

VICTOR ENRIQUE GOMEZ LEÓN

**HORMONAL MECHANISMS REGULATING FOLLICULAR WAVE
DYNAMICS: INSIGHTS FROM THE SUBORDINATE FOLLICLES DURING
DIAMETER DEVIATION**

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae*.

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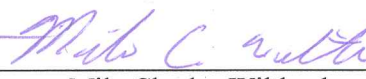
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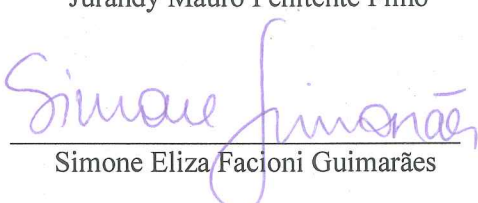
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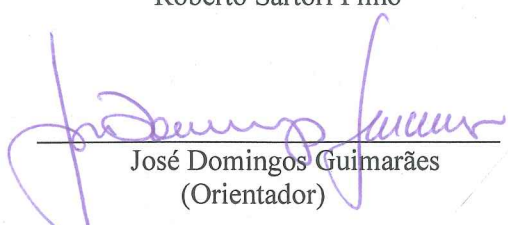

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ABSTRACT

GOMEZ-LEÓN, Victor Enrique, D.Sc., Universidade Federal de Viçosa, december, 2018. **Hormonal mechanisms regulating follicular wave dynamics: Insights from the subordinate follicles during diameter deviation.** Advisor: José Domingos Guimarães. Co-advisors: Eduardo Paulino da Costa, José Dantas Ribeiro Filho and Laércio dos Anjos Benjamin.

Follicle diameter deviation has been identified as the pivotal morphological manifestation of follicle selection. Complexities in diameter deviation (F1 ~ 8.5 mm) have been reported based on the diameter of the largest subordinate follicle (F2) at time of follicle deviation (conventional F2 ≥ 7 or undersized < 7 mm). However, many of the hormonal mechanisms leading to this key event and regulating variation between individual follicular waves remain undefined. Our main objective was to characterize and categorized the variations during the follicular diameter deviation. Thus, based on the subordinate follicles point of view, a biological model for future follicle selection studies could be proposed. Paper 1 compared circulating FSH, LH, and P4 with the follicular dynamics during wave 1 vs 2, spontaneous vs induced wave 2, and conventional vs undersized deviations. Values were normalized to the day of expected diameter deviation (day 0) and compared using SAS PROC MIXED. The effect of different hormonal conditions on follicle dynamics was observed for F2 and not for F1. Increased frequency of undersized deviation was associated with high P4 and FSH but lower LH concentration. Although, the causes for conventional and undersized remains unclear. Paper 2 directly evaluated the role of P4 in the diameter deviation complexities by manipulating P4 (Experiment 1). Experiment 2 tested whether decreased LH was the mechanism whereby elevated P4 increases undersized deviations. Data were normalized to F1 ≥ 7.5 mm (day 0) and compared using SAS PROC MIXED. Elevated P4 was linked to undersized deviation but this occurred either in increased or decreased LH activity and, surprisingly, increased or decreased circulating FSH. Paper 3 evaluated whether deficient LH activity was the underlying mechanism causing inhibition of follicular deviation and follicle growth only until 8.5mm in GnRH-antagonist (Acyline) treated heifers. Holstein heifers (n=24) were randomized: Control (n=8; Saline

treatments), Acyline (n=8; 5 μ g/kg Acyline), or Acyline+hCG (n= 8; 5 μ g/kg Acyline plus hCG; 50 IU of hCG at start then 100 IU every 12h). Data were normalized to F1 ~ 7.5mm (day 0) and compared using SAS PROC MIXED. Dominant follicle selection and growth after deviation was inhibit by Acyline treatment and associated with ablation of LH pulses. However, restoration of this process happened after replacement of LH action by hCG treatment. Future molecular studies are in need to determinate the cause of the differences observed in the follicular deviation of waves during high and low progesterone.

RESUMO

GOMEZ-LEÓN, Victor Enrique, D.Sc., Universidade Federal de Viçosa, dezembro 2018. **Mecanismos hormonais que regulam a dinâmica das ondas foliculares: Aporte dos folículos subordinados durante o desvio do diâmetro folicular.** Orientador: José Domingos Guimarães. Coorientadores: Eduardo Paulino da Costa, José Dantas Ribeiro Filho e Laércio dos Anjos Benjamin.

O desvio do diâmetro dos folículos foi identificado como a principal manifestação morfológica da seleção do folículo dominante (F1). Complexidades no momento do desvio dos diâmetros (F1 ~ 8.5 mm) foram relatadas com base no diâmetro do maior folículo subordinado (F2) no momento do desvio folicular (convencional $F2 \geq 7$ ou F2-subdesenvolvido < 7 mm). No entanto, muitos dos mecanismos hormonais que levam a este evento chave e regulam a variação entre as ondas foliculares individuais permanecem indefinidos. O objetivo principal deste estudo foi analisar e categorizar as variações que acontecem durante o processo do desvio folicular. Assim, baseado no ponto de vista dos folículos subordinados, um novo modelo para o estudo da seleção folicular poderia ser proposto. O artigo 1 comparou as concentrações de FSH, LH e Progesterona (P4) circulantes e a dinâmica folicular durante a primera vs a segunda onda, uma segunda onda folicular espontânea vs induzida, e os desvios convencionais vs F2-subdesenvolvidos. Os valores foram normalizados para o dia do desvio de diâmetro esperado (dia 0) e comparados usando o SAS PROC MIXED. O efeito de diferentes condições hormonais na dinâmica folicular foi observado para F2 e não para F1. Um aumento da frequência de desvios F2-subdesenvolvidos foi associado com alta concentração de P4 e FSH e baixo LH. Contudo, as causas para convencional e subdimensionada permanecem incertas. O artigo 2 avaliou diretamente o papel da P4 nas complexidades do desvio dos folículos pela manipulação de P4 (Experimento 1). Já o Experimento 2 testou se a diminuição de LH é o mecanismo pelo qual a alta P4 aumenta os desvios F2-subdesenvolvidos. Os dados foram normalizados para $F1 \geq 7,5$ mm (dia 0) e comparados usando o SAS PROC MIXED. O aumento de P4 foi associado a desvios F2-subdesenvolvido, mas isso ocorreu tanto alta quanto em baixa atividade de LH e, surpreendentemente, em alta e baixa concentração de FSH circulante. O artigo 3 avaliou se a deficiência da atividade de LH era o mecanismo que

causa a inibição do desvio e crescimento folicular apenas até 8,5 mm em novilhas tratadas com um antagonista de GnRH (Acyline). Novilhas da raça Holandesa (N=24) foram randomizadas em: Controle (n=8; Solução salina), Acyline (n=8; 5 µg/kg Acyline), ou Acyline+hCG (n= 8; 5 µg/kg Acyline mais hCG; 50 IU de hCG no começo e logo 100 IU cada 12h). Os dados foram normalizados para F1 ~ 7,5 mm (dia 0) e comparados usando o SAS PROC MIXED. A seleção do folículo dominante e o crescimento após o desvio foram inibidos pelo tratamento com Acyline e associados à ablação de pulsos de LH. No entanto, a restauração desse processo aconteceu após a substituição da ação da LH pelo tratamento com hCG. São necessários futuros estudos na área molecular para determinar a causa das diferenças no processo do desvio folicular de ondas em alta e baixa concentração de progesterona.

GENERAL INTRODUCTION

Studying and understanding reproductive physiology has allowed to development new biotechnologies that improved animal production and helped in the solution of classic physiological problems that affect the reproduction of domestic animals [1-3]. Rajakoski [4] proposed for the first time, in the 60's, that follicle development in cattle occurs in a follicular wave pattern. The study based on the observation of slaughterhouse animal ovaries, concluded that two dominant follicles develop in the interval of an estral cycle. However, many discrepancies have come around this model, and it was only validated on *in-vivo* studies that were done in the 80's [5]. The number of follicular waves that occur during the bovine estrous cycle can vary between two or three waves for *Bos taurus* animals [6], whereas, several authors have reported the incidence of four or more follicular waves in *Bos indicus* animals [7]. For mono-ovulatory species, such as humans, horses and cattle, only one single follicle is selected as dominant within each follicular wave and it is able to ovulate [8].

In this regard, the ultrasonography technique has been used as a great research tool in the study of physiological and applied reproduction of numerous domestic species [9, 10]. The initial studies proposed that the follicles grew at different rates (mm/day) according to its ranking, and that the selection process occurred by a gradual divergence among the follicles [11]. In 1996 a conceptual change was proposed and the model of follicular selection for the bovine specie was then considered in a different way [12]. In this study, the authors observed that after wave emergence at about 4 mm the follicles grow at similar rates among them for approximately 2 days. At this point, the largest follicle continues to develop and the subordinate ones decrease their growth

rate or regress. This process was termed "Deviation" to emphasize the difference of the old "Divergence" concept [13]. The abrupt growth rate difference between the selected dominant follicle and the subordinate ones was only observed when the follicular waves were normalized to F1 closest value to 8.5 mm but not when they were compared from the time of ovulation.

It has been shown that antral follicles (≥ 4 mm) are responsive to the follicle stimulation hormone (FSH) and emerges by an increase of the circulating FSH concentration in blood [14, 15]. As the follicles grow, from 3 to 5 mm, their production of inhibin increased, decreasing the FSH concentrations in blood [12], which is a critical factor to limit the growth of the future subordinate follicles. Finally, follicular deviation between the future dominant follicle and the future subordinate follicles occurs at 8.5 mm in diameter in Bos Taurus and 7 mm in Bos Indicus heifers. Thus, the deviation process has been proposed as the morphological manifestation of the dominant follicle selection [16, 17].

It was possible to study the acquisition of Lutropin Hormone (LH) receptors in the granulosa cells of the dominant follicle by centralizing the data in the follicular deviation during this phase [12, 18, 19]. These receptors induce the steroidogenic capacity in the dominant follicle which cause the final inhibition on the circulating FSH concentration, therefore, limiting the growth of future follicles [20]. Haughian et al. [21] administered a GnRH antagonist in heifers before the time of follicular diameter deviation, suppressing the LH pulses and stopping the follicle growth after ~ 8 mm in diameter. Thus, follicle diameter deviation was abolished. These results confirmed the importance of the LH in the selection of the dominant follicle and its subsequent

development. However, the exogenous replacement of LH in this model for re-establish of follicle selection and growth after diameter deviation has never been done.

The first follicular wave has been the most study one because of its synchronization after ovulation although it is the only anovulatory wave. [12]. In addition, variation among different hormonal environments happen during the follicle selection process. For example, FSH during the first follicular wave has at least two components, (1) a GnRH-dependent component (pre-ovulatory peak) and (2) an FSH increase (constitutive) because of the lack of inhibitory factors, whereas the second and third wave only present one component, the constitutive FSH [14, 21-23]. Also. the second follicular wave shows high concentration of progesterone accompanied by a decrease in LH when compared to the first follicular wave [24]. Recently, some of these variations have started to be characterized in order to improve the knowledge of the mechanisms that control ovarian follicular dynamics.

Four classes for different follicular deviation types have been proposed based on the diameter of the largest subordinate follicle (F2) when the dominant follicle reaches ~ 8.5 mm [25]: 1) Conventional deviation ($F2 \geq 7$ mm); 2) F2-Undersized (< 7 mm); 3) Double-dominants, $F1$ and $F2 \geq 10$ mm; and 4) Switching ($F1$ and $F2$ switched their diameter 24 hours before or after 8.5 mm) [26]. Based on the study of almost 200 follicular waves, Ginther et al. [27] reported a higher ($P < 0,0001$) frequency (40 %) of F2-undersized class during the second follicular wave, when compared to the frequency (15 %) during the first one. However, the hormonal profiles associated with this frequency variation was not studied on this or subsequent papers.

It is necessary to fill the knowledge gap generated by the complexities of a dynamic process such as the selection of the dominant follicle. Thus, the study of variations in follicular deviation from the stand point of view of the subordinate follicles could provide new models for the study of the selection process. These models could represent advances in the understanding the follicular, hormonal and molecular selection of the dominant follicle in the mono-ovulatory species.

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Hormonal mechanisms regulating follicular wave dynamics I: Comparison of follicle growth profiles under different physiological conditions in heifers

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Abstract

Follicle diameter deviation has been identified as the pivotal morphological manifestation of follicle selection, however, many of the hormonal mechanisms leading to this key event and regulating variation between individual follicular waves remain undefined. This study compared circulating FSH, LH, and P4 with the follicular dynamics during three different physiological conditions. We hypothesized that these end-points would: 1) be similar for a spontaneous wave 2 vs one induced by follicular aspiration, 2) but would differ between wave 1 and 2, and 3) between conventional vs undersized deviations in either wave. Holstein dairy heifers (N = 24) were studied daily during an interovulatory interval. All heifers were evaluated during wave 1 and randomized 6 days after ovulation into an induced wave 2 and a spontaneous wave 2. Values were normalized to the day of expected diameter deviation (day 0) and compared for day -2 to 0 and 0 to 2. Hypothesis 1 was supported that an induced wave 2 from ablation of follicles of wave 1 and spontaneous wave 2 have similar follicle dynamics. However, the peak FSH surge was more prominent at emergence of an induced wave 2 ($P < 0.003$). Hypothesis 2 was supported that waves 1 and 2 differ in follicle and hormone events. Circulating P4 was lower and LH was greater ($P < 0.01$) with no difference in diameter of F1 but with a greater ($P < 0.01$) diameter of F2 on day 0 in wave 1 (7.3 ± 0.2 mm) than in wave 2 (6.6 ± 0.2 mm). Differences between waves were not found when each follicular wave was categorized into conventional vs undersized deviation and analyzed separately. Hypothesis 3 was supported as there were differences in circulating hormone between conventional and undersized deviations. Growth rate of F2 differed ($P < 0.0005$) during days -2 to 0 (conventional, 2.6 ± 0.2

mm/2d; undersized, 1.4 ± 0.3 mm/2d). However, circulating FSH and P4 concentration on days -1 and 0 tended to be greater ($P < 0.06$) in undersized than conventional deviations. In conclusion, the effect of different hormonal conditions on follicle dynamics was observed for F2 and not for F1. Furthermore, understanding the physiology that produces conventional vs undersized deviations is crucial since these categories explained most differences in follicular dynamics and circulating FSH observed in these different physiological conditions. In addition, future studies of wave 2 may be facilitated by using an induced wave 2 since it was similar to a spontaneous wave 2.

Keywords: Diameter complexities; Diameter deviation; Follicle selection; Wave synchronization.

1. Introduction

Research on the basic mechanisms that regulate follicle dynamics and the relationships with circulating concentrations of gonadotropins and steroids has been fundamental to our recent progress in understanding basic reproductive physiology in cattle and in the optimization of reproductive technologies including timed artificial insemination, superovulation, and embryo transfer programs. The interovulatory interval (IOI) has two or three follicular waves (waves 1, 2, 3). Emergence of each wave is stimulated by a surge in circulating FSH (surges 1, 2, 3) [1]. Wave emergence of 4-mm follicles is followed by a cohort of follicles in a common growth phase and a decline in the FSH surge. Inhibitory factors secreted by the growing follicles (inhibin, estradiol) directly suppress the pituitary release of FSH during the surge decline [2, 3]. When the future dominant follicle (DF or F1) nears a diameter of 8.5 mm, the FSH surge reaches a nadir concentration and a difference in follicle growth rate between F1 and subordinate follicles (F2, F3, etc) begins at diameter deviation. This has been proposed as the primary morphologic manifestation of DF selection [4, 5]. However, individual follicular waves show surprising variability in follicle growth patterns and hormone profiles especially as diameter deviation approaches [6]. An understanding of the contribution of the hormonal and physiologic environment to the variation in deviation characteristics between animals and between waves may allow for a better understanding of the biology of follicle selection and the development of methods to practically control this process.

Although studies have been done on the periovulatory wave 1 [7] and wave 2 [8], direct comparisons of the follicular and hormonal characteristics of waves 1 and 2

in the same IOI are needed. Previous studies have primarily focused on the characteristics of the DF of these two waves including the reduced diameter of F1 of an anovulatory wave 2 in three-wave IOI [9-12]. Differences in follicle dynamics between waves 1 and 2 that encompassed deviation have been reported, but the underlying physiology that produces these differences needs further study [12]. One obvious difference is that wave 1 occurs in the presence of low progesterone (P4) concentration, whereas wave 2 occurs during the mid-luteal phase when circulating P4 is elevated. This difference in P4 may underlie the observed differences in circulating LH concentration with a minor LH surge encompassing deviation in wave 1 but not in wave 2 [10]. Two distinct FSH surges (preovulatory and periovulatory) are components of surge 1 whereas a single FSH surge is observed prior to the second follicular wave [13, 14]. Comparison of follicle characteristics of waves 1 and 2, FSH concentration of surges 1 and 2, and a transient increase in LH concentration encompassing deviation could provide fresh insight into the relationship between follicle growth profiles and circulating gonadotropins.

Emergence of 4-mm follicles in wave 2 occurs 8 to 11 days after ovulation in individual cattle. This variability is related to the duration of DF of the wave 1, perhaps influenced by circulating LH, P4, or FSH concentrations. A synchrony of wave 2 among cattle can be induced by ablation of the DF of wave 1 [15]. A previous study indicated that the day of ablation by aspiration of follicle content may alter the follicle selection process, as indicated by an increased percentage of heifers with codominant follicles when follicle aspiration was done 4 days after ovulation but not at 7 days [16]. However, comparison of follicles and hormones between spontaneous wave 2 and

induced wave 2 are needed especially for the days that encompass deviation. Production of a synchronous wave 2 may be useful for future studies since wave 2 is of substantial physiologic importance as it gives rise to the ovulatory DF in two-wave IOI.

Recent studies have defined four classes of diameter deviation based on distinct differences in F2 diameter near deviation (conventional, F2-undersized, F1,F2-switched and double dominant or codominant) [17]. In conventional deviation, F2 is at least 7.0 mm at diameter deviation when F1 is 8.5 mm. Deviation is abrupt in growth rate difference between F1 and F2. In undersized deviation, F2 is less than 7.0 mm when F1 is 8.5 mm and deviation occur gradually over 1 or 2 days. In wave 1, the frequency is greater for conventional (eg, 59%) than for undersized deviations (15%) [12]. In wave 2, no frequential difference was found between conventional (35%) and undersized deviations (40%). In addition to comparisons between waves 1 and 2, the physiologic processes underlying the two distinct classes provide a new model for studying the relationships between follicle dynamics and gonadotropin concentrations.

The present study compared follicle and hormonal dynamics during: (1) spontaneous vs induced second follicular wave; (2) wave 1 vs wave 2; and (3) conventional vs undersized deviations either within a follicular wave or independent of follicular wave. One goal was to establish a model that facilitates the study of wave 2 by using induced synchronization of wave 2. Study of the mechanisms underlying the differences between waves 1 and 2 and between conventional vs undersized deviations were expected to advance our understanding of the fundamental mechanisms that regulate follicle dynamics and selection of a DF. It was hypothesized that (1) the dynamic of F1 and F2 and the associated concentrations of gonadotropins and P4 in

wave 2 would be similar between a spontaneous wave and a wave induced by follicular aspiration, (2) wave 1 would differ from wave 2 in diameters of F1 and F2 and the associated hormones, and (3) there would be differences in follicle growth dynamics and hormone profiles between waves with conventional deviation vs undersized deviation. It was speculated that the findings would allow development of a biological model explaining the interplay of hormones and follicles.

2. Material and methods

Holstein dairy heifers (n=24) between 16 and 26 months old, weighing 350 - 450 kg, and with no apparent abnormalities in the reproductive organs were used. The experiment was conducted during April to June in the northern temperate zone. The heifers were kept under natural light in an open shelter and provided with *ad libitum* access to water, mineralized salt, and grass hay. The heifers were not synchronized and were managed in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research.

2.1 Experimental design

An Aloka ultrasound SSD 3500 (Aloka America, Wallingford, CT, USA) was used with a multi-frequency 7.5-MHz finger transducer. Follicle diameter was based on two perpendicular measurements (height and width) at the apparent maximal area of the follicle antrum from well-focused gray scale images. Daily ultrasound scan was started 16 days after the last ovulation and continuing through the subsequent IOI. Since the heifers were not synchronized, the day of the IOI was determined by a daily ultrasound

ovulation record. Diameter of the four largest follicles of each follicular wave were measured daily and identity was maintained from day to day as described before [18]. All heifers were used to study wave 1 from ovulation to day 6 of the IOI. On day 6, all the heifers were randomized into two groups: (1) induced group with heifers submitted to follicle (≥ 4 mm) ablation 6 days after ovulation (presumably before the second spontaneous FSH surge) to induce a synchronous wave 2 as described [15] and (2) spontaneous group of untreated heifers. In the induced group, re-aspiration of follicles was done 12 hours later if there was identification of antral refilled follicles as suggested by apparent blood spots (echoic areas) or fluid (anechoic areas) with irregular periphery.

2.2 Follicles and deviation classes

The diameters of F1 and F2 were plotted daily from first detection at 4 mm (emergence) until regression to less than 4 mm or ovulation. The follicle that reached 10 mm was designated the dominant follicle (F1) and the day it was closest to 8.5 mm was designated the day of expected diameter deviation (day 0). The F2 was identified as the largest subordinate follicle on day 0. The F1 and F2 identity was maintained throughout the wave including before and after diameter deviation. Each follicular wave was assigned into four reported [17] deviation classes based on diameter of F2 at expected diameter deviation: (1) conventional deviation ($F2 \geq 7$ mm), (2) F2-undersized deviations ($F2 < 7$ mm), (3) F1, F2-switched (F2 were larger than F1 on days - 1, 0, or both), and (4) double dominant follicles (both F1 and F2 reached 10 mm).

2.3 Hormonal assays

Plasma concentrations of FSH, LH, and P4 were determined in blood samples that were collected into heparinized tubes from the coccygeal vein immediately prior to each ultrasound examination. After collection, the blood sample was immediately placed in ice water until plasma separation by centrifugation (2,000 g for 10 minutes). Plasma samples were frozen and stored at -20°C until assayed. The FSH and LH concentrations were determined by validated RIA [19, 20] with the modifications reported by our laboratory for FSH [1] and LH [21]. The intra-assay CV and the sensitivity were 3.0% and 0.02 ng/mL for the FSH and 2.2% and 0.04 ng/mL for LH. The P4 concentration was assayed using a solid-phase RIA kit containing antibody-coated tubes and ^{125}I -labeled P4 (ImmuChem Coated Tube Progesterone ^{125}I RIA Kit, MP Biomedicals, Costa Mesa, CA, USA) as described before [22]. The intra- and inter-assay CV and the sensitivity were 3.0%, 1.0%, and 0.06 ng/mL, respectively.

2.4 Data handling and statistical analysis

Data from switched deviations were excluded from the analyses because of inadequate numbers (4 from wave 1 and 3 from wave 2); whereas, no double dominant deviations were recorded during this experiment. Values were transformed into natural logarithms or ranks when they were not normally distributed. Analyses used the SAS PROC MIXED procedure (Version 9.4; SAS Institute). For comparison of spontaneous vs induced wave 2, wave 1 vs wave 2, waves 1 and 2 within conventional and undersized deviation, and conventional vs undersized deviation, separate analyses were

done for days -2 to 0 and 0 to 2. This was done so that changes leading to a difference before and on day 0 were given special attention owing to the establishment of conventional and undersized deviation by day 0. In addition, the difference in the FSH surge between spontaneous and induced wave 2 were compared by normalizing to the FSH peak. Frequency of deviation class between waves were compared using Fisher's exact test and frequency within a wave was compared using a Chi-Square Goodness of Fit. A probability of $P \leq 0.05$ indicated that a difference was significant, and a probability of $P > 0.05$ to $P \leq 0.1$ indicated a trend or that significance was approached. Data are presented as the mean \pm standard error of the mean (SEM).

3. Results

3.1 Spontaneous vs induced follicular waves

One heifer in the spontaneous group was removed from the experiment due to a prolonged IOI (> 30 days). The follicle emergence ($F1 \geq 4\text{mm}$) occurred on day -0.3 ± 0.1 for wave 1; day 7.8 ± 0.1 for induced wave 2 and day 9.6 ± 0.2 for spontaneous wave 2 (day 0 = ovulation; $P < 0.001$). The day of wave emergence was placed on days 8 and 10 for the induced and spontaneous waves 2 respectively (**Fig. 1; first panel**). The interval from F1 emergence (≥ 4 mm) to expected deviation was similar between the spontaneous and induced waves 2 (2.5 ± 0.2 days).

The day of peak of the FSH surge associated with the wave 2 emergence, occurred on day 7.3 ± 0.2 for the induced and day 9.2 ± 0.4 for the spontaneous group ($P < 0.0001$); thus, were placed on days 7 and 9 on the second panel of the Fig. 1. The induced wave 2 had greater FSH concentration when compared to the spontaneous

wave 2 on the day of the peak and one day after it (**Fig. 1; third panel**). The diameters of F1 relative to the FSH peak was similar between the induced and spontaneous wave 2.

The concentration of LH increased during the FSH surge for both the spontaneous and induced wave 2, and the maximum LH concentration was similar between the groups (**fourth panel**). Although the interaction of group-by-day was not significant, separate analysis indicated that the LH increase occurred one day earlier in the spontaneous than in the induced wave 2 ($P < 0.05$). The percentage FSH increase (111%) during the induced surge was greater ($P < 0.0001$) than the corresponding LH increase (42%). The P4 concentration (not shown) was similar between the spontaneous and the induced wave 2 when normalized to follicle emergence ($P = 0.12$).

3.2 Follicle and hormone dynamics of wave 1 vs combined wave 2

The induced vs spontaneous wave 2 had a similar frequency of conventional (38.9%) and undersized (61.1%) deviations and therefore both types of wave 2 were combined for comparison to wave 1 (**Table 1**). The frequency of conventional deviations was significantly greater for wave 1 (15/19, 78.9%) than for wave 2 (7/18, 38.9%). Also, the frequency of undersized deviations was significantly lower for wave 1 (4/19, 21.0%) than for combined wave 2 (11/18, 61.1%). The frequency of conventional deviation was significantly greater than undersized deviations within wave 1, but not within wave 2.

The F1 diameter was similar between wave 1 and wave 2 (**Fig. 2; first panel**), whereas the F2 diameter had a trend for an interaction of wave-by-day during days -2 to 0. This was reflected in a decreased growth rate of F2 and smaller diameter on day 0

in wave 2 when compared to wave 1. Also, during days 0 to 2, the F2 in wave 2 had a smaller overall diameter than the F2 in wave 1.

The FSH concentration (**Fig. 2; second panel**) had an interaction of wave-by-day during days 0 to 2, primarily from greater concentration in wave 1 than 2 (specifically on day 2). The LH concentration (**third panel**) had an interaction of wave-by-day during days -2 and 0, from greater concentration on day 0 in wave 1 than 2. Also, the overall LH concentration during days 0 to 2 was significantly greater in wave 1 (0.39 ± 0.02 ng/mL) than 2 (0.31 ± 0.03 ng/mL). The P4 concentration (**fourth panel**) increased during days -2 to 2 in both groups but was significantly lower in wave 1 than in wave 2.

3.3 Follicle and hormone dynamics of conventional and undersized deviations within waves

Comparison of Wave 1 and wave 2 for animals with conventional deviation (**Fig. 3; two upper panels**) indicated no significant difference for the F1 diameter during days -2 to 0 and a trend for greater F1 overall diameter in wave 1 than 2 during days 0 to 2. The F2 diameter and the FSH concentration were similar between the groups, although there was a significant FSH interaction for wave-by-day during days 0 to 2. The differences for the LH and P4 concentration between waves 1 and 2 within conventional deviation (not shown) were similar to those between waves 1 and 2 regardless the deviation class.

Comparison of waves 1 and 2 for animals with undersized deviations (**Fig. 3; two lower panels**) indicated similar F1 & F2 diameters at all days and similar FSH &

LH (not shown) concentrations. However, there was a trend for greater LH concentrations in wave 2 than 1 on day -2 ($P = 0.09$). The P4 concentrations (not shown) were greater in wave 2 than 1 regardless of the deviation class.

3.4 Conventional and undersized deviation dynamics

The comparison of conventional and undersized deviations combining the data of waves 1 and 2 indicated a trend ($P = 0.078$) for greater overall F1 diameter in conventional than undersized deviations during days -2 to 0 but not during days 0 to 2. (**Fig. 4; first panel**). Comparison of F2 diameter (**second panel**) indicated smaller diameter for undersized than conventional deviation class on days -1, 0 and 1.

The FSH concentration (**Fig 4; third panel**) was similar between the conventional and undersized deviations during the days -2 to 0. However, an analysis of individual days indicated that FSH concentrations were greater ($P < 0.05$) for undersized compared to conventional deviation on day -1 and 0.

The LH concentration (**fourth panel**) was similar between conventional and undersized deviations, but the circulating P4 (not shown) concentration tended ($P < 0.06$) to be greater in undersized (3.47 ± 0.49 ng/mL) than in conventional (1.69 ± 0.34 ng/mL) deviation during days -2 to 0.

3.5 Growth rates of F1 and F2 during the three different physiological conditions

The comparison between spontaneous and induced wave 2, indicated similar F1, and F2 growth rates during the 2-day interval before and after diameter deviation (**Fig. 5, first panel**). The comparison between wave 1 and combined wave 2, indicated similar F1 but not F2 growth rates. Thus, the F2 growth rate was greater for wave 1 than

wave 2 during the 2-day interval before deviation (**second panel**). Finally, the comparison between conventional and undersized deviations, indicated a similar F1 growth rate, and a greater F2-growth rate in conventional than undersized deviations during the 2-day interval prior to diameter deviation but lower F2 growth rate in conventional than undersized deviations during the 2-day interval after diameter deviation (**third panel**).

4. Discussion

This research focused on the mechanisms that regulate specific relationships between follicular wave dynamics and circulating hormone concentration during three distinct physiologic situations. Hypothesis 1 was partially supported that diameters of F1 and F2 and the associated concentrations of gonadotropins and P4 in wave 2 were similar between a spontaneous wave and a wave induced by follicle aspiration. Support was from the similar growth rate of F1 and F2 between a spontaneous and induced wave 2. However, FSH surge 2 was more prominent for the induced than the spontaneous wave. Hypothesis 2 that a spontaneous wave 1 is dissimilar to a spontaneous wave 2 in diameters of F1 and F2 and the associated hormones was supported by the greater circulating P4 and lower LH concentration in wave 2. Differences in F2 growth was shown by greater growth rate on days 2 to 0 in wave 1. However, the F2 growth characteristics were similar between waves 1 and 2 within conventional deviation and separately within undersized deviation. The hypothesis was not supported by the similarity between waves of F1 growth characteristics during the 5 days that encompassed deviation. Hypothesis 3 that there would be differences in

follicle growth dynamics and hormone profiles between waves with conventional deviation vs undersized deviation was supported for F2 and FSH but not for F1 and LH. The greater activity of F2 on days 2 to 0 was demonstrated by greater growth rate and increased diameter in conventional than in undersized deviations. Although no interaction was found on days -2 to 0, examination of individual days indicated greater FSH concentrations in heifers with undersized deviations on day -1 and 0. Results provided substantial insight into the functional relationships of FSH and LH with follicular dynamics during different physiological conditions and during specific phases of a follicular wave including emergence at 4 mm, the following common growth phase, transition to diameter deviation, and diameter deviation.

An FSH surge is critical in the emergence of both waves 1 and 2 based on early research studies [1, 23] and continuing through the most recent published research [6, 24]. Multiple FSH surges constitute different components of surge 1. The preovulatory component of FSH surge 1 is not regulated by inhibin, and is induced by a surge of GnRH from the hypothalamus with simultaneous induction of an LH surge [25]. A second periovulatory FSH surge then occurs independently of GnRH and is attributed to a decrease in follicular inhibin and estradiol following the LH surge [25]. The periovulatory FSH surge is related to FSH secretion that perhaps emanates from a gonadotropic cell-type in the anterior pituitary that differs from the cell-type that secretes the GnRH-stimulated LH and FSH surges [25-29]. In contrast, wave 2 is initiated by a single FSH surge that arises in response to decreasing FSH inhibitors from the DF of wave 1. In the present study, the FSH surge associated with wave 2 was more prominent when it was induced by follicle aspiration than when it arose spontaneously.

This observation agrees with a previous report from our laboratory [16] and suggests that rapid removal of follicular inhibitory factors by aspiration of follicle content allows greater FSH secretion than when the DF undergoes a slower, spontaneous atresia.

Future studies with greater frequency of blood sampling are warranted to study whether the differences in FSH during induced vs spontaneous waves represent rapid vs slow loss of follicular inhibitors, respectively.

Another finding was that the greater prominence of the induced FSH surge 2 did not enhance the growth rate of F1 or F2 during the common growth phase when normalized to the peak of the FSH [6]. However, a study with ablation 4 days after ovulation compared to 6 days in the present project resulted in an increased frequency of codominant follicles [16]. Despite the less prominent spontaneous FSH surge 2, the F1 of spontaneous wave 2 tended to have a greater growth rate than an F1 in an induced wave 2. In this regard, follicle growth is not altered by the extensive variation in the wave-stimulating FSH surge [6]. Although the peak of the FSH surge varies substantially among waves, the absolute circulating FSH concentration apparently is not the primary driver of follicle growth during the emergence near the FSH peak and the subsequent common growth phase of a follicular wave.

To analyze which factors alter follicle growth rate and follicle diameter during the common growth phase that ends at diameter deviation, data were analyzed for changes during the 2 days prior to expected diameter deviation. The growth rate of the F1 was not different between waves 1 and 2 even though follicles in wave 2 grew in the presence of greater circulating P4 and lower circulating LH. The major difference in follicle growth between wave 1 and 2 was found for the F2 with much greater growth

rate in wave 1 than in wave 2. This difference in F2 growth was not observed when the comparisons of wave 1 and wave 2 were done separately for conventional and undersized deviations, demonstrating that the greater growth rate of F2 in wave 1 may have been related to the greater frequency of conventional diameter deviation. Initial comparisons between wave 1 and 2 failed to show a difference in the F2 diameter between the two waves [9, 30], but results of a more extensive study that considered deviation class [12] are consistent with the present results. The lack of differences in the declining portion of pre-deviation FSH for surges 1 vs 2, suggests that changes in FSH, at least when based on daily blood sampling, do not explain the dramatic decrease in F2 growth rate that was observed in wave 2 compared to wave 1. Perhaps the increased P4 and/or the decreased LH concentration during wave 2 altered the growth characteristics of F2, even though there was no detected effect on F1 growth characteristics. A previous study found lower circulating LH when physiologic doses of P4 (50 mg every 8 h; im) were administered after F1 reached >6.0 mm; however, no difference in F2 diameter was observed and the decrease in F1 follicle growth was detected only 31 hours after diameter deviation [31]. Similarly, in the spontaneous wave 2 of the three-wave IOI, diameter of the anovulatory F1 decreases about 2 days after deviation apparently from low LH concentration [10]. Previous studies have focused on effect of LH concentration on follicle selection [31, 32] with particular attention to the growth rate of F1 during the dominant phase [11, 33-38], but previous studies have not found effects of LH during the common growth phase [25].

As the analyses proceeded, it became clear that consideration of conventional vs undersized deviations was of critical importance to understand the physiology driving

follicular wave dynamics. Again, the major differences were found in the growth rate of the F2 in contrast to the minor, generally non-significant, differences in diameter or growth rate of the F1. For example, there was a dramatically smaller F2 in undersized compared to conventional deviations at days -1, 0, and 1 with undersized deviations having half the growth rate of the F2 from day -2 to 0 compared to conventional deviations. In attempting to understand this difference in F2 growth rate, circulating FSH seemed like a candidate for potential hormonal stimulus. However, completely inconsistent with this idea, circulating FSH was greater in the follicular waves with undersized deviation compared with conventional deviation on the day before and on the day of deviation. Thus, the smaller F2 follicle permits increased FSH rather than changes in FSH preventing the F2 from reaching greater follicle size during undersized deviations. It is likely that the smaller F2 and other subordinate follicles in undersized deviations provide less inhibitory FSH signals resulting in greater FSH concentration before deviation. This result emphasizes the close temporal role of two-way functional coupling between FSH and follicles [2].

As expected, circulating P4 concentration was much greater and LH concentration was lower during deviation in wave 2 than wave 1, and in undersized than in conventional deviations, indicating a need for manipulative studies. A goal in future studies should include the possible effects of P4 on FSH and LH concentration or on their follicular receptors during the expected diameter deviation and during the transition period prior to deviation. An understanding of the mechanisms that underlie the reduced growth rate of F2 during undersized deviation may provide key insights into the nature of the deviation mechanism.

4.1 Conclusions

During the period encompassing diameter deviation under very different physiological conditions, differences were consistently found in the growth rate of F2 with little change in F1 growth rate. That is, the deviation process and the different hormonal mechanisms that drive follicle growth may be best viewed from the perspective of the subordinate follicles rather than the dominant follicle. Designating individual follicular waves into the categories of either conventional or undersized deviation, based on the maximal size of the F2, provided the most revealing results during the different physiologic conditions that were examined. Follicle ablation during wave 1 induced emergence of an earlier wave 2 with follicle growth dynamics similar to the spontaneous wave 2. This may facilitate future studies of wave 2. However, the ablation was followed by a more prominent FSH surge at emergence of the induced wave 2 than observed at the emergence of the spontaneous wave 2. Interpretations were not forthcoming from these results to account for the reduced growth rate of the F2 during undersized deviation. It was assumed that the reduced diameter of F2 and other subordinate follicles produced the temporally associated increase in FSH, contradicting the consideration that the reduced growth of F2 is caused by reduced FSH concentration. The increased frequency of undersized deviations during wave 2 was temporally associated with greater concentration of P4 and lower LH concentration but other support for the association was not obtained.

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Table 1. Frequency of conventional and undersized deviation classifications in wave 1 and combined^a spontaneous and induced waves in wave 2.

| Deviation class | Wave 1 ^b | Wave 2 | Probability |
|-----------------|---------------------|------------|-------------|
| Conventional | 15 (78.9%) | 7 (38.9%) | P < 0.01 |
| Undersized | 4 (21.1%) | 11 (61.1%) | P < 0.01 |
| Total | 19 (100%) | 18 (100%) | — — — |

^a No significant difference between spontaneous and induced wave 2 in frequencies of conventional and undersized deviations.

^b The frequency of conventional deviation was greater than undersized deviation within wave 1 (P = 0.01) but not within wave 2 (P = 0.35).

Figure Legends

Fig. 1. Mean \pm SEM for (1) the diameter of the dominant follicle (F1) of wave 1 and the spontaneous and induced wave 2, (2) FSH surge associated with the emergence of spontaneous and induced wave 2, (3) FSH concentration normalized to the FSH peak and F1 normalized to the day of emergence for spontaneous and induced wave 2, and (4) LH concentration associated with the emergence of wave 2 for spontaneous and induced groups. The mean day of F1 emergence for wave 2 (first panel) and the mean day of the FSH peak (second panel) are indicated by their placement on the day-scale. The FSH (third panel) and LH (fourth panel) concentration for the two groups are superimposed and normalized to the FSH peak. Significant probabilities for a main effect of day (D) and an interaction group-by-day (GD) are shown. An asterisk (*) indicates a day with a difference between groups. Diameters of F1-wave 2 for the two groups involved placement of emergence relative to the corresponding FSH surge, probabilities for main effects of the day (D) are shown. The LH concentration associated with wave 2 and its probability for a main effect of day (D) are shown. A circle indicates the day of a significant increase within groups.

Fig. 2. Mean \pm SEM for diameters of the future dominant follicle (F1) and subordinate largest follicle (F2) and concentration of FSH, LH, and P4 associated with follicle diameter deviation in wave 1 and wave 2. Values were normalized to expected diameter deviation (F1 closest value to 8.5 mm). Probabilities for the significant main effect of the wave (W) and day (D) and an interaction wave-by-day (WD) are shown for days -2

to 0 and 0 to 2 in each panel. An asterisk (*) indicates a day with a difference between groups.

Fig. 3. Mean \pm SEM for diameter of the dominant follicle (F1) and subordinate largest follicle (F2) and concentration of FSH associated with wave 1 vs 2 within conventional diameter deviation (upper two panels) and within undersized deviation (lower two panels). Values were normalized to expected diameter deviation (F1 closest value to 8.5 mm). Probabilities for the significant main effect of the wave (W) and day (D) and an interaction wave-by-day (WD) are shown for days -2 to 0 and 0 to 2 in each panel.

Fig. 4. Mean \pm SEM for diameter of the dominant follicle (F1), subordinate largest follicle (F2), concentration of FSH, and concentration of LH associated with follicle diameter deviation for conventional and undersized deviations (combined waves 1 and 2). Values were normalized to expected diameter deviation (F1 closest value to 8.5 mm). Probabilities for the significant main effect of the classification (C) and day (D) and an interaction class-by-day (CD) are shown for days -2 to 0 and 0 to 2 in each panel. An asterisk (*) indicates a day with a difference between groups.

Fig. 5. Mean \pm SEM for the growth rate on days 2 to 0 and 0 to 2 for the dominant follicle (F1) and largest subordinate follicle (F2) for (1) spontaneous vs induced wave 2, (2) wave 1 vs wave 2, and (3) conventional vs undersized deviations. Probabilities for a difference between groups are shown for each of days 2 to 0 and 0 to 2.

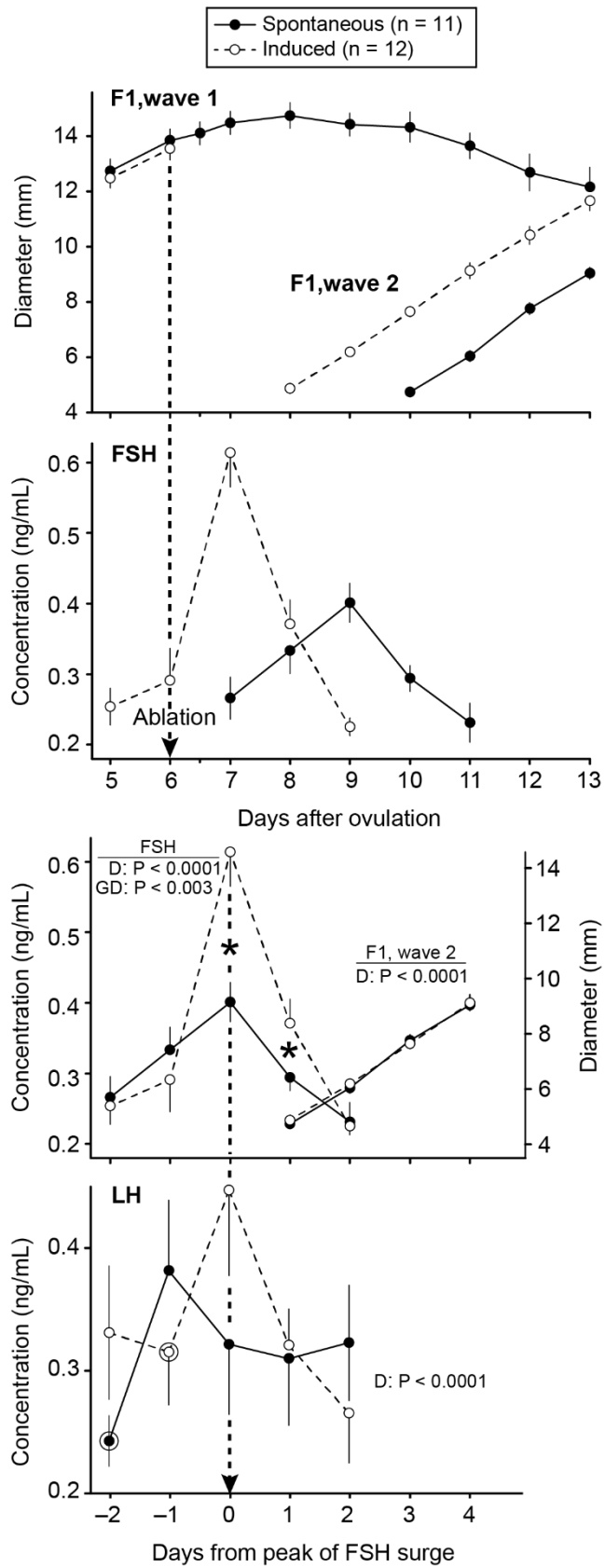


Fig. 1.

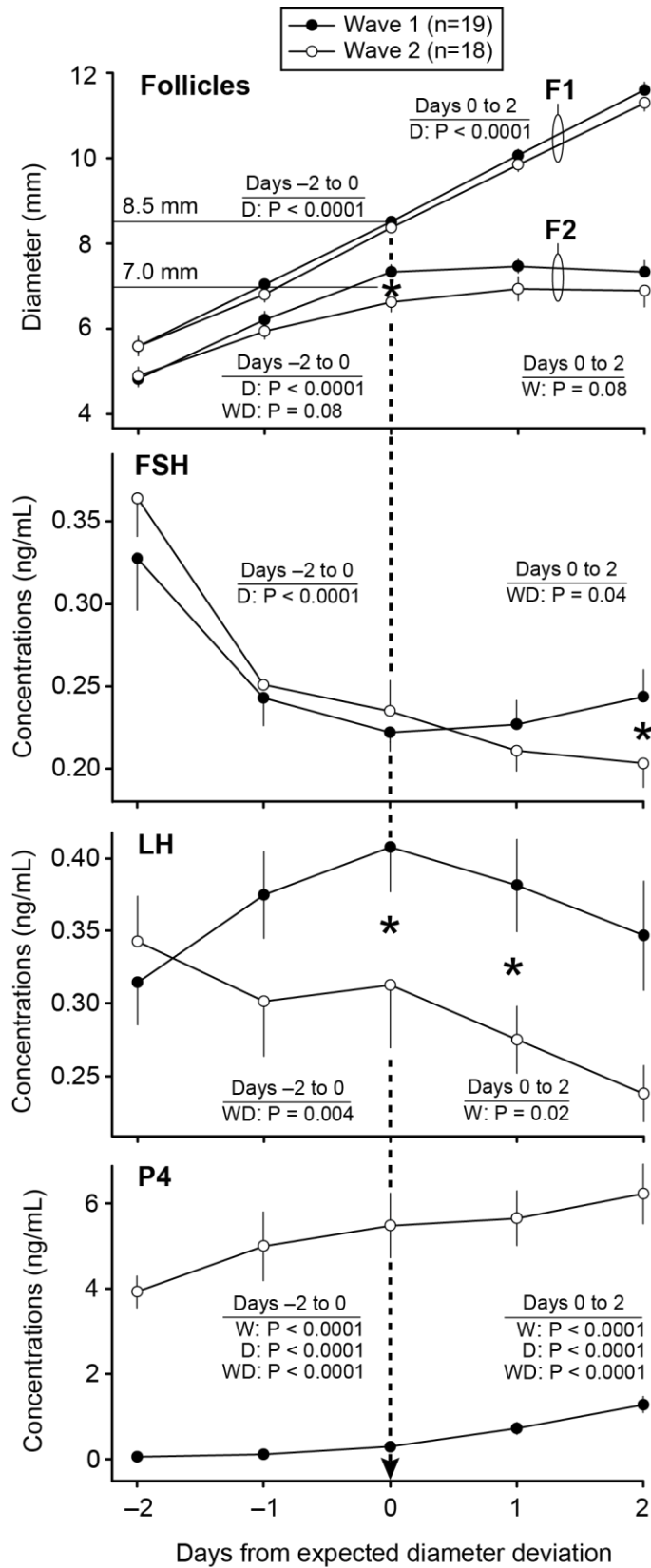


Fig. 2.

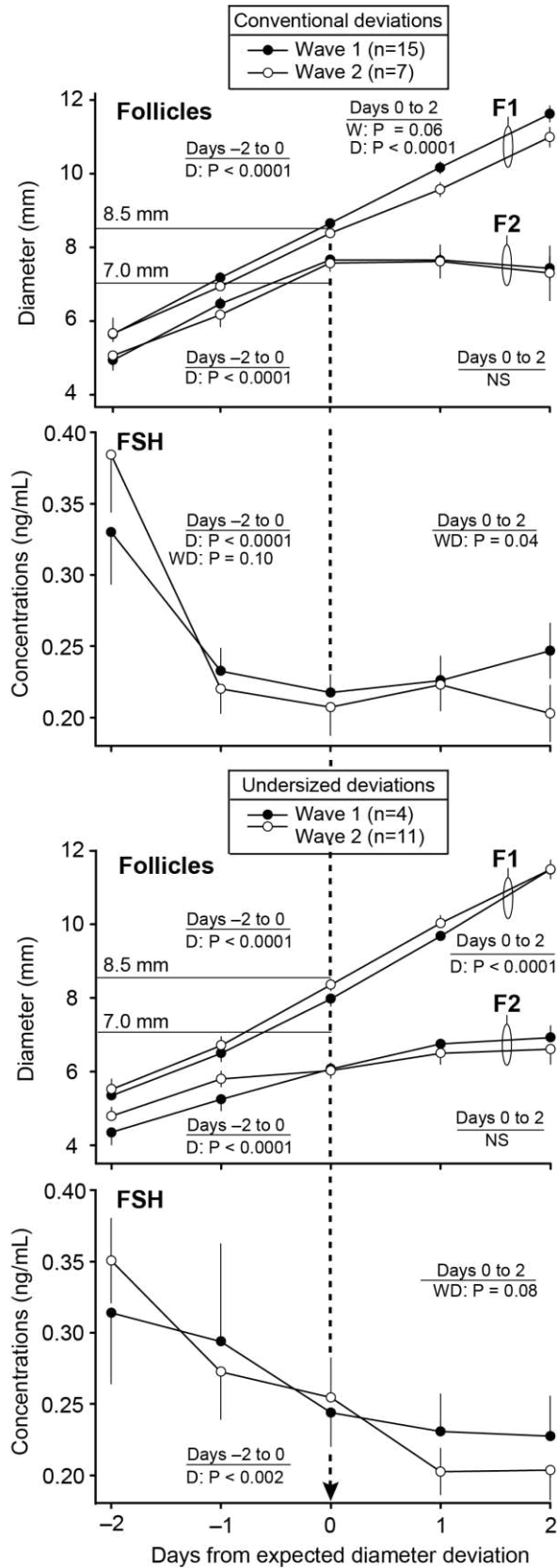


Fig. 3.

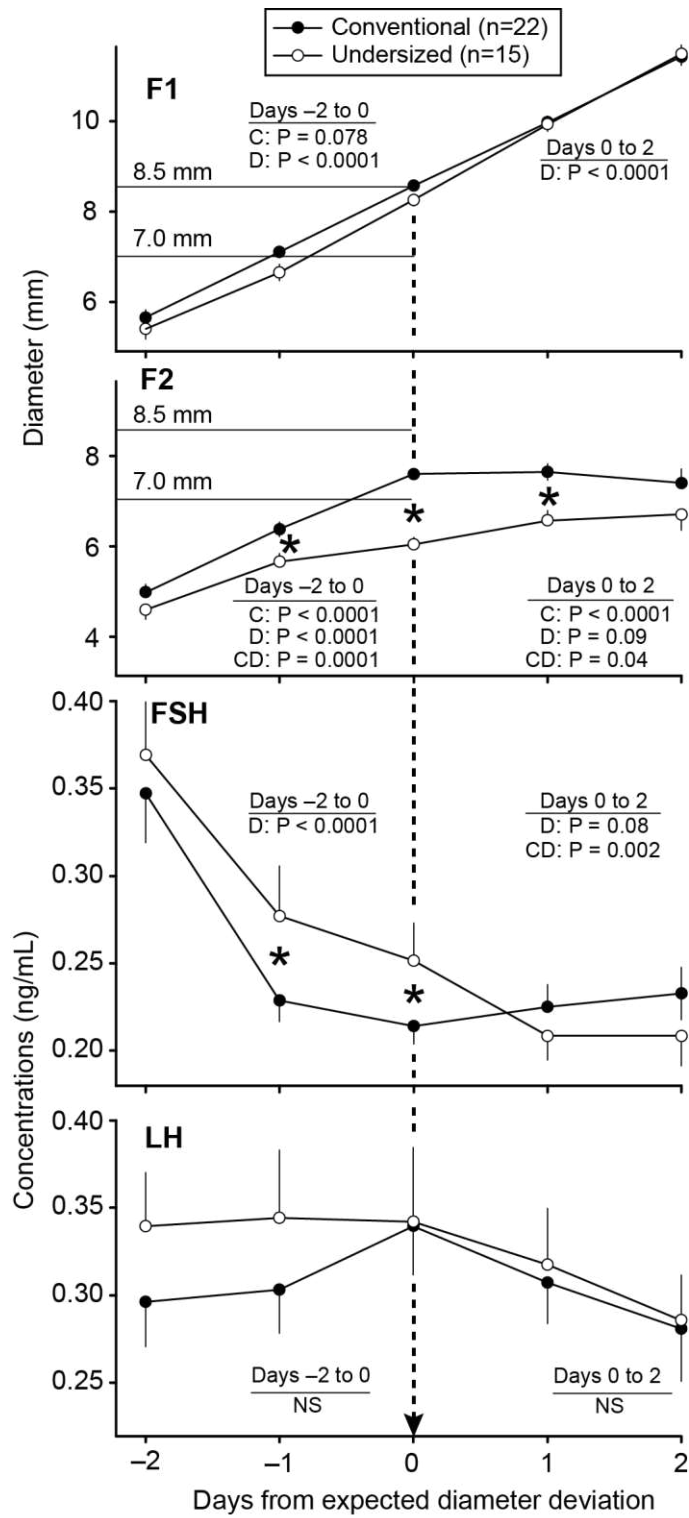


Fig. 4.

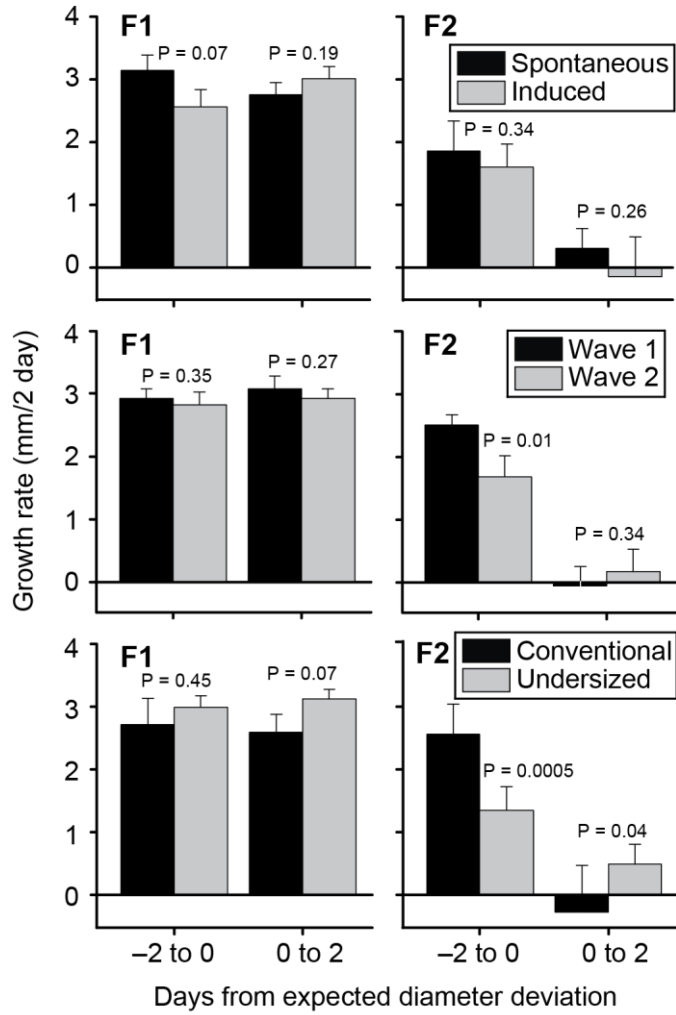


Fig. 5

Hormonal mechanisms regulating follicular wave dynamics II: Progesterone changes the diameter at follicle selection regardless of low or high circulating LH or FSH.

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Abstract

Complexities in diameter deviation ($F1 \sim 8.5$ mm) have been reported based on the diameter of the largest subordinate follicle ($F2$) at time of follicle deviation (conventional $F2 \geq 7$ or undersized < 7 mm). Experiment 1 directly manipulated P4 to test the role of P4 in these complexities. Experiment 2 tested whether decreased LH was the mechanism whereby elevated P4 increases undersized deviations. Wave 2 emergence was synchronized by follicle ablation on day 6 (D6) after ovulation. Ultrasound evaluations and blood sample collections were performed every 12 h from D6.5 in both experiments. Data were normalized to $F1 \geq 7.5$ mm (day 0) and compared by group and treatment using SAS PROC MIXED. In experiment 1, Holstein heifers ($n=20$) had CL regressed on D5 and were randomized to: 1) Control: absence of P4; 2) P4-emergence: P4 treatment starting at D6.5 (time of emergence), and 3) P4-6mm: P4 treatment began when $F1 \geq 6$ mm (P4 treatment = 12 i.m. P4, 75 mg every 12 h). Treatment with P4 increased frequency of undersized deviations (54%), compared to Control (0%). Heifers treated with P4 had lower LH than Control (0.24 ± 0.01 vs. 0.38 ± 0.03 ng/mL) but tended to have greater FSH (0.20 ± 0.01 vs. 0.14 ± 0.01 ng/mL). Thus, hypothesis 1 was supported, i.e. increased P4 will increase frequency of undersized deviation, decrease LH, but increase FSH. In experiment 2, Holstein heifers ($n=27$) on D6 were randomized to: 1) Control: saline; 2) pLH: porcine pituitary LH (1.25 mg/every 12 h), or 3) hCG: initial 160 IU hCG and subsequent 96 IU/every 24 h. Circulating P4 was greater from days 0 to 1.5 (day 0 = $F1 \geq 7.5$ mm) in hCG (8.6 ± 0.8 ng/mL), intermediate in pLH (6.4 ± 0.3 ng/mL), and lower in Control (4.6 ± 0.3 ng/mL). The pLH/hCG-treatments had lower FSH from days -2.5 to 0 (0.34 ± 0.02 vs 0.46 ± 0.03

ng/mL) and days 0 to 1.5 (0.17 ± 0.01 vs 0.23 ± 0.01 ng/mL). Despite differences in P4 and FSH, the F1 and F2 dynamics, as well as the frequency of conventional (37%) and undersized (48%) deviations, were similar between treatments. Hence, hypothesis 2, that in the presence of elevated circulating P4, an increase in circulating LH activity would directly stimulate the subordinate follicles and thereby increase the likelihood of conventional rather than undersized deviations was not supported. Thus, elevated P4 is linked to undersized deviation but this can occur either in increased or decreased LH activity and, surprisingly, increased or decreased circulating FSH.

Keywords: Deviation complexities; Follicle selection; FSH; LH; Progesterone.

1. Introduction

The most-utilized current reproductive technologies are based on the creative translation of basic biological information and techniques [1-4]. One key observation was made by using transrectal ultrasound to examine the morphological manifestation of follicle selection termed diameter deviation [5]. It was proposed that when the largest follicle (F1) of a growing cohort nears 8.5 mm, it deviates from the future subordinate follicles (F2, F3, etc) due to a major decrease in subordinate follicle growth rate attributed to reduced circulating FSH [6]. However, many studies have reported surprising variation in the size of F1 and F2 at time of diameter deviation based on breed [7, 8], animal category [9], high fecundity or single ovulation genotypes [10, 11], and between first and second follicular wave within individual animals [12, 13]. In spite of the clear variation in follicle wave dynamics, the role of the FSH surge in initiating the follicular wave and the function of the FSH nadir in the deviation process continue to occupy fundamental positions in the current follicle selection models [14-16].

Recent results have begun to disentangle some of the complexities in the follicular selection process, providing evidence for four different classes of diameter deviations. Based on the F2 diameter or capacity to become dominant at the time that the F1 reaches 8.5 mm [17, 18] the classes are termed: conventional ($F2 \geq 7\text{mm}$); undersized ($F2 < 7\text{mm}$); switched (F1 & F2 switch in ranking); and co-dominant ($F1 \& F2 \geq 10\text{mm}$). The first manuscript in this series [13] and other recent studies [12, 19] demonstrated the key association of circulating progesterone (P4) concentrations with deviation class such that there was an increased ($P < 0.01$) frequency of undersized deviation associated with greater P4 (61%) when compared to lower circulating P4

(21%) which was primarily associated with conventional deviation [13]. Nevertheless, the compelling association of greater circulating P4 with increased undersized deviation is based on observational studies and has not been confirmed in studies that specifically manipulated the circulating P4 concentration to precisely determined its effect on the follicular deviation complexities.

One important hormonal relationship that may underlie the effect of circulating P4 on the dynamics of the follicular deviation process is the well-known impact of circulating P4 on circulating luteinizing hormone (LH) concentration. For example, increased P4 decreased the frequency of GnRH pulses from the hypothalamus [20] leading to a decreased frequency of LH pulses [21, 22]. In addition, greater circulating P4 acts directly on the pituitary to decrease the amplitude of LH released in response to a given dose of GnRH [23]. In our previous study, it was not possible to separate the effect of elevated P4 from the potential role of circulating LH on the follicular deviation process because increased circulating P4 was accompanied by decreased LH [13]. Previous studies have shown that increased circulating P4 can decrease the growth rate of the F1 after follicle deviation and decrease maximum F1 diameter, possibly due to effects of P4-induced changes in LH acting on the dominant follicle [24]. However, a role for LH in follicle growth during the common growth phase, prior to deviation, or effects of LH on the variations in follicle deviation dynamics have not been previously described. In agreement with a role for LH after deviation, LH receptor expression in granulosa cells increased only near the time of diameter deviation, and only in the granulosa cells of the selected dominant follicle [12, 25, 26]. Nevertheless, there are LH receptors on thecal cells throughout follicle development [27] and, therefore, a follicle

growth effect of LH, acting through stimulation of thecal cells, is theoretically possible prior to the deviation, although currently untested. Consistent with this idea, *in vitro* experiments found that androgens directly increased expression of FSH receptor mRNA in granulosa cells from small follicles [28, 29], making thecal androgens a possible mediator of LH action prior to the acquisition of LH receptors on granulosa cell. Hence, decreased circulating LH is an obvious pathway by which increased circulating P4 may alter F2 growth dynamics and thereby change the follicular deviation dynamics.

However, this physiological concept remains to be tested, *in vivo*.

Thus, the objectives of this study were to test: 1) the role of P4 in conventional and undersized deviations by using direct manipulation of circulating P4 and 2) the role of decreased LH in producing the undersized deviations that are observed in elevated circulating P4. Two experiments were performed to evaluate these ideas. Experiment 1 directly manipulated circulating P4 during the second follicular wave to test the hypothesis that increased circulating P4 would increase the likelihood of undersized deviation and alter circulating LH (decreased) and FSH (increased) concentrations, as observed in our observational studies [13]. Experiment 2 tested the hypothesis that, in the presence of elevated circulating P4, an increase in circulating LH activity would directly stimulate the subordinate follicles and thereby increase the likelihood of conventional rather than undersized deviation. We utilized two methods for elevating LH activity, treatment with human chorionic gonadotropin (hCG), a direct and specific agonist of the bovine LH receptor [30], and treatment with purified LH from porcine pituitaries, although this preparation could have some FSH contamination [31].

2. Material and methods

2.1 General heifer management

The studies were performed in the northern temperate zone using Holstein dairy heifers between 16 and 26 months old and weighing 350 to 450 kg. Only heifers having single ovulations and no apparent abnormalities in the reproductive tract (as evaluated by ultrasound) were used for the studies. The heifers were kept under natural light in open shelters provided with *ad libitum* access to water, mineralized salt, and grass hay. The heifers were managed in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research.

2.2 Experiment 1

Holstein dairy heifers (n=20) at random days of the estrous cycle were synchronized using the following protocol: d0, 200 µg GnRH i.m. (gonadorelin acetate; Gonabreed®, Parnell Pharmaceuticals, Overland Park, KS, USA) and insertion of an intravaginal Controlled Internal Drug Release device (CIDR) with 1.38 grams of P4 (Eazi-Breed CIDR, Zoetis, Florham Park, NJ, USA); d5, 500 µg i.m. of cloprostenol (Estroplan®, Parnell Pharmaceuticals); d6, second treatment with 500 µg i.m. of cloprostenol and withdrawal of the CIDR. Starting on d7, the heifers had their ovaries scanned every 12 hours for detection of ovulation (D0). On D5, the heifers received two doses (12 h apart) of 500 µg of cloprostenol (Estroplan®, Parnell Pharmaceuticals) for regression of the corpus luteum (CL). It was anticipated that on D6 the heifer should have a 60% decrease in CL blood flow, and a CL diameter \leq 15mm, as well as

circulating P4 concentration ≤ 0.5 ng/mL on D6.5. Wave 2 emergence was synchronized by follicle (≥ 4 mm) ablation on D6 and re-aspiration of re-filled follicles on D6.5 [13].

The experimental phase (**Fig. 1**) started on D6.5. On this day, the heifers were randomized into 3 different groups: (1) the Control (Non-P4 treated) group consisted of heifers that were untreated, so the new follicular wave emerged (after follicle aspiration) and grew in minimal P4 concentrations; (2) the P4-emergence group had heifers receiving P4 treatment starting on D6.5, during the inclining portion of the FSH surge (F1 < 4 mm), so the new follicular wave emerged and grew in high P4 concentrations; and (3) the P4-6 mm group had heifers receiving P4 treatment starting when F1 reached or exceeded 6.0 mm so the new follicular wave emerged in low P4 but grew in high P4 concentrations. All P4 treatments consisted of i.m. doses of 75 mg of P4 in sunflower oil (1mL; 75 mg/mL) every 12 h for 5 d. Treatment with P4 was initiated at two different times to allow evaluation of the P4 effect during the common-growth phase in the presence of chronic (P4-emergence) or acute (P4-6 mm) P4 treatment.

2.3 Experiment 2

Holstein dairy heifers (n=27) on d16 of the estrous cycle had daily ultrasound evaluation of their ovaries for detection of ovulation (D0). Wave 2 emergence was synchronized by follicle (≥ 4 mm) ablation on D6 and re-aspiration of re-filled follicles on D6.5 [13]. The experimental phase began on D6.5 (**Fig. 1**) and the heifers were

randomized into three groups: (1) the Control group (Saline-treated), received i.m. saline solution every 12 h; (2) the pLH group received 1.25 mg of porcine pituitary luteinizing hormone i.m. every 12 h (Lutropin®-V, Vetoquinol N.-A. Inc., QC, Canada); and (3) hCG group received human Chorionic Gonadotropin (Chorulon®, Merck Sharp & Dohme Corp, Kenilworth, NJ, USA) treatment with an initial i.m. dose of 160 IU and subsequent i.m. doses of 96 IU every 24 h. All the treatments were done from D6.5 until the F1 reached or exceeded 10 mm.

2.4 Ultrasound examinations during experiments

Starting on D6.5, both experiments (**Fig. 1**) used transrectal ultrasonography to track, map, and record the diameter of the 4 largest follicles of each follicular wave from their emergence (≥ 4 mm) until the end of the experimental phase (subsequent ovulation for experiment 1, and $F1 \geq 10$ mm for experiment 2). Follicle diameter was based on two perpendicular measurements (height and width) at the apparent maximal area of the follicle antrum as described before [32]. Ultrasound examinations were done using an Aloka SSD 3500 (Aloka America, Wallingford, CT, USA) with a multi-frequency 7.5-MHz finger transducer. The dual-B-mode of the ultrasound was used to obtain a video of each ovary on each side of the screen. The video recorded each individual ovary with a slow ultrasound scan from the outer to the inner most part of the ovary (left ovary) or from the inner to the outer most part of the ovary (right ovary). Videos were saved and evaluated, frame-by-frame, to allow identification, tracking, and subsequent mapping of individual follicles from examination to examination. Initial

measurements were done at the time of the ultrasound examination, with the heifer still in the chute, allowing a second ultrasound video to be recorded, if necessary.

2.5 Hormonal assays

Plasma concentrations of FSH, LH, and P4 were determined in blood samples collected into heparinized tubes from the coccygeal vein, prior to each ultrasound examination. After collection, the blood samples were immediately placed on ice water until centrifugation at 2,000 x g for 10 min, to separate plasma from blood cells. Plasma samples were frozen and stored at -20 °C until assayed. The FSH and LH concentrations were determined by validated RIA [33, 34] with the modifications reported by our laboratory for FSH [14] and LH [35]. The P4 concentration was assayed using a solid-phase RIA kit containing antibody-coated tubes and 125I-labeled P4 (ImmuChem Coated Tube Progesterone 125I RIA Kit, MP Biomedicals, Costa Mesa, CA, USA) as described before [36].

For experiment 1, the intra- and inter-assay CV and the sensitivity for the FSH assay were 7.6%, 3.2%, and 0.019 ng/mL; for the LH assay were 1.9%, 1.4%, and 0.054 ng/mL; and for the P4 assay were 4.5%, 15.3%, and 0.06 ng/mL, respectively.

For experiment 2, the intra-assay CV and the sensitivity for the FSH assay were 2.7%, and 0.032 ng/mL; whereas for the P4 assay the intra- and inter-assay CV and the sensitivity were 3.5%, 24.0%, and 0.12 ng/mL, respectively. The LH concentrations were not assayed in experiment 2.

2.6 Data handling and statistical analysis

The diameters of the four largest follicles of each follicular wave were plotted, starting from their first detection at 4 mm (emergence). The follicle that reached more than 10 mm was designated as the dominant follicle (F1). Then, the largest subordinate follicle (F2) was identified on the day that the F1 was closest to 8.5 mm in diameter (expected diameter deviation). After assignment, the identities of the F1 and F2 were tracked throughout the follicular wave. Subsequently, each follicular wave was assigned into one of four reported [18] deviation classes, based on the diameter of F2 on the day of expected diameter deviation: (1) conventional deviation ($F2 \geq 7$ mm), (2) F2-Undersized deviations ($F2 < 7$ mm), (3) F1, F2-switched (F2 was larger than F1 on days - 1, 0, or both), and (4) co-dominant follicles (both F1 and F2 reached 10 mm). The frequency of each of the four classes was compared among the groups or between the treatments for each experiment by using the Fisher's exact test. Data from switched and co-dominant deviations were excluded from subsequent analyses.

All the end-points, F1 and F2 diameter, and the FSH, LH, and P4 concentrations were compared among the groups within experiment 1 (Control, P4-emergence, and P4-6 mm) and within experiment 2 (Control, pLH, and hCG). After initial examination to determine any potential differences between the three experimental groups in each study, it was previously designated that the two treated groups within each experiment would be combined if no major differences were identified between the two experimental group, i.e. comparisons Non-P4 treated vs P4-treated heifers in experiment 1 and comparison of saline-treated vs pLH/hCG treated heifers in experiment 2 (**Fig. 1**).

The length of time for F1 to proceed through specific stages of growth (F1 – days) and the growth rate of F2 during the given stages (F2 – mm of growth during the stage) were compared (**Table 1 and 2**) using one-way Tuckey test. Specific stages were selected based on physiological classification, i.e., during the FSH peak surge (timing from ablation to F1 emergence at ≥ 4.0 mm); during the FSH declining portion (F1 ≥ 4 to ≥ 6 mm); during the end of the common growth phase (F1 ≥ 6 to ≥ 7.5 mm); during the transitional period to expected diameter deviation (F1 ≥ 7.5 to ≥ 8.5 mm); and during expected diameter deviation (F1 ≥ 8.5 to ≥ 10 mm). These analyses were done to validly determine the day of diameter deviation, based on the time when there was a change in growth rate for the F2.

For comparison among groups or between treatments, the data were normalized to F1 ≥ 7.5 mm (day 0). Separate analyses for all the end-points were done from day – 1.5 to 0 and 0 to 2 in experiment 1. In experiment 2, the F1 & F2 diameters were analyzed from day –1.5 to 0 and 0 to 1.5. Whereas, the circulating FSH and P4 concentrations were analyzed from day –2.5 to 0 and 0 to 1.5. The objective of the experiment was focused on whether P4 treatments would affect circulating LH and FSH, and, therefore, it was determined a priori that analyses would be done among the groups for each individual day. All values were transformed into natural logarithms or ranks when they were not normally distributed and analyzed using the SAS PROC MIXED (Version 9.4; SAS Institute). Results were considered significant when P -value ≤ 0.05 and trending when $0.05 < P$ -value ≤ 0.10 . Data are presented as the mean \pm standard error of the mean (SEM).

3. Results

For experiment 1, one heifer was excluded from the analysis because of the lack of a dominant follicle. For experiment 2, three heifers (one in each group) were excluded during the experimental phase because of failure to synchronize emergence of the second follicular wave. Therefore, three different heifers were added to the experimental groups to achieve the sample size. Analysis of the frequency of diameter deviation included the four classes (**Table 1 and 2**) but data from switched (n=5) and co-dominant (n=1) deviations were excluded from the subsequent experimental analyses because of insufficient number of observations to allow valid analyses of the physiology within these two classes.

3.1 Experiment 1: Timing of P4 treatment (emergence vs 6mm) on follicular dynamics

The frequency of undersized deviations (4/7 vs 3/6) and the incidence of switched or co-dominant deviations (0%) were similar ($P = 1.0$) between the P4-emergence and the P4-6 mm groups as well as the length of time for F1 to proceed through specific stages of growth, i.e. the timing from ablation to emergence (1.9 ± 0.2 vs 1.7 ± 0.1 d/stage); 4 to 6 mm (1 ± 0 vs 1.2 ± 0.1 d/stage); 6 to 7.5 mm (0.6 ± 0.1 vs 0.7 ± 0.1 d/stage); 7.5 to 8.5 mm (0.6 ± 0.2 vs 0.3 ± 0.1 d/stage); and 8.5 to 10 mm (1.7 ± 0.5 vs 1.4 ± 0.1 d/stage). The growth rate of F2 during the given stages was also similar between the P4-emergence and the P4-6 mm groups. In other words, the timing of the decrease in F2 growth rate occurred in both groups during the same F1 growth stage (7.5 mm to 8.5 mm).

3.2 Experiment 1: Circulating P4 (Non-P4 vs P4-treated) on follicular and hormonal dynamics

Based on the similarities of the P4-emergence and the P4-6 mm, the two groups were combined (P4-treated) and compared with the Non-P4 treated heifers (Control).

Table 1 summarizes the main comparisons between the two treatments. The frequency of conventional deviation was similar in Non-P4 (4/6) and P4-treated (6/13) heifers. However, the frequency of undersized deviations was lower in the Non-P4 treated (0/6) than in the P4-treated heifers (7/13).

The length of time for F1 to proceed through specific stages of growth prior to 8.5 mm (Table 1) and for the total time from ablation to 8.5 mm was similar in Non-P4 (3.7 ± 0.4 d) and P4-treated heifers (4.1 ± 0.6 d). Although, from 8.5 to 10 mm P4-treated tended to require a longer time, suggesting a slower growth rate for the F1 after 8.5 mm in the P4-treated compared to the Non-P4 treated heifers. The F2 in P4-treated heifers had an earlier decrease in growth rate, as evidenced during stage 7.5 to 8.5 mm (0.2 vs 1.1 mm/stage). The F2 grew at a similar rapid rate in both groups prior to F1 \geq 7.5 mm and at a slow and similar rate after 8.5 mm. Within each treatment, the F2 growth rate decreased when the F1 grew from 8.5 to 10 mm in the Non-P4 treated ($P = 0.016$), whereas, in P4-treated heifers, this decrease ($P = 0.0001$) occurred when the F1 grew from 7.5 to 8.5 mm. Thus, the F2 growth rate decreased earlier in the P4-treated heifers and only differed ($P = 0.0006$) between the treatments during the stage of F1 \geq 7.5 mm to \geq 8.5 mm.

The first difference among the three groups (Control, P4-emergence, and P4-6 mm; $P = 0.07$) or between the two treatments (Non-P4 vs P4-treated; Table 1; $P =$

0.0006) was detected during the stage when the F1 grew from 7.5 to 8.5 mm. Thus, it was decided to normalize the F1 and F2 diameters, and the FSH, LH, and P4 concentrations to the values when F1 reached or exceeded 7.5 mm in diameter. This day was designated as day 0 and the dynamics during days -1.5 to 2 are presented in **Fig. 2**. The F1 diameter comparisons among all three groups (**left top panel**) indicated similar F1 dynamic before day 0 with only a main effect of day. From day 0 to 2, the analysis only indicated a tendency ($P = 0.095$) for a main effect of group. The F1 comparison using combined P4 treatments (**right top panel**) resulted in an interaction of treatment-by-day due to smaller F1 diameter in the P4-treated than in the Non-P4 treated heifers after day 0.5. When analysed separately the F1 in the Control group had a larger diameter ($P = 0.029$) than in the two P4-treated groups on day 2 but not before (12 ± 0.5 vs 10 ± 0.4 vs 10.3 ± 0.4 mm).

The F2 diameter was statistically similar among the three groups (**Fig. 2; left second panel**). However, comparison between the two treatments (**right second panel**) revealed a tendency for an interaction of treatment-by-day explained by the larger ($P \leq 0.05$) F2 diameter in the Non-P4 treated heifers on days 0.5, 1, and 1.5.

The circulating FSH concentrations were similar among the three groups during days -1.5 to 2 (**Fig. 2; left third panel**). However, the two-treatment comparison (**right third panel**) resulted in a tendency for overall lower FSH concentration in the Non-P4 treated (0.14 ± 0.01) than in the P4-treated heifers (0.20 ± 0.01) during days 0 to 2. Analysis of individual days indicated lower FSH concentrations in Non-P4 than in P4-treated heifers on days 0.5 and 1 ($P = 0.07$) and on days 1.5 and 2 ($P = 0.03$).

Comparison of circulating LH concentration (**Fig. 2; left fourth panel**) among the three groups indicated greater LH concentration on day -1.5 in P4-6 mm (0.47 ± 0.03 ng/mL), medium concentration in Control (0.31 ± 0.08 ng/mL), and lower concentration in the P4-emergence (0.22 ± 0.05 ng/mL). On days -1 and -0.5 the three groups were similar, and finally during days 0 to 2, the overall LH concentrations were greater for Control (0.38 ± 0.02 ng/mL) than P4-emergence (0.26 ± 0.02 ng/mL) or P4-6 mm (0.21 ± 0.01 ng/mL). For the direct comparison between treatments (**right fourth panel**), the overall LH concentrations were 58% greater in the Non-P4 (0.38 ± 0.04 ng/mL) than in the P4-treated heifers (0.24 ± 0.01 ng/mL).

The overall P4 concentrations (**Fig. 2; bottom panels**) during days -1.5 to 0 were greater for the heifers in the P4-emergence group (1.7 ± 0.2 ng/mL) than P4-6 mm (0.26 ± 0.08 ng/mL) or Control (0.08 ± 0.07 ng/mL). During days 0 to 2, P4 concentrations increased in P4-6 mm (1.0 ± 0.1 ng/mL), remained high in the P4-emergence (2.1 ± 0.2 ng/mL) and were lower in the Control (0.05 ± 0.01 ng/mL). As expected, comparison between the two treatments demonstrated greater overall P4 concentration from days 0 to 2 in P4-treated (1.4 ± 0.11 ng/mL) than Non-P4 treated heifers (0.08 ± 0.04 ng/mL).

3.3 Experiment 2: pLH vs hCG on follicular dynamics

The frequency of conventional (3/9 vs 4/9), undersized (5/9 vs 4/9), switched (1/9 vs 1/9) and co-dominant (0) deviations were similar ($P = 1.0$) between the pLH and hCG groups. The length of time for F1 to proceed through specific stages was similar ($P > 0.14$) between the pLH and the hCG groups i.e., the timing was similar from ablation

to emergence (1.6 ± 0.2 vs 1.2 ± 0.2 d/stage); 4 to 6 mm (0.9 ± 0.1 vs 1.1 ± 0.1 d/stage); 6 to ≥ 7.5 mm (1.0 ± 0.1 vs 0.9 ± 0.1 d/stage); 7.5 to 8.5 mm (0.6 ± 0.1 vs 0.5 ± 0.1 d/stage); and 8.5 to 10 mm (1.1 ± 0.1 vs 1.0 ± 0.1 d/stage). The growth rate of F2 during the given stages was also similar between the two groups, resulting in decreased growth rate (-0.2 ± 0.1 mm/stage) during the stage of F1 7.5 to 8.5 mm.

3.4 Experiment 2: LH activity (Saline vs pLH/hCG-treated) on follicular and hormonal dynamics

Based on the similarities of the pLH and hCG groups, the two groups were combined and directly compared to the Saline-treated heifers (**Table 2**). Similar frequency of conventional (3/9 vs 7/18) and undersized (4/9 vs 9/18) deviations were observed for the Saline and pLH/hCG-treated heifers.

The length of time for F1 to proceed through specific stages of growth was similar between Saline and LH/hCG-treated heifers (Table 2). The growth rate of F2 during the given stages was similar between the treatments, except for a tendency for a negative growth rate in the LH/hCG-treated (-0.1 ± 0.1 mm/stage) compared to just a decreased growth rate in the Saline-treated heifers (0.3 ± 0.3 mm/stage) during the stage of F1 7.5 to 8.5 mm. The F2 growth rate of both treatments decreased during this stage. Thus, the F1 and F2 diameters, and the FSH, LH, and P4 concentrations were normalized to the value when F1 reached or exceeded 7.5 mm. This day was designated as day 0 and the dynamics during days -2.5 to 1.5 are presented in **Fig. 3**. The F1 diameter (**top panels**) was similar among the Control, pLH, and hCG groups (**left panel**), and between the Saline and pLH/hCG-treated heifers (**right panel**), despite a

detected interaction of group-by-day and treatment-by-day. The interaction is explained by a switch in diameter ranking on day -0.5 with Saline-treated being greater than pLH/hCG-treated groups. The F2 diameter (**second panels**) was similar among the groups and between the treatments during days -1.5 to 1.5 . In other words, only a main effect of day was observed with no treatment effect or treatment-by-day interaction.

The overall circulating FSH concentrations (**Fig. 3; third panels**) were greater during days -2.5 to 0 in Control (0.45 ± 0.03 ng/mL), medium in the hCG group (0.36 ± 0.02 ng/mL), and lower in the pLH group (0.33 ± 0.03 ng/mL). From day 0 to 1.5 , overall circulating FSH concentration was greater in Control (0.22 ± 0.01 ng/mL) than pLH (0.18 ± 0.01 ng/mL) or hCG groups (0.17 ± 0.01 ng/mL). In agreement, the direct comparison between Saline and pLH/hCG-treated heifers, showed greater overall circulating FSH from day -2.5 to 0 (0.46 ± 0.04 vs 0.34 ± 0.02 ng/mL) and 0 to 1.5 (0.22 ± 0.01 vs 0.17 ± 0.01 ng/mL). The P4 concentrations (**bottom panels**) were similar between groups prior to day 0 but from day 0 to 1.5 , greater overall P4 concentration was observed in the hCG treatment (8.6 ± 0.8 ng/mL), with medium P4 in the pLH group (6.4 ± 0.3 ng/mL), and lower P4 in the Control (4.6 ± 0.3 ng/mL). Similarly, direct comparison of the two treatments indicated lower overall circulating P4 for Saline-treated (4.7 ± 0.3 ng/mL) than for LH/hCG-treated heifers (7.5 ± 0.5 ng/mL) during days 0 to 1.5 .

4. Discussion

This research reports the results of two studies that characterized the hormonal and follicular wave dynamics and, particularly, the process of diameter deviation during

direct manipulation of circulating P4 (experiment 1) or direct manipulation of LH activity in the presence of high P4 (experiment 2). These studies used an experimental approach in which emergence of the second follicular wave was synchronized using follicular ablation of all follicles (≥ 4 mm) on day 6 of the estrous cycle during the first follicular wave. Our previous study found similar follicular dynamics during a spontaneous second follicular wave and one induced by follicular aspiration [13]. Diameter deviation is considered the key period when a single dominant follicle (F1) is morphologically selected from the subordinate follicles (F2, F3) which begin atresia [5, 15]. However, there continue to be critical questions about the mechanisms involved in this process and about the surprising variability in the diameters of the F1 and F2 at the time of deviation in different breeds, genotypes, and between or within individual cattle [18]. One objective of this series of experiments is to understand the physiologic conditions and the hormonal and cellular mechanisms that produce the complexity of differences in diameter deviation.

Designating a particular follicular wave as having conventional or undersized deviation is based on the diameter of the F2 (larger or smaller than 7.0 mm) at expected deviation (F1 ≥ 8.5 mm) [13, 17]. Conventional deviation (F2 ≥ 7.0 mm) was more likely during the first follicular wave, when circulating P4 is low, whereas the second follicular wave, when circulating P4 is elevated, had a greater likelihood for undersized deviation [12, 13]. Thus, high circulating P4 is associated with increased undersized deviation, however, these previous observational studies may have been confounded because low P4 was only present during the first follicular wave and high P4 during the second follicular wave [13]. The current study demonstrated, by direct manipulation of

circulating P4, that the differences in the follicular deviation process are directly related to the circulating P4 concentrations.

The dose of P4 used in this study was selected, based on previous reports demonstrating that treatment with 150 mg of P4/day produced circulating P4 concentrations that were similar to mid-luteal phase concentrations [14, 37]. This same dose of 150 mg/day has been delivered either every 8 or 12 h to produce a more constant P4 profile [38, 39]. In the current experiment we utilized 75 mg of P4 given every 12 h to match our times of ultrasound evaluations and blood sample collections and to stabilize the pattern of P4 during the experimental period. Previous studies using 150 mg of P4/day reported decreased size of the dominant follicle, probably due to reduced circulating LH [14, 37-39]. Use of other methods to elevate circulating P4 also indicate that increased P4 leads to decreased growth rate of the dominant follicle and decreased size of the ovulatory follicle [40-42]. In our experiment, we also found that elevated P4 reduced F1 growth after deviation in association with a reduction in circulating LH. However, in agreement with previous studies [14, 24, 37-39], we found no detectable effect of elevated P4 on F1 growth prior to deviation. This would be consistent with the idea that increased P4 only alters F1 growth after acquisition of LH receptors in granulosa cells of the F1 (post-deviation) and that this effect is likely mediated by decreased LH during P4 treatment.

One of the most intriguing effects of elevated P4 on follicular dynamics was the earlier decrease in F2 growth during P4 treatment, as evidenced: 1) increased undersized deviations (53.8% vs 0%), 2) smaller F2 diameter on day 1 (one day after F1 ≥ 7.5 mm; 8.0 ± 0.5 vs 7.2 ± 0.2 mm), and 3) reduced growth of F2 during stage of F1

growth from 7.5 to 8.5 mm (**Table 1**). Previous studies have suggested that undersized deviation may represent a slower follicle divergence process rather than an abrupt deviation, as clearly documented for conventional deviations [12, 18]. However, the abrupt cessation of F2 growth rate when the F1 is growing from 7.5 to 8.5 mm during elevated P4 indicates a distinct deviation process that is clearly occurring at a smaller F1 and F2 diameter than in heifers with low circulating P4. In low P4, F2 growth continues to be elevated during this stage (F1 growing from 7.5 to 8.5) but then there is an abrupt decrease in F2 growth (deviation) during F1 growth from 8.5 to 10 mm, as previously reported for conventional deviation [18]. This earlier deviation in F2 growth rate seems to be the basis for undersized deviation and is clearly produced by elevating P4 into physiological concentrations.

Even more dramatic growth of the F2 can be observed when co-dominant follicles are selected and it is also associated with a low P4 concentrations [43, 44]. This has been particularly apparent in lactating dairy cows that have elevated steroid metabolism leading to decreased circulating P4, increased circulating LH and FSH, and much greater likelihood for co-dominance and double ovulation [42, 45]. It seems possible that there is a continuum of F2 growth from the diminished size observed during undersized deviation (< 7.0 mm), increased F2 growth during conventional deviation ($F2 \geq 7.0$), and F2 growth until acquisition of the dominant phenotype during co-dominant deviation.

The second experiment tested whether the decreased F2 growth rate in an elevated P4 environment (undersized deviation) was caused by the decreased circulating LH that is clearly associated with elevated P4. Our hypothesis was that elevating LH

activity in the presence of high circulating P4 would change the deviation process from primarily undersized to primarily conventional deviation. The observation that treatment with hCG or pLH caused a dramatic increase in circulating P4 is consistent with other studies [46-48] and validates that the hCG/pLH treatments produced elevated LH activity in our model. Nevertheless, treatment with pLH/hCG did not alter F1 or F2 growth rates, as demonstrated by similar follicle diameter throughout follicle growth (**Fig. 3**), similar time of diameter deviation, and similar timing of an abrupt decrease in F2 growth rate during the stage when the F1 was growing from 7.5 to 8.5 (**Table 2**). Contrasting with our hypothesis, treatment with hCG/pLH not only did not improve F2 growth during this critical period but resulted in a tendency ($P = 0.07$) for an even lower F2 growth rate in heifers treated with hCG/pLH compared to Saline (-0.1 ± 0.1 vs 0.3 ± 0.3). Thus, our second hypothesis can be clearly rejected. The increase in undersized deviations caused by elevated P4 is not due to diminished LH activity, if anything, increased LH activity in the presence of elevated P4 produced a more robust undersized deviation process with a greater suppression of F2 growth. It should be stated that the focus of this experiment and the timing of hCG/pLH treatment was related to the pre-deviation period and the diameter deviation dynamics and therefore did not evaluate the role of LH in the maximum diameter of the F1, as has been already shown in previous studies [42, 49, 50].

Circulating LH is primarily the result of GnRH/LH pulses, whereas, circulating FSH is a combination of constitutive FSH secretion, that is independent of acute GnRH action, and GnRH-stimulated FSH secretion [51, 52]. Thus, the panoramic view of gonadotropin concentrations provided by the 12-hour sampling, while clearly indicating

decreased LH caused by elevated P4, is probably most informative in evaluating circulating FSH concentrations. In the presence of elevated P4, while undersized deviation was occurring, there was an elevation in circulating FSH (**Fig. 2**). This result is consistent with previous reports that P4 treatment caused a decrease in LH but an increase in circulating FSH [39]. This contrasts with *in vitro* studies that show a direct inhibition by P4 of both basal and GnRH-stimulated FSH production by primary pituitary cell cultures [53-55]. The small but clear-cut elevation in FSH after deviation caused by elevated P4 has been hypothesized to result from diminished FSH inhibitors emanating from the smaller subordinate follicles [13]. However, in Experiment 2, treatment with hCG/pLH maintained and even may have accentuated undersized deviation while clearly reducing the circulating FSH (**Fig. 3**). Thus, it seems more likely, in our opinion, that it is the dominant follicle responding to LH action that is responsible for the differences in circulating FSH detected during the three physiologic conditions observed in this study. During elevated P4, a reduction in circulating LH could reduce production of estradiol and perhaps other FSH inhibitors produced by the dominant follicle. This is consistent with a previous study in which heifers treated with P4 appeared to have reduced functionality of the F1, as evidenced by changes in the molecular composition of the follicular fluid such as reduced estradiol, androstenedione, estrone, and free IGF-1 [39]. In contrast, treatment with activators of the LH receptor (hCG/pLH), in the presence of elevated P4, could stimulate the F1 as it acquires LH receptors on the granulosa cells leading to increased F1 function, including increased estradiol production. Thus, greater LH action would increase FSH inhibition and decrease circulating FSH (hCG/pLH-treatment), while less LH activity (elevated P4)

could lead to less FSH inhibitors from the F1 and therefore greater circulating FSH after deviation. In either circumstance, elevated P4 lead to a decrease in F2 growth at a smaller diameter and undersized deviation. Obviously, the various aspects of this physiologic model remain to be tested.

4.1 Conclusions

This research clearly demonstrated that elevated P4 leads to an earlier inhibition of the growth rate of the F2, i.e. undersized deviation. Although an inhibition of circulating LH was associated with the elevation in circulating P4, this was not the mechanistic cause of undersized deviation because direct treatment with LH receptor agonists, hCG and pLH, stimulated function of the CL but did not change the early deviation in F2 growth rate. In contrast, the changes in circulating FSH that occur after deviation do appear to be related to the action of LH on the F1, since reduced LH (elevated P4) allowed increased FSH, whereas, increased LH activity decreased circulating FSH after deviation. Regardless, the nadir in FSH occurs near the time of deviation during conventional (nadir at 8.5 mm) or undersized deviation in the presence or absence of LH agonists (nadir at 7.5 mm). Thus, the primary cause of undersized deviation remains obscure since it can happen in the presence of elevated or reduced FSH concentration and elevated or reduced LH activity. It is unmistakable from the current research that elevated circulating P4 is the most noticeably hormone linked to undersized deviation.

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Table 1. Deviation class frequency, length of time for F1 to proceed through specific stages of growth, and F2 growth rate during the given F1 stages in experiment 1.

| End-Points | Experiment 1 | | |
|-------------------------------------|-----------------------------|--------------------------|----------------|
| | Non-P4 treated (n=6) | P4-treated (n=13) | P value |
| Deviation class frequency | | | |
| Conventional | 4 (66.7%) | 6 (46.15%) | 0.628 |
| Undersized | 0 (0%) | 7 (53.85%) | 0.0341 |
| Switched | 1 (16.7%) | 0 (0%) | – |
| Co-dominant | 1 (16.7%) | 0 (0%) | – |
| Length of time (days/stage): | (n=4) | (n=13) | |
| Stage: Ablation to emergence | 1.6 ± 0.1 | 1.8 ± 0.1 | 0.529 |
| Stage: F1 4 to 6 mm | 1.0 ± 0.2 | 1.1 ± 0.1 | 0.638 |
| Stage: F1 6 to 7.5 mm | 0.6 ± 0.1 | 0.7 ± 0.1 | 0.838 |
| Stage: F1 7.5 to 8.5 mm | 0.5 ± 0.1 | 0.5 ± 0.1 | 0.797 |
| Stage: F1 8.5 to 10 mm | 0.9 ± 0.2 | 1.6 ± 0.3 | 0.085 |
| F2 Growth Rate (mm/stage) | | | |
| Stage: F1 4 to 6 mm | 1.4 ± 0.6 | 1.6 ± 0.2 | 0.478 |
| Stage: F1 6 to 7.5 mm | 1.1 ± 0.3 | 0.7 ± 0.2 | 0.267 |
| Stage: F1 7.5 to 8.5 mm | 1.1 ± 0.2 | 0.2 ± 0.1 | 0.0006 |
| Stage: F1 8.5 to 10 mm | 0.3 ± 0.3 | 0.2 ± 0.3 | 0.882 |

All heifers were submitted to 2 doses (12 h apart) of 500 µg i.m. of cloprostenol on day 5 day after ovulation and follicular ablation 24 h after for regression of the corpus luteum and synchronization of the wave 2 emergence, respectively. Non-P4 consisted in untreated heifers (absence of P4); P4-treated consisted in 12 i.m. injections of P4 every 12 h (75 mg/each) starting 12 h after follicle ablation or at F1 ≥ 6mm. Deviation class frequency was compared between treatments by using a Fisher's exact test.

Table 2. Deviation class frequency, length of time for F1 to proceed through specific stages of growth, and F2 growth rate during the given F1 stages in experiment 2.

| End-Points | Experiment 2 | | |
|---|-------------------------|-------------------------------|---------------|
| | Saline-treated (n=9) | pLH/hCG -treated (n=18) | P value |
| Deviation class frequency | | | |
| Conventional | 3 (33.3%) | 7 (38.9%) | 0.3169 |
| Undersized | 4 (44.5%) | 9 (50%) | 1.0 |
| Switched | 2 (22.2%) | 2 (11.1%) | – |
| Co-dominant | 0 (0%) | 0 (0%) | – |
| Length of time (days/stage): | (n=7) | (n=16) | |
| Stage: Ablation to emergence | 1.3 ± 0.2 | 1.4 ± 0.1 | 0.593 |
| Stage: F1 4 to 6 mm | 1.1 ± 0.1 | 1.0 ± 0.1 | 0.398 |
| Stage: F1 6 to 7.5 mm | 0.9 ± 0.1 | 1.0 ± 0.1 | 0.475 |
| Stage: F1 7.5 to 8.5 mm | 0.5 ± 0.2 | 0.6 ± 0.1 | 0.702 |
| Stage: F1 8.5 to 10 mm | 1.4 ± 0.2 | 1.1 ± 0.1 | 0.141 |
| F2 Growth Rate (mm/stage) | | | |
| Stage: F1 4 to 6 mm | 1.4 ± 0.2 | 1.5 ± 0.2 | 0.981 |
| Stage: F1 6 to 7.5 mm | 1.0 ± 0.2 | 1.1 ± 0.2 | 0.755 |
| Stage: F1 7.5 to 8.5 mm | 0.3±0.3 | –0.1 ± 0.1 | 0.070 |
| Stage: F1 8.5 to 10 mm | –0.4 ± 0.3 | –0.3 ± 0.2 | 0.679 |

All heifers were submitted to follicle ablation 6 days after ovulation, so wave 2 emergence was synchronized. Saline-treated consisted in i.m. injections every 12 h whereas pLH/hCG-treatment consisted in pLH injections (1.25 mg) every 12 h or hCG injections (first dose of 160 IU and subsequent doses of 96 IU) every 24 h. Treatments started 12 h after follicle ablation and were done until F1 ≥ 10 mm.

Deviation class frequency was compared between groups by using a Fisher's exact test.

Figure Legends

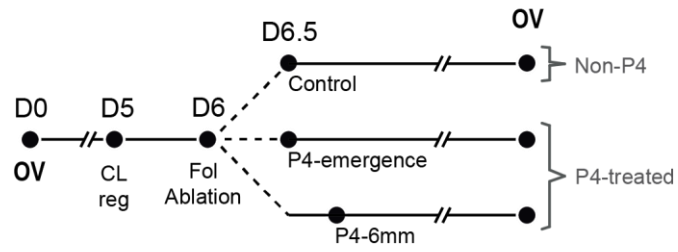
Fig. 1. Schematic experimental design. Ultrasound scans were performed to detect ovulation (D0). On D5 heifers in Experiment 1 but not in Experiment 2, received two doses (12 h apart) of 500 µg of cloprostenol for regression of the corpus luteum. Both experiments had emergence of the second follicular wave synchronized by follicle (≥ 4 mm) ablation on D6 and re-aspiration of re-filled follicles on D6.5. On D6.5 heifers (n=20) in Experiment 1 were randomized into: (1) Control, absence of P4; (2) P4-emergence and (3) P4-6mm treatment with 12 i.m. injections of P4 every 12 h (75 mg/each) from D6.5 or F1 ≥ 6 mm. On D6.5 heifers (n=27) in Experiment 2 were equally randomized into: (1) Control, i.m. saline solution; (2) pLH, i.m. porcine pituitary LH (1.25 mg/ every 12 h) and (3) hCG, i.m. hCG (160 and subsequent 96 IU/ every 24 h). After analysis by group, the data was compared by treatment within each experiment, i.e. Non-P4 vs P4-treated heifers and Saline vs pLH/hCG-treated heifers. Ultrasound scans and blood sample collections were done every 12 h from D6.5 until ovulation in Experiment 1 and until F1 ≥ 10 mm in experiment 2.

Fig. 2. Mean \pm standard error of the mean for the diameter of the dominant follicle (F1) and the subordinate largest follicle (F2), the FSH, LH, and P4 circulating concentrations in Experiment 1. All heifers received two doses (12 h apart) of 500 µg of cloprostenol for regression of the corpus luteum and had emergence of the second follicular wave synchronized by follicle (≥ 4 mm) ablation. The Control consisted in no-treatment; the P4-emergence consisted in P4 treatment starting on 12 h after ablation and P4-6mm consisted in P4 treatment starting when F1 ≥ 6 mm. P4 treatments were i.m. doses of 75

mg of P4 in sunflower oil (1mL; 75 mg/mL) every 12 h for 5 d. Comparisons among groups are presented on the left panels whereas between treatments are presented in the right panels. Significant probabilities for main effect of group (G) / treatment (T), main effect of day (D) and an interaction group-by-day (GD) / treatment-by-day (TD) are shown for each of the days -1.5 to 0 and 0 to 1.5 into each panel. Superimposed letters indicate significant differences among/between the groups/treatments.

Fig. 3. Mean \pm standard error of the mean for the diameter of the dominant follicle (F1) and the subordinate largest follicle (F2), the FSH and P4 circulating concentrations in Experiment 2. All had emergence of the second follicular wave synchronized by follicle (≥ 4 mm) ablation. The Control consisted in i.m. saline solution; the pLH, consisted in i.m. porcine pituitary LH (1.25 mg/ every 12 h) and the hCG consisted in i.m. hCG (160 and subsequent 96 IU/ every 24 h). Comparisons among groups are presented on the left panels whereas between treatments (Saline and pLH/hCG-treated) are presented in the right panels. Significant probabilities for main effect of group (G) / treatment (T), main effect of day (D) and an interaction group-by-day (GD) / treatment-by-day (TD) are shown for each of the days -1.5 to 0 and 0 to 1.5 into the follicle panels and for each of the days -2.5 to 0 and 0 to 1.5 into the hormonal panels. An asterisk (*) indicates a day with a difference among/between the groups/treatments.

Experiment 1:



Experiment 2:

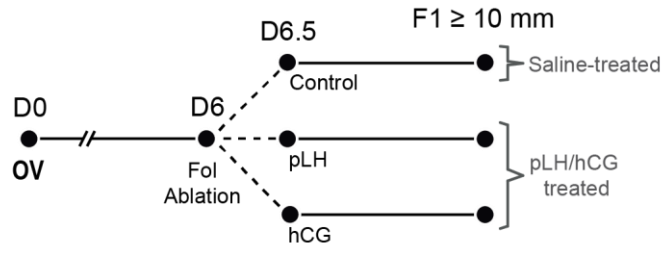


Fig. 1.

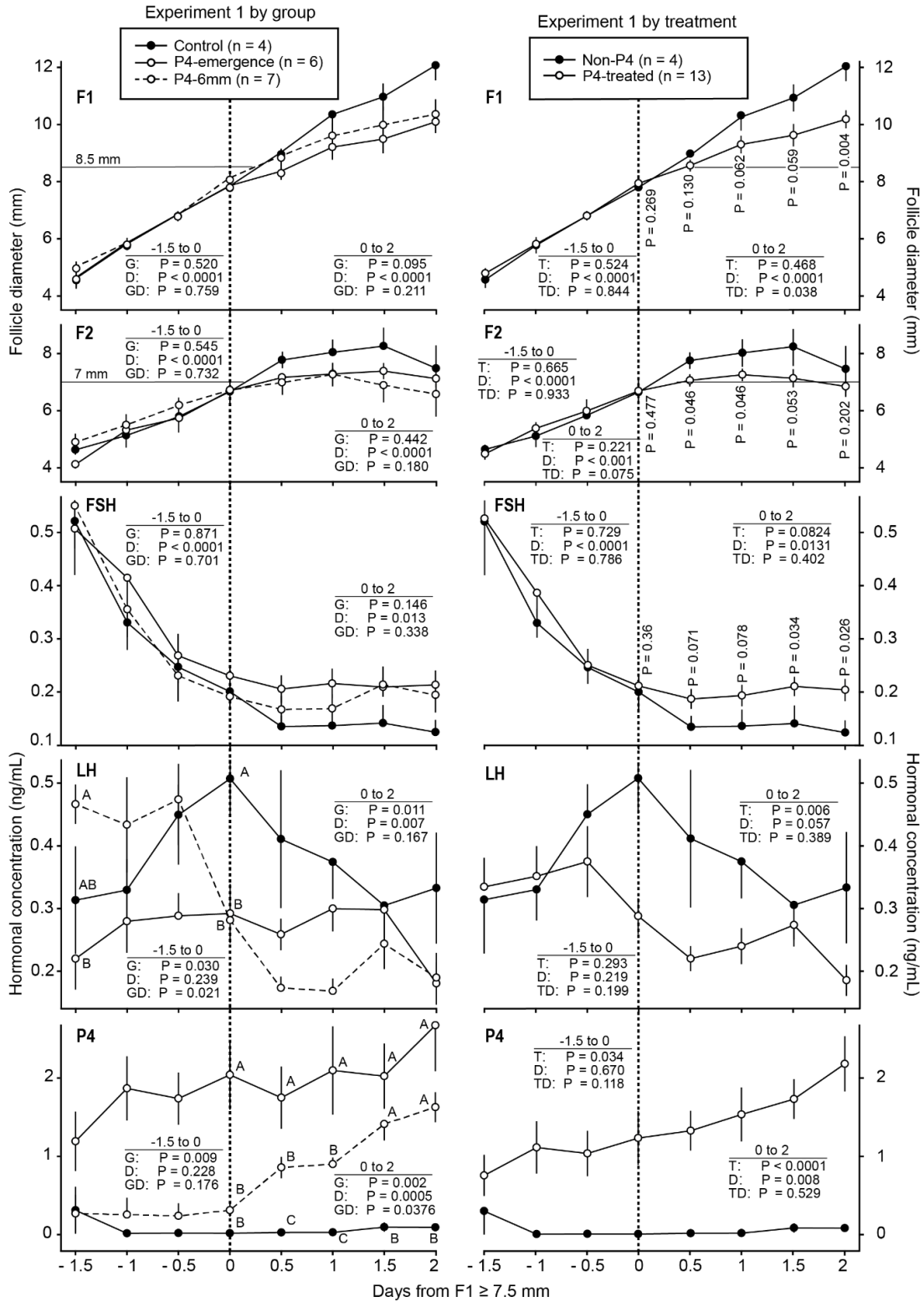


Fig. 2.

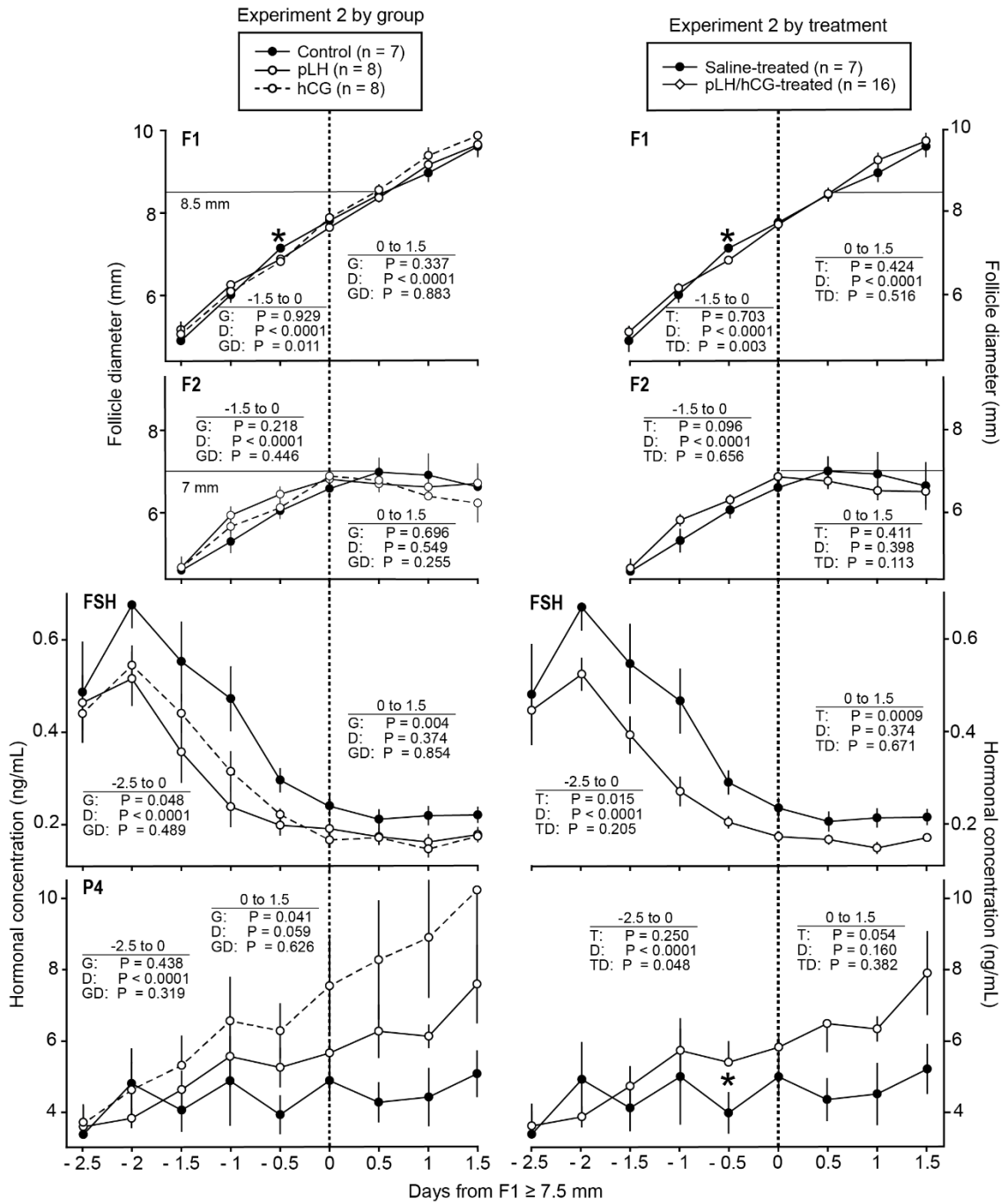


Fig. 3.

To be submitted to BOR

Hormonal mechanisms regulating follicular wave dynamics III: Insights on the role of LH in follicle selection from studies using hCG and GnRH-antagonist

Running Title: GnRH antagonist and hCG on follicle development

Summary sentence: Dominant follicle selection is linked to LH action, as shown by follicle growth inhibition at time of normal selection in heifers treated with GnRH antagonist and restored growth of single dominant follicle after replacement of LH action using hCG.

Keywords: Cattle; dominant follicle; selection; GnRH antagonist; Acyline; luteinizing hormone.

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ABSTRACT

Previous research demonstrated that treatment with GnRH-antagonist (Acyline) allowed follicle growth until 8.5mm but no dominant follicle was selected, i.e. there was inhibition of follicular deviation. This study evaluated whether deficient LH was the underlying mechanism by replacing LH action, using human chorionic gonadotropin (hCG) during Acyline treatment. Holstein heifers (n=24) during the first follicular wave (starting at ovulation) were evaluated by ultrasound and randomized into one of three treatments when largest follicle reached 5.5 mm diameter: Control (n=8; Saline treatments), Acyline (n=8; 5µg/kg Acyline), or Acyline+hCG (n= 8; 5µg/kg Acyline plus hCG; 50 IU of hCG at start then 100 IU every 12h). Pulses of LH were present in control heifers (9 Pulses/10 h) but eliminated by Acyline treatment. Follicular deviation occurred when largest follicle (F1) reached 8.5 ± 0.5 mm in Control heifers but did not occur in Acyline-treated (maximum diameter of 8.7 ± 0.5 mm). Heifers treated with Acyline+hCG demonstrated selection of a single dominant follicle, although deviation occurred at smaller F1 (7.6 ± 0.1 mm) and F2 (largest subordinate follicle; 6 ± 0.3 mm) . Circulating FSH was greater after day 2 in Acyline-treated than controls but lower than controls in Acyline+hCG after day 3. Circulating LH was lower in Acyline and Acyline+hCG than controls from days -0.5 to 3. Thus, dominant follicle selection and growth after deviation is due to LH action as shown by inhibition of this process during ablation of GnRH/LH pulses and restoration of the process after replacement of LH action by hCG treatment.

INTRODUCTION

Classical endocrinology experiments removed the endocrine gland of interest and then treated with the expected active constituent from the gland to determine physiologic hormone activity. For example, removal of the ovary or ablation of the corpus luteum (CL) led to pregnancy loss [1-3]. Replacement of the active hormone by treatment with an ethanol-extract of the CL allowed maintenance of the CL [4] and eventually led to discovery of progesterone (P4)[5]. Specific ablation of the pituitary gonadotropins, LH and FSH, is more difficult but has been tried using hypophysectomy [6], hypothalamo-pituitary disconnection [7], immunization against GnRH [8], inhibition of GnRH receptors using a GnRH receptor antagonist [9, 10], or downregulation of GnRH receptors by chronic treatment with a potent GnRH agonist [11]. Unfortunately, subsequent physiologic replacement of LH and FSH after these different ablation methods has not always been adequately performed.

Diameter deviation has been proposed as the main morphological manifestation of follicle selection [12] although all of the mechanisms involved in this process have not yet been defined. A surge in circulating FSH is clearly the stimulus for initiation of a follicular wave and the nadir in circulating FSH is, on average, observed near the time of selection of the single dominant follicle [13]. Near the time of follicle deviation, the granulosa cells of the future dominant follicle acquire LH receptors [14] and it has been postulated that LH pulses now drive growth of the dominant follicle, whereas the subordinate follicles undergo atresia in the presence of low circulating FSH [13]. Our previous research found that short-term treatment with the GnRH antagonist, Acyline, during the first follicular wave led to an increase in circulating FSH, even though

GnRH-stimulated LH and FSH secretion were completely suppressed [10]. In association with these results, follicle growth occurred until 8.5mm but no dominant follicle was selected, i.e. there was inhibition of follicular deviation. Similar results have been observed during chronic treatment with a potent GnRH agonist [11]. However, replacement of LH during acute GnRH antagonist treatment has not yet been performed to determine whether deficient LH is the reason for the lack of follicle dominance.

Controlled replacement of gonadotropin is a routine approach in human reproductive medicine during GnRH antagonist treatment, usually using recombinant FSH or urine-derived human menopausal gonadotropin to produce ovarian stimulation and a high dose of human chorionic gonadotropin (hCG) to stimulate ovulation [15-17]. Of particular interest, completion of the final stages of normal follicle development was possible in women receiving GnRH antagonist treatment by using low doses (non-ovulatory) of hCG to stimulate LH activity on the dominant follicle, without the need for exogenous FSH treatment [18]. In cattle that had long-term immunization against GnRH, normal follicle development only occurred in heifers treated with both FSH and LH and not in heifers treated with LH or FSH alone [8]. However, high doses of recombinant FSH did not appear to optimally induce the final stages of follicle maturation, as evidenced by lower circulating estradiol and ovulation failure in some animals [19]. Thus, in humans and cattle the final stages of follicle maturation may primarily depend on LH activity, if follicles had reached deviation by completing the common growth phase under sufficient FSH activity. It seems clear that greater understanding of the hormonal regulation of follicle wave dynamics and the hormonal

and molecular control of the follicle deviation process could provide valuable insights for human and veterinary medicine and reproductive physiology.

Our previous studies found that a smaller diameter of the subordinate follicles was associated with elevated circulating P4 and may be related to an earlier acquisition of dominance in the largest follicle (F1 or future dominant follicle). In agreement, treatments that increased circulating LH activity, such as porcine LH or hCG treatment, resulted in greater suppression of the largest subordinate follicle (F2) growth. Previous studies have not yet evaluated the variability of diameter deviation [20] during LH ablation (Acyline) and replacement (hCG treatment).

This research focused on the role of LH in follicular wave dynamics and particularly in the follicular deviation process. Three specific hypotheses were tested related to the central idea that dominant follicle selection, subsequent growth, and functionality depends on LH activity. Specific hypotheses are: 1) Treatment with a potent GnRH antagonist will eliminate LH pulses, inhibit dominant follicle growth after follicular deviation (~8.5mm), and increase circulating FSH, confirming previous results [10]. 2) Replacement of LH action in Acyline-treated heifers using a specific LH receptor agonist, hCG, will restore follicular deviation, selection of a single dominant follicle, and normal growth rate of the dominant follicle. 3) Heifers from the Acyline+hCG group will have earlier F2 growth rate decrease and smaller follicle diameter at deviation, i.e. undersized deviation, as a result of reduced circulating FSH and removal of FSH pulses, when compared to the Control group.

MATERIAL AND METHODS

Heifers, Pre-Synchronization and Ultrasound Exams

The present experiment used Holstein dairy heifers in the range of 16 to 26 months old and 508 ± 12 kg of body weight, with no apparent abnormalities in the reproductive tract as evaluated by ultrasound. The heifers were kept under natural light in open shelters provided with *ad libitum* access to water, mineralized salt, and grass hay in the northern temperate zone. All experimental procedures and heifer management were done in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research.

Prior to the experimental phase, heifers at random days of the estrous cycle were synchronized using the following protocol: d0, 200 μ g GnRH i.m. (gonadorelin acetate; Gonabreed®, Parnell Phameuticals, Overland Park, KS, USA) and insertion of an intravaginal Controlled Internal Drug Release device (CIDR) with 1.38 grams of P4 (Eazi-Breed CIDR, Zoetis, Florham Park, NJ, USA); d5, 500 μ g i.m. of cloprostenol (Estroplan®, Parnell Pharmaceuticals); d6, second treatment with 500 μ g i.m. of cloprostenol and withdrawal of the CIDR. Starting on d7, the heifers had their ovaries scanned every 12 hours to detect ovulation. After ovulation, heifers were included in the experimental phase for 7 days.

During the experimental phase, ultrasound examinations were done every 12 hours using a Mindray M5-Vet machine (Mindray North America, Mahwah, NJ, USA) with a multi-frequency 5.0-MHz linear transducer. The dual-B-mode of the ultrasound was used to obtain a video of each ovary on each side of the screen. The video recorded each individual ovary with a slow ultrasound scan from the outer- to the inner-most part

of ovary (left ovary) or from the inner- to the outer-most part of ovary (right ovary). Videos were evaluated, frame-by-frame, to allow identification, tracking, and subsequent mapping of individual follicles from examination to examination. Initial measurements were done at the time of the ultrasound examination, with the heifer still in the chute, allowing a second ultrasound video to be recorded, if necessary. The follicle diameter was based on two perpendicular measurements (height and width) at the apparent maximal area of the antrum, as described previously [21].

Experimental Treatments

Each heifer was randomized to one of three experimental groups (n = 8/group) when the largest growing follicle, present at the time, reached or exceeded 5.5 mm in diameter. The treatment groups were: 1) **Control** heifers received saline treatments to match treatments (1 mL initially and subsequent doses of 2 mL of saline solution i.m. every 12 hours); 2) **Acyline** heifers received a single dose of 5 μ g/Kg of Acyline (2.2 mg/mL), a GnRH antagonist kindly donated by NIH/NICHD (Lot RDZ1007) in 1 mL and subsequent saline treatments of 2 mL i.m. every 12 hours; 3) **Acyline+hCG** heifers received single treatment of 5 μ g/Kg of Acyline in 1 mL with initial hCG dose of 50 IU (1 mL) and subsequent doses of 100 IU hCG (2 mL) i.m. every 12 hours (50 IU/mL; Chorulon®, Merck Sharp & Dohme Corp, Kenilworth, NJ, USA). The single treatment with 5 μ g/kg of Acyline was based on complete inhibition of the LH and FSH response to 100 μ g of GnRH by treatment with either 3 or 10 μ g/kg of Acyline [10]. The hCG and saline treatments were administered until the end of the experimental phase (d7 after ovulation).

Hormone Assays

Blood samples were collected into heparinized tubes from the coccygeal vein prior to each ultrasound examination and immediately placed on ice until centrifugation (2,000 x g for 10 min). Plasma samples were collected and pipetted into 7 mL vials for storage at -20°C until hormonal assay. Additionally, 1.5 days after ovulation, intrajugular catheters were placed in 4 heifers per group in order to perform blood sample collections every 15 minutes for 10 hours.

The FSH and LH concentrations were determined using a validated radioimmunoassay technique [22, 23] with modifications reported previously [24, 25]. The FSH intra- and inter-assay CV and the mean assay sensitivity were 8.5%, 4.4% and 0.035 ng/mL; The LH intra- and inter-assay CV and the mean assay sensitivity were 3.2%, 0.6% and 0.052 ng/mL, respectively.

Data Handling and Statistical Analysis

The follicle that reached the largest diameter (F1) during the experimental phase was plotted in retrospective throughout the days until its emergence at 4 mm. For experimental purposes, it was determined that the second and third largest follicles (F2 and F3) would be identified on the day that the F1 diameter was closest to 7.5 mm, since an absence of diameter deviation was expected in the Acyline-treated heifers. In addition, this diameter has been considered as a transition period into diameter deviation [26]. The follicular waves that had an $F1 \geq 12$ mm were classified into one of four deviation classes as previously described [20], based on the F2 diameter on the day that F1 was closest to 8.5 mm in diameter (expected diameter deviation) as follows: 1)

conventional deviation ($F2 \geq 7$ mm), 2) F2-undersized deviations ($F2 < 7$ mm), 3) F1, F2-switched (F2 was larger than F1 on days -1, 0, or both), and 4) co-dominant follicles (both F1 and F2 reached 10 mm) [20]. The deviation class frequency was compared among the groups by using the Fisher's exact test. Data from switched deviations ($n = 1$) were excluded from all subsequent analyses.

The F1 and F2 diameters (mm), as well as the FSH and LH concentrations (ng/mL), were normalized to the time when the F1 was closest to 7.5 mm (day 0) and compared from day -1.5 to 0 and separately from day 0 to 4, among the groups. The F1 and F2 growth rate was calculated and compared among the groups during each 12 hours range from day -1.5 to 0.5 and as an overall group from day 0.5 to 4 (mean 12-hour growth rate). The FSH data were analyzed for each individual day separately since it was expected that the hormonal concentrations would be different among the groups.

All values were transformed into natural logarithms or ranks when they were not normally distributed. Analyses were performed using the SAS PROC MIXED procedure (Version 9.4; SAS Institute). A probability of $P \leq 0.05$ indicated that a difference was significant, whereas a probability of $P > 0.05$ to $P \leq 0.1$ indicated a trend or that significance was approached. Data are presented as the mean \pm standard error of the mean (SEM).

RESULTS

Representative heifers from each treatment group are shown in Figures 1 (Controls), 2 (Acyline), and 3 (Acyline+hCG) displaying follicular dynamics, time of treatment (largest growing follicle ≥ 5.5 mm), circulating FSH and LH concentrations (every 12 hours), plus the LH and FSH pulse profiles from blood samples collected

every 15 min for 10 h on day 1.5 post-ovulation. Control heifers (Figure 1) had conventional deviation with the F2 reaching a diameter of more than 7 mm near the time when the F1 reached 8.5 mm. Controls also had suppressed FSH near the time of deviation and had high frequency of LH pulses, as would be typical of the first follicular wave. Pulses of FSH were less distinct than LH pulses and of much smaller amplitude. The Acyline heifers (Figure 2) did not have F1 growth past the typical time of deviation, had a rebound in circulating FSH about 24 h after Acyline treatment, and had no evidence of LH or FSH pulses. The Acyline+hCG heifers (Figure 3) had much smaller growth of the F2 with likely earlier follicular deviation, suppressed FSH, and no evidence of LH or FSH pulses. The LH pulse frequency differed ($P < 0.0001$) among the Controls (9 ± 0.7 pulses/10 h) and the Acyline and Acyline+hCG groups (0.1 ± 0.5 pulses/10 h).

The frequency of conventional deviations in the Control group (6/8) was greater ($P = 0.02$) compared to the Acyline+hCG (1/8), whereas the frequency of undersized deviation was greater ($P = 0.005$) in the Acyline+hCG (7/8) compared to the Controls (1/8). There was one switched deviation in the control group and this heifer was excluded from subsequent analyses. The Acyline heifers could not be accurately classified for class of follicular deviation.

Figure 4 shows the average follicular dynamics for each treatment group, normalized to time that the F1 was closest to 7.5 mm. The F1 maximum diameter was reached on day 4 for Controls and the Acyline+hCG groups (14.2 ± 0.4 mm), whereas for Acyline heifers maximum F1 diameter was reached on day 1 (8.7 ± 0.5 mm). The F1 was similar ($P > 0.8$) among groups between days -1.5 to 0 but had ($P < 0.0001$) a

smaller diameter in the Acyline group during days 0.5 to 4. The F2, from day -1.5 to 0 was only different on day 0, with smaller diameter of F2 in the Acyline+hCG-treated heifers than the other two treatments, whereas during days 0 to 4 there was only a main effect of group ($P = 0.008$) explained by the smaller F2 diameter in the Acyline+hCG ($6.1 \pm 0.1\text{mm}$) compared to the Control and Acyline heifers ($7 \pm 0.1\text{mm}$). The mean day for the start of treatments (largest growing follicle ≥ 5.5 mm) was similar among the three groups (-0.9 ± 0.1 days before F1 reaching value closest to 7.5 mm) and therefore was placed on day -1 in Figure 4.

Figure 5 (upper panel) shows a similar overall growth rate ($1.0 \pm 0.1\text{mm}/12$ h) among the three groups during each of the 12 h ranges from day -1.5 to 0. However, there was a decreased growth rate from day 0 to 4 ($0.08 \pm 0.1\text{mm}/12$ h) in the Acyline treatment compared to the other two treatment groups that had similar F1 growth rates. The lower panel shows similar F2 growth rate ($0.8 \pm 0.1\text{mm}/12$ h) among the three groups from each of the 12 h periods from day -1.5 to -0.5. However, from day -0.5 to 0, there was a decreased F2 growth rate in the Acyline+hCG ($0.1 \pm 0.2\text{mm}/12$ h) compared to the other two groups ($0.9 \pm 0.1\text{mm}/12$ h). The F2 growth, in the range from day 0 to 0.5, decreased to a similar rate in all groups ($0.3 \pm 0.1\text{mm}/12$ h) and averaged a similar negative growth rate (-0.1 ± 0.05 mm/12 h) from day 0.5 to 4 in the three groups.

Figure 6 shows the circulating FSH and LH concentrations associated with the follicular dynamics normalized to the time when the F1 reached a value closest to 7.5 mm for the three experimental treatment groups. The FSH (upper panel) concentration was similar among the three treatments during the early declining portion of the FSH

surge, in other words, there was no main effect of treatment or group-by-day interaction during days -1.5 to 0. During days 0 to 4, the Acyline group had greater overall FSH concentrations (0.26 ± 0.01 ng/mL) compared to the concentrations in the Control (0.17 ± 0.01 ng/mL) and Acyline+hCG treatments (0.13 ± 0.01 ng/mL). There was a group-by-day interaction after day 0 for circulating FSH resulting from greater FSH in the Acyline-treated heifers from days 1.5 to 4 and lower FSH in the Acyline+hCG compared to the Controls during days 2.5 to 4. Separate analyses using t-tests indicated that circulating FSH was lower in Acyline+hCG than Controls from day 2 to 4.

Circulating LH concentration decreased faster after Acyline treatment (Figure 6). As a result, an interaction of group-by-day was observed during days -1.5 to 0. Circulating LH concentrations (lower panel) were much lower in the Acyline and Acyline+hCG groups (0.095 ± 0.0002 ng/mL) after day 0 compared to the Controls (0.18 ± 0.01 ng/mL).

DISCUSSION

Numerous studies have attempted to understand the molecular, cellular, paracrine, hormonal, and follicular dynamics that produce selection of a single dominant follicle from the cohort of follicles that are growing during a follicular wave [12, 27]. This study focused on the role of circulating LH in this process. Specifically, the present study used precise characterization of follicular wave dynamics using ultrasound to analyze the effect of ablation of LH pulses using the GnRH antagonist, Acyline, and subsequent replacement of LH action, using hCG, to gain insight into the hormonal control of follicle selection. The bovine model is particularly informative because of the extensive previous research using sequential ultrasound to characterize

the follicle dynamics and the complexities of the follicular deviation process under different physiological conditions [20, 28].

Our first hypothesis was that Acyline treatment would eliminate LH pulses and inhibit follicle growth after the point of follicle selection (~8.5 mm in *Bos taurus* cattle). This hypothesis was clearly supported, confirming our previous study that used similar methods [10]. There were essentially no detectable LH pulses during the 10 h frequent sampling period in heifers treated with Acyline compared to about one LH pulse per hour in the Control group (9 pulses per 10 h), similar to previous studies evaluating LH pulses during the early luteal phase [29, 30]. In spite of the lack of LH pulses, growth rate of the F1 was identical until the time when the F1 reached 7.5 mm (~1 mm/12 h; Figure 5). Thus, follicle growth until 7.5 mm does not depend on LH pulses. This is consistent with our previous study using Acyline [10] and is consistent with a previous study in which hCG or porcine LH treatment did not alter F1 follicle growth prior to deviation. However, studies that inhibited circulating FSH led to reduced follicle growth during the common growth phase (prior to 7.5 mm) [10, 12, 27, 31]. Thus, follicle growth prior to follicular deviation is dependent upon circulating FSH, whereas follicle growth after deviation is dependent upon LH pulses, as evidenced by lack of growth of the follicles past deviation during Acyline treatment.

Our second hypothesis represented the most important reason for this research, to determine whether the lack of follicle growth after GnRH antagonist treatment was resolved by treatment with LH. To assure specificity and sufficient duration of LH activity, we utilized hCG based on the specificity of hCG for the LH receptor [32-34] and increased half-life of hCG compared to LH [35]. As can be observed in follicle data

from individual animals (Figures 1 and 3), in the data on the average growth of the F1 for Control and Acyline+hCG, or as a direct comparison of growth rate for the F1 during all of the periods of follicle growth (Figure 5) there is a complete restoration of normal F1 follicle growth after replacement of LH activity during Acyline treatment. The dose of hCG was chosen, based on previous studies in *Bos indicus* cattle showing that this dose restored follicle growth in suckled beef cattle and that higher doses of hCG produced premature ovulations [36]. Previous studies reported greater maximum diameter of the dominant follicle and greater growth rate of the dominant follicle due to increased circulating LH or treatment with LH agonists, such as hCG [37]. This contrasts with the results of the present study in which there was no change in growth rate of the F1 in response to hCG treatment. However, previous studies utilized heifers with elevated P4 for the control group, whereas, this study was during the first follicular wave, a period of the cycle with low P4 and higher circulating LH concentrations [38]. Thus, the similar growth rate for Control and Acyline+hCG heifers in our study may relate to already elevated LH activity during the first follicular wave or potentially the hCG dose that we utilized closely simulated the physiologic LH activity. Either way, the F1 growth rates in Control and Acyline+hCG were 0.8 to 1.2 mm/12 h periods during follicle growth for two days prior to deviation and for four days after deviation (Figure 5). Thus, this study clearly demonstrates that the explanation for lack of F1 growth after 8.5 mm and for lack of follicular selection in the Acyline-treated heifers is insufficient LH activity.

Our third hypothesis related to growth of the F2 and the relationships of follicle growth with FSH. Treatment with Acyline was expected to suppress GnRH-stimulated

FSH secretion, however, in ruminants, circulating FSH is primarily driven by the GnRH-independent component, termed constitutive FSH secretion [54]. Acyline treatment did not suppress circulating FSH in previous studies with heifers unlike in mares or women [10, 55, 56]. In agreement, GnRH agonist treatment only suppressed circulating FSH after 40 days of chronic treatment [11].

The first key observation on this relationship in our study is that Acyline treatment not only produced a lack of dominant follicle selection but also increased circulating FSH concentrations, confirming previous observations [10]. In our previous study [10], the increase in circulating FSH began at about 6 h after Acyline treatment and FSH continued to be elevated, compared to controls, up to 4 days after Acyline. In the present experiment, we observed a delay in the Acyline-induced increase in FSH with significant increases only observed at 2 days after Acyline treatment (Figure 6). In the previous experiment, Acyline treatment began prior to wave emergence (~22 h before ovulation) compared to the later time in the follicular wave (≥ 5.5 mm) utilized in this experiment. In the present study, the elevation in FSH appears to be due to lack of dominant follicle selection and perhaps the observed FSH increase is a premature FSH surge that will prematurely initiate a new follicular wave. The results of both studies support the idea that periovulatory FSH is primarily the result of constitutive and not GnRH-stimulated FSH secretion from the pituitary gland in ruminants [54, 57], as evidenced by similar or elevated circulating FSH during inhibition of GnRH action by Acyline treatment.

On the other hand, treatment with Acyline+hCG had similar circulating FSH as controls from the start of treatment (≥ 5.5 mm) until 3 days after treatment (~14 mm).

After this time, Acyline+hCG had reduced FSH compared to controls, possibly due to elevated FSH inhibitors from the dominant follicle that continued to be stimulated by the hCG treatment. This study was not designed to definitively evaluate FSH pulse patterns, due to low numbers of animals that were evaluated. However, the FSH pulses were clearly less obvious in control heifers compared to the LH pulses and are likely to have little impact on the average FSH concentrations that are monitored during 12 h blood sampling experiments. Thus, the results of this study and previous studies are consistent with periovulatory FSH concentrations in the bovine being primarily related to constitutive FSH secretion regulated by follicular inhibitors and not by acute regulation by GnRH.

One of the most interesting observations in this study is that heifers treated with Acyline+hCG primarily had undersized deviation, compared to the control heifers (7/8 vs 1/8) that primarily exhibited conventional deviation. Consistent with this observation diameter deviation occurred earlier in the Acyline+hCG group than in the Control one, despite the similarity in the growth dynamics of the F1 between the groups. This idea is clearly demonstrated by the decrease in F2 growth rate (~ 0 mm/12 h) during the 12 h period prior to the F1 reaching 7.5 mm, whereas, the F2 growth rate in both the control and Acyline-treated heifers were both ~ 0.8 mm/12 h, similar to the F1 growth rate for all groups during this period (Figure 5). Prior to the experiment, we postulated that treatment with hCG would cause undersized deviation (see Hypothesis 3), based on our previous observations during hCG or pLH treatment during the second follicular wave. In that previous study, treatment with LH agonists decreased circulating FSH and growth rate of F2. In contrast, the decrease in F2 growth rate in the Acyline+hCG

treatment in this study was not associated with a detectable decrease in circulating FSH, compared to controls or Acyline treatment. The underlying basis for the earlier deviation of the F2 during Acyline+hCG remains unclear but may relate to changes in FSH pulses, as we speculated in hypothesis 3. Future studies should be designed to clearly test the regulation and physiological action of FSH pulses in follicle growth and diameter deviation.

In conclusion, this study provides strong support for the role of LH activity in follicle selection and diameter deviation based on: (1) completely inhibition of LH pulses/activity by the Acyline treatment, leading to (2) inhibition of follicle selection and diameter deviation; and (3) reestablishment of dominant follicle selection by the hCG treatment during Acyline treatment. The earlier decrease in the F2 growth rate and the increased frequency of undersized deviations in the Acyline+hCG group, as well as the later decrease in circulating FSH suggest that there may be molecular differences in the F1 between the controls and the Acyline+hCG groups, inhibiting F2 growth and FSH during hCG treatment. Future studies are needed to determine the precise molecular mechanisms that cause induction of LH responsiveness, i.e. LH receptors, in the granulosa cells of the dominant follicle near deviation and that permit LH to stimulate selection and continued growth of the dominant follicle.

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FIGURE LEGENDS

Figure 1. Data of two representative individual heifers in the Control group. Data collected every 12 h from ovulation until 6 days after, belong to: (a) diameter of the largest follicle or future dominant follicle (F1), the second largest follicle (F2), and the third largest follicle (F3). Open, inverted triangles indicate the day of saline treatment; b) FSH and LH circulating concentration. Data collected every 15 minutes for a 10 h period starting two hours after day 1.5 post-ovulation, belongs to: c-d) FSH and LH pulse patterns.

Figure 2. Data of two representative individual heifers in the Acyline group. Data collected every 12 h from ovulation until 6 days after, belong to: (a) diameter of the largest follicle (F1), the second largest follicle (F2), and the third largest follicle (F3). Open, inverted triangles indicate the day of Acyline treatment ($5\mu\text{g}/\text{Kg}$); b) FSH and LH circulating concentration. Data collected every 15 minutes for a 10 h period starting two hours after day 1.5 post-ovulation, belongs to: c-d) FSH and LH pulse patterns.

Figure 3. Data of two representative individual heifers in the Acyline+hCG group. Data collected every 12 h from ovulation until 6 days after, belong to: (a) diameter of the largest follicle or future dominant (F1), the second largest follicle (F2), and the third largest follicle (F3). Open, inverted triangles indicate the day of Acyline treatment ($5\mu\text{g}/\text{Kg}$) and beginning of hCG treatment (initial dose of 50 IU and subsequent ones of 100 IU/12h); b) FSH and LH circulating concentration. Data collected every 15 minutes for a 10 h period starting two hours after day 1.5 post-ovulation, belongs to: c-d) FSH and LH pulse patterns.

Figure 4. Mean \pm SEM for the diameter of the largest follicle or future dominant (F1), the second largest follicle (F2), and the third largest follicle (F3) for each of the Control, Acyline and Acyline+hCG group. Open, inverted triangles indicate (respectively) the mean day of saline treatment, Acyline treatment (5 μ g/Kg), and Acyline treatment (5 μ g/Kg) plus beginning of hCG treatment (initial dose of 50 IU and subsequent ones of 100 IU/12h). Data were normalized to F1 closest value to 7.5mm. Graphic scale from 4 to 9mm is larger than 9.1 to 14.5mm in order to visually assess the F2 and F3 dynamics.

Figure 5. Mean \pm SEM for the growth rate (mm/12h) of the largest follicle or future dominant (F1) and the second largest follicle (F2) for each of the Control, Acyline and Acyline+hCG group. When $P \leq 0.05$, an asterisk would be an indicative of the group that differs.

Figure 6. Mean \pm SEM for the FSH and LH circulating concentrations for each of the Control, Acyline and Acyline+hCG group. Open, inverted triangles indicate (respectively) the mean day of saline treatment, Acyline treatment (5 μ g/Kg), and Acyline treatment (5 μ g/Kg) plus beginning of hCG treatment (initial dose of 50 IU and subsequent ones of 100 IU/12h). Data were normalized to F1 closest value to 7.5mm. Probabilities for main effect of group (G), main effect of day (D) and interaction group-by-day (GD) are indicated for each hormone, and separately for days -1.5 to 0 and 0 to 4. When $GD \leq 0.05$, letters would be an indicative of difference among the groups.

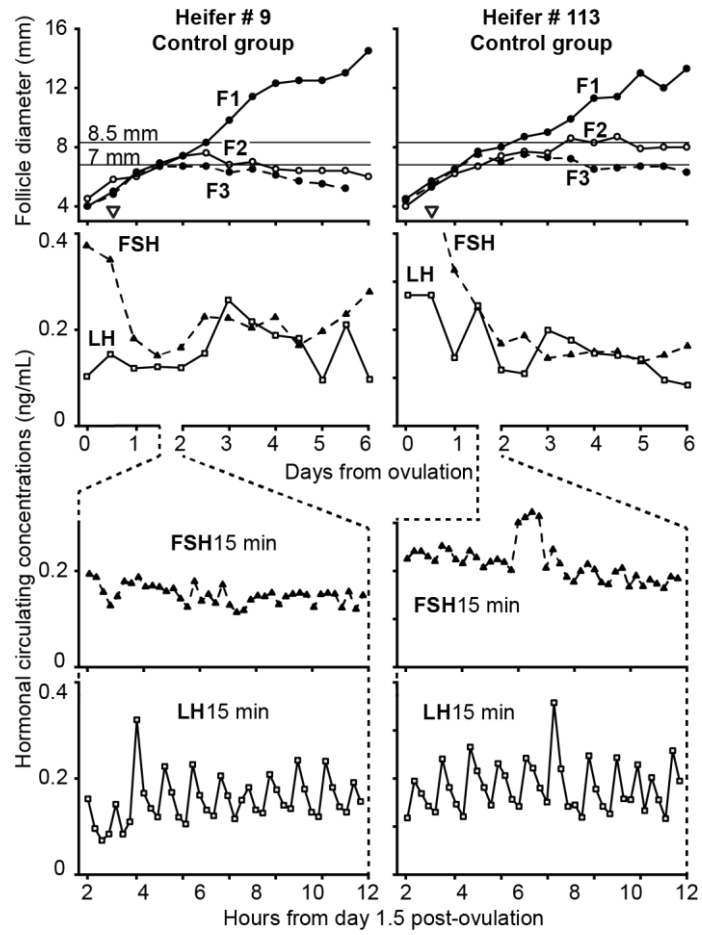


Figure 1.

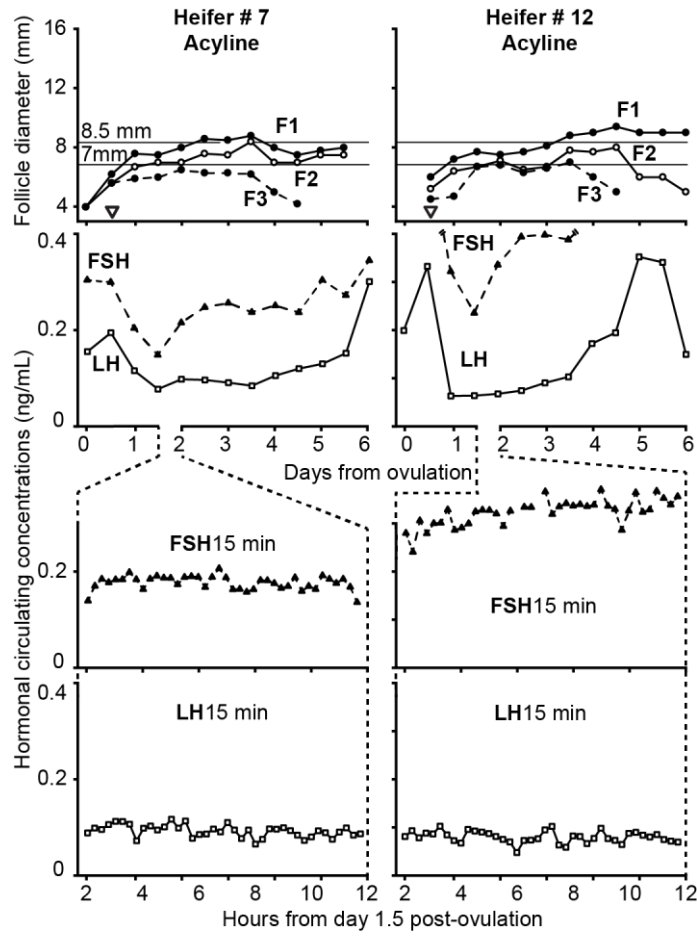


Figure 2.

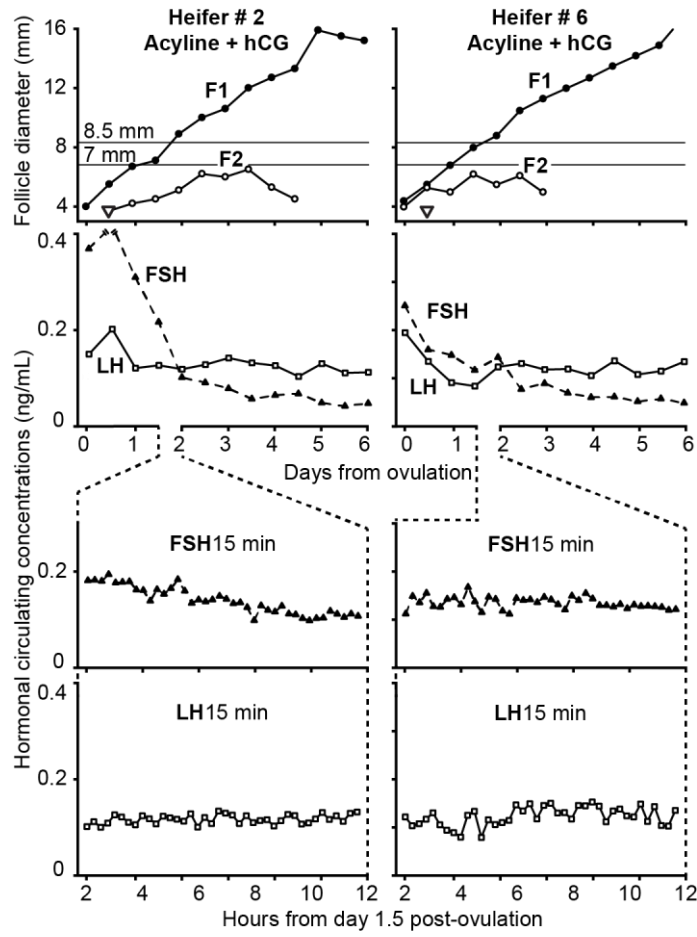


Figure 3.

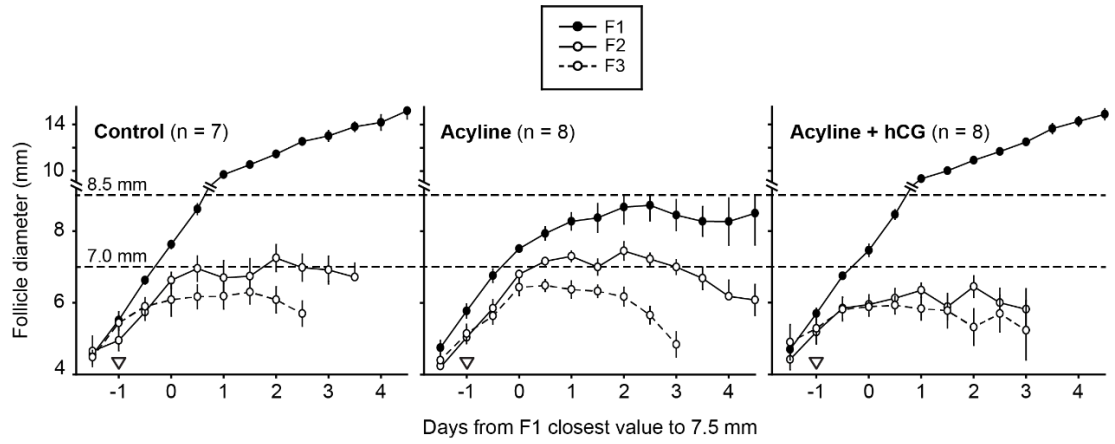


Figure 4.

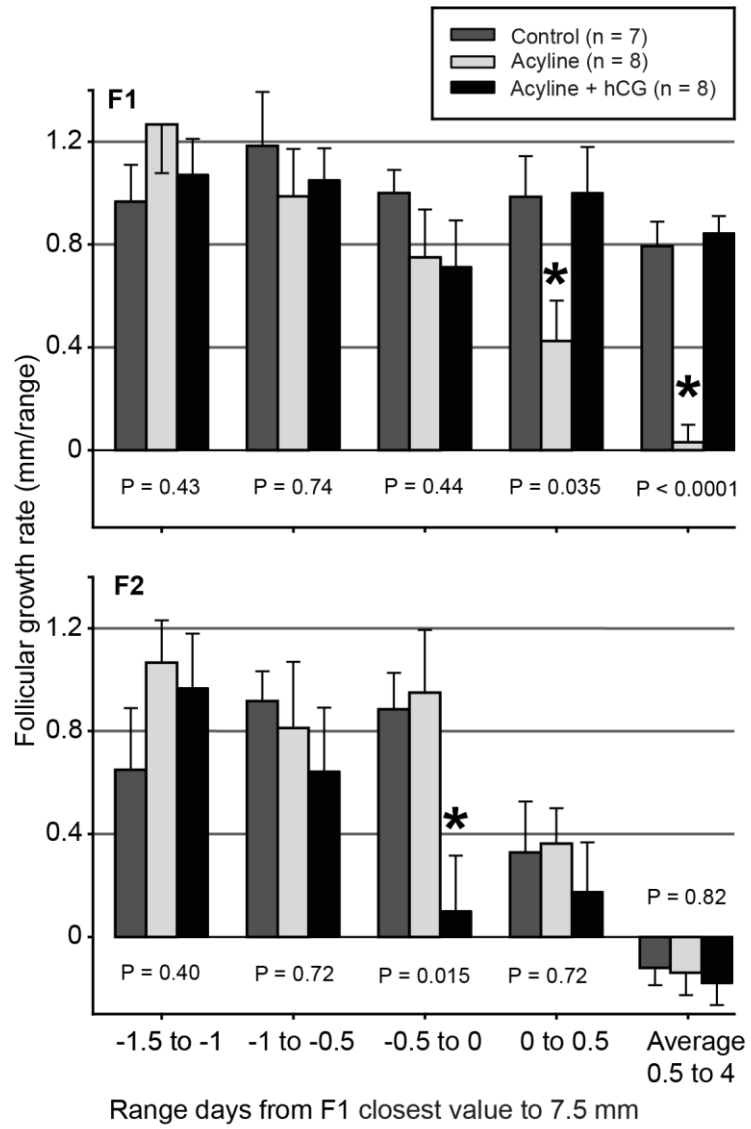


Figure 5.

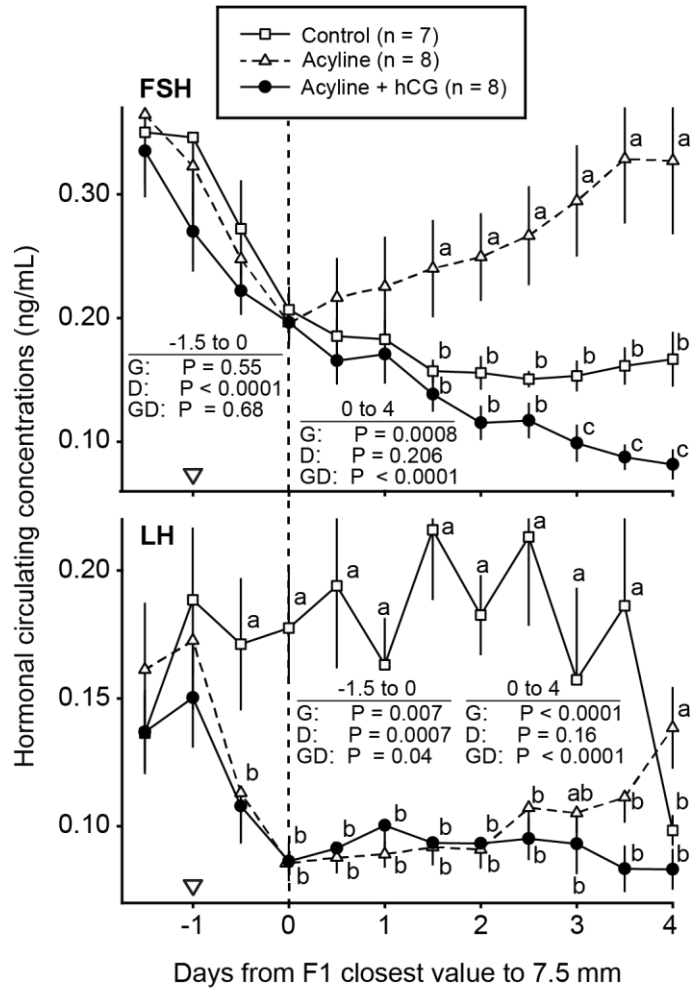


Figure 6.

FINAL CONSIDERATIONS

The present study represents a progress on our knowledge of the physiologic reproduction dynamics during the different stages of the antral follicles. The hormonal and antral follicular interactions profiles were characterized during different physiologic environments resulting in:

✓ The second follicular wave presented higher progesterone concentration associated to lower LH but higher FSH concentration during the expected time of follicular diameter deviation (F1 ~ 8.5 mm), as well as a smaller diameter of F2 when compared to the first follicular wave. Thus, an increased in the frequency of F2-undersized deviations was observed in the second follicular wave when compared to the first one.

✓ The spontaneous and induced second follicular wave presented similar follicle and hormonal profiles besides a greater FSH peak in the induced one. This result will aid the studies of the second follicular wave, which in most cases is an ovulatory wave.

✓ Higher LH activity was associated with lower FSH concentration and presumably with a greater dominant follicle activity producing more inhibitory factors to the FSH concentration, although a difference in the F1 diameter or growth rate was not observed.

✓ FSH nadir was associated with the time of diameter deviation, regardless the diameter of the follicles at this time or the concentration reached by the FSH. That is, diameter deviation occurred earlier and at a smaller diameter (F1 ~ 7.5 mm) in the presence of high progesterone concentration when compared to a later and larger diameter (F1 ~ 8.5 mm) in the absence of it. Nevertheless, the FSH nadir was greater in the presence of high circulating progesterone concentration than in the absence of it.

✓ Re-establishment of diameter deviation, follicle selection and growth during the dominance phase was done by the use of hCG in heifers treated with a potent GnRH antagonist (ablation of LH pulses), reassuring the function of LH during the deviation – selection process.

It was clearly shown on these experiments that high circulating progesterone concentration is associated to a smaller follicle size during the diameter deviation process regardless of greater or lower LH and FSH concentrations. Thus, this represents a new follicular model to be studied. However, the causes of this model and the molecular biology characterization, such as interactions among intrafollicular factors and genes, remains as a subject of our future studies to elucidate and confirm if follicle selection is happening at a smaller diameter in the presence of high progesterone concentration.