Universidade Estadual de Londrina

FÁBIO MOROTTI

# INFLUÊNCIA DA CONTAGEM DE FOLÍCULOS ANTRAIS NA DINÂMICA FOLICULAR OVARIANA E NOS PARÂMETROS DE MÉRITO GENÉTICO DE FÊMEAS BOVINAS

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Tese de Doutorado apresentada ao Programa de Pós-graduação em Ciência Animal da Universidade Estadual de Londrina, Área de Concentração Produção Animal, como requisito para obtenção do título de Doutor em Ciência Animal.

Orientador: Prof. Dr. Marcelo Marcondes Seneda.

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Londrina, 29 de Julho de 2016.

## DETICATÓRIA

Dedico este trabalho aos meus país, Onivaldo e Dizolina, pelo imenso apoio, carinho, amor e inestimável fonte de perseverança para que eu pudesse prosseguir firme e confiante em minha caminhada, árdua, porém recompensadora!

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“O começo da sabedoria é encontrado na dúvida, duvidando começamos a questionar, e procurando podemos achar a verdade.”

Pierre Abelard

“Senhor, dai-me força para mudar o que pode ser mudado... Resignação para aceitar o que não pode ser mudado... E sabedoria para distinguir uma coisa da outra.”

Francisco de Assis

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## RESUMO

O objetivo deste trabalho foi I) avaliar a influência da baixa e alta contagem de folículos antrais (CFA) sobre a dinâmica folicular ovariana de vacas Nelore (*Bos indicus*) durante a sincronização da ovulação, e II) determinar a repetibilidade da CFA e se há correlação das características de mérito genético com a CFA em novilhas Braford (*Bos indicus-taurus*). No experimento I, 250 vacas multiparas foram avaliadas por ultrassom para determinação da CFA e seleção dos grupos de alta (≥ 45 folículos, N = 43) ou baixa CFA (≤ 15 folículos, N = 32). Em um dia aleatório do ciclo estral (D0), as vacas receberam um protocolo convencional de sincronização da ovulação (3 mg norgestomet e 2 mg benzoato de estradiol no D0 e remoção do implante + 0,150 mg D-cloprostenol + 300 UI de gonadotrofina coriônica equina + 1 mg cipionato de estradiol no D8) para o monitoramento da dinâmica folicular ovariana. Os dados foram submetidos a ANOVA e teste de Tukey ($p < 0,05$). No experimento II, a CFA de novilhas (N = 137) foi monitorada por ultrassom (a cada 60 dias) do desmame (9 meses) até o sobreano (20 meses). Posteriormente, a CFA destes animais e de novilhas contemporâneas (N = 270) foi correlacionada com registros de programa de seleção genética utilizando quatro modelos estatísticos: Modelo 1 - efeito do grupo contemporâneo e covariáveis idade, ganho de peso do nascimento ao desmame e escores visuais de características de carcaça ao desmame; Modelo 2 - efeito do grupo contemporâneo e covariáveis idade, ganho de peso do desmame ao sobreano e escores visuais de características de carcaças ao sobreano. Os efeitos, variáveis e covariáveis dos Modelo 1 e 2 foram combinados no Modelo 3. O Modelo 4 foi estabelecido pelo Modelo 3 adicionado do efeito touro. No experimento I, o número de folículos antrais do grupo de alta foi superior ($p = 0,01$) ao de baixa em todas as avaliações. O diâmetro, o perímetro e a área do ovário foram maiores ($p = 0,0001$) nas vacas de alta do que nas de baixa CFA (28,3 ± 3,9 *vs.* 20,5 ± 3,2 mm, 100,9 ± 13,2 *vs.* 73,8 ± 13,9 mm e 67,5 ± 16,4 *vs.* 37,7 ± 11,7 $mm^2$; respectivamente). A taxa de crescimento folicular, o tempo de ovulação e o diâmetro do corpo lúteo foram semelhantes ($p > 0,05$) entre os grupos. Os diâmetros foliculares foram maiores ($p < 0,05$) nas vacas de baixa do que nas de alta CFA: no D4 (7,3 ± 2,2 *vs.* 6,2 ± 1,4 mm; $p = 0,069$, tendência), no D8 (11,2 ± 1,8 *vs.* 9,5 ± 1,8 mm), no D9 (12,3 ± 1,7 *vs.* 10,6 ± 1,7 mm), no D10 (13,4 ± 1,3 *vs.* 12,2 ± 1,8 mm) e tendência ($p = 0,08$) para um maior diâmetro do folículo pré-ovulatório (14,4 ± 1,5 *vs.* 13,4 ± 2,1 mm). No experimento II, a CFA foi variável entre as novilhas (3 a 64 folículos), mas repetível nas fêmeas do mesmo grupo (0,89-0,92). Os quatro modelos (1, 2, 3 e 4) apresentaram baixa correlação com a CFA (0,072, 0,056, 0,082 e 0,172; respectivamente). O modelo com efeito touro foi o que melhor explicou a influência genética com a CFA (17%). A precocidade de acabamento ao desmame revelou uma correlação linear negativa com a CFA ($p < 0,05$). No estudo I, vacas Nelore com alta CFA apresentaram maiores mensurações ovarianas. Entretanto, fêmeas de baixa CFA apresentaram maior diâmetro folicular durante o protocolo de sincronização da ovulação. No estudo II, a CFA de novilhas Braford apresentou baixa correlação com as características de mérito genético. No entanto, a CFA demostrou ser influenciada pelo escore de precocidade ao desmame, além de sugerir alguma relação com o efeito paterno.



**Palavras-chave:** Contagem folicular antral. Ultrassom. Dinâmica folicular. Mérito genético. Bovinos.

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## ABSTRACT

The aim of this study was I) to evaluate the influence of high and low antral follicle count (AFC) on the ovarian follicular dynamics in Nelore (*Bos indicus*) cows during synchronization of ovulation, and II) to determine the repeatability of the AFC and if the AFC is correlated with genetic merit characteristics in Braford (*Bos indicus-taurus*) heifers. In the experiment I, 250 multiparous cows were evaluated by ultrasound to determine AFC and selection of groups with high (≥ 45 follicles, n = 43) or low AFC (≤ 15 follicles, n = 32). On a random day of the estrous cycle (D0), cows received a conventional protocol to synchronization of ovulation (3 mg norgestomet and 2 mg estradiol benzoate in D0 and implant removal + 0,150 mg D-cloprostenol + 300 IU of equine chorionic gonadotropin + 1 mg cypionate estradiol in D8) for monitoring of ovarian follicular dynamics. Data were analyzed by ANOVA and Tukey test ($p < 0.05$). In the experiment II, the AFC in heifers (n = 137) were monitored by ultrasound (60 day intervals) from weaning (9 months) to yearling ages (20 months). After, AFC in these animals and contemporary heifers (n = 270) were correlated with records of genetic program using four statistical models: Model 1 - effects of contemporary group and covariates age, weight gain from birth to weaning and visual scores for carcass traits at weaning; Model 2 - effects of contemporary group, age, weight gain from weaning to yearling and visual scores for carcass traits at yearling. The effects, variables and covariates of Model 1 and 2 were combined to form Model 3. The Model 4 included the Model 3 with additon of bull effect. In the experiment I, the number of antral follicles was higher ($p = 0.01$) in cows with high count compared to the low AFC during all evaluations. Ovary diameter, perimeter and area were higher ($p = 0.0001$) in cows with high than low AFC (28.3 ± 3.9 *vs.* 20.5 ± 3.2 mm, 100.9 ± 13.2 *vs.* 73.8 ± 13.9 mm and 67.5 ± 16.4 *vs.* 37.7 ± 11.7 $mm^2$, respectively). The follicular growth rate, time of ovulation and *corpus luteum* diameter were similar ($p > 0.05$) between groups. Follicular diameters were greater ($p < 0.05$) in cows with low than in high AFC: at D4 (7.3 ± 2.2 *vs.* 6.2 ± 1.4 mm; $p = 0.069$, tendency), at D8 (11.2 ± 1.8 *vs.* 9.5 ± 1.8 mm), at D9 (12.3 ± 1.7 *vs.* 10.6 ± 1.7 mm), at D10 (13.4 ± 1.3 *vs.* 12.2 ± 1.8 mm) and tendency ($p = 0.08$) to a larger diameter of preovulatory follicle (14.4 ± 1.5 *vs.* 13.4 ± 2.1 mm). In the experiment II, AFC varied from 3 to 64 follicles among females but was repeatable (0.89 - 0.92) within individuals in the same group. The four models tested showed low correlations with AFC: 0.072, 0.056, 0.082 and 0.172 for models 1, 2, 3 and 4, respectively. The model with bull effect provided the best explanation for the genetic influence on AFC (17%).The finishing precocity at weaning showed a negative linear correlation with AFC ($p < 0.05$). In experiment I, Nelore cows with high AFC presented high ovarian measurements. However, the low AFC showed large follicular diameter during the synchronization of ovulation. In experiment II, the AFC in Braford heifers showed low correlation with the genetic merit characteristics. However, the AFC was influenced by finishing precocity at weaning and suggests some relationship with the paternal effect.



**Keywords:** Antral follicles count. Ultrasound. Follicular dynamics. Genetic merit. Cattle.

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## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| AFC / CFA | *antral follicles count* / contagem de folículos antrais |
| AG | age |
| AMH | *anti-Müllerian hormone* - hormônio anti-Mülleriano |
| ANOVA | análise de variância |
| FGF | *fibroblast growth factor* - fator de crescimento de fibroblasto |
| CL | corpo lúteo - *corpus luteum* |
| CG | *contemporary group* |
| CW | conformation at weaning |
| CY | *conformation at yearling* |
| BCS | *body-condition score* |
| D | dia - *day* |
| DF | dominant follicle |
| DP/SD | desvio padrão / *standard deviation* |
| EB | *estradiol benzoate* - benzoato de estradiol |
| EC | *estradiol cypionate* - cipionato de estradiol |
| eCG | *equine chorionic gonadotropin* - gonadotrofina coriônica equina |
| EPDs | *expected progeny differences* |
| ET / TE | *embryo transfer* - transferência de embriões |
| FOPA | folículos ovarianos pré-antrais |
| FSH | *follicle stimulant hormone* - hormônio folículo estimulante |
| GDF-9 | *growth and differentiation factor* - fator de crescimento e diferenciação |
| GFP | *green fluorescent protein* - proteína verde fluorescente |
| GW | *weight gain from birth to weaning* |
| h | *hour* |
| IA/AI | inseminação artificial / *artificial insemination* |
| IATF/TAI | inseminação artificial em tempo fixo / *timed artificial insemination* |
| IVF/ FIV | *in vitro fertilization* - Fecundação in vitro |
| IVP/ PIVE | *in vitro embryo production* - produção in vitro de embriões |
| kDa | kilodalton |
| Kg | quilograma |
| LH | *luteinizing hormone* - hormônio luteinizante |
| LIF | *leukemia inhibitor factor* - fator inibidor de leucemia |

| | |
|---|---|
| M | *mean* – média |
| mg | miligrama |
| mm | milímetro |
| MW | *musculature at weaning* |
| MY | *musculature at yearling* |
| N° | número |
| OPU | *ovum pick up* - aspiração folicular guiada por ultrassom |
| PGF2α | prostaglandina F2α |
| PW | *precocity at weaning* |
| PY | *precocity at yearling* |
| R2 / r | *coefficient of determination* |
| rRNA | *ribosomal ribonucleic acid* |
| SE | *standard error* |
| SOV | superovulação |
| STAR | *steroidogenic acute regularoty protein* - proteína de regulação aguda da esteroidogênese |
| TGF- β | *transforming growth factor* - fator transformador do crescimento |
| UI / IU | unidade internacioal / *international unit* |

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# 1 INTRODUÇÃO

O Brasil se destaca por possuir o maior rebanho comercial de bovinos do mundo com aproximadamente 212 milhões de cabeças (IBGE, 2014), das quais 80 a 85% são compostas por raças zebuínas e cruzamentos, animais altamente adaptados as nossas condições de clima e mantidos praticamente sob pastejo. Frente a este panorama promissor, estudos que foquem a reprodução e a produção animal são considerados de extrema importância tanto por agregar maior conhecimento sobre o manejo e biotécnicas reprodutivas, quanto por permitir o desenvolvimento de novas estratégias que visem o aumento da produtividade animal. Neste contexto, um fator crucial para o aumento na produção e maior eficiência pecuária está diretamente relacionado ao uso de animais de alto valor genético.

Animais de alto potencial genético podem ser multiplicados de forma eficiente com o emprego do manejo reprodutivo associado ao uso de biotécnicas reprodutivas, tais como a inseminação artificial (IA) convencional, a inseminação artificial em tempo fixo (IATF) e a produção *in vivo* (SOV/TE) e *in vitro* de embriões (PIVE). Estas ferramentas são consideradas fundamentais para o progresso do setor pecuário, pois favorecem a seleção, multiplicação e disseminação de animais de alta qualidade genética e elevado potencial produtivo em um curto espaço de tempo (HANSEN, 2014).

A produção na bovinocultura de corte e de leite tem se tornado cada vez mais eficiente nos últimos anos, e isto certamente deve-se ao maior conhecimento da fisiologia do ciclo estral na espécie bovina, que associado ao uso de estratégias farmacológicas específicas, possibilitaram o controle das fases de desenvolvimento folicular, facilitando o manejo reprodutivo e a implementação das biotécnicas reprodutivas (BARUSELLI et al., 2004; LAMB et al., 2010).

Nos últimos anos, houve um número expressivo de estudos aplicados à população folicular ovariana e sua influência sobre o desempenho reprodutivo de bovinos, bem como sobre a eficiência das biotécnicas reprodutivas (IRELAND et al., 2011; RICO et al., 2012; BATISTA et al., 2014; SILVA-SANTOS et al., 2014; SANTOS et al., 2016). No entanto, apesar dos frequentes avanços, muitos aspectos relacionados à fisiologia reprodutiva da fêmea permanecem desconhecidos, principalmente aqueles ligados às diferenças entre as subespécies (*Bos taurus vs. Bos indicus*), bem como as características relacionadas à população folicular ovarina e sua influência com a fertilidade do rebanho bovino (MOROTTI et al., 2015).

A alta variabilidade na população folicular ovariana (pré-antral e antral) é uma característica marcante em bovinos (ERICKSON, 1966; BURNS et al., 2005; SILVA-SANTOS et al., 2014). Apesar desta alta variabilidade entre as fêmeas, existe uma alta repetibilidade (0,85 a 0,95) da contagem folículos antrais (CFA; folículos ≥ 3 mm) no mesmo indivíduo durante as ondas de crescimento folicular (BURNS et al., 2005; IRELAND et al., 2007; 2008). Portanto, realizando apenas um exame ultrassonográfico para contagem de todos os folículos ≥ 3 mm de diâmetro é possível classificar as fêmeas em grupos de baixa, intermediária ou alta CFA.

Estudos europeus e norte americanos que avaliaram a correlação da CFA e o desempenho reprodutivo em fêmeas bovinas *Bos taurus* revelaram que o alto número de folículos antrais está diretamente relacionado a melhor *performance* reprodutiva. Em contrapartida, fêmeas com baixa CFA foram associadas com várias características relacionadas à baixa fertilidade (BURNS et al., 2005; IRELAND et al., 2007; 2008; 2009; 2011; MOSSA et al., 2012; EVANS et al., 2012; WALSH et al., 2014; MARTINEZ et al., 2016).

No Brasil, estudos realizados especificamente com gado *Bos indicus* e *Bos indicus-taurus*, também revelaram que a população de folículos antrais influencia positivamente a produção de embriões tanto no método *in vivo* quanto *in vitro* (SILVA-SANTOS et al., 2014; SANTOS et al., 2016). Nestes estudos, vacas de alta CFA apresentaram melhor *performance* para produção embrionária quando comparadas com vacas de baixa contagem folicular. Entretanto, avaliando a taxa de concepção à IATF, Rodrigues et al. (2013; 2015) e Santos et al. (2016) revelaram que o número de folículos antrais não exerce influência sobre a taxa de concepção de fêmeas Nelore com alta, intermediária ou baixa CFA. Em suma, estes resultados já se revelaram intrigantes, pois as vacas de baixa CFA, diferentemente dos estudos europeus e norte americanos, não apresentaram desempenho reprodutivo inferior aos demais grupos, como relatado em gado *Bos taurus*. Adicionalmente, outros trabalhos do mesmo grupo de pesquisa, conduzidos especificamente com fêmeas Nelore, revelaram taxa de concepção semelhante (MENDONÇA et al., 2013; MORAES, 2016) e até mesmo superior em vacas de baixa CFA (SANTOS et al., 2013; MORETTI, 2016).

Questões como o efeito da CFA na fertilidade de fêmeas bovinas precisam de uma atenção especial, particularmente em bovinos de corte (*Bos indicus*) em que esta relação ainda não é bem estabelecida. Além disso, considerando o impacto da seleção de fêmeas com alta CFA e o grande número de descendentes que podem ser produzidos *in vitro* a partir de uma única doadora, esta questão ganha um destaque altamente relevante, pois a relação entre a CFA e as características de mérito genético utilizadas nos programas de melhoramento é pouco conhecida. Um único estudo avaliou a herdabilidade e o impacto dos efeitos ambientais sobre a CFA em bovinos de leite, o qual sugeriu que a CFA possui de baixa à moderada herdabilidade, sendo influenciada pela idade, pelo *status* de lactação e pela qualidade do leite, mas não pelo nível de produção da mãe durante a gestação (WASLH et al., 2014). Sob outro aspecto, alguns estudos revelam indícios de que a baixa CFA parece ser mais vantajosa quando se pretende fazer o uso da IATF no rebanho de corte zebuíno. No entanto, não há publicações que avaliaram o comportamento da dinâmica folicular ovarina em fêmeas com diferentes CFAs.

Apesar dos inúmeros estudos sobre população folicular, as razões para as diferenças no número de folículos antrais em bovinos e a sua relação com a fertilidade ainda não são totalmente compreendidas. Várias lacunas precisam ser preenchidas para melhorar a compreensão das características reprodutivas de animais *Bos indicus* e *Bos taurus*, a fim de permitir ajustes no manejo e nas biotécnicas reprodutivas e, consequentemente, melhorias nos sistemas de produção.

Neste contexto, o objetivo deste trabalho foi avaliar se a contagem de folículos antrais I) exerce influência nos parâmetros de dinâmica folicular ovariana de vacas Nelore (*Bos indicus*) durante o tratamento hormonal; e II) se há correlação com parâmetros de mérito genético utilizados em programa de melhoramento de novilhas Braford (*Bos indicus-taurus*).

# 2 REVISÃO DE LITERATURA

## 2.1 Oogênese e a foliculogênese

Nas espécies de animais domésticos, as fêmeas nascem com um estoque de oócitos formado ainda durante a vida fetal, como consequência de dois eventos: a oogênese e a foliculogênese (SAUMANDE, 1991). Em ruminantes, a oogênese pode ser definida como o desenvolvimento e a diferenciação das células germinativas primordiais da fêmea, culminado com a formação do oócito haplóide fecundado (RUSSE, 1983). A foliculogênese é um evento fisiológico que se inicia com a formação do folículo primordial e culmina com o estágio de folículo maturo, também conhecido como folículo pré-ovulatório (SAUMANDE, 1981).

A foliculogênese é finalizada no momento da ovulação do folículo maturo, enquanto a oogênese se encerra somente após a fecundação (FIGUEIREDO et al., 2002). Durante o desenvolvimento fetal, as células germinativas primordiais migram do saco vitelínico para região das cristas gonadais e sofrem sucessivas mitoses, originando as oogônias. Nesta etapa, células somáticas do mesonefron circundam as oogônias, formando os cordões corticais, que são os precursores dos folículos primordiais. Os cordões corticais, também chamados de cordões ovígeros, são descritos como estruturas alongadas contendo células germinativas circundadas por células da pré-granulosa que ficam sobre a lâmina basal (JUENGEL et al., 2002).

As oogônias sofrem sucessivas mitoses e se diferenciam em oócitos, estes iniciam o processo de divisão meiótica, o qual é interrompido em prófase da meiose I no estágio de diplóteno (SOTO-SUAZO; ZORN, 2005; VAN DEN HURK; ZHAO, 2005). A formação dos folículos primordiais ocorre quando os oócitos são individualizados a partir da separação dos cordões de células germinativas (BRISTOL-GOULD et al., 2006).

Na espécie bovina, o oócito primário ou imaturo permanece no estágio de prófase I até imediatamente antes da ovulação. O processo de meiose é retomado em resposta ao estímulo do hormônio folículo estimulante (FSH) e hormônio luteinizante (LH; BUCCIONE et al., 1990), passando pelas fases de metáfase I, anáfase I e telófase I, ocorrendo a liberação do primeiro corpúsculo polar e formação do oócito secundário (BETTERIDGE et al., 1989). O processo *in vivo* de maturação meiótica pode ocorrer apenas no oócito do folículo pré-ovulatório, resultante, dentre outros fatores, da estimulação específica do pico pré-ovulatório de LH e FSH (ERICKSON, 1986).

No estágio de metáfase II, ocorre uma segunda interrupção da meiose (BETTERIDGE et al., 1989), sendo que, na maioria das espécies domésticas, o oócito permanece em metáfase II até ser ovulado e transportado para a tuba uterina, onde poderá ser fecundado. Caso a fecundação ocorra, o oócito retoma a meiose (BETTERIDGE et al., 1989; BUCCIONE et al.,1990) e culmina com a extrusão do segundo corpúsculo polar (GORDON, 1994), marcando assim o fim da oogênese.

Apesar do conceito do estoque finito e não renovável de células germinativas (ZUCKERMAN, 1951) ser amplamente aceito, estudos de Johnson et al. (2004 e 2005) surpreenderam a comunidade científica ao demonstrarem indícios de continuidade da oogênese e foliculogênese no período pós-natal, apontando as células-tronco da medula óssea como responsáveis pela renovação dos gametas femininos. Estes pesquisadores sugeriram a ocorrência de células germinativas nos ovários, na medula óssea e no sangue periférico. Os trabalhos de Johnson et al. (2004 e 2005) geraram muito debate no meio científico devido à ocorrência da menopausa e a inatividade ovariana em fêmeas senis. As críticas também estão relacionadas à ausência de sinais de início e término da primeira prófase meiótica e subsequente fase de diplóteno nos supostos oócitos derivados de células-tronco. Além disso, o curto período para crescimento dos novos folículos permitiu o questionamento da eficácia da esterilização química. A teoria de neo-oogênese/foliculogênese proposta por Johnson et al. (2004 e 2005) continua sendo polêmica e desafia um conceito de muitos anos de estudo. Ainda há muita contestação, principalmente após o relato de nascimento de camundongos a partir de oócitos neoformados depois do cultivo de células germinativas de ovários de camundongos adultos transgênicos para proteína verde fluorescente (GFP) e transferência para ovários de fêmeas esterilizadas quimicamente (ZOU et al., 2009). Após os primeiros relatos do grupo de Johnson et al. (2004), diversos estudos mostraram-se a favor da neo-foliculogêne (BUKOVSKY et al., 2005; ABBAN; JOHNSON, 2009; CELIK et al., 2009; ZOU et al., 2009; DE FELICI, 2010; PACCHIAROTTI et al., 2010; PARTE et al., 2010; VIRANT-KLUN, 2010; SKUTELLA, 2010; VIRANT-KLUN et al., 2011), enquanto outros sustentam o conceito de não haver renovação folicular (BRISTOL-GOULD et al., 2006; EGGAN et al., 2006; LIU et al., 2007; BEGUM et al., 2008; FADDY; GOSDEN, 2009; ZHANG et al., 2010; BYSKOV et al., 2011).

Semelhantemente ao que ocorre em camundongas adultas, White et al. (2012) relataram que ovários provenientes de mulheres que atingiram a maturidade sexual possuem células germinativas mitoticamente ativas que podem ser propagadas *in vitro* e gerar oócitos *in vitro* e *in vivo*. Apesar da questão permanecer em discussão, os trabalhos sugerem revisão dos conceitos sobre foliculogênese.

## 2.2 População folicular ovariana

A população folicular ovariana é influenciada por diversos fatores tais como a espécie, a raça (CAHILL et al., 1979), a genética (ERICKSON, 1966; SMITH et al., 1994), a idade, os níveis hormonais (PETERS, 1976; RUSSE, 1983; ROY; TREACY, 1993) e o estado reprodutivo do animal (ERICKSON et al., 1976). Além destes, deve-se ressaltar que existe uma variação individual na população folicular ovariana, que em bovinos é de 0 e 720.000 folículos por ovário (ERICKSON, 1966).

Ao nascimento, estima-se que a população folicular ovariana seja de aproximadamente 235.000 folículos na vaca (ERICKSON, 1966), 160.000 folículos na ovelha (DRIANCOURT et al., 1991) e 2.000.000 de folículos na mulher (ERICKSON, 1986). Já foi relatada a estimativa da população folicular pré-antral em ovários de fêmeas bovinas de diferentes idades e raças, com valores de 143.929 e 285.155 folículos para fetos, 76.851 e 109.673 folículos para novilhas, e 39.438 e 89.577 folículos para vacas, em animais *Bos indicus* e *Bos taurus* respectivamente (SILVA-SANTOS et al., 2011).

### 2.2.1 Folículos ovarianos

O folículo é considerado a unidade morfofuncional do ovário e constituído por um oócito circundado por células somáticas da granulosa e tecais. O folículo possui uma função endócrina (produção e liberação de hormônios esteroides e outros peptídeos) e exócrina ou gametogênica, apresentando-se como elemento essencial para a manutenção da viabilidade oocitária. Dessa forma, o folículo proporciona um ambiente ideal para o crescimento e o desenvolvimento do oócito imaturo, permitindo que o oócito maturo alcance a ovulação (FIGUEIREDO et al., 2002).

Na espécie bovina, os folículos ovarianos localizam-se no córtex do ovário e de acordo com o grau de desenvolvimento folicular eles podem ser classificados em folículos pré-antrais ou não cavitários (primordiais, em transição, primários e secundários) e folículos antrais ou cavitários (terciários e pré-ovulatórios; Figura 1).

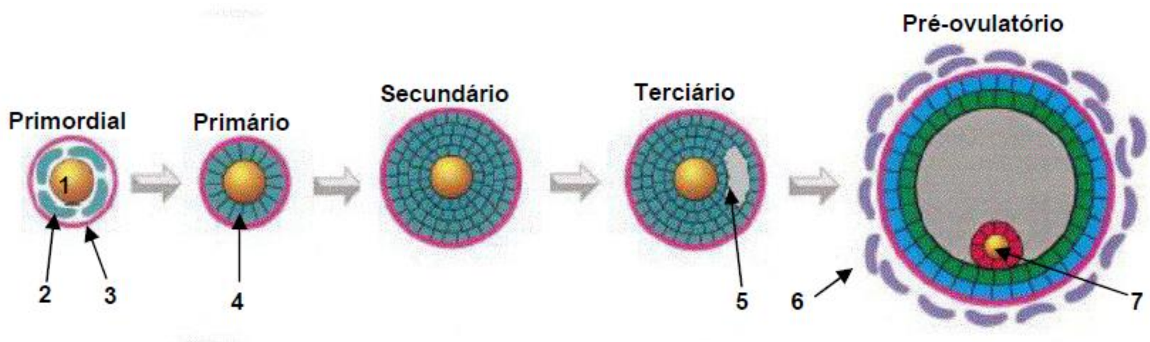


Figura 1. Representação esquemática do desenvolvimento dos folículos ovarianos (Adaptado de Rodgers et al., 1999). 1) Oócito primário; 2) Célula da Pré-granulosa; 3) Membrana basal; 4) Células da Granulosa; 5) Cavidade antral; 6) Célula da teca e 7) Oócito secundário.

No início do crescimento folicular, admite-se que os folículos se desenvolvam predominantemente por uma ação local (fatores intraovarianos) e, somente após a puberdade, por uma ação gonadotrófica. Vários fatores de crescimento foram identificados nas primeiras modificações foliculares, tais como o *Kit Ligand* (PARROT; SKINNER, 1999), o fator de crescimento e diferenciação - GDF-9 (VITT et al., 2000), o fator de crescimento de fibroblasto - FGF (NILSSON et al., 2001) e o fator inibidor de leucemia - LIF (NILSSON et al., 2002).

Os folículos ovarianos pré-antrais (FOPA) representam 90% da população folicular (SAUMANDE, 1991) e são responsáveis pela renovação contínua de folículos antrais no ovário (GUILBAULT et al., 1986). No entanto, aproximadamente 99,9% dos folículos pré-antrais presentes nos ovários não chegam até a ovulação (CARROL et al., 1990) porque sofrem um processo degenerativo ou apoptótico conhecido por atresia folicular. Portanto, embora o ovário tenha um alto potencial produtivo, ele é considerado um órgão de baixa produtividade (IRELAND, 1987).

Ainda há muita controvérsia com relação ao momento em que aparecem os folículos primordiais no ovário de fetos bovinos, com relatos ao redor de 74 dias (TANAKA et al., 2001), 90 dias (RUSSE, 1983) e até 150 dias de gestação (MOUSTAFA; HAFEZ, 1971). Além disso, os mecanismos envolvidos com o recrutamento e a ativação desses folículos também são pouco compreendidos, embora após sua ativação, estima-se que em cerca de 100 dias o folículo primordial bovino já alcance o estágio pré-ovulatório (BRITT, 1991).

O folículo primordial se caracteriza por apresentar uma única camada de células somáticas (células da pré-granulosa) de formato achatado que circunda o oócito primário ou imaturo (HIRSHFIELD, 1991). Uma vez recrutado, o folículo primordial se desenvolve até folículo primário, que também apresenta uma única camada de células da granulosa de formato cuboide (HULSHOF et al., 1994). Este período trata-se mais de um processo de maturação folicular, pois o oócito praticamente permanece com o mesmo diâmetro. No entanto, uma característica marcante dessa fase é o surgimento da zona pelúcida, estrutura ao redor do oócito que é mantida por todo o período de desenvolvimento folicular (FIGUEIREDO et al., 2002; VAN DEN HURK; ZHAO, 2005).

O folículo secundário se caracteriza por apresentar o oócito mais desenvolvido, a zona pelúcida e as primeiras células da teca já formadas (VAN DEN HURK; ZHAO, 2005) e pelo menos duas camadas de células da granulosa (HULSHOF et al., 1994). Ao fim deste estágio, pode ser detectada uma maior atividade de hormônios gonadotróficos (FSH e LH) para conduzir os folículos secundários até as fases antrais (VAN DEN HURK; ZHAO, 2005).

A categoria de folículos antrais compreende os folículos terciários e os folículos pré-ovulatórios. Com a intensa proliferação das células da granulosa, surge o antro folicular, uma área preenchida por fluido folicular e considerada característica principal do folículo antral (FIGUEIREDO et al., 2002). O surgimento dos primeiros folículos terciários em bovinos é observado aproximadamente com 230 dias de gestação (ERICKSON, 1966; RUSSE, 1983). A partir de então, os folículos com diâmetros entre 0,14 e 0,28 mm começam a desenvolver a cavidade antral (LUSSIER et al., 1987). Nos bovinos, os folículos primordiais crescem em tamanho e diâmetro, passando de 0,020 a 0,040 mm para mais de 10 mm antes da ovulação (IRELAND, 1987). Para um folículo crescer do início da formação do antro (0,13 mm) ao tamanho pré-ovulatório são necessários dois ciclos estrais (LUSSIER et al., 1987).

## 2.3 Contagem de folículos antrais (CFA) e sua repetibilidade

O número de folículos antrais é uma característica altamente variável no ovário da fêmea bovina (BURNS et al., 2005; IRELAND et al., 2008; 2011; EVANS et al., 2012), porém existe uma alta repetibilidade no mesmo indivíduo (BURNS et al., 2005; IRELAND et al., 2008; SILVA-SANTOS et al., 2014). Devido a esta característica de alta repetibilidade, originalmente em rebanho taurino e através do exame ultrassonográfico, foi demonstrado ser possível classificar as fêmeas em baixa, intermediária e alta CFA de acordo com o número de folículos antrais (folículos $\geq$ 3 mm) identificados no ultrassom, independentemente da estação do ano, do número de ondas de crescimento folicular por ciclo, da idade e do status lactacional (BURNS et al., 2005).

O mesmo comportamento de repetibilidade da CFA foi demonstrado em rebanho zebuíno cruzado, com correlação de 0,90 a 0,92 em fêmeas *Bos indicus-taurus*, cuja CFA foi monitorada do desmame até o sobreano (SILVA-SANTOS et al., 2014). Portanto, empregando apenas um exame ultrassonográfico de rotina é possível identificar fêmeas com baixa, intermediária ou alta CFA.

## 2.4 Contagem de folículos antrais e sua relação com o hormônio anti-Mülleriano (AMH)

O AMH é uma glicoproteína de 140 kDa que pertence à família do fator transformador do crescimento (TGF-β), sendo produzido pelas células da granulosa de folículos saudáveis em crescimento e expresso somente nas gônadas (VIGIER et al., 1984; CATE et al., 1986; TAKAHASHI et al., 1986; LEE et al., 1996; MONNIAUX et al., 2008). Sua expressão é elevada nas células da granulosa de folículos antrais pequenos e diminui durante o crescimento folicular, inibindo o recrutamento de folículos primordiais para o *pool* de folículos em crescimento, além de reduzir a responsividade dos folículos em crescimento à ação do FSH (DURLINGER et al., 1999; 2001; 2002). Portanto, o AMH é considerado um ótimo marcador endócrino para o número de folículos em desenvolvimento (BARUSELLI et al., 2015).

As altas concentrações de AMH estão positivamente associadas com o tamanho da população folicular em ovários de ratas, mulheres e vacas (VISSER et al., 2006; KWEE et al., 2008; RICO et al., 2009; 2012). Na fêmea bovina, a concentração de AMH está altamente correlacionada com a CFA e o número de oócitos saudáveis, sendo considerado um bom marcador endócrino do número de folículos antrais nesta espécie (IRELAND et al., 2011; BATISTA et al., 2014; BARUSELLI et al., 2015).

Em rebanho taurino, fêmeas com alta CFA (> 25 folículos) apresentam maiores concentrações circulantes de AMH em comparação com fêmeas de baixa CFA (< 15 folículos; $p < 0{,}01$). Adicionalmente, uma alta correlação pode ser encontrada entre a concentração média de AMH e a média de CFA ($r = 0{,}88$, $p < 0{,}001$; IRELAND et al., 2008). Comparando fêmeas taurinas *vs.* zebuínas, de corte e leite, a concentração de AMH também foi positivamente correlacionada com a CFA de animais zebuínos (Nelore, variação de 0,56 a 0,68) e taurinos (Holandês, variação de 0,73 a 0,90). Independentemente do grupo genético, fêmeas com alta CFA apresentaram maior concentração de AMH (*Bos taurus* 0,57 ng/mL e *Bos indicus* 1,20 ng/mL) do que fêmeas com baixa CFA (0,06 e 0,78 ng/mL, respectivamente). Além disso, há evidências de que a CFA em gado *Bos indicus* (baixa = 28 e alta = 48 folículos) é maior do que a de *Bos taurus* (baixa = 13 e alta = 34 folículos; BATISTA et al., 2014).

Atualmente, o AMH é reconhecido como um marcador fidedigno para identificar fêmeas com melhor resposta ovariana à superovulação, melhor potencial para produção oocitária, além de estar associado a várias características relacionadas à fertilidade (BARUSELLI et al., 2015). Portanto, a determinação das concentrações de AMH em vacas doadoras pode ajudar a prever a resposta folicular e ovulatória para tratamentos gonadotróficos (RICO et al., 2009). Em estudo com vacas Holandesas ao longo de um ano, as concentrações de AMH, determinadas por teste de ELISA, foram constantes e fortemente correlacionadas com a CFA. As doadoras com concentrações de AMH abaixo de 87 pg/mL apresentaram menos de 15 folículos grandes por ciclo estral e baixa eficiência na produção de embriões. Deste modo, a determinação da concentração de AMH no plasma em bovinos pode ser considerada um procedimento regular antes da OPU/FIV e SOV para identificação dos animais com melhor potencial de produção embrionária (RICO et al., 2012).

## 2.5 Contagem de folículos antrais e sua relação com a produção embrionária (*in vivo* e *in vitro*)

Há uma grande variabilidade no número de embriões produzidos por doadora, tanto no método *in vivo* quanto *in vitro* (PONTES et al., 2009; 2010; 2011). Esta variabilidade está relacionada principalmente à eficiência na taxa de recuperação de oócitos e à resposta superovulatória, fatores estes dependentes do número de folículos antrais da doadora e que podem afetar significativamente o sucesso na produção de embriões (TANEJA et al., 2000; IRELAND et al., 2007; 2011; SILVA-SANTOS et al., 2014; SANTOS et al., 2016).

Vários estudos com animais *Bos taurus* demonstraram que a maior CFA está relacionada com benefícios quantitativos para o sucesso da PIVE e da SOV (TANEJA et al., 2000; SINGH et al., 2004; IRELAND et al., 2008). Com o objetivo de comparar a produção embrionária de doadoras *Bos indicus-taurus* (Braford = 3/8 Brahman e 5/8 Hereford) com alta *vs.* baixa CFA, Silva-Santos et al. (2014) conduziram um estudo empregando tanto a produção *in vivo* (SOV) quanto *in vitro* (OPU/FIV) de embriões. Neste estudo, os animais foram avaliados por ultrassom seriado desde o desmame (nove meses) até completarem 24 meses de idade, quando foram submetidos às técnicas de produção embrionária. Os dados revelaram que as fêmeas de alta CFA apresentaram melhor *performance* na produção embrionária, tanto na SOV (6,95 ± 5,34 *vs.* 1,9 ± 2,13) quanto na OPU/FIV (6,10 ± 4,51 *vs.*

0,55 ± 0,83 embriões; p < 0,05; Tabela 1). Adicionalmente, o estudo constatou que um único exame ultrassonográfico realizado na idade de pré-puberdade (para determinação da CFA) pode ser usado como um preditor para produção embrionária. Portanto, este trabalho reforçou o conceito de que há benefícios quantitativos na produção de embriões conforme a CFA e que o ultrassom pode ser empregado como ferramenta preditora na seleção de doadora de embrião.

**Tabela 1**. Desempenho reprodutivo de fêmeas *Bos indicus-taurus* com alta (G-Alta, ≥ 40 folículos) ou baixa (G-Baixa, ≤ 10 folículos) contagem de folículos antrais submetidas à produção de embriões *in vitro* [*ovum pickup*/produção *in vitro* (OPU/PIV)] e *in vivo* [superovulação (SOV)].

| **Variáveis** | **G-Alta (N = 20)** | **G-Baixa (N = 20)** |
|---|---|---|
| Folículos antrais (n) | 47 ± 6 | 9 ± 3 |
| Total de oócitos recuperados | $738^{a}$ | $116^{b}$ |
| Proporção de oócitos viáveis (%) | 58,94 (435/738) | 55,17 (64/116) |
| Taxa de clivagem (%) | 61,25 (452/738) | 56,03 (65/116) |
| Taxa de blastocisto (%) | 16,53 (122/738) | 9,48 (11/116) |
| Total de embriões/ OPU/PIV (n) | $6{,}10 \pm 4{,}51^{aA}$ (122/20) | $0{,}55 \pm 0{,}83^{bB}$ (11/20) |
| Total de estruturas recuperadas (n) | $8{,}80 \pm 6{,}78^{a}$ (176/20) | $2{,}25 \pm 2{,}63^{b}$ (45/20) |
| Total de embriões/ colheita (n) | $6{,}95 \pm 5{,}34^{aA}$ (139/20) | $1{,}9 \pm 2{,}13^{bA}$ (38/20) |
| Proporção congelados (%) | $78{,}42^{a}$ (109/139) | $89{,}47^{a}$ (34/38) |

Os valores seguidos por letras diferentes sobrescritos (a, b) dentro da mesma linha (G-Alta *vs.* G-Baixa) ou (A, B) dentro da mesma coluna (OPU/IVP *vs.* SOV) foram significativamente diferentes (P ≤ 0,05). Adaptado de Silva-Santos et al. (2014).

Resultados semelhantes com doadoras *Bos taurus* foram obtidos por Ireland et al. (2007), com menor número médio de embriões *in vitro* para fêmeas de baixa CFA quando em comparação com animais de alta CFA (1,3 *vs.* 4,9 embriões). Para produção *in vivo* de embriões, doadoras de baixa CFA (< 15 folículos) produziram menor número de estruturas que fêmeas de alta CFA (> 25 folículos), com médias de 3,8 *vs.* 5,4 embriões, respectivamente.

Para avaliar a *performance* na produção embrionária com base na produção de oócito de doadoras, Santos et al. (2016) realizaram procedimentos de OPU em 66 vacas *Bos indicus* (Nelore) e com base na recuperação oocitária classificaram as doadoras em alta (N = 22, ≥ 40 oócitos), intermediária (N = 25, 18-25 oócitos) ou baixa (N = 19, ≤ 7 oócitos) produção de oócitos. Após procedimento de FIV com sêmen de mesmo touro, a taxa de blastocisto e o número de embriões viáveis foram maiores no grupo de alta recuperação oocitária (41,9% e 18,4 ± 6,7 embriões) em relação aos grupos de produção intermediária (32,4% e 6,1 ± 3,6) e baixa (13,0% e 0,6 ± 0,7; respectivamente; $p < 0,05$).

Utilizando ovários de abatedouro oriundos de uma amostragem de 356 vacas Nelore (alta CFA > 92 folículos, intermediária 46 a 76 folículos e baixa < 31 folículos), Rosa (2015) constatou que a *performance* do grupo de alta CFA, ao avaliar as taxas embrionárias, não se repetiu como em outros trabalhos de campo. Neste estudo, não houve diferença ($p > 0,05$) na taxa de clivagem (76,6%, 77,5% e 79,5%) e nem na taxa de blastocisto (40,6%, 36,3% e 38,6%) para os grupos de alta, média e baixa CFA, respectivamente. Todavia, cabe ressaltar que os limites numéricos de folículos que estabelecem os grupos de CFA foram bem maiores que os de trabalhos de campo previamente discutidos. Tal variabilidade na CFA entre os estudos e os resultados distintos reforça a complexidade do tema e ressalta a necessidade de mais investigações.

Uma melhor resposta da doadora para a OPU/FIV ou SOV ainda não é completamente compreendida, e certamente a CFA não é a única variável que interfere quantitativamente na produção de embriões. Apesar das variações individuais conforme a técnica utilizada, é consenso considerar a vantagem quantitativa das doadoras de alta CFA. Desta forma, a variação da CFA entre as doadoras é um fator muito importante para os programas comerciais de produção de embriões, sendo comum fazer uma avaliação ultrassonográfica prévia como método de seleção das doadoras potenciais. As doadoras selecionadas geralmente são aquelas com alta CFA ou com elevado número de oócitos viáveis (MOROTTI et al., 2015), uma vez que o impacto no número final de prenhez apresenta grande variação, conforme ilustrado na Tabela 2.

Apesar da inquestionável vantagem quantitativa (maior número de embriões) em selecionar a doadora com base na CFA, o impacto deste critério sobre outros aspectos da fertilidade e da produtividade em bovinos de corte e de leite ainda não é bem conhecido, particularmente nos animais *Bos indicus*. Desta forma, até que mais estudos sejam realizados, a escolha da doadora pela CFA deve ser considerada após a avaliação do mérito genético da mesma (MOROTTI et al., 2015).

**Tabela 2**. Produção de oócitos, embriões e prenhez conforme número de oócitos obtidos por OPU/PIV (N = 656) a partir de doadoras da raça Nelore (N = 317), conforme o número de oócitos. Os valores (média ± DP) são apresentados por doadora.

| Doadoras conforme N° de oócitos | N° oócitos viáveis | N° embriões viáveis | N° prenhez com 30 dias | N° prenhez com 90 dias |
|---|---|---|---|---|
| Elevada (N= 78) | 47,06 ± 1,6[a] | 15,06 ± 0,86[a] | 5,62 ± 0,54[a] | 5,52 ± 0,81[a] |
| Alta (N= 80) | 24,95 ± 0,33[b] | 9,17 ± 0,63[b] | 3,63 ± 0,36[b] | 3,32 ± 0,33[b] |
| Intermediária (n= 79) | 15,57 ± 0,26[c] | 6,00 ± 0,39[c] | 2,10 ± 0,21[c] | 1,92 ± 0,20[b] |
| Baixa (N= 80) | 6,31 ± 0,38[d] | 2,42 ± 0,25[d] | 0,92 ± 0,13[d] | 0,85 ± 0,13[c] |

a-d Valor médio sobrescrito dentro de uma coluna difere estatisticamente ($P \leq 0,05$). Adaptado de Pontes et al. (2011).

## 2.6 Contagem de folículos antrais e sua relação com a progesterona

A produção de progesterona tem sido vinculada à atividade fisiológica do corpo lúteo (CL), a funcionalidade do ovário e útero e ao desenvolvimento embrionário e prenhez nos bovinos (DISKIN; MORRIS, 2008). Portanto, a baixa concentração de progesterona proporciona uma alta taxa de mortalidade embrionária, menor quantidade de oócitos saudáveis e menor desenvolvimento endometrial (DISKIN; MORRIS, 2008; EVANS et al., 2012).

Um estudo que avaliou a relação do número de folículos antrais com a progesterona em fêmeas taurinas revelou que a baixa CFA proporciona menor concentração de progesterona durante o ciclo estral, cerca de 30 a 50% menor em comparação com fêmeas de alta contagem folicular (JIMENEZ-KRASSEL et al., 2009). Neste estudo, as menores concentrações circulantes de progesterona em vacas com baixa CFA foram atribuídas principalmente à função reduzida do CL devido alteração na capacidade de resposta das células luteínicas ao LH, uma redução na proteína de regulação aguda da esteroidogênese (STAR) no CL, a capacidade de resposta diminuída da granulosa e células luteais ao 25-hidroxicolesterol, bem como uma menor responsividade das células da granulosa do folículo dominante em sofrer luteinização. Portanto, sugeriu-se que a alta CFA influencia positivamente a espessura do endométrio, favorecendo melhor fertilidade.

## 2.7 Contagem de folículos antrais e sua relação com parâmetros de fertilidade em novilhas e vacas

A baixa CFA em fêmeas taurinas com aptidão para produção de leite (raça Holandesa) foi associada com várias características negativas para fertilidade, tais como ovários menores (IRELAND et al., 2008), menor chance de prenhez ao final da estação reprodutiva (MOSSA et al., 2012), reduzida capacidade de resposta ao tratamento de SOV, menor número de embriões viáveis (SINGH et al., 2004; IRELAND et al., 2007), menores concentrações circulantes de progesterona e AMH (IRELAND et al., 2011; EVANS et al., 2012) e reduzida espessura do endométrio (JIMENEZ-KRASSEL et al., 2009). Neste contexto, animais com alta CFA têm se mostrado superiores em todos os aspectos mencionados, de modo que, pelo menos para fêmeas taurinas na Europa e América do Norte, parece haver uma correlação linear entre o alto número de folículos antrais e importantes aspectos da eficiência reprodutiva.

Tal contexto tem sido pouco estudado em animais zebuínos (corte e leite) e não há relato de estudo com *performance* reprodutiva (alta *vs.* baixa CFA) em rebanho Holandês conduzido no Brasil. Estudos do nosso grupo de pesquisa têm revelado que as fêmeas de baixa CFA, particularmente animais zebuínos e cruzamentos, não estão necessariamente associadas às características de baixa fertilidade (SANTOS et al., 2013; MENDONÇA et al., 2013; MORAES; 2016; MORETTI, 2016; SANTOS et al., 2016). Estudos preliminares com dinâmica folicular ovariana em gado *Bos indicus-taurus* (Braford) revelaram que as maiores taxas de crescimento do folículo dominante e os maiores diâmetros não foram encontrados em fêmeas de alta CFA (SANTOS et al., 2012). Em rebanho *Bos indicus*, não há relato na literatura sobre o efeito da CFA na dinâmica folicular ovariana.

Considerando a relação da CFA com a taxa de prenhez após a IATF, quatro estudos foram conduzidos pelo nosso grupo de pesquisa em fêmeas *Bos indicus* (Nelore) e também revelaram achados diferentes de trabalhos europeus e norte americanos. Estes estudos foram conduzidos em novilhas cíclicas de 24 meses (estudo I) ou em vacas multíparas no pós-parto (estudo II a IV). As fêmeas foram avaliadas por ultrassonografia no dia 0 ou dia 8 do protocolo de IATF para classificação dos grupos de alta, média ou baixa contagem segundo a CFA média mais ou menos um desvio padrão. Posteriormente, a taxa de concepção foi avaliada por ultrassonografia transretal 30 dias após a IATF. Os dados, resumidos na Tabela 3, não demonstram haver influência da CFA sobre a taxa de concepção nos estudos I a III, mas no estudo IV a concepção foi melhor para o grupo de baixa CFA.

**Tabela 3**. Taxa de concepção de fêmeas Nelore (*Bos indicus*) com alta, média ou baixa contagem de folículos antrais (CFA) após inseminação artificial em tempo fixo.

| Estudo/Categoria (Autor) | Alta (n) | Intermediária (n) | Baixa (n) | Total/Média (n) |
|---|---|---|---|---|
| I / Novilhas (Mendonça et al., 2013) | 30 folículos (38) | 13-29 folículos (143) | ≤ 12 folículos (27) | (208) |
| CFA (M ± DP) | $37{,}7 \pm 7{,}0^{a}$ | $19{,}2 \pm 4{,}2^{b}$ | $10{,}5 \pm 2{,}1^{c}$ | $21{,}4 \pm 9{,}4^{b}$ |
| Taxa concepção (%) | 44,7 | 43,3 | 51,8 | 44,7 |
| II / Vacas (Santos et al., 2016) | ≥ 25 folículos (183) | 11-24 folículos (183) | ≤ 10 folículos (181) | (547) |
| CFA (M ± DP) | $30{,}7 \pm 6{,}5^{a}$ | $18{,}6 \pm 1{,}6^{b}$ | $7{,}8 \pm 2{,}4^{c}$ | $17{,}6 \pm 10{,}7^{b}$ |
| Taxa concepção (%) | 51,9 | 48,6 | 58,5 | 53,0 |
| III / Vacas (Moraes, 2016) | ≥ 30 folículos (119) | 11-29 folículos (314) | ≤ 10 folículos (303) | (736) |
| CFA (M ± DP) | $37{,}5 \pm 12{,}3^{a}$ | $17{,}7 \pm 3{,}4^{b}$ | $7{,}6 \pm 2{,}6^{c}$ | $16{,}8 \pm 11{,}7^{b}$ |
| Taxa concepção (%) | 47,9 | 50,0 | 57,4 | 52,7 |
| IV / Vacas (Moretti, 2016) | ≥ 45 folículos (167) | 20-40 folículos (295) | ≤ 50 folículos (144) | (606) |
| CFA (M ± DP) | $51{,}9 \pm 6{,}2^{a}$ | $29{,}4 \pm 6{,}0^{b}$ | $11{,}4 \pm 2{,}8^{c}$ | $30{,}1 \pm 15{,}1^{b}$ |
| Taxa concepção (%) | $49{,}3^{b}$ | $54{,}2^{b}$ | $67{,}1^{a}$ | 56,6 |

a-c Valor sobrescrito dentro de uma linha difere estatisticamente ($P \leq 0{,}05$). Os dados são apresentados como média (M) ± desvio padrão da média (DP).

Apesar destes estudos não esclareceram completamente a influência da CFA sobre a fertilidade do rebanho zebuíno, é valido ressaltar que nesta subespécie a relação da CFA e a fertilidade (concepção a IATF) não parece seguir o mesmo padrão descrito para fêmeas taurinas. Enquanto os estudos I (novilha cíclicas com 24 meses), II e III (vacas multíparas no pós-parto) revelaram não haver influência, o estudo III (vacas multíparas no pós-parto) mostrou melhor taxa de concepção para as vacas de baixa contagem folicular, o que foge totalmente do comportamento reprodutivo descrito em rebanho *Bos taurus* com aptidão para leite fora do Brasil.

Além das clássicas diferenças entre as subespécies (*Bos taurus vs. Bos indicus*) e aptidão de produção (leite *vs.* corte), outro aspecto crítico para se estabelecer comparação entre os estudos se refere à variabilidade do número de folículos antrais entre os indivíduos avaliados, os rebanhos amostrados, bem como os limites numéricos de CFA para se estabelecer os grupos de alta, intermediária e baixa contagem folicular. Na tabela 4, encontra-se um resumo das diferentes CFAs utilizadas em alguns trabalhos como critério para divisão dos grupos experimentais.

**Tabela 4**. Número limite de folículos antrais utilizados na classificação dos grupos de alta, intermediária e baixa contagem folicular antral (CFA) em fêmeas *Bos taurus* e *Bos indicus* de diferentes estudos.

| **Autor** | **Subespécie** | **CFA média** | **Grupos** | | |
|---|---|---|---|---|---|
| | | | Alta | Intermediária | Baixa |
| Burns et al. (2005) | *Bos taurus* | 21,5 | ≥ 25 | 16 a 20 | ≤ 15 |
| Ireland et al. (2011) | *Bos taurus* | 21,5 | ≥ 25 | 16 a 24 | ≤ 15 |
| Mossa et al. (2012) | *Bos taurus* | 18.5 ± 9.0 | ≥ 25 | 16 a 24 | ≤ 15 |
| Santos et al. (2013) | *Bos indicus* | 17,9 ± 8,4 | ≥ 25 | 11 a 24 | ≤ 10 |
| Rodrigues et al. (2015) | *Bos indicus* | 44,2 ± 0,8 | ≥ 48 | 32 a 48 | ≤ 32 |
| Rosa (2015) | *Bos indicus* | 61,1 ± 30,4 | ≥ 92 | 46 a 76 | ≤ 31 |
| Martinez et al. (2016) | *Bos taurus* | 25,7 | ≥ 30 | 21 a 29 | ≤ 20 |
| Santos et al. (2016) | *Bos indicus* | 19,6 ± 10,7 | ≥ 25 | 16 a 20 | ≤ 10 |
| Martinez (2016) | *Bos indicus* | 16,8 ± 11,7 | ≥ 30 | 11 a 29 | ≤ 10 |
| Moraes (2016) | *Bos indicus* | 16,8 ± 11,7 | ≥ 30 | 11 a 29 | ≤ 10 |
| Moretti (2016) | *Bos indicus* | 30,1 ± 15,1 | ≥ 45 | 20 a 40 | ≤ 15 |

A grande variabilidade das CFAs entre os estudos, os diferentes tipos de rebanhos e categorias de fêmeas, as distintas práticas de manejo, e principalmente, a falta de uma padronização nos critérios de classificação dos grupos tornam ainda mais desafiador o pleno conhecimento da CFA sobre a fertilidade da fêmea bovina.

## 2.8 Contagem folicular antral e sua relação com a nutrição e a saúde materna

Uma vez que os folículos primordiais são estabelecidos durante o segundo trimestre de gestação nos bovinos (ERICKSON, 1966), é provável que o tamanho da reserva folicular seja influenciado pelo ambiente uterino em que o feto é formado (EVANS et al., 2012). Neste contexto, o estado nutricional da mãe tem sido apontado como importante fator de influência sobre o número de folículos primordiais formados ainda durante a vida fetal. Mossa et al. (2009) avaliaram vacas que receberam alimentação de mantença ou foram submetidas à restrição alimentar (0.6 das necessidades energéticas de mantença) imediatamente antes da concepção até os primeiros 110 dias de gestação e, posteriormente, avaliaram os ovários de suas bezerras com 7, 18 e 35 semanas de idade. A CFA e a concentração de AMH das bezerras com diferentes idades, cujas vacas foram submetidas à restrição alimentar, foram em média 60% menor do que a das bezerras do grupo das vacas que receberam alimentação de mantença.

Também há evidências indicando que o comprometimento da saúde materna durante a gestação possa afetar negativamente o tamanho da reserva folicular. Vacas com alta contagem de células somáticas no leite, o que é indicativo de infecção da glândula mamária, pariram bezerras que apresentaram concentração de AMH quase 50% menor em comparação às bezerras nascidas de vacas com baixa contagem de células somáticas (0,01 ± 0,075 *vs.* 0,13 ± 0,03 ng/mL; p < 0,05; IRELAND et al., 2011; EVANS et al., 2012). Portanto, como o nível sérico de AMH em fêmeas bovinas tem sido correlacionado com a CFA e o tamanho da reserva folicular ovariana, os resultados destes estudos indicam que tanto a nutrição quanto a saúde materna durante a gestação podem afetar o número de folículos formados durante a vida fetal.

## 2.9 Contagem folicular antral e sua relação com as características de mérito genético

Apesar de conhecer a influência da nutrição e da saúde materna sobre a CFA da prole, o papel que as características de seleção genética e os fatores ambientais exercem sobre a variabilidade da CFA e o tamanho da reserva folicular ovariana é pouco compreendido, principalmente devido à escassez de trabalhos sobre este assunto.

Em bovinos, a maioria das características relacionadas à fertilidade se caracteriza por apresentar uma herdabilidade baixa, frequentemente inferior a 5% (CAMMACK et al., 2009). Isto ocorre porque o desempenho reprodutivo em bovinos pode sofrer influência de diversos fatores, tais como aqueles ligados ao meio ambiente, às práticas de manejo do rebanho, às falhas nos registros de dados, bem como às diferentes estratégias reprodutivas que podem ser aplicadas na reprodução animal (CAMMACK et al., 2009; WALSH et al., 2011).

Uma alta correlação entre as características fenotípicas e o melhor desempenho reprodutivo em bovinos poderia ser uma estratégia eficiente para escolher os animais com melhor potencial de fertilidade no rebanho. Em rebanho de leite, por exemplo, tem sido notada uma relação antagônica entre o mérito genético para produção de leite e o desempenho reprodutivo de vacas (PHILIPSSON, 1981; LUCY, 2001). Isto pode ser percebido pelo fato da capacidade para produção de leite ter aumentado significativamente nos últimos anos, mas em contrapartida, a fertilidade das vacas (taxa de concepção) reduziu dos 65% para aproximadamente 30 a 40% (WALSH et al., 2011). Neste contexto, a descoberta de traços fenotípicos com alta correlação para com a fertilidade nas fêmeas, certamente pode ser relevante não só para o sistema produtivo e práticas de manejo reprodutivo, mas também para o desenvolvimento de novas estratégias de melhoramento genético para o rebanho, potencializando a cadeia produtiva da carne e do leite.

A CFA tem sido amplamente utilizada em programas de produção de embrião, tanto *in vivo* quanto *in vitro*, com o objetivo de identificar as doadoras com maior potencial para produção embrionária (PONTES et al., 2009; 2010; 2011; SILVA-SANTOS et al., 2014). Em suma, realiza-se um exame ultrassonográfico prévio de todas as doadoras para seleção das fêmeas que apresentam maior número de folículos antrais. As fêmeas com maior CFA apresentam vantagens quantitativas na *performance* de produção embrionária (MOROTTI et al., 2015), o que parece ser algo muito evidente. No entanto, a relação entre o mérito genético e a CFA é um assunto pouco estudado. Por exemplo, não se sabe se o número de folículos antrais pode sofrer influência das características de seleção genética utilizadas em programa de melhoramento. Em rebanho de corte (taurinos, zebuínos ou cruzas) não há qualquer relato sobre a relação da CFA com as características de mérito genético.

Um único estudo conduzido por Waslh et al. (2014) em vacas (N = 445) e novilhas (N = 522) da raça Holandesa, avaliou a herdabilidade da CFA, uma possível associação entre as características de mérito genético e a CFA, bem como se o *status* lactacional e o nível de produção durante a gestação poderiam influenciar a CFA das filhas. Neste estudo, a CFA apresentou herdabilidade de 0,31 ± 0,14 para vacas e de 0,25 ± 0,13 para novilhas. A CFA apresentou correlação negativa com o mérito genético para concentração de gordura no leite, ou seja, para o aumento de uma unidade na porcentagem de gordura, há redução de 0,4 folículos na CFA. Além disso, vacas em lactação pariram bezerras com maior CFA (19,2 ± 0,6) do que vacas que não estavam em lactação (15,9 ± 1; $p < 0,01$). Portanto, este estudo sugeriu que, ao menos em fêmeas da raça Holandesa, a CFA apresenta traços genéticos de herdabilidade baixa a moderada, sendo que a CFA pode ser influenciada pela concentração de gordura no leite e pelo *status* lactacional durante a gestação.

Certamente, muitos aspectos relacionados a presente revisão necessitam de mais investigações, e apesar dos inúmeros estudos sobre população folicular ovariana, as razões para as diferenças no número de folículos antrais em bovinos e a sua relação com a fertilidade do rebanho e com a eficiência das biotécnicas reprodutivas ainda não são totalmente compreendidas. Além disso, várias lacunas relacionadas às particularidades reprodutivas de *Bos indicus* e *Bos taurus* precisam de melhor compreensão, a fim de permitir ajustes no manejo dos rebanhos e melhorar a eficiência das biotécnicas reprodutivas.

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# 3 HIPÓTESES

- Vacas Nelore (*Bos indicus*) com baixa contagem de folículos antrais apresentam um padrão de dinâmica folicular ovariana distinto das vacas com alta contagem de folículos antrais; e

- As características genotípicas e fenotípicas utilizadas em programa de melhoramento genético influenciam o número de folículos antrais de Novilhas Braford (*Bos indicus-taurus*).

# 4 OBJETIVOS

## 4.1 Objetivo geral

- Avaliar a influência da contagem de folículos antrais sobre a dinâmica folicular ovariana e sobre os parâmetros de mérito genético de fêmeas bovinas.

## 4.2 Objetivos específicos

- Avaliar a influência da baixa e alta contagem de folículos antrais sobre a dinâmica folicular ovariana de vacas Nelore (*Bos indicus*) durante o tratamento hormonal para sincronização da ovulação; e

- Avaliar se há correlação das características fenotípicas e genotípicas com a contagem de folículos antrais em novilhas Braford (*Bos indicus-taurus*).

# 5 ARTIGOS PARA PUBLICAÇÃO

# ARTIGO 1

Ovarian follicular dynamics in *Bos indicus* (Nelore) cows with high or low antral follicles count during hormonal treatment for synchronization of ovulation

*O artigo foi redigido conforme as normas do periódico Theriogenology.*

Ovarian follicular dynamics in *Bos indicus* (Nelore) cows with high or low antral follicles count during hormonal treatment for synchronization of ovulation

Abstract

The aim of this study was to evaluate the influence of high or low antral follicular count (AFC) on the ovarian follicular dynamics during the synchronization of ovulation in *Bos indicus* cows. Seven days before the starting tratments ($D_{-7}$) 250 Nelore cows were evaluated by ultrasonography to determine AFC (follicles ≥ 3 mm). Then, 75 multiparous cows (average 45 days pospartum and without *corpus luteum* - CL) were allocated in two groups: with a consistently high (G-High, ≥ 45 follicles; n = 43) or low AFC (G-Low, ≤ 15 follicles; n = 32). In random day of the estrous cycle (day 0), the animals received a conventional protocol of synchronization of ovulation (3 mg norgestomet + 2 mg estradiol benzoate on day 0 and ear implant withdrawal + 0.150 mg D-cloprostenol + 300 IU equine chorionic gonadotropin + 1 mg estradiol cypionate on day 8) for monitoring ovarian follicular dynamics. Ultrasound exams were performed at $D_{-7}$, $D_4$ and $D_{18}$ to determine AFC; at $D_0$ and $D_4$, and daily ($D_8$ to $D_{10}$) to monitoring the follicular diameter; every 12 h ($D_{10}$ to $D_{12}$) to evaluate the ovulation; and at $D_{18}$ to evaluate the CL. Data were analyzed using ANOVA followed by the Tukey test ($P < 0.05$). The AFC at $D_{-7}$, $D_4$ and $D_{18}$ were 48.9 ± 5.8, 49.5 ± 5.7 and 49.7 ± 6.9 follicles for G-High and 12.6 ± 4.1, 12.5 ± 4.4 and 12.9 ± 3.8 follicles for G-Low, respectively (P = 0.01). The ovrian diameter, perimeter and area were higher (P = 0.0001) in G-High than G-Low (28.3 ± 3.9 *vs.* 20.5 ± 3.2 mm, 100.9 ± 13.2 *vs.* 73.8 ± 13.9 mm, and 67.5 ± 16.4 *vs.* 37.7 ± 11.7 $mm^2$; respectively). The follicular growth rate, time of ovulation and CL diameter were similar (P > 0.05) for cows with low or high AFC. Dominant follicles showed greater diameter in G-Low than G-High at D4 (7.3 ± 2.2 *vs.* 6.2 ± 1.4 mm, P = 0.069, tendency), D8 (11.2 ± 1.8 *vs.* 9.5 ± 1.8 mm, P = 0.004), D9 (12.3 ± 1.7 *vs.* 10.6 ± 1.7 mm, P = 0.002), and D10 (13.4 ± 1.3 *vs.* 12.2 ± 1.8 mm, P = 0.019), and the estimated diameter of the ovulatory follicle tended (P = 0.08) to be higher in G-Low than G-High (14.4 ± 1.5 *vs.* 13.4 ± 2.1 mm). In the present, Nelore cows with high AFC showed high ovarian measurements. However, cows with low AFC had large follicular diameteres during the hormonal treatment for synchronization of ovulation.

Keywords: antral follicle count, synchronization, follicular dynamics, follicular diameter, cattle.

## 1. Introduction

The relationship between antral follicles count (AFC) and fertility has considerable importance for bovine production, because many physiological aspects remain unknown in cattle, and negative influence on biotechnologies such as artificial insemination (AI), timed artificial insemination (TAI) and *in vivo* and *in vitro* embryo production can dramatically impact strategies to provide genetic gain and improvement of livestock production. Therefore, the relationship between the number of antral follicles/AFC and reproductive performance in cattle, as well as its application in reproductive biotechnologies has been the focus of several studies in the last years [1-8].

The high variability in the number of antral follicles/AFC (follicles with diameter ≥ 3 mm) is an outstanding characteristic in cattle. Despite the high variability, there is high repeatability in the number of antral follicles observed in same individual in consecutive AFC evaluations [8, 9]. Therefore, this constancy of AFC in the same individual becomes a strategic resource by the possibility of classifying cattle with low, intermediate or high number of follicles performing a single ultrasound examination during the estrous cycle [2, 8-10].

The low AFC in *Bos taurus* females, especially with dairy ability, was associated with various features associate to low reproductive performance, such as small ovaries [10], less probability of pregnancy to the end of the breeding season [7], reduced responsiveness to superovulatory treatment, less potential for embryo production and few viable embryos [9, 12], low circulating concentrations of progesterone and anti-Müllerian hormone (AMH) [4-6] and reduced endometrial thickness [13]. Therefore, females with high AFC have demonstrated to be superior in all aspects mentioned, which suggests a linear correlation between high number of antral follicles and important aspects of reproductive fertility in *Bos taurus* cattle.

This context is poorly understood in *Bos indicus* cattle (both beef and dairy) due to the limited number of studies with this subspecie. Similar to North American and European studies, first studies in Brazil with *Bos indicus-taurus* and *Bos indicus* cattle revealed that at least embryo production is more efficient in donors with high AFC [1, 2]. However, studies that evaluated the association between AFC and pregnancy in *Bos indicus* (Nelore) cattle showed no influence of the number of antral follicles on the pregnancy to TAI [1, 14]. Although these studies have not completely clarified the influence of AFC in zebu cattle, it is noteworthy that the relationship between AFC and fertility (pregnancy TAI) in this subspecie does not seem to follow the same pattern described for taurine females, since there was no association between low AFC and low pregnancy.

To the best of our knowledge there is no study that evaluated the effect of AFC on ovarian follicular dynamics in *Bos indicus* cattle. Certainly, the behavior of ovarian follicular dynamics in females with different AFC can contribute to a better understanding of ovarian and follicular features in this subspecie. This study hypothesized that cows with high or low AFC present distinct pattern of follicular dynamics when subjected to a conventional TAI protocol. Therefore, the aim of this study was to evaluate the influence of low or high AFC on the ovarian follicular dynamics during hormonal treatment for synchronization of ovulation in Nelore cows.

## 2. Materials and methods

This study was conducted according to the standards of the Ethics Committee for Animal Experimentation of the State University of Londrina based on Federal Law 11.794 of 08 October 2008 and approved by number 5898.2014.76.

### *2.1. Location, animals and management*

This study was performed during the 2013/2014 breeding season in South America at latitude 24°12'18" and longitude 50°56'56" in a single commercial beef farm in South Brazil. The climate in this region is humid subtropical, with an average temperature above 25°C during summer and a rainy season from November to February.

The animals were selected from 250 Nelore females (*Bos indicus*) after ultrasound examination to determine AFC seven days (Day $_{-7}$) prior to the beginning of the TAI protocol. Then, a total of 75 multiparous cows (48 to 96 mo. old), with 30 to 50 days postpartum (average 45 days), without *corpus luteum* (CL) and large follicles (< 8 mm) [15], body condition score (BCS) between 2.5 and 4.0 (2.8 ± 0.2) on a scale of 1 to 5 [16] and maintained in continuous grazing of *Urochloa spp*. with mineralized mix and water *ad libitum* were used in this study.

### *2.2 AFC and experimental design*

To determine AFC, the ovaries from each animal were scanned with a 7.5-convex intravaginal array transducer (Aquila PRO, Pie Medical, Maastricht, the Netherlands) and antral follicles (all follicles ≥ 3 mm) were counted as previously described [8, 10] for allocating cows (n = 75) in two experimental groups according the number of antral follicles. Cows with consistently high (G-High, ≥ 45 follicles; n = 43) or low AFC (G-Low, ≤ 15 follicles; n = 32) were defined based on the overall mean of AFC (about 30 follicles) more or less one standard deviation (about 15 follicles). Animals with intermediate AFC (16 to 44 follicles) were not included in this study.

Seven days after this evaluation, animals were subjected to a synchronization protocol of ovulation at random day of the estrous cycle (Day 0) for monitoring ovarian follicular dynamics. The protocol consisted of the insertion of an ear implant containing 3 mg of norgestomet (Crestar, MSD Animal Health, São Paulo, Brazil) and intramuscular (i.m.) administration of 2 mg of estradiol benzoate (EB; Gonadiol®, MSD Animal Health, São Paulo, Brazil). On Day 8, the implants were removed, and the animals received i.m. injections containing 250 μg of cloprostenol (Ciosin®, MSD Animal Health, São Paulo, Brazil), 300 IU of equine chorionic gonadotropin (eCG; Novormon®, MSD Animal Health, São Paulo, Brazil) and 1.0 mg of estradiol cypionate (EC; ECP®, Zoetis, São Paulo, Brazil) as illustrated in Figure 1.

*2.3. Ovarian follicular dynamics*

Evaluations of follicular dynamics were performed by the same operator on a blinded assessment on days 0, 4, 8 to 10 (daily, toevaluation of follicular diameter), 10 to 12 (every 12 h, to monitoring of ovulation) and on day 18 (to evaluate the CL). All follicles ≥ 5 mm were identified and measured by ultrasonography and mean follicular diameter was calculated by two cross-sectional linear measurements of the follicular antrum captured on the ultrasound monitor; then, diameter were drawn on an ovarian map [17]. The diameter, perimeter and area of both ovaries for animals in the low and high groups were also determined by consecutive ultrasonography exams. After scanned from end to end, the largest ovary image was captured on the ultrasound monitor and the measurements of each ovary image were determined using an internal calibrator.

During each evaluation of follicular dynamics parameters such as AFC on days -7 (prior to the beginning of the TAI protocol), 4 and 18 (7 days after ovulation); ovarian diameter, perimeter and area on day 4; diameter of the largest follicle on days 4, 8, 9 and 10;

time of ovulation after ear implant removal; and diameter of the CL on day 18 were determined. All data were obtained for both ovaries and individually recorded for subsequent monitoring.

The dominant follicle (DF) was defined as the follicle that grew to at least > 8 mm in diameter and exceeded the diameter of all other follicles [18]. Ovulation was monitored at every 12 h between 48 and 96 h after progesterone device removal and previously detected by the absence of the DF and confirmed later by the presence of a CL in the same ovary. On the seventh day after ovulation, the diameter of the CL was determined in the same ovary that previously contained the preovulatory follicle. The diameter of the CL was determined by the average of two linear measurements of the cross-section area of the CL [17].

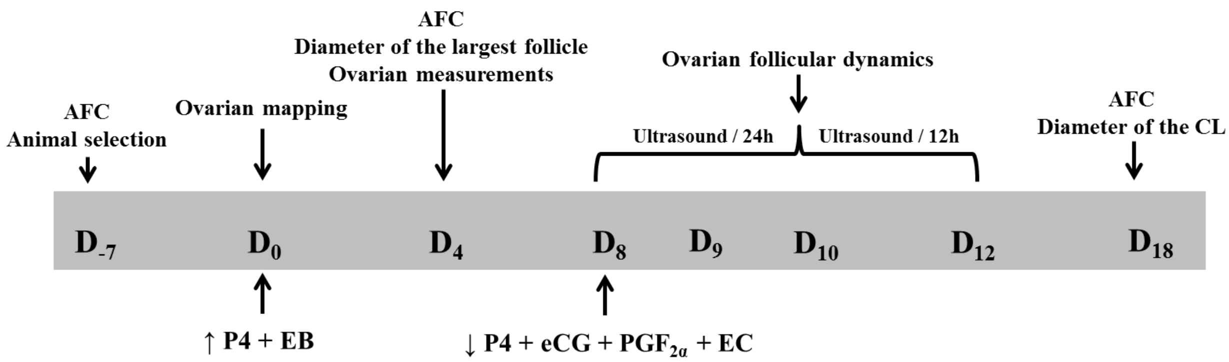


Figure 1. Experimental design to ovarian follicular dynamics and hormonal treatment used for synchronization of ovulation in Nelore cows with consistently high (G-High, ≥ 45 follicles; n = 43) or low AFC (G-Low, ≤ 15 follicles; n = 32). AFC - antral follicle count; P4 – progesterone/Norgestomet; EB - estradiol benzoate; eCG - equine chorionic gonadotropin; $PGF_{2\alpha}$ - cloprostenol; EC - estradiol cypionate; and CL - *corpus luteum*.

*2.4. Evaluated variables and statistical analysis*

The AFC in cows from groups was evaluated on days 0, 4 and 18; the ovarian diameter (mm), perimeter (mm) and area ($mm^2$) on day 4; the diameter of the largest follicle on day 4 (mm); the diameter of the DF on days 8, 9 and 10 (mm); and the diameter of the CL on day 18 (mm). The average of the follicular growth rate (mm/day), estimated diameter of the preovulatory follicle (mm) and the time of ovulation (h) were also determined.

Individual information of each animal was tabulated into a data set for statistical analysis. The daily follicular growth rate was calculated for each cow and each group according to Figueiredo et al. [18].

Quantitative variables were tested for the normal distribution of data, homogeneity of variances and therefore assessed using an analysis of variance (one-way ANOVA). If differences between groups were significant, the data were examined using the Tukey test. All data were analyzed using the Statistical Software MNITAB16 program. The significance level for rejecting the H0 (null hypothesis) was 5%; and, therefore, a level of significance ≤ 0.05 was considered to indicate an effect of the categorical variables and their interactions. The parametric data are presented as the mean values more or less one standard deviation (M ± SD).

## 3. Results

The AFC remained stable during the hormonal treatment for synchronization of ovulation in both the groups, and the number of antral follicles was consistently higher (P = 0.001) in G-High (48.9 ± 5.8, 49.5 ± 5.7 and 49.7 ± 6.9 follicles) compared to G-Low (12.6 ± 4.1, 12.5 ± 4.4 and 12.9 ± 3.8 follicles) at days -7, 4 and 18 (Table 1).

The ovarian measurements were higher in G-High than in G-Low (diameter 28.3 ± 3.9 *vs.* 20.5 ± 3.2 mm, P = 0.0001; perimeter 100.9 ± 13.2 *vs.* 73.8 ± 13.9 mm, P = 0.0001; area 67.5 ± 16.4 *vs.* 37.7 ± 11.7 $mm^2$, P = 0.0001).

Table 1. Parameters evaluated during the ovarian follicular dynamics in Nelore cows with consistently high (G-High, ≥ 45 follicles) or low AFC (G-Low, ≤ 15 follicles) following a TAI protocol.

| Variables | G-High AFC | | G-Low AFC | | P-value |
|---|---|---|---|---|---|
| | n | Mean ± SD | n | Mean ± SD | |
| Body condition score (1-5) | 43 | 2.8 ± 0.2 | 32 | 2.9 ± 0.2 | 0.423 |
| Synchronization rate (%) | 43 | 51.2 | 32 | 62.5 | 0.327 |
| AFC Day $_{-7}$ (n) | 43 | 48.9 ± 5.8 | 32 | 12.6 ± 4.1 | 0.0001 |
| AFC Day $_{4}$ (n) | 43 | 49.5 ± 5.7 | 32 | 12.5 ± 4.4 | 0.0001 |
| AFC Day $_{18}$ (n) | 43 | 49.7 ± 6.9 | 32 | 12.9 ± 3.8 | 0.0001 |
| Ovarian diameter (mm) | 43 | 28.3 ± 3.9 | 32 | 20.5 ± 3.2 | 0.0001 |
| Ovarian perimeter (mm) | 43 | 100.9 ± 13.2 | 32 | 73.8 ± 13.9 | 0.0001 |
| Ovarian area ($mm^2$) | 43 | 67.5 ± 16.4 | 32 | 37.7 ± 11.7 | 0.0001 |
| Follicular growth D4 to D8 (mm) | 22 | 3.3 ± 0.5 | 20 | 4.0 ± 0.4 | 0.304 |

| | | | | | |
|---|---|---|---|---|---|
| Follicular growth D8 to D9 (mm) | 22 | 1.2 ± 0.2 | 20 | 1.1 ± 0.2 | 0.737 |
| Follicular growth (mm/day) | 22 | 1.4 ± 0.2 | 20 | 1.1 ± 0.1 | 0.160 |
| Time of ovulation (h) | 22 | 69.8 ± 7.1 | 20 | 70.2 ± 5.9 | 0.851 |
| CL diameter (mm) | 22 | 18.2 ± 3.1 | 20 | 19.4 ± 1.7 | 0.129 |

The follicular diameter was greater in cows with low AFC than those with high follicle count at Day 4 (7.3 ± 2.2 *vs.* 6.2 ± 1.4 mm, P = 0.069), Day 8 (11.2 ± 1.8 *vs.* 9.5 ± 1.8 mm, P = 0.004), Day 9 (12.3 ± 1.7 *vs.* 10.6 ± 1.7 mm, P = 0.002), and Day 10 (13.4 ± 1.3 *vs.* 12.2 ± 1.8 mm, P = 0.019). However, the follicular growth was similar for G-Low and G-High from D4 to D8 (4.0 ± 0.4 *vs.* 3.3 ± 0.5 mm, P = 0.304), D8 to D9 (1.1 ± 0.2 *vs.* 1.2 ± 0.2 mm, P = 0.737), as well as the daily follicular growth rate from D8 to D10 (1.1 ± 0.1 *vs.* 1.4 ± 0.2 mm/day, P = 0.16; Figure 2).

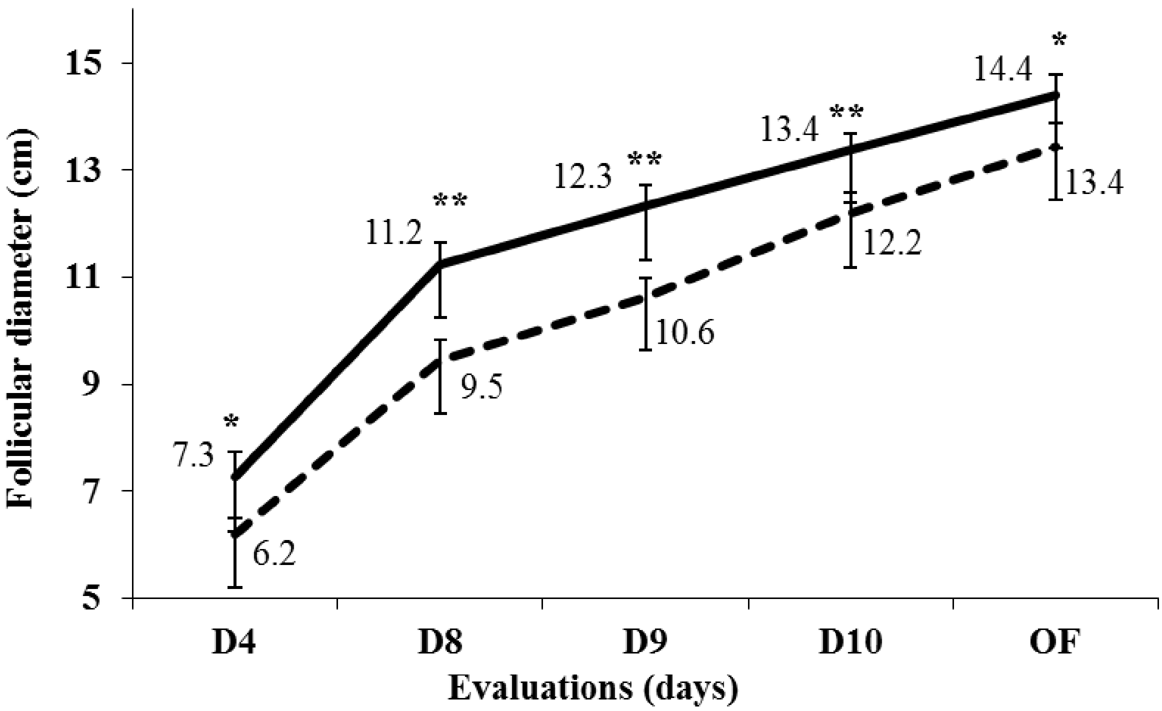


Figure 2. Follicular diameter (mm) and estimated diameter of the ovulatory follicle (OF; mm) during ovarian follicular dynamics in Nelore cows with consistently high AFC (≥ 45 follicles; dashed line) or low AFC (≤ 15 follicles; continuous line) synchronized with TAI protocol. (** P < 0.05 and * D4/P = 0.06 and OF/P = 0.08).

The time of ovulation after ear implant removal was similar between cows with low and high AFC (69.8 ± 7.1 *vs.* 70.2 ± 5.9 h; P = 0.85) as well as the CL diameter at Day 18

(19.4 ± 1.7 *vs.* 18.2 ± 3.1 mm; P = 0.129). However, the estimated diameter of the ovulatory follicle showed a tendency to be greater in G-Low compared to G-High (14.4 ± 1.5 *vs.* 13.4 ± 2.1 mm; P = 0.087).

## 4. Discussion

To our knowledge, this is the first study evaluating ovarian follicular dynamics in *Bos indicus* cows with high or low AFC subjected to synchronization of ovulation. This study showed that ovarian measurements (diameter, perimeter and area) in cows with high AFC were higher than in cows with low AFC. In accordance with our hypothesis, the present study also showed that follicular diameters since the fourth day of the protocol for synchronization of ovulation until the time of estimated diameter of the ovulatory follicle were higher in cows with low AFC than in those with high AFC. However, the follicular growth rate during this period was similar between groups.

The present study provides applicable information about AFC and its relationship with ovarian follicular dynamics in *Bos indicus* cattle, suggesting that differences in follicular diameter between groups of high and low AFC may affect the pregnancy rate in cows this subespecie submitted to TAI. Additionally, it contributes for a better understanding of antral follicular population in *Bos indicus*, which was different from results obtained in others studies conducted with *Bos taurus* cattle outside Brazil.

Many studies on the AFC and fertility in bovine female associated low AFC with several negative characteristics to fertility, especially in *Bos taurus* cattle with dairy ability [4-7, 10, 19]. Small ovaries [10], less probability of pregnancy to the end of the breeding period [7], reduced responsiveness to treatment SOV, few viable *in vivo* and *in vitro* produced embryos [5, 9, 12], low circulating concentrations of progesterone and AMH [6, 13] and reduced endometrial thickness during estrous cycles [13] were associated to females with low AFC.

In the present study, cows with high AFC showed greater ovarian diameter (28.3 ± 3.9 mm), perimeter (100.9 ± 13.2 mm) and area (67.5 ± 16.4 $mm^2$) than cows with low AFC (20.5 ± 3.2 mm, 73.8 ± 13.9 mm and 37.7 ± 11.7 $mm^2$, respectively). Although investigating different ovarian measurements, Ireland et al. [10] reported differences between ovarian height and length in cows with high *vs.* low AFC (15.5 ± 0.8 *vs.* 12.3 ± 1.1 mm, P < 0.05 and 28.3 ± 1.3 *vs.* 23.3 ± 1.4 mm, P < 0.05, respectively). Similarly, after assessement of lactating dairy cows with high (≥ 30 follicles), intermediate (21 to 29 follicles) or low AFC (≤ 20 follicles), Martinez et al. [19] found greater total ovarian area in animals with high AFC ($53.56 \pm 15.6$ mm$^2$) than intermediate and low AFC ($47.50 \pm 13.5$ and $43.04 \pm 11.3$ mm$^2$, $P = 0.001$, respectively). Therefore, data from this present study suggest that the largest size of ovary (diameter, perimeter and area) in *Bos indicus* cattle is also related with a greater number of antral follicles.

Cows with low AFC presented higher follicular diameter since day 4 of the hormonal treatment, remaining with a larger dominant follicle diameter over the following days until the presumed time to TAI. Additionally, the estimated diameter of the preovulatory follicle showed also a tendency to be higher ($P = 0.087$) in the group with low AFC compared to the high AFC. Conversely, in Holstein cows with low (≤ 15 follicles), intermediate (16 to 20 follicles), high (21 to 25 follicles) or very high (≥ 25 follicles) number of follicles during waves, Burns et al. [8] found no differences ($P > 0.1$) between groups regarding length of dominance, interval between ovulations, day of emergence, day of deviation, largest diameter of the dominant and subordinate follicles during dominance, and diameter of the dominant and subordinate follicles at deviation. It is noteworthy that these authors conducted the study in Holstein cows and the minimum number of follicles of the group with high AFC was lower (≥ 25 follicles) compared to the group with high AFC in the present study (≥ 45 follicles).

In our study, the diameter of the dominant follicle after 48h of progesterone implant removal (day that often TAI is performed) was higher ($P < 0.05$) in cows with low than high AFC ($13.4 \pm 1.3$ *vs.* $12.2 \pm 1.8$ mm). In Nelore cows in the postpartum using a conventional protocol to TAI (intravaginal device/EB and eCG/$PGF_{2\alpha}$/EC), Morotti et al [20] found dominant follicle of $11.4 \pm 1.4$ mm at TAI and $12.1 \pm 1.4$ mm to ovulatory follicle. Similarly, after induction of ovulation with EB and EC in *Bos indicus* cattle, Sales et al. [21] found a maximum diameter of dominant follicle of $13.4 \pm 0.4$ and $14.2 \pm 0.3$ and ovulatory follicle of $13.1 \pm 0.4$ and $13.9 \pm 0.4$ mm (for the respective inducers).

To the best of our knowledge, there is no report of studies that evaluated the effect of AFC on the ovarian follicular dynamics in *Bos indicus* cattle and even the information of AFC on the follicular diameter and growth rate is limited to *Bos taurus*. Although Burns et al. [8] showed no effect of AFC on the follicular behavior during the emergency, selection and dominance, many other studies, outlined especially with dairy cattle (*Bos taurus*), point to a low reproductive performance for cows with low AFC [4-7, 9, 10, 13]. It is certainly suggestive of different follicular behavior between cattle with low and high AFC.

The present experiment was aroused mainly by field results observed in other studies that found no effect of AFC on the conception rate following insemination of Nelore cows [1, 14]. The absence of an effect reveals very intriguing and completely different information from studies conducted outside Brazil, which associate cows with low AFC and low fertility [4, 5, 7]. Comparing the conception rates for TAI and *in vitro* embryo production in Nelore cows with different AFC (G-High ≥ 25; G-Intermediate 16 to 20; and G-Low ≤ 10 follicles) females with high AFC had larger embryo production than intermediate and low groups. However, there was no difference in the conception rates after TAI between cows with high, intermediate or low AFC (51.9 *vs.* 48.6 *vs.* 58.6%) [1].

Despite the larger follicular diameter in cows with low AFC, the follicular growth rate was similar between groups of high and low AFC (Table 1). Studies performed with Nelore cattle reported an average rate of follicular growth (0.9 to 1.0 mm/day) similar to those observed the presenty study, both during hormonal treatment for TAI [20], such as during the last follicular wave in cows without control of the estrous cycle [18].

The ovulatory follicle in cattle may have variability from 12 to 20 mm of diameter [22, 23] depending on factors such as use of hormonal treatment to control of the estrous cycle and subspecies particularities, which in *Bos indicus* smaller diameter can be found [18]. The dominant follicle that is able to reach a larger diameter after follicular deviation has a better ovulatory potential [24] and result in larger CL and, consequently, higher progesterone concentration [25]. In *Bos indicus* females has been already reported that the largest ovulatory follicle detected at TAI provides higher probability of pregnancy [26-28].

The influence of the ovulatory follicle diameter (classes: 10-12, 13-15, 16-18, 19-21 and > 22 mm) on the reproductive performance of lactating beef cows (*Bos taurus*, 60-80 days postpartum) subjected to TAI [25] was reported cows which presented an ovulatory follicle > 19 mm resulted in a larger CL, but cows with an ovulatory follicle between 13-15 mm had higher pregnancy. Therefore, although larger ovulatory follicles result in larger CL and higher progesterone production, the optimal size of ovulatory follicles (13-15 mm) may result in positive benefits on pregnancy rate for cows subjected to TAI. In this context, considering the beneficial relationship between the largest follicular diameter and fertility, it can be suggested that in the present study, the largest follicle diameters in females with low AFC can influence the pregnancy rate of cows subjected to TAI.

In *Bos indicus* cattle another study evaluated whether changing the interval from progesterone removal to TAI according to the diameter of the preovulatory follicle would improve pregnancy per AI in cows [28]. In lactating Nelore cows (n = 412) the researchers performed TAI in blocks according to the diameter of the dominant follicle on the morning of Day 10 (time of TAI). After an ultrasound exam to determine the follicular diameter, cows with follicles ≥ 15 mm received TAI 0 h; 13 to 14.9 mm, TAI 6 h later; 10.1 to 12.9 mm, TAI 24 h later; and ≤ 10 mm, TAI 30 h later. The cows of the block group had greater pregnancy rates per AI than the control group (129/203, 63.5% vs. 102/209, 48.8%, respectively; $P < 0.01$). Clearly all of these studies indicate that the larger diameter of the dominant follicle at TAI provides improved pregnancy. Therefore, if it considers that cows with low AFC had larger diameters of the dominant follicle it can be suggested a time earlie of insemination compared to cows with high AFC during TAI programs.

Some hypotheses can be highlighted for greater follicular diameter in cows with low AFC, the first is an asynchrony in follicular emergency between the groups. If emergency of low group occurred before fourth day (time in that we defined to start follicular monitoring) indeed a higher growth time may have favored this group. Another possibility is that the high number of antral follicles provides a more competitive environment by hormonal action, and in this case, is more advantageous for the cows with low AFC that due to the lower number of follicles has a better gonadotropin use. In cattle for example, there is evidence that concentrations of FSH are inversely, rather than positively, associated to the number of follicles ≥ 3 mm diameter during follicular waves [8].

The time of ovulation after removal of the progesterone device and CL diameter on the seventh day after ovulation were similar between groups of high and low count. In *Bos indicus* cattle, after synchronization with TAI protocol, there are reports of CL with average diameter of 17.6 to 18.7 mm [20, 28-30] and without control of the estrous cycle an average size slightly lower of 15.5 to 17.8 mm [18].

Although the present study reveals different results from others conducted in *Bos taurus*, it should be highlighted that there are numerous physiological differences between the subspecies, being fully acceptable that reproductive behavior observed in subspecies to be different another. Certainly, the high variability of AFCs between studies, the different herds and categories of females, the different management practices adopted, and especially the absence of a standardization in the group classification become even more challenging the full knowledge of AFC on the fertility of female bovine.

In conclusion, Nelore cows with high AFC (≥ 45 follicles) had higher ovarian measurements (diameter, perimeter and area) than those with low AFC (≤ 15 follicles). However, the follicular diameter (dominant and ovulatory follicles) was higher in the group with low follicular count during the hormonal treatment for synchronization of ovulation. Additionally, this study suggests that AFC can influence the pregnancy rate in *Bos indicus* cows subjected to TAI.

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## ARTIGO 2

Correlation between phenotype, genotype and antral follicle population in beef heifers

*O artigo está sob avaliação no periódico Theriogenology.*

# Correlation between phenotype, genotype and antral follicle population in beef heifers


## Abstract

The present study was performed in *indicus-taurus* heifers 1) to determine if the antral follicle count (AFC) exhibits repeatability from puberty to yearling age and 2) to evaluate whether the phenotypic and genotypic parameters used in genetic improvement programs are correlated with AFC. In study I, Braford heifers (3/8 Brahman x 5/8 Hereford, n = 137) were serially examined by ultrasonography (with 60-day intervals) from weaning (9 ± 1 mo of age) to yearling ages (20 ± 1 mo of age) to monitor the numbers of antral follicles. In study II, the AFC of animals from experiment I and contemporary heifers (n = 270, 18-24 months) was correlated with the records of a genetic selection program using four statistical models with different covariates: i) model 1 considered effects of contemporary group and covariates age, weight gain from birth to weaning and visual scores for carcass traits at weaning, ii) model 2 covered contemporary group, age, weight gain from weaning to yearling and visual scores for carcass traits at yearling. The effects, variables and covariates of models 1 and 2 were combined to form model 3. Model 4 included the model 3 with addition of paternal effect. In study I, AFC varied from 3 to 64 follicles among females but was repeatable (0.89 to 0.92) within individuals in the same group. In study II, the four models tested showed low correlations with AFC: 0.072, 0.056, 0.082 and 0.172 for models 1, 2, 3 and 4, respectively. However, the model with paternal effect provided the best explanation for the genetic correlation of genotypic and phenotypic characteristics with AFC (17%). Models 1, 3 and 4 also showed that AFC in *indicus-taurus* heifers can be influenced by finishing precocity at weaning ($p < 0.05$). Based on these studies, AFC in heifers from weaning to yearling age is highly variable between individuals and repeatable within the same female. Additionally, there is no correlation between phenotypic or genotypic characteristics and the antral follicle population. However, AFC can be affected by finishing precocity at weaning.


Keywords: Antral follicles count; ultrasonography; genetic improvement; heifers; fertility.

1. Introduction

The use of reproductive biotechnologies in association with genetic improvement programs has been a widespread strategy for improving livestock production in a short time. For this purpose, artificial insemination and embryo production are the tools with the highest potential for disseminating superior genetic qualities and improving reproductive performance in both beef and dairy cattle [1, 2]. However, there is a greater emphasis on the *in vitro* production of embryos, which has been the most used technique in the world during the last decade [3], with highly effective results reported in many large-scale studies in cattle [4-7]. Nevertheless, the number of antral follicles (follicles ≥ 3 mm of diameter visualized on ultrasound) or antral follicle count (AFC) is closely related to the number of embryos produced by the donor, in both *in vitro* and *in vivo* methods [4, 6, 8], and can affect the reproductive efficiency of cattle.

AFC is an outstanding characteristic in the bovine female, and its influence on the efficiency of reproductive biotechnologies and reproductive performance in dairy cattle has been well documented in several studies performed especially in *Bos taurus* herds as revised by Ireland, Smith [9] and Evans, Mossa [10]. This count has high variation among animals and high repeatability within individuals, which allows the classification of females as low-, intermediate- or high-AFC by a simple ultrasonography exam during follicular waves [11-13].

For some reproductive biotechnologies, especially embryo production, ultrasonography has become a strategic tool in donor selection due to the constancy of AFC throughout the estrous cycles. Additionally, there is evidence that variability in the number of antral follicles in *Bos taurus* cattle can affect some characteristics of female fertility and compromise the reproductive performance of the herd [9, 10, 14]. Studies have shown that high AFC is positively correlated with characteristics linked to female fertility, such as the total number of morphologically healthy follicles and oocytes in the ovary [13], responsiveness to gonadotrophin treatment during superovulation (SOV) and the number of transferable embryos [9, 12], progesterone production during the estrous cycle [15], and a smaller interval between calving and improved reproductive performance [14]. In divergence, cows with low AFC (Holstein) have exhibited a negative impact on reproductive performance when several aspects related to fertility are compared with those of cows with a high follicle count [9, 10, 14].

Studies also have shown that AFC can be affected by maternal environmental conditions such as nutritional and health status during pregnancy [9, 10, 16, 17]. Additionally a single study has examined the relationship between AFC and genetic characteristics in dairy cattle (Holstein), which demonstrated that follicle count is also affected by lactation status and quality of milk; however, the level of milk production did not influence this count [18]. The effect of characteristics of genetic merit on AFC is an issue that needs to be better understood because the selection of females based on AFC has been a common practice before starting some reproductive biotechnologies; for example, due to the quantitative benefits, embryo production programs have prioritized donors with high follicle count. In this context, there is concern regarding whether AFC may be affected by characteristics linked to the genetic value of the female, especially in beef cattle, which has not been studied.

Considering the lack of information, we hypothesized that Braford heifers exhibit stable AFC from weaning to yearling age and as well as number of antral follicles can be influenced by characteristics of genetic merit. Therefore, the objectives of the present study in beef heifers were 1) to determine whether the number of antral follicles exhibits repeatability in its count between puberty and yearling age and 2) to evaluate whether the main phenotypic and genotypic parameters used in genetic improvement programs can be correlated with AFC in heifers.

## 2. Materials and Methods

### *2.1. Location, animals and feed management*

The experiments of the present study were performed in compliance with protocols approved by the Committee of Ethics in Animal Experimentation at the Universidade Estadual de Londrina, Parana, Brazil.

This study was performed in *Bos indicus-taurus* beef cattle that belonged to a large genetic improvement program (Conexão Delta G Group, Londrina, Parana, Brazil) during the 2012-2013 breeding season in South America at latitude 23° 17' 34" and longitude 51° 10' 24". The climate in this region is tropical, with an average temperature of 24°C and a rainy season from November to January.

Braford cattle (3/8 Brahman x 5/8 Hereford) with adequate body-condition score (BCS; between 3.0 and 4.0 on a scale of 1 to 5 according to Lowman, Scott [19] and normal health status were selected for the experiments. The animals were maintained by continuous grazing of *Urochloa brizantha* pasture and were given *ad libitum* access to mineralized mix and water.

*2.1.1. Study I - Antral follicle count and monitoring*

To evaluate AFC, Braford heifers (n = 137) were serially examined by ultrasonography from weaning (Mean ± SD; 9 ± 1 mo of age) to yearling ages (20 ± 1 mo of age). At 24 mo of age, the mean BCS was 3.0 ± 0.5, and the average live body weight was 360 ± 10 kg.

On a random day of the estrous cycle (Day 0), both ovaries from each animal were assessed (from end-to-end) using a real-time B-mode ultrasound scanner (Scanner 200 Vet; Pie Medical, Maastricht, The Netherlands) equipped with a 7.5-MHz convex intravaginal array transducer, and antral follicles (all follicles ≥ 3 mm in diameter) were counted as described previously [11, 13]. Additional similar ultrasound exams were performed at 60-day intervals (days 0, 60, 120, 180, 240 and 300) to monitor the numbers of antral follicles.

All of the ultrasound examinations were systematically performed by the same operator (without knowledge of the AFC of each animal - blind assessment), and all data were obtained in both ovaries and individually recorded for subsequent monitoring.

*2.1.2. Study II - Antral follicle count and variables of genetic merit*

Heifers from experiment I (n = 137) and another contemporary group of Braford heifers (n = 133) were included in the study to determine the correlation between AFC and genetic merit characteristics in beef cattle. The group of contemporary heifers belonged to the same property as experiment I and was established under the same conditions of health and nutritional management. Considering the previous results of study I, the AFCs of the contemporary animals were determined by intravaginal ultrasonography in a single assessment on a random day of the estrous cycle, under the same criteria adopted in the previous study and by the same operator. Characteristics of genetic selection (zootechnical characteristics) were evaluated in all heifers (n = 270) by the same inspector and in accordance with criteria adopted by the genetic improvement program (GenSys®, Porto Alegre, Brazil).

Data of birth, evaluations and ranking of productive characteristics at weaning and yearling ages were registered in the program database, and using corrections from the program itself, the data were adjusted for age of mother and time of birth. Phenotypic and genotypic characteristics considered in this study were age (AG); variables to weaning [weight gain from birth to weaning (GW) and visual scores for conformation at weaning

(CW), finishing precocity at weaning (PW) and musculature at weaning (MW)]; and variables to yearling [weight gain from weaning to yearling (GY) and visual scores for conformation at yearling (CY), finishing precocity at yearling (PY) and musculature at yearling (MY)]. The effects of contemporary group (CG) and sire of the heifer (effect of 23 bulls) were also considered depending on the models used (Figure 1).

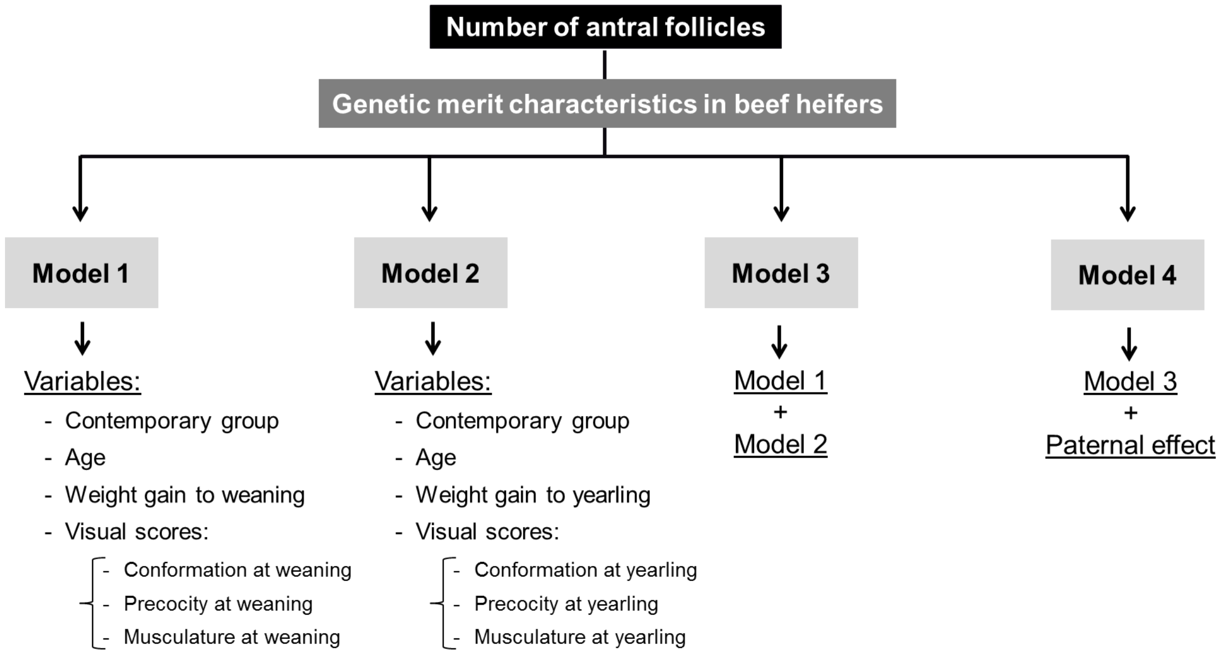


Figure 1. Experimental design for correlation of the parameters of genetic merit and number of antral follicles in beef heifers.

Model 1: Effects of contemporary group and covariates age, weight gain and visual scores at weaning (conformation, precocity and musculature).

Model 2: Effects of contemporary group and covariates age, weight gain and visual scores in the yearling (conformation, precocity and musculature).

Model 3: Effects, variables and covariates of models 1 and 2.

Model 4: Parameters of the model 3 and paternal effect (sire of the heifer).

The visual scores for conformation, precocity and musculature were individually assigned and ranged from 1 to 5 (Table 1) as described by Severo [20], i.e., for the conformation characteristic, the presence of muscle mass and total estimate of meat on the carcass were considered, in combination with good and strong physical structure and size (including good leg alignment). For precocity, early fat deposition capacity (finishing for slaughter) was considered, along with the animal biotype analysis (longilineal structure - late animal; median structure - early animal). For musculature, the development of muscle mass (shape and muscle activity) was considered by observing points such as the forearm, leg, palette, loin, croup, and especially the width and depth of the hindquarters.

Table 1. Weighting percentage applied to the expected progeny difference for ranking from Braford heifers (3/8 Brahman x 5/8 Hereford, n = 270) at weaning and yearling

| Productive characteristics | Weighting (%) |
|---|---|
| Weight gain from birth to weaning (GW, Kg) | 25 |
| Conformation at weaning (CW, score of 1 to 5) | 4 |
| Finishing precocity at weaning (PW, score of 1 to 5) | 8 |
| Musculature at weaning (MW, score of 1 to 5) | 8 |
| Weight gain from weaning to yearling (GY, Kg) | 25 |
| Conformation at yearling (CY, score of 1 to 5) | 4 |
| Finishing precocity at yearling (PY, score of 1 to 5) | 8 |
| Musculature at yearling (MY, score of 1 to 5) | 8 |

After yearling ratings, heifers (n = 235) inserted in the breeding program were ranked based on the Decas (1 to 10), an index often used in breeding programs that is determined based on the value of Expected Progeny Differences (EPDs) [21]. The weighting of animals in the Decas allows the quick and objective classification of program members. In the present study, the animals were ranked into groups of 10% according to the expected normal distribution, i.e., Deca 1 corresponded to the group of animals in the top 10% relative to their contemporaries, Deca 2 corresponded to the group between the top 10% and the top 20%, and so on, with the Deca 10 animals considered to be in the bottom 10% with respect to assessed production.

*2.2. Statistical analysis*

In the first study (on AFC and its repeatability), data were analyzed according to a normal distribution (parametric), and the females were assigned to three groups according to the number of antral follicles (follicles ≥ 3 mm diameters) and the standard deviation (SD), as follows: heifers with a consistently high (mean number of follicles of all the 137 heifers plus

1 SD; G-High, ≥ 41 follicles), intermediate (heifers with AFC closest to the mean number of follicles of all the 137 heifers; G-Intermediate, ≥ 12 and ≤ 40 follicles) or low AFC (mean number of follicles of all the 137 heifers minus 1 SD; G-Low, ≤ 11 follicles) in all ultrasound scans. Differences among assessments of AFC (days 0, 60, 120, 180, 240 and 300) were compared using analysis of variance (ANOVA) with repeated measures on Minitab® statistical 16.1.1 software. The significance level was set at $P \leq 0.05$, and the values were expressed as the mean ± one SD. Repeatability (proportion of the total variance that could be attributed to animal variance, range 0-1, 1 = perfect) was calculated according to Boni, Roelofsen [22].

To determine the association between characteristics of genetic merit (phenotypic and genotypic) and AFC in beef heifers, the data from the second study were analyzed using four different models, which considered different covariates. In model 1 (n = 270), the effect of CG and the covariates AG; GW; and visual scores for CW, PW and MW were considered. In model 2 (n = 270), the effects of CG and the covariates AG; GY; and visual scores for CY, PY and MY were considered. The effects, variables and covariates of models 1 and 2 were combined to form model 3 (n = 270). Model 4 (n = 270) included all the parameters of model 3 and added paternal effect (sire of the heifer). Additionally, the correlation between genetic ranking (Deca) and AFC was evaluated by Pearson correlation coefficient. All data were analyzed by linear regression using the GLM procedure of SAS, considering $P \leq 0.05$ to be significant.

## 3. Results

Out of 137 heifers monitored in study I, 25 (18.25%) showed higher numbers of antral follicles ≥ 41 follicles (G-higher) in all evaluations, 87 (63.50%) animals showed follicular count between 12 to 40 follicles (G-intermediate) and 25 (18.25%) animals had less than ≤ 10 follicles (G-Low) in all exams. The mean number of antral follicles varied ~21-fold among females (range 3 to 64 follicles) but was repeatable (G-High r = 0.90; G-intermediate r = 0.89 and G-Low r = 0.92) within individuals in the same group (Figure 2).

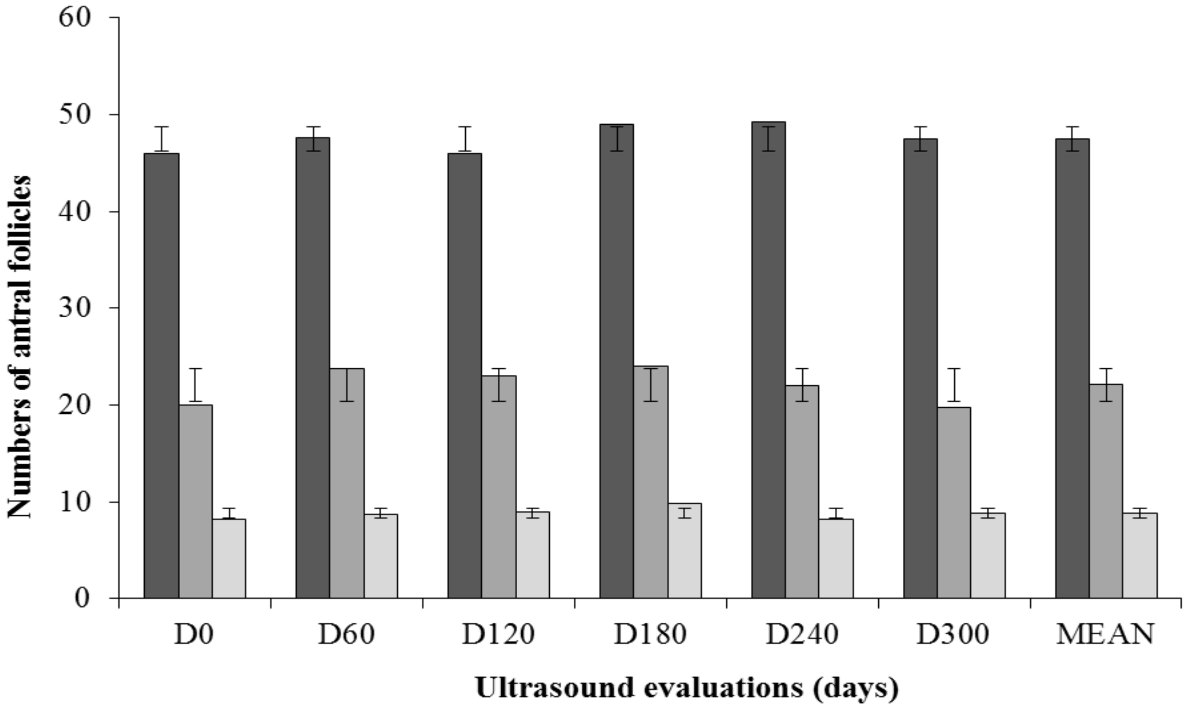


Figure 2. Antral follicle count during ultrasound evaluations from weaning to yearling ages in beef heifers with high- (Dark Gray column, G-High ≥ 41 follicles, n = 25), intermediate (Gray column, G-Intermediate 12-40 follicles, n = 87) or low-AFC (Light Gray column, G-Low ≤ 11 follicles, n = 25).

The average weight at the beginning of the experiment (weaning - Day 0) and at the end of the study (yearling - Day 300) did not differ between heifer groups: Low- (188 ± 27 and 300 ± 35 kg), Intermediate (194 ± 33 and 303 ± 27 kg) and High-AFC (186 ± 22 and 301 ± 28 kg; P > 0.05).

Out of a total of 270 heifers included in study II, the average AFC was 26.82 ± 15.74 follicles, with females presenting variability from 2 to 90 antral follicles. Among the four models tested in this study, model 2, which includes effects of CG and the covariates AG; GY; and visual scores for GY, PY and MY (yearling characteristics), showed no effect on the AFC ($p > 0.05$) and revealed a coefficient of determination ($R^2$) of 0.056. For the other models, the coefficients of determination were 0.072 (model 1 - effects, variables and covariates of weaning); 0.082 (model 3 - effects, variables and covariates of weaning and yearling); and 0.172 (model 4 - paternal effect, in addition to variables and covariates of weaning and yearling). Additionally, from all models that showed some correlation (models

1, 3 and 4), the visual scores for finishing precocity at weaning showed significant effects ($p < 0.05$; Table 2) on the numbers of antral follicles of the evaluated heifers.

Table 2. Coefficients of regression and p-values based on regression analysis for antral follicle count and characteristics of genetic merit in different models tested in *indicus-taurus* heifers

| Models | Variables and covariables | Coefficient of regression (±SE) | p-values |
|---|---|---|---|
| 1 | Contemporary group | 4.26 (10.9) | 0.137 |
| | Age | 0.20 (0.01) | 0.230 |
| | Weight gain to weaning | 0.07 (0.61) | 0.369 |
| | Conformation at weaning | 2.10 (1.66) | 0.765 |
| | Precocity at weaning | - 2.81 (1.43) | 0.018 |
| | Musculature at weaning | - 1.04 (1.65) | 0.528 |
| 2 | Contemporary group | 1.26 (9.44) | 0.091 |
| | Age | 0.02 (0.01) | 0.108 |
| | Weight gain to yearling | - 0.04 (0.08) | 0.531 |
| | Conformation at yearling | - 0.25 (1.42) | 0.926 |
| | Precocity at yearling | - 1.36 (1.57) | 0.603 |
| | Musculature at yearling | 1.68 (1.61) | 0.297 |
| 3 | Contemporary group | 0.55 (9.41) | 0.087 |
| | Age | 0.03 (0.01) | 0.106 |
| | Weight gain to weaning | 0.06 (0.07) | 0.396 |
| | Conformation at weaning | 2.92 (1.81) | 0.449 |
| | Precocity at weaning | - 3.25 (1.55) | 0.011 |
| | Musculature at weaning | - 1.02 (1.72) | 0.556 |
| | Weight gain to yearling | - 0.01 (0.10) | 0.714 |
| | Conformation at yearling | - 0.78 (1.57) | 0.739 |
| | Precocity at yearling | - 0.65 (1.59) | 0.886 |
| | Musculature at yearling | 1.26 (1.62) | 0.438 |
| 4 | Contemporary group | 1.66 (9.42) | 0.083 |
| | Age | 0.03 (0.01) | 0.087 |
| | Weight gain to weaning | 0.09 (0.08) | 0.131 |
| | Conformation at weaning | 2.71 (1.86) | 0.414 |
| | Precocity at weaning | - 2.93 (1.58) | 0.017 |
| | Musculature at weaning | - 0.65 (1.77) | 0.684 |
| | Weight gain to yearling | - 0.01 (0.10) | 0.614 |
| | Conformation at yearling | - 0.44 (1.63) | 0.680 |
| | Precocity at yearling | - 1.75 (1.65) | 0.379 |
| | Musculature at yearling | 1.13 (1.67) | 0.499 |
| | Bull | 4.99 (8.13) | 0.379 |

The genetic ranking (Deca) did not correlate with the number of antral follicles ($p > 0.05$) in Braford heifers. In addition, the proportion of heifers that received Deca 1 in the ranking (animals considered to be in the top 10% of the program) was similar between the groups of low, intermediate and high AFC (16.9, 15.3 and 15.6%, respectively; Figure 3; $P > 0.05$).

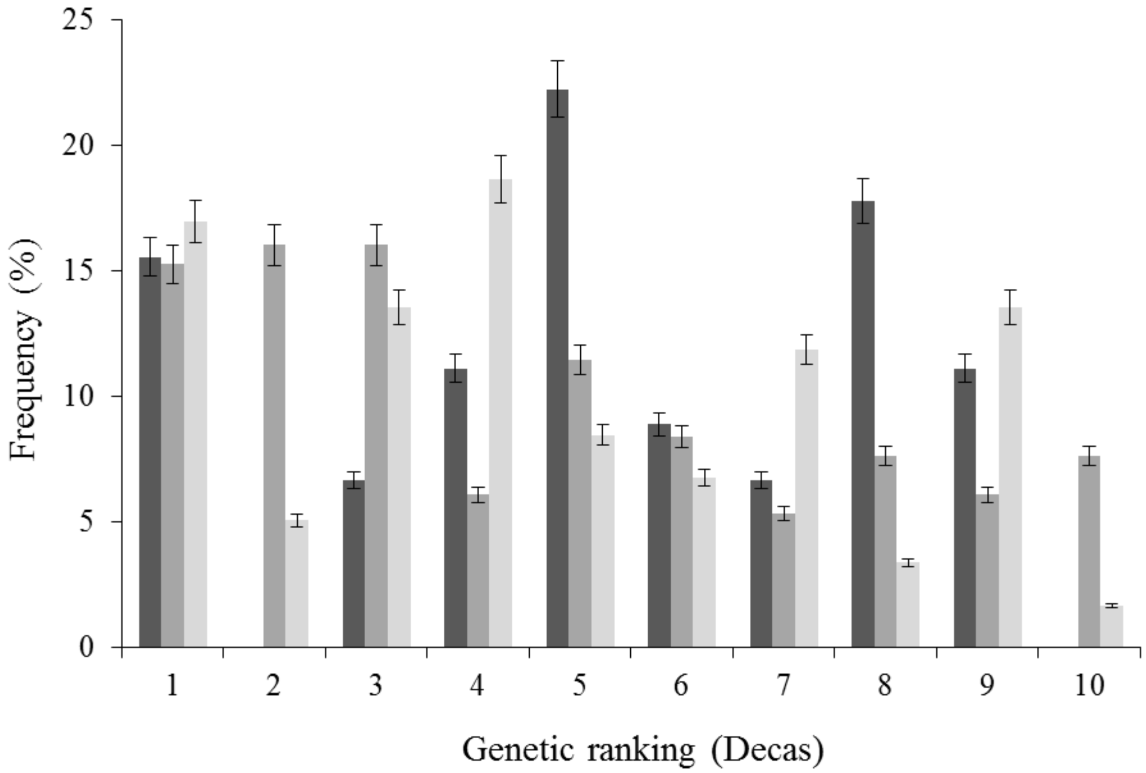


Figure 3. Frequency distribution of genetic ranking (Decas) in beef heifers according to the antral follicles count high (Dark Gray column, G-High ≥ 41 follicles, n = 45), intermediate (Gray column, G-Intermediate 12-40 follicles, n = 131) and low (Light Gray column, G-Low ≤ 11 follicles, n = 59).

Deca 1: Corresponded to the group of animals in the top 10% relative to their contemporaries.

Deca 2: Corresponded to the group between the top 10% and the top 20% relative to their contemporaries.

Deca 10: Animals considered to be in the bottom 10% relative to their contemporaries.

## 4. Discussion

The present study showed that in beef heifers, the numbers of antral follicles at weaning and yearling ages are highly variable between individuals but undergo only slight variation, showing a high repeatability (0.90 to 0.92) within the same female. Additionally, to our knowledge, this is the first study to evaluate the correlation between number of antral follicles and genetic merit characteristics in beef heifers that were actively participating in a genetic improvement program. The genetic models tested in this study did not demonstrate an effect on AFC; although (1) the bull variable genetically explained up to 17% of the variability of AFC in heifers, and (2) the visual scores for precocity at weaning exhibited a negative correlation with AFC.

The results of this study are interesting because they provide more information about using AFC as tool for selecting females in reproductive programs. This topic should be better discussed because full clarity is still lacking regarding the influence of AFC on bovine fertility when comparing studies with *Bos taurus* [12-15, 23] and *Bos indicus* [24, 25]; moreover, the relationship between the number of antral follicles and genetic merit characteristics in beef cattle. In this context, understanding the relationship between AFC, fertility and genetic characteristics is important to develop the best strategies for livestock management.

The high variability of AFC among beef heifers in the present study (range 3 to 64 follicles) and high repeatability (0.90 to 0.92) within the same animal were similar to results obtained in studies with *Bos taurus* that found variation from 8 to 56 follicles per wave among females and repeatability of 0.85 to 0.95 within the same individual, regardless of race, age, breeding season, lactation or pregnancy conditions [11, 12]. Therefore, although this characteristic is already known in *taurus* animals, we highlighted the fact that in *indicus-taurus* cattle, a single ultrasound examination during follicular waves can identify females with low, intermediate or high AFC even at weaning (9 ± 1 mo of age), due to the high degree of repeatability within the same female.

Currently, large-scale programs for the production of embryos in cattle perform ultrasound examination of the donor prior to the OPU or SOV procedure to select females with the highest number of antral follicles. This ultrasonography evaluation has become a widely used routine because the number of antral follicles has a close relationship with the number of embryos produced per donor, via both *in vitro* and *in vivo* methods [4, 6]. This relationship exists because the high variabilities in oocyte recovery rate and superovulatory response are important factors that affect the success of bovine embryo production [6, 8, 12, 26].

In this context, several studies involving *Bos taurus* [12, 26, 27], *Bos indicus* [4] and cattle with *indicus-taurus* blood [6, 8] have shown that a greater number of follicles has been associated with quantitative benefits to the success of OPU/IVP and SOV)/embryo collection. In a recent study, our research group also found these benefits to the evaluated reproductive performance of *indicus-taurus* females with high (≥ 40 follicles) and low AFC (≤ 10 follicles), comparing embryo production following *in vitro* (OPU/IVP) and *in vivo* (SOV/embryo collection) procedures. The study showed higher production of embryos in females with high than low AFC in both OPU/IVP (6.10 ± 4.51 *versus* 0.55 ± 0.83) and SOV/embryo collection (6.95 ± 5.34 *versus* 1.9 ± 2.13) [8].

For *Bos indicus* (Nelore) cattle we also showed that high numbers of antral follicles are positively associated with *in vitro* embryo production but not with conception rate in females subjected to TAI. The number of viable embryos was 18.4 from donors with high AFC, 6.1 from females with intermediate AFC and 0.6 from those with low AFC ($P < 0.05$). However, there was no difference in the conception rates after TAI between cows with high, intermediate or low AFC (51.9 vs. 48.6 vs. 58.6% [28].

Really, it is well established that donors with higher numbers of follicles are associated with higher numbers of IVF embryos than females with few follicles. This value is a numerical indicator that is strongly considered for improving the efficiency of the IVF industry. However, little was known about genetic merit and the number of antral follicles, particularly in beef cattle. Here, we present consistent data to clarify this topic. We found low correlation between the number of antral follicles and the most important parameters of evaluation in beef cattle. It is also important to emphasize that the animals of the three follicular categories (low, intermediate and high AFC) were similarly distributed among the best 10% of females (Deca 1) that were selected based on genetic merit for beef cattle. Thus, we consider that AFC may be an additional criterion for donor selection because no negative correlations were identified between number of follicles and characteristics of genetic gain. Therefore, considering the importance and impact of embryo production for the genetic gain and reproductive performance of livestock, the present study, which adopts a genetic improvement perspective, reinforces the obvious concept that AFC is a useful tool for embryo production in cattle due to the quantitative benefits of females with high follicle counts.

In general, all models tested in this study showed a low correlation with the number of antral follicles (0.072, 0.056, 0.082 and 0.172 for models 1, 2, 3 and 4, respectively). However, it is noteworthy that the inclusion of paternal effect (model 4 - doubled the coefficient of determination) was the correlation with AFC that had the highest score (17%) among the examined genotypic and phenotypic characteristics, suggesting some paternal influences on the number of antral follicles in heifers. Furthermore, considering all models that were statistically significant, the covariate visual scores for finishing precocity at weaning showed a negative correlation with the number of antral follicles, i.e., for each point that increases the visual scores for finishing precocity at weaning, there is a reduction of ~ 3 antral follicles (coefficient of regression - 2.81, - 3.25 and - 2.93 for models 1, 3 and 4, respectively). However, as there was no effect of other variables in the tested models, it is plausible that this small number of follicles (~ 3 antral follicles) is not indicative of a dramatic reduction in the AFC of the offspring. Additionally, assessing the heritability and impact of environmental effects during pregnancy on AFC in dairy cattle, [18] also found a negative ($p < 0.05$) linear relationship between milk fat concentration and AFC, with AFC decreasing by 0.4 per 1-percentage-unit increase in fat concentration (genetic correlation of - 0.53).

In the global context of embryo production in cattle, AFC has been widely used as a donor selection criterion for both SOV and IVP. A traditional research group supervised by Evans and collaborators has revealed that several characteristics related to female fertility are positively correlated with a high number of antral follicles mostly in *Bos taurus* cattle [9, 10, 14, 15, 17, 18, 28-30]. Despite the differences among the herds (beef cattle with *indicus* cross breeding), this study did not show a correlation between AFC and genetic merit characteristics used in breeding programs for beef cattle. Therefore, the inconstancy of results between different studies highlights the need to always prioritize genetic merit in donor selection, not just the number of antral follicles. Particularly in donors of oocytes, the latter strategy seems to be the most widely adopted due to the quantitative advantage, which tends to favor the selection of donors with high AFC because they make the IVP program more efficient and profitable [25, 28]. Additionally, considering the impact of this reproductive biotechnology in livestock production, we suggest that the use of AFC does not promote genetic demerit if a female was selected within an improvement program. However, even given that females with high AFC exhibit quantitative superiority, it is more plausible to evaluate the genetic merit of the donor first.

In conclusion, the number of antral follicles in beef heifers from weaning to yearling age is highly variable between individuals and repeatable within the same female. Additionally, there was no correlation between the number of antral follicles and the most important genetic merit characteristics used in improvement programs for beef cattle. Nevertheless, we demonstrated that the paternal effect was the variable that best explained the model used, and visual scores for precocity at weaning showed a negative correlation with AFC in heifers.

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# 6 CONCLUSÕES

- Vacas Nelore (*Bos indicus*) com alta contagem de folículos antrais apresentam mensurações ovarianas (diâmetro, perímetro e área) superiores às vacas de baixa contagem;

- Durante o tratamento hormonal para sincronização da ovulação, os diâmetros foliculares (folículo dominante e pré-ovulatório) foram maiores em vacas Nelore (*Bos indicus*) com baixa contagem de folículos antrais do que em vacas com alta contagem;

- Novilhas Braford (*Bos indicus-taurus*) apresentam alta variabilidade na contagem de folículos antrais, mas repetível na mesma fêmea desde o desmame até o sobreano;

- Não foi observado correlação entre o número de folículos antrais e as características de mérito genético (avaliadas por quatro modelos estatísticos diferentes) em novilhas Braford (*Bos indicus-taurus*);

- A variável escore visual de precocidade ao desmame apresentou uma correlação linear negativa com a contagem de folículos antrais em novilhas Braford (*Bos indicus-taurus*); e

- A inclusão do efeito paterno dobrou o coeficiente de determinação do modelo quatro, sugerindo alguma influência paterna na população folicular antral.

**ANEXOS**

Anexo A - *Publicações relacionadas ao tema da tese*

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# High numbers of antral follicles are positively associated with *in vitro* embryo production but not the conception rate for FTAI in Nelore cattle

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ABSTRACT

The objective was to compare the conception rates for FTAI and *in vitro* embryo production between Nelore cows with different antral follicle counts (AFC = number of follicles ≤3 mm in diameter in the ovaries). Nelore cows ($n$ = 547) were subjected to ovulation synchronization. Randomly during the estrous cycle (D0), cows received an intravaginal device containing 1.9 g P4 (CIDR®) and 2 mg BE (Estrogin®), IM. When the device was removed (D8), the cows received 500 μg PGF2α (Ciosin®), 300 IU eCG (Novormon®) and 1 mg EC (ECP®), IM. All cows were inseminated 48 h after P4 device removal. Antral follicles ≥3 mm were counted using an intravaginal microconvex transducer (D0), and the cows were assigned to high (G-High, ≥25 follicles, $n$ = 183), intermediate (G-Intermediate, 16–20 follicles, $n$ = 183) or low AFC groups (G-Low, ≤10 follicles, $n$ = 181). In another experiment, COCs were retrieved by OPU from Nelore cows ($n$ = 66), which were assigned to groups according to oocyte production: G-High ($n$ = 22, ≥40 oocytes), G-Intermediate ($n$ = 25, 18–25 oocytes) or G-Low ($n$ = 19, ≤7 oocytes). All COCs from the same cow were cultured individually (maximum of 25 COCs per drop) and then *in vitro* fertilized using thawed frozen sperm ($2 \times 10^6$/dose) from a Nelore sire of known fertility. The data were analyzed using a Kruskal–Wallis and a Chi-square test ($P \leq 0.05$). There was no difference in the conception rates after FTAI between Nelore cows with high, intermediate or low AFC (51.9 *vs.* 48.6 *vs.* 58.6%). The number of viable embryos was 18.4 ± 6.7 (G-High), 6.1 ± 3.6 (G-Intermediate) and 0.6 ± 0.7 (G-Low; $P < 0.05$). Therefore, AFC had no influence on the conception rates for FTAI; however, Nelore cows with high oocyte production exhibited better *in vitro* embryo production.



## 1. Introduction

The large number of oocytes obtained from *Bos taurus indicus* donor cows has stimulated *in vitro* embryo production commerce in Brazil. In this context, Nelore cattle are reported to have high oocyte production, with reports of hundreds of oocytes collected from a single *ovum pick up* (OPU; Santos et al., 2005). In recent years, follicular aspiration procedure has been largely and successfully performed in cattle (Pontes et al., 2011; Sanches et al., 2013; Silva-Santos et al., 2014a) but high individual variability in the number of oocytes has influenced

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# Is the number of antral follicles an interesting selection criterium for fertility in cattle?

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## Abstract

Recent studies indicate that the antral follicle population may be of paramount importance to improve reproductive performance in cows. There is already an agreement that the antral follicle count (AFC; follicles ≥3 mm in diameter) is a highly variable trait among animals, but with high repeatability in the same individual. Thus, females can be classified into low, intermediate or high AFC. Several studies in *Bos taurus* show a positive correlation between AFC and fertility parameters, such as increased quantity and quality of embryos, better pregnancy rates, higher progesterone levels, among others. However, there is still no consensus on AFC in *Bos indicus* females and *indicus-taurus*. This article aims to discuss the main aspects related to the population of antral follicles and its relation to the reproductive performance associated with the most common techniques in assisted reproduction (timed artificial insemination, *in vitro* embryo production, embryo transfer and superovulation).

**Keywords**: antral follicle count, embryo production, follicular dynamics, pregnancy rate, ultrasonography.

## Introduction

High genetic quality animals can be multiplied efficiently using reproductive biotechnologies such as artificial insemination and embryo production. These biotechnologies are useful strategies and known worldwide for improving genetics and productivity of flocks over a short period (Mapletoft and Hasler, 2005; Boni, 2012; Hansen, 2014).

Recently, there have been an increasing interest in studies concerning antral follicle count (AFC) and its influence on the reproductive performance in cattle, as well as its applications in reproductive biotechnologies (Ireland *et al.*, 2011; Pontes *et al.*, 2011; Rico *et al.*, 2012; Silva-Santos *et al.*, 2014a, b). Such fact may result in immense repercussions on the current scenario of animal reproduction, considering the significant increase in the world's embryo production. Despite several favorable results about AFC in *Bos taurus*, many aspects of reproductive physiology remain unknown. Considering AFC in *Bos indicus* there are many points to be addressed, particularly the impact on fertility when using *in vitro* embryo production (IVEP), timed artificial insemination (TAI) and timed embryo transfer (TET).

The high variability in the population of antral follicles is a hallmark in cattle (Burns *et al.*, 2005) with low, intermediate or high AFC (Santos *et al.*, 2012, 2013; Mendonça *et al.*, 2013). Despite the high variability among animals, there is a high repeatability of the number of follicles observed in the same individual through evaluations carried out during a period (Burns *et al.*, 2005; Ireland *et al.*, 2007, 2008, 2009).

This constancy in AFC in the same individual becomes a strategic resource for the possibility of classifying an animal by the AFC with a single ultrasound examination. For *taurus* animals, AFC is directly correlated with the size of the ovarian follicular reserve (Ireland *et al.*, 2011), which was not proven in indicus females, considering fetuses, heifers and cows (Silva-Santos *et al.*, 2011). However, other factors such as genetics (Walsh *et al.*, 2014), maternal environment, nutritional status and healthiness (Ireland *et al.*, 2011; Evans *et al.*, 2012) also appear to influence the AFC. For example, the nutritional status and the metabolic rate were mentioned as factors which affect the follicular growth, oocyte quality and secretion of reproductive hormones in cattle (Jimenez-Krassel *et al.*, 2009; Mossa *et al.*, 2010; Evans *et al.*, 2012).

The AFC may also influence the production of cattle embryos, both *in vivo* and *in vitro*, especially as the number of embryos produced by donor but also in process efficiency, with higher rates for high AFC animals (Ireland *et al.*, 2008; Santos *et al.*, 2014; Silva-Santos *et al.*, 2014a).

The importance of the AFC and its relationship with pregnancy rates must also be emphasized. Studies conducted with *taurus* females had higher pregnancy rates for high AFC females (Cushman *et al.*, 2009; Evans *et al.*, 2012; Mossa *et al.*, 2012). However, in recent studies with *indicus-taurus* and *indicus* animals, a better performance regarding pregnancy rates was not observed in high-AFC animals (Mendonça *et al.*, 2013; Santos *et al.*, 2014). Surprisingly, some data suggest a better performance regarding pregnancy rates for low AFC cows (Santos *et al.*, 2013).

In addition to ultrasound, the measurement of the concentration of anti-Mullerian Hormone (AMH)

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# Antral Follicle Populations and Embryo Production – *In Vitro* and *In Vivo* – of *Bos indicus–taurus* Donors from Weaning to Yearling Ages

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## Contents

Interest in *indicus–taurus* cattle has been increasing, as these animals are likely to present the best characteristics of Zebu and European bovine breeds. The aim of this study was to compare the embryo production of *indicus–taurus* donors with high vs low antral follicle counts obtained by *ovum pickup*/*in vitro* production (OPU/IVP) and superovulation (SOV)/embryo collection. Braford females at weaning age (3/8 Nelore × 5/8 Hereford, n = 137, 9 ± 1 month old) were subjected to six serial ovarian ultrasonographs and were assigned to two groups according to the number of antral follicles ≥3 mm as follows: G-High antral follicular count (AFC, n = 20, mean ≥40 follicles) and G-Low AFC (n = 20, mean ≤10 follicles). When the females (n = 40) reached 24 months of age, they were subjected to both OPU/IVP and SOV/embryo collection. The average number of follicles remained highly stable throughout all of the ultrasound evaluations (range 0.90–0.92). The mean number of COCs recovered (36.90 ± 13.68 vs 5.80 ± 3.40) was higher ($p < 0.05$) for females with high AFC, resulting in higher ($p < 0.05$) numbers of total embryos among females with high vs low AFC (6.10 ± 4.51 vs 0.55 ± 0.83). The mean number of embryos per collection was also higher ($p < 0.05$) for G-High vs G-Low (6.95 ± 5.34 vs 1.9 ± 2.13). We conclude that a single ultrasound performed at pre-pubertal ages to count antral follicles can be used as a predictor of embryo production following IVP and SOV/embryo collection in *indicus–taurus* females.

## Introduction

Interest in *indicus–taurus* bovine breeds has been increasing due to their adaptability to produce meat and milk under stressful conditions, such as high temperature, parasites and poor pastures (Pontes et al. 2011). These characteristics are maintained in Nelore or Bhraman – Hereford cross-bred animals, usually referred to as 'Braford', which are popular beef cattle in Central and South America and other tropical and non-tropical areas.

Considering the importance of embryo production for genetic improvement, it is important to note the high variability in embryo production per donor following *ovum pickup* (OPU)/IVP and superovulation (SOV)/embryo collection (Pontes et al. 2009). For IVP-derived embryos, variation in oocyte yield reportedly influences the final number of embryos produced (Pontes et al. 2011). Additionally, some females produce their highest embryo yield following OPU/IVP or SOV/embryo collection (Pontes et al. 2009).

Some studies have reported high variability in the number of pre-antral and antral follicles among individual adult cattle (Erickson 1966; Burns et al. 2005; Silva-Santos et al. 2011). However, the number of antral follicles is repeatable in individuals during follicular waves (Burns et al. 2005; Ireland et al. 2007). Therefore, it is possible to identify bovine females with low, intermediate or high numbers of follicles during waves by ultrasonography. It is unknown whether variation in the ovarian follicular reserve and the antral follicular count (AFC) can affect *in vitro* or *in vivo* embryo production in cattle. Comparisons between *in vitro* and *in vivo* embryo production have revealed higher numbers of recovered and transferable embryos with high AFC vs low AFC, lower proportions of transferable embryos per animal after SOV of beef cattle, higher numbers of oocytes and blastocysts following follicular aspiration of abattoir ovaries with high numbers of antral follicles and similar proportions of blastocysts between groups (Ireland et al. 2007). However, *in vitro* and *in vivo* embryo productions in the same cattle have not been assessed. The aim of this study was to compare the efficiency of OPU/IVP vs SOV/embryo collection on embryo production in the same donors with consistently high vs low AFC. This comparison was performed after counting antral follicles by ultrasonography in *indicus–taurus* cattle from weaning to yearling ages.

## Materials and Methods

### Animals and AFC

Braford females (3/8 Nelore × 5/8 Hereford, n = 137) maintained in *Brachiaria brizantha* pasture supplemented with mineral salt *ad libitum* were serially examined by ultrasonography from weaning (9 ± 1 months of age) to yearling ages (20 ± 1 months of age). At 24 months of age, the mean body condition score was 3.0 ± 0.5 (scale, 1–5; Lowman et al. 1976), and the average live body weight was 360 ± 10 kg. Several of the females were selected based on the AFC. The ovaries from each animal were monitored with a 7.5-convex intravaginal array transducer (Áquila PRO, Pie Medical, Maastricht, the Netherlands) spaced 60 day apart (days 0, 60, 120, 180, 240 and 300), and antral follicles were counted as described previously (Burns et al. 2005; Ireland et al. 2008). After six ultrasound evaluations performed by the same operator, females were assigned to two groups according to the number of antral follicles ≥3 mm (AFC – antral follicle count) as follows: females with a consistently high (G-High, ≥40 follicles; n = 20)

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# Comparison of Antral and Preantral Ovarian Follicle Populations Between *Bos indicus* and *Bos indicus-taurus* Cows with High or Low Antral Follicles Counts

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**Contents**

The objective was to compare populations of antral and preantral ovarian follicles in *Bos indicus* and *Bos indicus-taurus* cows with high and low antral follicle counts. Nelore (*Bos indicus*, n = 20) and Nelore X Angus (1/2 *Bos indicus-taurus*, n = 20) cows were subjected to follicular aspiration without regard to the stage of their oestrous cycle (day of aspiration = D0) to remove all follicles ≥3 mm and induce growth of a new follicular wave. Ovaries were examined by ultrasonography on D4, D19, D34, D49 and D64, and antral follicles ≥3 mm were counted. Thereafter, cows were assigned to one of two groups: high or low antral follicular count (AFC, ≥30 and ≤15 antral follicles, respectively). After D64, ovaries were collected after slaughter and processed for histological evaluation. There was high repeatability in the numbers of antral follicles for all groups (range 0.77–0.96). The mean (±SD) numbers of antral follicles were 35 ± 9 (*Bos indicus*) and 38 ± 6 (*Bos indicus-taurus)* for the high AFC group and 10 ± 3 (*Bos indicus*) and 12 ± 2 (*Bos indicus-taurus*) follicles for the low AFC. The mean number of preantral follicles in the ovaries of *Bos indicus-taurus* cows with high AFC (116 226 ± 83 156 follicles) was greater ($p < 0.05$) than that of *Bos indicus* cows (63 032 ± 58 705 follicles) with high AFC. However, there was no significant correlation between numbers of antral and preantral follicles.

## Introduction

The number of bovine embryos produce by IVF is increasing in Brazil. This situation has been attributed to the large Brazilian cattle population (~200 million animals), composed largely of Nelore – *Bos indicus* – cows (80%). This breed has much higher rates of oocytes recoverable by ultrasound-guided follicular aspiration (OPU) than European breed – *Bos taurus* – (Stroud 2010). In that regard, the mean number of oocytes recovered per OPU procedure is 18–25 oocytes in *Bos indicus* breeds (Watanabe et al. 1999; Thibier 2004; Pontes et al. 2011) and 4–14 oocytes in *Bos taurus* breeds (Machado et al. 2003; Rubin et al. 2005; Martins et al. 2007).

There has been an increasing interest on crossbred cattle (*Bos indicus* × *Bos taurus*), because they may provide the best characteristics of the two types, namely disease and weather resistance (*indicus*) and meat and milk production (*taurus*; Pontes et al. 2010). However, there are apparently no reports regarding antral and preantral follicle population on *taurus-indicus* donors.

There is high variability in the population of ovarian preantral follicles (Erickson 1966a,b; Silva-Santos et al. 2011). Conversely, the number of antral follicles per follicular wave is repeatable within animals (Burns et al. 2005), and some cows present remarkably high versus low antral follicle populations during follicular waves (Ireland et al. 2008). However, there is a lack of information comparing preantral and antral follicular populations, particularly on *indicus* and *indicus-taurus* cross cows.

The objective of this study was to compare the number of preantral and antral follicles between *Bos indicus* and *Bos indicus-taurus* cows with high versus low antral follicle counts.

## Materials and Methods

### Cattle

Healthy, non-pregnant, non-lactating, cycling *Bos indicus* (Nelore, n = 20, 72–96 months old) and *Bos indicus-taurus* cross (1/2 Nelore X Angus, n = 20, 72–96 months old) cows were subjected to follicular aspiration without regard to the stage of the oestrous cycle (day of aspiration = D0). All follicles ≥3 mm were removed. Cows were maintained in *Brachiaria brizantha* pastures and given *ad libitum* access to mineral salt. The mean body condition score of cows was 3.5 ± 0.5 (scale of 1 to 5), and the average body weight was 450 ± 10 kg (Lowman et al. 1976).

### Antral follicular counting

On D4, D19, D34, D49 and D64, ovaries were examined by ultrasonography (Áquila PRO, Pie medical, Maastricht, The Netherlands) using a 7.5-convex array transducer. Antral follicles ≥3 mm were counted as described (Burns et al. 2005; Ireland et al. 2008). After five evaluations, cows were assigned to one of two groups: high antral follicle count (AFC, mean ≥30 follicles, *Bos indicus*, n = 7; *Bos indicus-taurus*, n = 6) or low AFC (mean ≤15 follicles, *Bos indicus*, n = 6; *Bos indicus-taurus*, n = 6; Ireland et al. 2008). From 40 animals evaluated by ultrasonography, cows with intermediate AFC (16–31 follicles, n = 15) were eliminated from any further studies.

### Collection of ovaries

After the last evaluation (D64), cows were slaughtered and ovaries were collected for histological evaluation. Following collection, ovaries were washed in 0.9% saline, cut longitudinally into halves and fixed in Bouin's fixative for 24 h. After being immersed in fixative, ovaries were transported to the laboratory. Ovaries were then placed in 70% alcohol. Only ovaries without corporea lutea (CL) were used to ensure good histological processing, and only one ovary per female was analysed. Ovarian halves were dehydrated in alcohol, *Chapter*

# THE ANTRAL OVARIAN FOLLICLE POPULATION AND ITS APPLICATION TO FERTILITY AND LIVESTOCK BREEDING

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## ABSTRACT

An efficient production system is needed to improve the animal production per unit, for which the most effective strategies are investment in animal feed, genetic improvement, and enhancing reproductive efficiency. Recent studies to improve the reproductive performance of cattle through the management of female fertility have been performed. The main advances included a better understanding of the physiological basis of fertility and the use of reproduction biotechnologies, such as estrus synchronization, superovulation and *in vitro* embryo production.

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*Chapter*

# CURRENT ACHIEVEMENTS IN THE OVARIAN FOLLICULAR RESERVE: IMPLICATIONS FOR APPLIED BIOTECHNOLOGIES AND FERTILITY IN CATTLE

*Katia Cristina Silva-Santos, Gustavo MartinsGomes dos Santos, Fabio Morotti,Fernanda Zandonadi Machado, Suellen Miguez González, Marcelo Marcondes Seneda*[*]
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## ABSTRACT

In recent years, interest in folliculogenesis has been increasing due to its current application in reproductive biotechnology. However, several aspects of the regulation of early follicular development and establishment of the ovarian follicular reserve remain unclear. The concept of formation of the ovarian follicular pool during the fetal stage has been challenged, and some new intriguing aspects have yet to be well understood. Evidence of female gamete formation and development in adult ovaries has provided interesting debates about follicle origin and the

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Anexo B - *Outras publicações realizadas durante o período de doutorado*

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*Chapter 3*

# ADVANCES OF ARTIFICIAL INSEMINATION IN CATTLE

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## ABSTRACT

Artificial insemination is considered one of the oldest biotechnologies of animal reproduction, which is frequently recommended in order to increasing reproductive efficiency and genetic improvement of cattle. This biotechnology has many advantages compared to natural breeding such as health benefits (control of infectious diseases), genetic improvement (use of genetically improved sires) and improvement of reproductive, zootechnical and economic features of herd (standardized management and animals). This biotechnology also enables a large number of offspring from a single sire, increasing the dissemination of genetically improved sires. Furthermore, it is an essential tool for other reproductive biotechnologies such as timed artificial insemination, resynchronization of estrous or ovulation, multiple ovulation and embryo transfer, and *in vitro* embryo production. The use of artificial insemination reduces the cost of purchase maintenance of animals, because is not necessary to keep sires on the stock. In this context, semen cryopreservation is a biotechnology which contributes to dissemination of artificial insemination in many domestic animal species, because sperm can be stored for a relatively low cost on the stock. In addition, pharmacological control of the estrous cycle is other strategy that has also contributed to the dissemination of artificial insemination, because the use of hormonal protocols for estrous synchronization enabled management and insemination of large number of females. Therefore, the aim of this chapter was to present the main points of reproductive biotechnologies, mainly the technical parameters, advantages and influencing factors, and to discuss practical strategies to improvement of herd.

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*Chapter 2*

# MALE REPRODUCTIVE PHYSIOLOGY: CONCEPTS AND ADVANCES

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## ABSTRACT

Understanding of physiological processes of male reproduction is an important issue for reproductive events in domestic animals, because the physiological basis contributes for advances in reproductive biotechnologies, to the improvement of reproductive efficiency, for treatment of infertility beyond being used as a model for andrological trials in humans. Despite anatomical and physiological peculiarities in different species, the reproductive system of male consists of different organs, which are responsible for steroidogenesis and spermatogenesis. These events are controlled by the central nervous system along with synchronic neuroendocrine actions involving the hypothalamic-pituitary-testicular axis and local growth factors. Spermatogenesis involves the production of spermatozoa in the seminiferous tubules from spermatogonia, mitosis and meiosis, and cell differentiation. All events are dependent of the functionality of Sertoli and Leydig cells beyond gonadotropin and testicular hormonal support. Fertility capacity and the ability to identify subfertility in males have increased importance for future generations in a production system, because potential sire can be mated with hundreds of females using reproductive biotechnologies. However, several factors can affect the male reproductive capacity, such as physiological factors, intrinsic or extrinsic reproductive conditions, health, nutritional status and sexual behavior. Studies on male reproductive physiology have been performed to improve the reproductive performance and better understand events related to different phases of reproduction. These researches have stimulated advances, such as improvement of fertilization capacity of spermatozoa, use of frozen semen and sperm sexing to control sex ratio, enhancing the use of the sire. Herein,

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*Chapter 4*

# ULTRASONOGRAPHY IN BREEDING OF CATTLE AND HORSES

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## ABSTRACT

In cattle and horses, ultrasonography is an important diagnostic tool, particularly for evaluation of reproductive system. Among the many advantages that this technology offers, there is easily and practicality of its application, non-invasive examination and mainly the accuracy of evaluation that allows a detailed real-time examination. Ultrasonography has also contributed to a better understanding of physiological bases and the use of reproduction biotechnologies such as artificial insemination, estrus synchronization, superovulation and *in vitro* embryo production. In cattle, ultrasound can be used to evaluate the reproductive system; for diagnosis of some diseases of the ovaries, uterus, testis and accessory glands; and as an auxiliary tool in the breeding management or in the implementation of reproductive biotechnologies such as early pregnancy diagnosis for estrus resynchronization, to evaluate embryonic losses, fetal sex identification, to evaluate the puberty occurrence in heifers, determine the healthy condition in embryo donors and to assist ovum pick-up procedures for *in vitro* embryo production. In horses, the ultrasound examination is an excellent tool for reproductive evaluation of healthy or pathological conditions (ovarian and uterine), in the identification of cyclicality, in the follicular control for artificial insemination, in early pregnancy diagnosis, diagnosis and management of twin pregnancy, monitoring of pregnancy development, fetal sex identification, and aiding in some biotechnologies applied to mare, for example, the ovum pick-up. Considering the benefits and advances

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*Chapter 5*

# METHODS AND ADVANCES IN SEMEN ANALYSIS

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## ABSTRACT

Sperm fertility is considered one of the main factors for successful breeding of domestic animals, either through natural mating or after using reproductive biotechnologies. Kinetics (motility and sperm trajectory) and the morphological characteristics of spermatozoa evaluation are essential for ejaculate analyses and are correlated with male fertility. Due to technical and scientific progress and the need to ensure the maximum fertility potential of sperm cells, several semen evaluation methods have been established over time. This analysis aims to identify key factors for semen quality, using sperm trial parameters and obtain the most accurate results, which can be correlated with fertility, infertility or subfertility. Subjective semen assessment by optical microscopy was one of first methods to evaluate sperm cells and is still the most commonly used method. However, due to its subjectivity (high variability in results), other methods such as computer-aided semen analysis (CASA) are greatly required. Semen analyses using CASA systems associated with flow cytometry have reduced the subjectivity of evaluations, increasing the safety and speed of obtaining data. In addition, these methods have high potential for clinical applications, mainly due to the significant correlation between sperm motility characteristics and *in vitro* or *in vivo* fertilization. However, their use is still limited due to equipment costs, data validation requirements, quality control and analysis standardization, especially between laboratories and research centers. Recently, molecular biology studies, such as genomics and proteomics applied to sperm cells and seminal plasma, have also contributed as tools for seminal assessment and identification of breeding males with improved fertility potential. In this context, the

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# Use of sexed sorted semen for fixed-time artificial insemination or fixed-time embryo transfer of *in vitro*–produced embryos in cattle

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A B S T R A C T

Artificial insemination and *in vitro* embryo production are powerful tools for disseminating superior genetic qualities and improving the reproductive performance of dairy and beef cattle. In conjunction with these biotechnologies, sexed-sorted semen has been used to obtain offspring of a predetermined sex. This study compared the pregnancy rates obtained using in vitro fertilization/timed embryo transfer (IVF/TET) and timed artificial insemination (TAI), both performed using sexed-sorted (Y-chromosome-bearing) semen obtained from the same bull. For the in vitro embryo production, the ovaries of 250 Nelore cows with known histories were collected in the slaughterhouse and used for IVF. After evaluation of the recipients (IVF/TET group; n = 974), the resultant embryos were transferred to the females with corpus luteum (n = 822). The pregnancy-related data for this group were compared with those for the TAI group (n = 974). Ultrasonography was performed at 60 days to determine the pregnancy status and confirm the sex of the fetus. A total of 2008 oocytes produced 1050 embryos, with 52% of them reaching the blastocyst stage. The pregnancy rate and the accuracy in determining the fetal sex were 35.4% (345/974) and 95.07% (328/345), respectively, for the IVF/TET group and 30% (293/974; P < 0.05) and 94.88% (278/293), respectively, for the TAI group. In the present study, we concluded that male calves could be better obtained using IVF/TET rather than TAI; therefore, this strategy can be considered to increase the pregnancy rate of beef cattle.


## 1. Introduction

Considering the worldwide aspiration to increase meat consumption as well as the need for sustainable livestock, beef cattle producers must adapt more efficient and economical management practice methods. Using reproductive biotechnologies together with genetic selection is the best strategy to meet this goal because reproducing genetically superior animals could rapidly increase the productivity of the livestock [1,2].

Artificial insemination and embryo production are powerful tools for disseminating superior genetic qualities and improving the genetics and reproductive performance of dairy and beef cattle. The biotechnology of embryo production through *in vitro* fertilization (IVF) has been constantly improved over the last decade [3], and its efficacy has been reported in large-scale studies of cattle [4,5], resulting in its application on a commercial

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# Asynchronous embryo transfer as a tool to understand embryo–uterine interaction in cattle: is a large conceptus a good thing?

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**Abstract.** The aim was to examine the effect of embryo–uterine synchrony on conceptus elongation and pregnancy rate in cattle. In Study 1, crossbred beef heifers each received 10 Day-7 *in vitro*-produced blastocysts on either Day 5, 7 or 9 after oestrus. A proportion of Day 5 recipients were supplemented with progesterone, via a progesterone-releasing intravaginal device from Days 3–5 plus either 750 IU equine chorionic gonadotrophin or 3000 IU human chorionic gonadotrophin on Day 3. At embryo age Day 14, all heifers were slaughtered and the uterus was flushed. Fewer recipients yielded conceptuses ($P < 0.05$) and fewer conceptuses were recovered ($P < 0.05$) following transfer on Day 5 compared with Day 7 or 9. Supplementation with progesterone resulted in short cycles in approximately 50% of recipients. Mean conceptus length was greater ($P < 0.05$) following transfer to an advanced uterus. In Study 2, overall pregnancy rate following the fresh transfer of a single *in vitro*-produced blastocyst was 43.5% (2065/4749). Transfer of a Day 7 embryo to a synchronous Day-7 uterus resulted in a pregnancy rate of 47.3%. Transfer to a Day-5 (40.8%) or a Day-8 (41.3%) uterus moderately impacted pregnancy rate ($P < 0.01$) while transfer to a uterus 2 days in advance (Day-9, 24.4%) or 3 days behind (Day-4, 27.0%) reduced ($P < 0.001$) pregnancy rate compared with synchronous transfers. In conclusion, transfer of an embryo into an advanced uterus results in an acceleration of conceptus development, but does not result in greater pregnancy rates.

**Additional keywords:** conceptus elongation, cow, pregnancy establishment, progesterone.

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## Introduction

Establishment of pregnancy in ruminants is dependent on an optimal interaction between the developing embryo–conceptus and the maternal uterine environment. The early bovine embryo is somewhat autonomous for the first week or so after fertilisation as evidenced by the fact that blastocysts can be produced in large numbers *in vitro* in the absence of any interaction with the female reproductive tract. However, following hatching from the zona pellucida, the rapid conceptus elongation that is characteristic of ruminant (and porcine) embryos is driven by the uterine environment; it has not been reproduced *in vitro* despite numerous attempts (Brandão *et al.* 2004; Alexopoulos *et al.* 2005; Vejlsted *et al.* 2006) and does not occur *in vivo* in the absence of secretions from uterine glands (Gray *et al.* 2002).

Similarly, in order to establish a pregnancy, the reproductive tract of the cow does not require exposure to an embryo up until approximately Day 16, when luteolysis is initiated in the absence of conceptus-derived interferon-tau. The commercial bovine embryo-transfer industry is based on the ability to transfer Day-7 embryos, recovered from a donor female, to the uteri of synchronised recipients that, before the transfer, had not been exposed to an embryo. Indeed, studies have demonstrated that pregnancy can be established following transfer of an appropriately staged conceptus up to Day 16 after oestrus (Betteridge *et al.* 1980), although the recovery and handling of such elongated conceptuses is challenging and impractical. In support of this, global gene expression studies have shown that before maternal recognition on Day 16, the temporal changes

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JVS

# Influence of category—heifers, primiparous and multiparous lactating cows—in a large-scale resynchronization fixed-time artificial insemination program

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This study was conducted to evaluate the influence of category (heifers, primiparous or multiparous cows) on pregnancy rates in a large scale resynchronization ovulation program. Nelore heifers (n = 903), primiparous lactating cows (n = 338) and multiparous lactating cows (n = 1,223) were synchronized using a conventional protocol of estradiol/P4-based fixed-time artificial insemination (FTAI). Thirty days after ultrasonography, females who failed the first FTAI were resynchronized with the same hormonal protocol prior to a second FTAI. The pregnancy status of each cohort was evaluated by ultrasonography 30 days after each FTAI. The average conception rate after the first FTAI and resynchronization was 80.5%. Heifers had a higher conception rate (85%) than primiparous (76%) or multiparous cows (78%; $p = 0.0001$). The conception rate after the first FTAI was similar among heifers (57%), primiparous cows (51%) and multiparous cows (56%; $p = 0.193$). After the second FTAI, heifers exhibited a higher conception rate (66%) than primiparous or multiparous cows (51%; $p = 0.0001$). These results demonstrate the feasibility of resynchronization in large beef herds for providing consistent pregnancy rates in a short period of time. We also demonstrated that ovulation resynchronization 30 days after FTAI is particularly effective for heifers, providing a conception rate of up to 66%.

**Keywords:** *Bos indicus*, conception rate, fixed-time artificial insemination, hormonal treatment, resynchronization

## Introduction

Reproductive efficiency is an important strategy for maximizing the productivity of cattle herds. Among the current strategies used to improve reproductive performance, ovulation synchronization has become the best technique for optimizing herd management and providing genetic improvements with greater efficiency.

Among beef cattle, approximately 40 to 60% of inseminated females become pregnant after the first cycle of fixed-time artificial insemination (FTAI), with slight variations in rates depending on several factors including the animals, farm management, body condition score, postpartum time and hormonal treatments [2,3,11]. Therefore, females that do not conceive after the first service need to be re-inseminated as soon as possible to increase the reproductive performance of the herd [4,6,7,19]. If these females are not re-inseminated in a short period of time, the interval between calving and conception increases, decreasing the reproductive efficiency of the herd.

The resynchronization of females who failed the first FTAI is an effective strategy to increase the number of pregnancies achieved by AI during a short breeding season [19]. Ovulation resynchronization can increase the percentage of non-pregnant cows that are subjected to a second insemination [13] and be an advantageous strategy when the number of bulls is insufficient to mate with all of the cows that did not become pregnant after the first FTAI [8]. During resynchronization, all cows inseminated in the first round of FTAI should be evaluated by ultrasonography, and those that did not become pregnant receive the same hormonal protocol for a second round of FTAI [7,18].

One of the most common hormonal protocols for FTAI or resynchronization in cattle involves the use of a progesterone

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