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# Relationship between growth of the preovulatory follicle and its steroidogenic activity on the onset and expression of estrus behavior in CIDR-treated *Bos indicus* cows: An observational study

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## HIGHLIGHTS

- The pattern of follicular growth contributes to the delay in the onset of estrus.
- Cows that delay the onset of estrus have an increased steroidogenic activity.
- Estradiol levels and P450arom expression are reduced in cows not detected in estrus.
- Preovulatory follicle growth and its steroidogenic activity affect sexual behavior.

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## ABSTRACT

Estrus synchronization induces cows to gather in sexually active groups (SAGs) composed of females displaying mounting activity. Although this technique promotes the enhancement of sexual behavior, there are cows in estrus (CE) that delay estrus expression and also cows not displaying estrus (CNDE) even in the presence of a preovulatory follicle (PF). To elucidate the physiological mechanisms of the delay in the onset of estrus or absence of estrus behavior, an observational study was undertaken in 17 *Bos indicus* cows treated with exogenous progesterone (CIDR) to synchronize estrus and to monitor follicular growth and its steroidogenic activity. After SAGs formation, cows were ovariectomized at 24, 48, and 72 h post-CIDR. Among ovariectomized groups there were only 9 CE which: 1) showed differences in the onset of estrus; 2) displayed distinctive follicular growth patterns; and 3) at 72 h produced the highest intrafollicular estradiol concentration, and showed a linear trend to increase expression of P450scc and P450arom. Comparison of CE vs. CNDE showed that: 1) both groups had progesterone levels indicative of cyclic activity, and a PF which grew at a similar rate and size; 2) CE showed a stronger association between time and growth; and 3) CE produced more intrafollicular estradiol and progesterone, together with the expression of higher levels of P450arom. Results suggest that pending on the pattern of growth of the PF and its steroidogenic potential to produce estradiol, the onset and expression of estrus behavior may be delayed probably until the establishment of the appropriate conditions to ensure ovulation.



## 1. Introduction

Accurate estrus detection in cattle remains the single most important limitation to the use of assisted reproductive techniques [1]. Estrus synchronization using pharmacological manipulation of the estrous cycle therefore constitutes a reliable approach to overcome this problem. However, the development of an efficient synchronization program depends on the physiological changes and behavior during the estrous cycle [2].

A behavioral response to estrus synchronization is the formation of sexually active groups (SAGs) which consist of a variable number of females displaying mounting activity [3]. This kind of grouping in cows may result from their tendency to manifest synergistic sexual behavior produced by a social facilitation effect [4]. There are several factors that can advance, delay or completely inhibit estrus. For instance, pharmacological treatments for synchronization of estrus in *Bos indicus* cattle produce a low and variable response in the expression of sexual behavior (reviewed in [5]). We have found that there are cows in estrus (CE) accelerating or delaying the appearance of

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overt signs of estrus [6–10] due to the tendency of these cows to cluster sexual behavior, sometimes without apparent preovulatory follicle (PF) support [11,12]. Even with the presence of a PF at the expected moment of sexual activity, there are cows not displaying estrus (CNDE) as well as CE failing to ovulate [11–14]. This effect is possibly associated with insufficient production of steroid hormones by the growing PF [13], which is normally characterized by a high steroidogenic synthesis by the cytochrome P450scc and P450arom enzymes. In any case, the result is a lower than expected response in the expression of estrus, accompanied by reduced fertility.

The behavioral response to the synchronization of estrus requires two main events, the growth of the PF and the development of its steroidogenic activity enabling the production of sex hormones to sustain the expression of estrus after the pharmacological stimuli. Consequently, a thorough evaluation of these factors should improve our understanding of the physiological requirements for sustaining estrus behavior. However, previous studies have considered neither the entire pattern of follicular growth nor the production of sex hormones as physiological factors that could be affecting the onset and expression of estrus behavior in synchronized cows. Therefore to elucidate the mechanisms producing the delay in the onset or lack of estrus expression in CIDR-synchronized *B. indicus* cows, we tested the hypotheses that 1) CE delaying the onset of estrus have different PF growth characteristics than those CE accelerating sexual behavior, and 2) in the CNDE the lack of estrus expression is associated with reduced intrafollicular production of estradiol, the hormone producing sexual activity.

## 2. Materials and methods

### 2.1. Animals and experimental design

The study was conducted in accordance with the Experimental Unit Directive of the University concerning the use of animals for experimentation.

Seventeen multiparous, non-lactating *B. indicus* (Brahman) cows aged 3–6 years exhibiting regular estrous cycles were randomly selected. Cows were allocated in one $n=5$ and two groups of $n=6$, and treated for nine days with a progesterone-releasing intravaginal device (CIDR, Pfizer, Mexico). CIDR was inserted with a 24 h difference between groups to produce an experimental model for expressing estrus sequentially [7]. To induce CL regression and start follicular growth, all cows received a 25 mg injection of $PGF_{2\alpha}$ i.m. (Lutalyse, Pharmacia & Upjohn Inc., Kalamazoo, MI, USA) after implant removal. Ovaries were removed from these cows at three different moments in which SAGs were formed at 24, 48, and 72 h post-CIDR; when mounting activity and sexual behavior were displayed by three or more cows [3]. Sexual behavior was recorded in the SAGs by continuous observation for 100 h using a previously proposed method [15]. Retrospective analysis of this information allowed us to determine the onset of estrus for the CE and to identify the cows not displaying estrus (CNDE). In any case, all cows were ovariectomized. To maintain the social structure [16] and to reduce the effect of the alteration of social rank within the herd [17], the cows remained together in the same paddock and handling was limited as much as possible. Animals were kept in the State of Veracruz, Mexico at 20°4′ N and 97°3′ W.

### 2.2. Surgery and collection of follicles and follicular fluid

Ovaries were obtained by transvaginal ovariectomy under epidural anesthesia (5% Xylocaine, 2 to 6 ml/cow) and transported to the laboratory in ice-cold saline solution (0.9 % NaCl). A sample of follicular fluid (200 μl) was aspirated from the PF, centrifuged to remove cellular debris and stored at −20 °C for later assay of steroid concentrations. Blocks of tissue (about 25 mm thick) containing the PF were dissected from the ovaries, embedded in Tissue-Tek OCT compound (Sakura, Torrance CA, USA), snap frozen in liquid $N_2$ fumes and stored at −80 °C until they were cut into 18 μm sections at −20 °C using a cryostat and mounted onto microscope slides (Superfrost Plus, Fisher Scientific, USA). Slides were air-dried and stored at −80 °C until immunofluorescence analysis.

### 2.3. Ultrasonography and blood sampling

Growth of individual follicles (>4 mm in diameter) in each animal was followed every 4 h from 0700 to 2000 h from implant removal until ovariectomy. This period was selected to avoid, as much as possible, interruptions in sexual behavior normally displayed during the interval from 2100 to 0600 h [1]. A 7.5 MHz transducer was used to perform transrectal ultrasonography (Aloka SSD -210DXII, Aloka, Tokyo, Japan) based on a previously described methodology for monitoring follicular growth [18]. The following variables were defined: 1) duration of growth, the number of days between the first measurement and its maximum recorded size; 2) initial diameter; 3) final diameter, maximum size measured before ovariectomy; 4) total growth, final diameter minus initial diameter; 5) daily growth rate, total growth divided by duration of growth; and 6) growth percentage, quotient of total growth between initial diameter multiplied by 100. Along with ultrasonography examination, coccygeal vein blood samples were taken from all animals by venipuncture to evaluate serum progesterone concentration. Blood samples were collected in heparinized tubes and centrifuged within 15 min of collection. Serum was stored at −4 °C until assayed.

### 2.4. Steroid hormone assays

For determination of serum progesterone concentration, 100 μl of unextracted aliquots was measured in duplicate via RIA according to the instructions of a commercial kit (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA, USA). The sensitivity of the assay was 0.1 ng/ml, and the intra-assay coefficient of variation (CV) was 3.6%. Samples were assayed together.

Concentrations of intrafollicular estradiol, testosterone and progesterone were measured in unextracted aliquots by ELISA standard technique. The following primary antibodies and concentrations were used: anti-estradiol (rabbit polyclonal R4972) 1/8000; anti-testosterone (rabbit polyclonal R-156/7) 1/35,000; and finally anti-progesterone (rabbit polyclonal R4859) 1/35,000. Samples were analyzed in triplicate within a single assay and diluted to the following concentrations: estradiol and progesterone, 1/300; and testosterone, 1/50. The following conjugate concentrations were used: estradiol (1/20,000), testosterone (1/40,000) and progesterone (1/50,000) all of them diluted in PBS. All antibodies, standards and conjugates were kindly donated by Dr. C. Munro (University of California, Davis, USA). The progesterone antibody cross-react 21% with 11α-hydroxyprogesterone, 29% with 5α-pregnene-3, 20-dione, and < 0.05% with other steroids, meanwhile estradiol antibody cross-reacted 100% with 17β-estradiol, 3.3% with estrone and < 1% with others steroids. Testosterone antibody cross-reacted 100% with testosterone, 57.4% with 5α-dihydrotestosterone, 0.27% with androstenedione and < 0.1% with others steroids. The sensitivity of the assay was 7.5, 2.4 and 0.4 ng/ml for estradiol, testosterone, and progesterone, respectively. For the same steroids, the intra-assay CV was 7.1, 8.1 and 4.8%.

### 2.5. Immunofluorescence

For immunofluorescence, a modification of a previously published method was used [19]. Rabbit anti-Cytochrome P450scc Enzyme (1:100; Ab1244; Chemicon, Millipore, Temecula, CA, USA) or rabbit anti-Aromatase (1:100; ab18995; Abcam, Cambridge, UK) primary antibodies, and goat anti-rabbit IgG (H+L) Alexa Fluor 488 or