



## Ultrasonographic and endocrine aspects of follicle deviation, and acquisition of ovulatory capacity in buffalo (*Bubalus bubalis*) heifers

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### ABSTRACT

The objectives of this study were to determine the interval from ovulation to deviation and the diameter of the dominant (DF) and largest subordinate (SF) follicles at deviation in buffalo (*Bubalus bubalis*) heifers. Two methods of evaluation (observed vs. calculated) were used. FSH and LH profiles encompassing follicle deviation (Experiment 1), and the follicular diameter when the DF acquired ovulatory capacity (Experiment 2) were also determined. The time of deviation and the diameter of the DF and the largest SF at deviation did not differ between observed and calculated methods. Overall, follicle deviation occurred  $2.6 \pm 0.2$  d (mean  $\pm$  SEM) after ovulation, and the diameters of the DF and SF at deviation were  $7.2 \pm 0.2$  and  $6.4 \pm 0.2$  mm, respectively. No changes in plasma levels of FSH or LH were observed ( $P=0.32$  and  $P=0.96$ , respectively). Experiment 2 was conducted in two phases according to the diameter of the DF during the first wave of follicular development at the time of LH challenge (25 mg of pLH). In the first phase, follicles ranging from 5.0 to 6.0 mm ( $n=7$ ), 6.1 to 7.0 mm ( $n=11$ ), or 7.1 to 8.0 mm ( $n=9$ ) were used, and in the second phase, follicles ranging from 7.0 to 8.4 mm ( $n=10$ ), 8.5 to 10.0 mm ( $n=10$ ), or 10.1 to 12.0 mm ( $n=9$ ) of diameter were used. After the pLH treatment, the DF was monitored by ultrasonography every 12 h for 48 h. No ovulations occurred in heifers in the first phase. However, in the second phase, an effect of follicular diameter was observed on ovulation rate [7.0–8.4 mm (0.0%, 0/10), 8.5–10.0 mm (50.0%, 5/10), and 10.0–12.0 mm (55.6%, 5/9)]. In summary, follicle deviation occurred 2.6 d after ovulation in buffalo (*B. bubalis*) heifers, when the diameters of the DF and SF were 7.2 and 6.4 mm, respectively. No significant changes in plasma concentrations of FSH or LH were detected. Finally, the acquisition of ovulatory capacity occurred when the DF reached 8.5 mm in diameter.

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### 1. Introduction

Studies on follicle deviation during waves of ovarian follicular development (Castilho et al., 2007; Gimenes et al., 2008; Ginther et al., 2003; Sartorelli et al., 2005) and ovu-

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latory capacity (Gimenes et al., 2008; Sartori et al., 2001) in *Bos taurus* and *Bos indicus* cattle have demonstrated great practical importance of these physiological events in cattle reproduction. This knowledge can be used to manipulate the estrous cycle of cattle, allowing the use of biotechnologies such as synchronization of ovulation for timed artificial insemination, or superstimulation for multiple ovulation and embryo transfer. However, precise information regarding follicular selection and the diameter when ovulation from the dominant follicle (DF) is able to occur in response to an exogenous stimulus in buffalo (*Bubalus bubalis*) is still missing.

Therefore, the objectives of the present study were (1) to determine the interval from ovulation to follicle deviation, and the diameters of the dominant (DF) and largest subordinate follicle (SF) at that time, comparing two methods of evaluation of follicle deviation (observed compared to calculated) in buffalo (*B. bubalis*) heifers; (2) to investigate the changes in circulating concentrations of FSH and LH encompassing follicle deviation; and (3) to determine the range of follicular diameters at which the DF acquires the capacity to ovulate in response to exogenous LH.

## 2. Materials and methods

### 2.1. Experiment 1: Follicle deviation

#### 2.1.1. Animal management and synchronization protocol

The study was conducted according to approved University of São Paulo Animal Care Committee protocol number 433/2004. Nineteen postpubertal Murrah (*B. bubalis*) heifers, 20–36 months of age, and weighing 340–518 kg were maintained on pasture (*Brachiaria decumbens* and *Brachiaria brizantha*), with corn silage supplementation and free access to water at APTA (Agência Paulista de Tecnologia dos Agronegócios, Registro, São Paulo, Brazil). Prior to initiating the study, all heifers were treated to synchronize time of ovulation: GnRH (Gestran Plus<sup>®</sup>, Tecnopec, Brazil) followed by PGF<sub>2α</sub> (Ciosin<sup>®</sup>, Intervet Schering-Plough, Brazil) 7 d later, and GnRH 48 h after PGF<sub>2α</sub>. This procedure was done to assure that all heifers were in the same phase of estrous cycle (first follicular wave).

#### 2.1.2. Ultrasound examinations

All heifers were examined by transrectal ultrasonography (Pie Medical – Scanner 100) every 12 h from the second GnRH to ovulation, to determine the time to ovulation (D0). Only heifers that ovulated ( $n = 12$ ) were used. From the time of ovulation, the heifers were examined every 8 h until D4, and then every 24 h until D6, in order to detect the time of follicle deviation, and the diameter of the two largest follicles at the time of deviation. All examinations were performed by a single operator.

Ovaries were mapped by recording the diameters of the three largest follicles at each examination. Observed deviation was determined retrospectively by tracking the two largest follicles and classifying them as the DF and the largest SF. Only follicular waves with a single DF were considered. The beginning of follicle deviation was defined as the end of a common growth phase, when differences in diameters between the two largest follicles were detected

(Ginther et al., 2001). All data were normalized to the day of observed deviation. Calculated deviation was determined using the same data used for observed deviation; however, a segmented linear regression model was applied to the data. The segmented regression model consists of two segments (1 and 2) and a Join Point. Basically, Segment 1 represented the common growth phase, Segment 2 represented the period of dominance thereafter, and the Join Point represented the beginning of deviation (Bergfelt et al., 2003).

#### 2.1.3. Blood collection and gonadotrophin assays

Blood samples were collected by jugular venipuncture into 10 mL heparinized tubes (Vacutainer<sup>®</sup>, Becton-Dickinson & Company) which were immediately refrigerated at 4 °C. Refrigerated samples were centrifuged (3000 × g for 20 min) within 1 h after collection, and the plasma was stored at –20 °C until assayed. Blood collections were done at 8-h intervals for the first 24 h post ovulation, then at 4-h intervals until D4, and then at 12-h intervals until D6. Plasma concentrations of FSH and LH were determined, using a validated RIA for cattle, according to methodology described by Bolt et al. (1990). A parallelism test was performed for validation in the buffalo species. The sensitivity was 0.1 ng/mL for FSH and 0.01 ng/mL for LH. Intraassay coefficients of variation (CV) were 13.5, 14.9, and 16.0% for FSH, and 15.0, 15.4, and 19.3% for LH. Interassay CV were 13.4% and 9.7%, respectively.

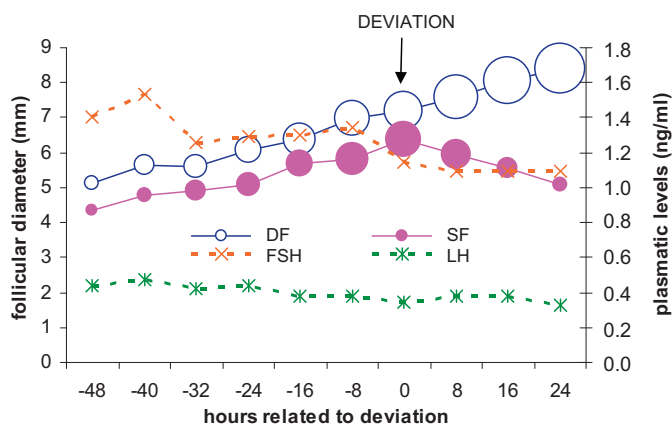
### 2.2. Experiment 2: Ovulatory capacity

#### 2.2.1. Animal management and synchronization of ovulation time protocol

This experiment was conducted in two phases. In the first phase, 38 Mediterranean buffalo heifers, 15–40 months of age and weighing 344–618 kg, were maintained on pasture (*B. decumbens*, *B. brizantha* and *Pennisetum* sp.) with free access to water at Santo Antônio dos Três Rios Farm (Caraguatatuba, São Paulo, Brazil). In the second phase, 59 crossbred Murrah × Mediterranean buffalo heifers, 16–45 months of age and weighing 359–610 kg, were maintained on pasture (*B. decumbens* and *B. brizantha*) with free access to water at Santa Eliza Farm (Dourado, São Paulo, Brazil). All animals had time of ovulation synchronized as described in Section 2.1.1. Only heifers that ovulated ( $n = 27$  in the first phase, and  $n = 29$  in the second one) were used.

#### 2.2.2. pLH treatment

In the first phase of Experiment 2, heifers were examined by ultrasonography after ovulation (D0) until the first-wave DF reached 5.0–6.0 mm ( $n = 7$ ), 6.1–7.0 mm ( $n = 11$ ), or 7.1–8.0 mm ( $n = 9$ ) in diameter. At this time, heifers were treated with 25 mg of pLH i.m. (Lutropin-V<sup>®</sup>, Bioniche Animal Health, Inc., Belleville, ON, Canada). In the second phase, heifers were treated with pLH when the first-wave DF reached 7.0–8.4 mm ( $n = 10$ ), 8.5–10.0 mm ( $n = 10$ ), or 10.0–12.0 mm ( $n = 9$ ) in diameter.



**Fig. 1.** Mean ( $\pm$ SEM) diameters of the dominant (DF) and largest subordinate (SF) follicles normalized to the day of deviation in 10 buffalo heifers (observed deviation). Heifers were examined by ultrasonography every 8 h from ovulation (Day 0) to Day 4. Overall, deviation occurred  $2.6 \pm 0.2$  d after ovulation, when the mean ( $\pm$ SEM) diameters for the DF and the largest SF were  $7.2 \pm 0.2$  and  $6.4 \pm 0.2$  mm, respectively. The growth rate of the DF differed from that of the largest SF 12 h after follicle deviation ( $P < 0.0001$ ). No changes in FSH or LH profiles were observed encompassing follicle deviation.

### 2.2.3. Ultrasonographic examinations

Ultrasonographic examinations were performed at second GnRH treatment and 48 h later, to identify the heifers that had ovulations. Then, the first-wave DF was monitored every 12 h until the time of the pLH treatment, and then for 48 h after the pLH treatment.

### 2.3. Statistical analysis

Prior to the analysis, normal distribution of the data was confirmed. A paired *t*-test was used to compare the methods (observed and calculated) of determining the diameter of the DF and largest SF at the time of follicle deviation. The slopes of the regression lines were used to reflect growth rate (mm/8 h) before (Segment 1) and after (Segment 2) follicle deviation for DF and SF. Growth rates were compared by ANOVA, using a Mixed Model.

FSH and LH plasma levels were also tested for normality and homogeneity of variances. Data were transformed to  $\log(\text{FSH}) - \log_{10}X$  – and square root (LH) –  $\text{SQRT } X$ , and analyzed by ANOVA using a Mixed Model.

Continuous variables (i.e., diameter of DF at the time of pLH treatment, follicle diameter at the time of ovulation, and interval from pLH treatment to ovulation) were analyzed by ANOVA using the GLM procedure of SAS. Groups were compared by the Tukey test. The proportion of animals that ovulated within groups was analyzed using the GENMOD procedure. All statistical analyses were done with the SAS software (SAS, Cary, NC, USA). Results are shown as mean  $\pm$  SEM. Strong evidence of effect is indicated by a probability of significance  $< 0.05$  and some evidence is indicated by probabilities between 0.05 and 0.10.

## 3. Results

### 3.1. Experiment 1: Follicle deviation

Nineteen heifers were pre-selected to use in the present study, however only 12 ovulated. The size of the follicle from which ovulation occurred in these 12 heifers

was  $11.9 \pm 0.6$  mm in diameter and ovulation occurred  $31.0 \pm 3.5$  h after the second GnRH treatment. Among these heifers, two were excluded because they had co-dominance (i.e., two dominant follicles). Thus, from 19 animals, only 10 were used in this study.

The future DF ( $4.0 \pm 0.2$  mm) was larger than the largest SF ( $3.4 \pm 0.1$  mm;  $P = 0.007$ ) on the day of ovulation (Day 0). There was no difference between observed and calculated deviations, thus data were combined. Overall, deviation occurred  $2.6 \pm 0.2$  d after ovulation, and the mean ( $\pm$ SEM) diameters of the DF and the largest SF at deviation were  $7.2 \pm 0.2$  and  $6.4 \pm 0.2$  mm, respectively.

The growth rate (mm/8 h) of the DF was similar before and after follicle deviation ( $0.42 \pm 0.04$  and  $0.43 \pm 0.07$ , respectively;  $P = 0.68$ ). However, the growth rate of the largest SF was greater before than after deviation ( $0.38 \pm 0.05$  and  $-0.47 \pm 0.08$ , respectively;  $P < 0.0001$ ).

There was no effect of time on plasma concentrations of LH ( $P = 0.96$ ) and FSH ( $P = 0.32$ ). LH concentrations 48 h and 24 h before deviation, at deviation, and 24 h after deviation were  $0.44 \pm 0.06$ ,  $0.44 \pm 0.14$ ,  $0.35 \pm 0.10$ , and  $0.32 \pm 0.07$  ng/mL, respectively. FSH levels at these same times were  $1.40 \pm 0.17$ ,  $1.29 \pm 0.13$ ,  $1.14 \pm 0.10$ , and  $1.10 \pm 0.08$  ng/mL, respectively. The follicular dynamics and the hormonal profiles, adjusted for 8 h interval, are shown in Fig. 1.

### 3.2. Experiment 2: Ovulatory capacity

In the first phase of this experiment, 27 of the 38 heifers ovulated  $24.7 \pm 1.5$  h after second GnRH treatment. The mean diameter of the ovulatory follicle was  $11.9 \pm 0.3$  mm. None of the heifers ovulated following pLH treatment, regardless of follicle size.

In the second phase, 29 of 59 heifers ovulated after the second GnRH treatment. The mean diameter of the follicle from which ovulation occurred was  $11.5 \pm 0.3$  mm. One heifer from the “10.0–12.0 mm group” was excluded because she developed a follicular cyst. Only heifers in the groups “8.5–10 mm” and “10.0–12.0 mm” responded to

pLH treatment. In the first group, 50% of heifers (5/10) had ovulations in a mean of  $37.2 \pm 2.9$  h after LH administration. In the second one, 55.6% of heifers (5/9) ovulated in a mean of  $30.0 \pm 3.8$  h. Overall, ovulations did not occur from follicles less than 8.5 mm in 37 heifers, while 52.6% (10/19) of ovulations did occur from follicles above that diameter at an average of 33.6 h after LH treatment.

#### 4. Discussion

The importance of the present study is that it describes for the first time the interval from ovulation to deviation, the diameter of DF and largest SF at deviation and the diameter at which the dominant follicle acquires the capacity for ovulation in *B. bubalis* heifers.

Both methods of evaluation of the diameter of the DF and the largest SF and time of follicle deviation were effective and statistically similar, as previously described by Bergfelt et al. (2003) in Holstein, and Sartorelli et al. (2005) and Gimenes et al. (2008) in Nelore cattle. Also, in the present study, the time of deviation was similar to that previously described in *B. indicus* (Castilho et al., 2007; Gimenes et al., 2008; Sartorelli et al., 2005) and *B. taurus* (Ginther et al., 1996, 1997) cattle.

A decrease in FSH concentrations and a transient rise in LH levels encompassing follicle deviation were not observed, contrary to that described in Holsteins (Ginther et al., 1998, 1999; Kulick et al., 1999). This could be attributed to the small number of animals used in the present study, which also showed heterogeneous patterns for both gonadotropins. Frequency of blood collection (each 4 h) probably was not the cause of our inability to detect LH changes, and possibly stress associated with handling also was not. Although stress may affect LH patterns mediated through effects at the hypothalamus or at the pituitary (Dobson and Smith, 2000), it was demonstrated that frequent blood collection (each 1 h during 24 h) in buffalo did not impair LH peak or ovulations (Porto Filho, 2000).

In the present study, 8.5 mm was the threshold diameter for ovulation after treatment with pLH. This can be related to an increase in follicle diameter and presence of LHR on granulosa cells, as recently reported in *B. indicus* (Simões et al., 2010). In this study, the relationship among follicular diameter, ovulation rate, and gene expression of LH receptor was investigated in Nelore cattle. The authors observed an increase in ovulation rate according to follicle diameter when 6.25 mg of LHP was used (group 7.0–8.0 mm: 9%, group 8.1–9.0 mm: 36%, and group 9.1–10.0 mm: 90%). Besides that, an increase in LHR in granulosa cells of follicles at the same range diameters was observed (16.5%, 21.0%, and 37.6%, respectively). Nevertheless, there are reports in multiparous Mediterranean buffalo (Campanile et al., 2007, 2008) in which ovulations following administration of hCG or GnRH agonist were observed in follicles ranging from 4.2 to 13.0 mm. We are not fully convinced that ovulations can be induced in follicles of 4 mm, as studies in cattle seem to indicate expression of LHR gene in granulosa cells around the time of follicle selection (Bao et al., 1997; Barros et al., 2010; Beg et al., 2001; Xu et al., 1995). No information seems to be available concerning expression of the LHR gene in buffalo follicles.

Finally, studies on follicular selection are important in basic research, and can be applied to improve efficiency of timed artificial insemination and superovulation (reviewed in Lucy, 2007; Barros et al., 2010). Concerning capacity for ovulation, the major role may be associated with follicular diameter and pregnancy rates. In cattle, some reports correlate a larger pre-ovulatory follicle with higher pregnancy rates, due a greater progesterone concentration from the newly formed corpus luteum. This greater progesterone stimulates the conceptus to produce higher concentrations of IFN- $\delta$ , which prevents luteolysis (reviewed in Binelli et al., 2001; Perry et al., 2007). However, similar information in buffaloes does not exist. Thus, novel research will be necessary to elucidate the relationship between follicular diameter and fertility in the buffalo species.

In conclusion, follicle deviation occurred 2.6 d after ovulation in buffalo (*B. bubalis*) heifers. At that time, diameters of the DF and the largest SF were 7.2 and 6.4 mm, respectively. No significant changes in plasma concentrations of FSH or LH were observed. There was an analogous efficacy of both methods (observed and calculated) to determine deviation. Finally, the acquisition of capacity for ovulation occurred when the DF reached 8.5 mm in diameter in these buffalo heifers.

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