mean   Std Dev  Std Dev
 e                    1     43    8.1645  8.3721    8.5797    0.5561   0.6745
 e                    2     15    10.183  10.373    10.563    0.2513   0.3432
 e         Diff (1-2)             -2.367  -2.001    -1.636     0.514   0.6088
                                Statistics
                              Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                    1    0.8573     0.1029        6.7        9.8
      e                    2    0.5413     0.0886        9.6       10.9
      e         Diff (1-2)      0.7468     0.1826

                                   T-Tests
    Variable    Method           Variances      DF    t Value    Pr > |t|
    e           Pooled           Equal          56     -10.96      <.0001
    e           Satterthwaite    Unequal      48.1     -14.74      <.0001

                           Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F        42        14       3.86    0.0086
```

5.17. bFSH data from -24 to 24 h after follicle deviation – Figure 2.4.

Heifer	Group	-12	0	6	12	18	24
94.0	**1**	0.19	0.16	0.16	0.11	0.13	0.13
99.0	**1**	.	0.20	0.17	0.20	0.18	0.17
302.0	**1**	0.18	0.19	0.16	0.17	0.16	0.16

520.0	**1**	.	0.18	0.14	0.15	0.13	0.17
524.0	**1**	.	.	.	.	.	0.11
99.1	**1**	0.31	0.22	0.23	0.19	0.16	0.14
38.1	**1**	0.23	0.17	0.13	0.13	0.14	0.17
43.0	**1**	0.21	0.16	0.19	0.19	0.20	0.20
44.1	**1**	0.19	0.20	0.16	0.14	0.15	0.13
99.2	**2**	0.51	0.34	0.25	0.17	0.19	0.17
26.0	**2**	0.22	0.29	0.24	0.15	0.14	0.11
6280.0	**2**	0.42	0.25	0.18	0.15	0.13	0.12

5.18. Statistical analysis of the bFSH data from -24 to 24 h after follicle deviation – Figure 2.4.

The UNIVARIATE Procedure
Variable: Resid Le (Residual)

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.989941	Pr < W	0.8786
Kolmogorov-Smirnov	D	0.044687	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.015876	Pr > W-Sq	>0.2500
Anderson-Darling	A-Sq	0.122899	Pr > A-Sq	>0.2500

The Mixed Procedure

Number of Observations

Number of Observations Read	72
Number of Observations Used	65
Number of Observations Not Used	7

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
heifer(group)		0.01064
AR(1)	heifer(group)	0.4685

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
Residual		0.02937

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
group	1	10	3.92	0.0758
time	5	43	16.01	<.0001
group*time	5	43	4.30	0.0029

---------- time=-12 ----------

The MEANS Procedure
Analysis Variable : e

group	Obs	N	Mean	Std Error
1	9	6	0.2183333	0.0197343
2	3	3	0.3833333	0.0856997

---------- time=0 ----------

Analysis Variable : e

group	Obs	N	Mean	Std Error
1	9	8	0.1850000	0.0075593
2	3	3	0.2933333	0.0260342

---------- time=6 ----------

Analysis Variable : e

group	Obs	N	Mean	Std Error
1	9	8	0.1675000	0.0109789
2	3	3	0.2233333	0.0218581

---------- time=12 ----------

Analysis Variable : e
N

group	Obs	N	Mean	Std Error
1	9	8	0.1600000	0.0114953
2	3	3	0.1566667	0.0066667

---------- time=18 ----------

Analysis Variable : e
N

group	Obs	N	Mean	Std Error
1	9	8	0.1562500	0.0086474
2	3	3	0.1533333	0.0185592

---------- time=24 ----------

Analysis Variable : e
N

group	Obs	N	Mean	Std Error
1	9	9	0.1533333	0.0092796
2	3	3	0.1333333	0.0185592

---------- time=-12 ----------

The TTEST Procedure
Statistics

Variable	group	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev
e	1	6	0.1676	0.2183	0.2691	0.0302	0.0483
e	2	3	0.0146	0.3833	0.7521	0.0773	0.1484
e	Diff (1-2)		-0.314	-0.165	-0.016	0.059	0.0892

Statistics
Upper CL

Variable	group	Std Dev	Std Err	Minimum	Maximum
e	1	0.1186	0.0197	0.18	0.31
e	2	0.9329	0.0857	0.22	0.51
e	Diff (1-2)	0.1816	0.0631		

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
e	Pooled	Equal	7	-2.61	0.0347
e	Satterthwaite	Unequal	2.22	-1.88	0.1890

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
e	Folded F	2	5	9.43	0.0402

---------- time=0 ----------

The TTEST Procedure
Statistics

Variable	group	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev
e	1	8	0.1671	0.185	0.2029	0.0141	0.0214
e	2	3	0.1813	0.2933	0.4053	0.0235	0.0451
e	Diff (1-2)		-0.152	-0.108	-0.065	0.0195	0.0284

Statistics
Upper CL

Variable	group	Std Dev	Std Err	Minimum	Maximum
e	1	0.0435	0.0076	0.16	0.22
e	2	0.2834	0.026	0.25	0.34
e	Diff (1-2)	0.0519	0.0192		

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
e	Pooled	Equal	9	-5.63	0.0003
e	Satterthwaite	Unequal	2.35	-4.00	0.0437

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
e	Folded F	2	7	4.45	0.1133

---------- time=6 ----------

The TTEST Procedure

Statistics

Variable	group	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev
e	1	8	0.1415	0.1675	0.1935	0.0205	0.0311
e	2	3	0.1293	0.2233	0.3174	0.0197	0.0379
e	Diff (1-2)		-0.106	-0.056	-0.006	0.0225	0.0327

Statistics

Variable	group	Upper CL Std Dev	Std Err	Minimum	Maximum
e	1	0.0632	0.011	0.13	0.23
e	2	0.2379	0.0219	0.18	0.25
e	Diff (1-2)	0.0597	0.0221		

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
e	Pooled	Equal	9	-2.52	0.0326
e	Satterthwaite	Unequal	3.08	-2.28	0.1044

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
e	Folded F	2	7	1.49	0.5794

---------- time=12 ----------

The TTEST Procedure

Statistics

Variable	group	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev
e	1	8	0.1328	0.16	0.1872	0.0215	0.0325
e	2	3	0.128	0.1567	0.1854	0.006	0.0115
e	Diff (1-2)		-0.041	0.0033	0.048	0.0201	0.0292

Statistics

Variable	group	Upper CL Std Dev	Std Err	Minimum	Maximum
e	1	0.0662	0.0115	0.11	0.2
e	2	0.0726	0.0067	0.15	0.17
e	Diff (1-2)	0.0533	0.0198		

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
e	Pooled	Equal	9	0.17	0.8698
e	Satterthwaite	Unequal	8.96	0.25	0.8076

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
e	Folded F	7	2	7.93	0.2331

---------- time=18 ----------

The TTEST Procedure

Statistics

Variable	group	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev
e	1	8	0.1358	0.1563	0.1767	0.0162	0.0245
e	2	3	0.0735	0.1533	0.2332	0.0167	0.0321
e	Diff (1-2)		-0.037	0.0029	0.0433	0.0181	0.0264

Statistics

Variable	group	Upper CL Std Dev	Std Err	Minimum	Maximum
e	1	0.0498	0.0086	0.13	0.2
e	2	0.202	0.0186	0.13	0.19
e	Diff (1-2)	0.0481	0.0178		

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
e	Pooled	Equal	9	0.16	0.8738
e	Satterthwaite	Unequal	2.92	0.14	0.8960

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F

```
        e           Folded F         2         7       1.73    0.4912

---------------------------------- time=24 ----------------------------------

                           The TTEST Procedure
                                Statistics
                              Lower CL           Upper CL  Lower CL
 Variable  group            N      Mean    Mean      Mean    Std Dev  Std Dev
 e                  1       9    0.1319  0.1533    0.1747    0.0188   0.0278
 e                  2       3    0.0535  0.1333    0.2132    0.0167   0.0321
 e         Diff (1-2)             -0.023    0.02    0.0627    0.0201   0.0288

                                    Statistics
                               Upper CL
      Variable  group          Std Dev    Std Err    Minimum    Maximum
      e                   1    0.0533     0.0093       0.11        0.2
      e                   2     0.202     0.0186       0.11       0.17
      e         Diff (1-2)     0.0505     0.0192

                                  T-Tests
    Variable    Method           Variances      DF    t Value    Pr > |t|
    e           Pooled           Equal          10       1.04      0.3213
    e           Satterthwaite    Unequal      3.08       0.96      0.4046

                           Equality of Variances
        Variable    Method      Num DF    Den DF    F Value    Pr > F
        e           Folded F         2         8       1.33    0.6328
```

5.19. bFSH data from -24 to 24 h after peak of LH surge – Figure 2.4.

Heifer	Group	-24	-18	-12	-6	0	6	12	18	24
94.0	**1**	0.16	0.14	0.15	0.13	0.32	0.23	0.12	0.29	0.42
99.0	**1**	0.17	0.20	0.16	0.18	0.38	0.19	0.14	0.30	0.53
302.0	**1**	0.16	0.16	0.16	0.18	0.29	0.14	0.19	0.37	0.48
520.0	**1**	0.17	.	0.13	0.15	0.34	0.13	0.10	0.23	0.34
524.0	**1**	0.09	0.09	0.11	0.06	0.42	0.14	0.10	0.39	0.36
99.1	**1**	0.14	0.16	0.16	0.12	0.52	0.27	0.15	0.28	0.49
38.1	**1**	0.15	0.16	0.13	0.16	0.43	0.15	0.18	0.34	0.43
43.0	**1**	0.19	0.22	0.19	0.24	0.37	0.26	0.14	0.23	0.37
44.1	**1**	0.21	0.15	0.18	0.20	0.78	0.18	0.24	0.32	0.37
99.2	**2**	0.16	0.19	0.15	0.17	0.45	.	0.17	0.22	0.50
26.0	**2**	0.12	0.11	0.11	0.12	0.40	0.16	0.10	0.18	0.23
6280.0	**2**	0.10	0.13	0.13	0.16	0.61	0.14	0.21	0.40	0.47

5.20. Statistical analysis of the bFSH data from -24 to 24 h after peak of LH surge – Figure 2.4.


```
                        The UNIVARIATE Procedure

                     Variable:  Resid  (Residual)
                         Tests for Normality

      Test                  --Statistic---    -----p Value------
      Shapiro-Wilk          W     0.989751    Pr < W      0.6038
      Kolmogorov-Smirnov    D     0.048096    Pr > D     >0.1500
      Cramer-von Mises      W-Sq  0.030645    Pr > W-Sq  >0.2500
      Anderson-Darling      A-Sq  0.224324    Pr > A-Sq  >0.2500
                        The Mixed Procedure
                      Number of Observations

             Number of Observations Read            108
             Number of Observations Used            106
             Number of Observations Not Used          2

                      Covariance Parameter Estimates
             Cov Parm          Subject          Estimate
             heifer(group)                       0.02007
```

AR(1)	heifer(group)	0.1258
Residual		0.05320

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
group	1	10	0.50	0.4977
time	8	78	37.26	<.0001
group*time	8	78	0.67	0.7120