Follicle and hormone dynamics in single versus double ovulating heifers

M P Palhao^{1,2}, M A Beg³, M T Rodrigues² and O J Ginther^{1,3}

¹Eutheria Foundation, Cross Plains, Wisconsin 53528, USA, ²Departamento de Zootecnia, Universidade Federal de Viçosa, 36570 Viçosa, Minas Gerais, Brazil and ³Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, 1656 Linden Drive, Madison, Wisconsin 53706, USA

Correspondence should be addressed to O J Ginther at Eutheria Foundation, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison; Email: ginther@vetmed.wisc.edu

Abstract

Follicles ≥ 5 mm were ablated at 4 day post-ovulation in heifers to induce a follicular wave, and prostaglandin $F_{2\alpha}$ was given at day 6 to increase the incidence of double ovulations. Follicle diameters and plasma hormone concentrations were compared between single ovulators ($n=12$) and double ovulators ($n=8$). In double ovulators, the interval from follicle deviation to the peak of the pre-ovulatory LH surge was shorter (1.9 ± 0.2 vs 2.5 ± 0.2 days; $P < 0.02$) and diameter of the largest pre-ovulatory follicle was smaller (12.2 ± 0.5 vs 13.3 ± 0.3 mm; $P < 0.02$). The LH concentrations of the pre-ovulatory surge did not differ between single and double ovulators for 24 h on each side of the peak. When data were normalised to LH peak, the peak of the pre-ovulatory FSH and oestradiol (E_2) surges occurred in synchrony with the peak of LH surge for both groups. Concentration of FSH for 24 h on each side of the peak showed a group effect ($P < 0.0001$) from lower concentration in the double ovulators. A group-by-hour interaction ($P < 0.008$) for E_2 reflected greater concentration in the double ovulators before and at the peak. Results indicated that two pre-ovulatory follicles resulted in an earlier and greater E_2 increase, leading to lower FSH concentration, an earlier LH surge, and ovulation at a smaller diameter. In conclusion, the difference in hormone concentrations during the pre-ovulatory period was an effect rather than a cause of double ovulations.

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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 & Campbell 2007, Adams *et al.* 2008, Aerts & Bols 2009, Mihm & Evans 2008). Follicular growth in a wave is characterised 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 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 (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 (E_2) 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 E_2 around the time of ovulation was found. In another series of studies (Echternkamp *et al.* 1990, 2004), cows were genetically selected for double ovulations and twin births (defined as twinning) and were compared with controls at 0, 1, 2, and 3 days after induction of luteolysis with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Cows were slaughtered on the indicated days, precluding determination of ovulation rate or normalising to ovulation or to the LH surge. No differences were found after $PGF_{2\alpha}$ 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 pre-ovulatory difference in hormone concentrations between single and double ovulations was an effect rather than a cause of double ovulations (Ginther *et al.* 2008, 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 $PGF_{2\alpha}$ 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 $PGF_{2\alpha}$ 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 $PGF_{2\alpha}$ 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.

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. 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 $PGF_{2\alpha}$, 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 synchronised 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.

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 1).

Figure 1 Diagrammatic outcome for single and multiple dominant follicles and single and multiple ovulations after ablating all follicles (≥ 5 mm) and treatment with $PGF_{2\alpha}$.

Table 1 Mean $\pm$ S.E.M. for intervals between follicle events in single and double ovulators^a.

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

^aOvulatory wave induced by ablating all follicles ≥ 5 mm 4 days after ovulation and treating with PGF_{2 α} 6 days after ovulation.

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 pre-treatment 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). 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 $\pm$ 0.1 mm/day) and between the LH peak and pre-ovulation (0.1 $\pm$ 0.1 mm/day) were not different between single and double ovulators.

Data and results of the analyses for diameters of F1, F2, and F3 normalised to deviation and to the LH peak are shown (Fig. 2). When normalised to deviation, only the main effect of hour, representing a gradual diameter increase, was significant for each follicle. For F1 normalised 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 normalised 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 normalised to the LH peak.

Normalised to the beginning of deviation, there were no significant effects for LH, but a main effect of hour for FSH represented declining concentrations (Fig. 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 normalised 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 normalised 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 (0.25 $\pm$ 0.01 vs 0.22 $\pm$ 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*<0.06) and –6 h (*P*<0.03) before the peak and at the peak (*P*<0.01).

The hour of the periovulatory peak in FSH concentration was not significantly different between single and double ovulators (35.0 $\pm$ 3.3 and 32.3 $\pm$ 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 $\pm$ 0.03 vs 0.44 $\pm$ 0.04 ng/ml).

Table 2 Mean $\pm$ S.E.M. diameters of F1 and F2 in single and double ovulators^a.

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

^aOvulatory wave induced by ablating all follicles ≥ 5 mm 4 days after ovulation and treating with PGF_{2 α} 6 days after ovulation.

Figure 2 Mean ($\pm$ S.E.M.) 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.

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 normalised either to deviation or to the LH peak (not shown). For F2, a significant hour effect normalised to deviation reflected growth of the follicle averaged over subgroups (Fig. 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 normalised 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 normalised to deviation was significant, resulting from a continuing decrease averaged over subgroups (Fig. 4). However, the interaction was also significant, reflecting greater concentrations before deviation and 6 h after deviation

Figure 3 Mean ($\pm$ S.E.M.) 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.

Figure 4 Mean ($\pm$ S.E.M.) 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.

in the double-dominant subgroup, with no difference thereafter. Concentrations of FSH were not different between single and double ovulators normalised to the LH peak.

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 E_2 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 characterisation 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 (Ginther 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 $\text{PGF}_{2\alpha}$ 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 $\text{PGF}_{2\alpha}$ 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 $\text{PGF}_{2\alpha}$ 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 PGF_{2α} during dioestrus 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 postablation FSH surge.

In horses (Ginther *et al.* 2008, 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 (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 synchronised 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, 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, 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 predeviation 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 E_2 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 normalisation 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 PGF_{2 α} . 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 normalised to the LH peak, the peak of the pre-ovulatory FSH and E_2 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 E_2 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 E_2 concentration which led to lower FSH concentration, an earlier appearance of the LH surge, and ovulation of a smaller follicle.

Material and Methods

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 colour-Doppler ultrasound instrument (Aloka SSD 3500; Aloka American, Wallingford, CT, USA) equipped with a linear-array 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).

Experimental design

On day 4, all follicles ≥ 5 mm in 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 PGF_{2 α} (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.

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.

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°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.

Statistical analyses

Follicle diameter and hormone concentrations were divided into two data sets, one normalised (hour 0) to the beginning of deviation and encompassing -24 or -12 to 24 h and the second normalised (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 >5% to ≤10% were considered as approaching significance. Data are presented as the mean ± S.E.M. unless otherwise indicated.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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