



**Programa de pós-graduação em ciências veterinárias**  
**Universidade Federal de Uberlândia**

**Caracterização do desenvolvimento reprodutivo em  
fêmeas Nelore (*Bos taurus indicus*) em dois  
momentos da fase pré-puberal**

**Orientador: Maurício Machaim Franco**

**Co-orientador: Ricardo Alamino Figueiredo**

**Aluna: Taynan Stonoga Kawamoto**

**UBERLÂNDIA – MG 2020**



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Secretaria da Coordenação do Programa de Pós-Graduação em Ciências  
Veterinárias

BR 050, Km 78, Campus Glória, Uberlândia-MG, CEP 38400-902  
Telefone: (34) 2512-6811 - www.ppgcv.famev.ufu.br - mesvet@ufu.br



**ATA DE DEFESA - PÓS-GRADUAÇÃO**

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Reuniu-se na Sala 54, bloco 2D, Campus Umuarama, da Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Ciências Veterinárias, assim composta: Professores Doutores: Ricarda Maria dos Santos - UFU; José Octávio Jacomini - UFU; João Henrique Moreira Viana - EMBRAPA; Carlos Frederico Martins - EMBRAPA; Maurício Machaim Francoorientador(a) do(a) candidato(a).

Iniciando os trabalhos o(a) presidente da mesa, Dr(a). Maurício Machaim Franco, apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos(às) examinadores(as), que passaram a arguir o(a) candidato(a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

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Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.



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*“A menos que modifiquemos a nossa maneira de pensar, não seremos capazes de resolver os problemas causados pela forma como nos acostumamos a ver o mundo”.*

*Albert Einstein*

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## Resumo

O Brasil possui o maior rebanho comercial do mundo, composto em sua maioria por animais da raça Nelore. Além disso, é um dos maiores produtores de embriões *in vitro* do mundo, tendo essa tecnologia destaque por permitir acelerar o ganho genético dos rebanhos. Entretanto, zebuínos da raça Nelore possuem puberdade mais tardia se comparados à animais de raças taurinas, o que pode levar a um atraso no melhoramento genético. Neste contexto, a possibilidade de inclusão de bezerras como doadoras de ovócitos em programas de produção *in vitro* de embriões (PIVE), poderia ser um atrativo passo além, para diminuir o intervalo entre gerações, acelerar o ganho genético dos rebanhos e contribuir para melhorar a eficiência e a sustentabilidade (econômica, social e ambiental) de sistemas de produção de leite e carne. Porém, é sabido que ovócitos de bezerras são considerados menos competentes em gerar blastocistos e estabelecer gestações do que os de fêmeas adultas. Adicionalmente, observa-se que, a maioria dos trabalhos no tema são realizados em taurinos. Assim, mais estudos sobre a fisiologia endócrina e reprodutiva de bezerras zebuínas poderiam verificar a viabilidade de uso destes animais em programas de PIVE. Neste contexto, a presente proposta visou avaliar as concentrações hormonais de FSH/LH, a correlação entre características ovarianas e uterinas com o desenvolvimento e mudanças na geometria da garupa, bem como as características morfológicas e moleculares em ovócitos, e as taxas de PIVE a partir de fêmeas Nelore de 2 a 5 meses e de 8 a 11 meses de idade. Assim, o melhor entendimento do desenvolvimento reprodutivo, fornecerá subsídios para a tomada de decisões sobre o uso destes animais nessa fase, a proposição de protocolos hormonais em doadoras desta categoria e possíveis alterações nos meios de cultivo para a PIVE a partir de ovócitos obtidos de animais pré-púberes.

Palavras chave: Bezerras, PIVE, ovócitos, embriões, gonadotrofinas.

## Abstract

Brazil has the largest commercial herd in the world, being mostly composed of Nelore animals. In addition, it is one of the largest producers of *in vitro* embryos in the world, technology that allows to accelerate the herds' genetic gain. However, the Zebu cattle Nelore usually shows later puberty when compared to Taurines, that causes genetic improvement delays in Zebu. In this context, the possibility of including calves as oocyte donors for *in vitro* embryo production programs (IVEP), could be an attractive alternative, aiming to reduce the interval between generations and accelerate the genetic gain of herds, contributing to increase the efficiency and sustainability (economic, social and environmental) on milk and meat production systems. However, it is known that calves' oocytes are considered less competent to generate blastocysts and establish pregnancies than those obtained from adult females. In addition, it is observed that most of the studies addressing this topic have been carried out mainly with Taurine cattle, thus, further studies on the endocrine and reproductive physiology of Zebu calves could be important on this purpose. For this reason, the present study aims to evaluate the hormonal concentrations of FSH / LH, the correlation among ovarian and uterine characteristics with the rump development and its geometry changes, the oocytes morphological and molecular characteristics and the *in vitro* embryo production rates in 2 to 5 and 8 to 11 months old Nelore females. Thus, in addition to better understanding of the reproductive development, it would be possible to contribute with information to subsidize decisions about the use of this animal category in this phase, donors' hormonal protocols and alternative culture media changes for IVEP from prepubertal oocytes.

**Keywords:** Calves, IVEP, oocytes, embryos, gonadotropins.

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## **Apresentação**

Esta tese está subdividida em:

- Capítulo I: Considerações gerais.
- Capítulo II: Changes in reproductive and biometric development occur faster in 2 to 5 months of age than in 8 to 11 months old Nelore calves (*Bos taurus indicus*).
- Capítulo III: Features and developmental potential of oocytes collected from Non-stimulated Nelore (*Bos taurus indicus*) females in two prepubertal moments.
- Capítulo IV: Considerações Finais.

O capítulo I aborda os aspectos da fisiologia reprodutiva e estudos em fêmeas pré-púberes, que visam embasar os demais capítulos.

O capítulo II, em língua inglesa, tem formato de um artigo científico e aborda o desenvolvimento corporal, reprodutivo e suas relações, durante dois momentos da fase pré-púbere. Será submetido ao periódico *Animal* (Fator de impacto 2,026; em 2018).

O capítulo III também está no formato de artigo científico e em língua inglesa. Neste, são abordadas as características morfológicas e moleculares de ovócitos, produção *in vitro* de embriões e, transferência de embriões produzidos a partir de ovócitos de fêmeas pré-púberes de 2 a 5 meses e de 8 a 11 meses de idade. Esse será submetido à revista *Theriogenology* (Fator de impacto de 2,29; em 2018).

O capítulo IV traz os principais resultados encontrados ao longo de todos os experimentos.

# Capítulo I – Considerações Gerais

## 1. Introdução

O Brasil possui o maior rebanho comercial do mundo, com aproximadamente 213,5 milhões de bovinos (IBGE, 2019), sendo 87,7% do rebanho de corte de origem zebuína (*Bos taurus indicus*), dos quais se destaca a raça Nelore, que compõe 78% dos zebuínos do país (Rosa et al., 2016). O Brasil é considerado ainda, o maior exportador de carne bovina (ABRACOMEX, 2019), o quarto maior produtor de leite (EMBRAPA, 2019) e um dos maiores produtores de embrião *in vitro* do mundo (Viana et al., 2018).

Visando o incremento da produção e da produtividade na pecuária bovina, ao longo das últimas décadas, biotécnicas reprodutivas como a inseminação artificial (IA), inseminação artificial em tempo fixo (IATF), superovulação (SOV), produção *in vitro* de embriões (PIVE) e sexagem de sêmen têm sido crescentemente utilizadas na pecuária. Além disso, o Brasil tornou-se o segundo maior produtor de embriões PIVE do mundo (Viana et al., 2018), ou seja, utilizando uma biotecnologia que maximiza o uso de fêmeas de alto mérito genético no manejo reprodutivo das propriedades, proporciona o aumento da pressão de seleção e do ganho genético dos rebanhos. Este impacto pode ser alcançado porque, de modo geral pode-se afirmar que, se em condições ideais uma vaca (com capacidade reprodutiva em torno de 8 a 10 anos) poderia produzir uma única cria por ano, pela TE esta poderia produzir um bezerro por mês e, pela PIVE, um bezerro por semana.

Neste contexto, a inclusão de bezerras em programas PIVE poderia viabilizar a diminuição do intervalo entre gerações e acelerar ainda mais o ganho genético dos rebanhos (Louhis, 1995), podendo contribuir para melhorar a eficiência de sistemas de produção de leite e de carne. Esta possibilidade, do uso de bezerras na PIVE, tornou-se de interesse comercial também devido aos avanços na área de genômica ampla, que atualmente permitem a seleção de animais geneticamente superiores desde o nascimento. No entanto, é relatado que os ovócitos de bezerras são menos competentes apresentando menores taxas de produção de blastocistos (Oropeza et al., 2004; Bettegowda et al., 2008;

Patel et al., 2007), e com menor capacidade de estabelecer gestações a termo, quando comparados aos obtidos de animais adultos (Khatir et al., 1998). Assim, faz-se importante os estudos sobre o desenvolvimento hormonal, reprodutivo, e das características sexuais primárias e secundárias em bezerras.

## **2. Objetivos Gerais**

Assim, este estudo visou avaliar o desenvolvimento reprodutivo de fêmeas Nelore pré-púberes, associando características sexuais secundárias (medidas de garupa), dosagens hormonais de FSH/LH e desenvolvimento ovariano e uterino dos 2 aos 5 e dos 8 aos 11 meses de idade. Adicionalmente, nestes mesmos intervalos de idade, objetivou-se estudar, em ovócitos, a qualidade morfológica, o diâmetro, a expressão de genes relacionados à competência, a produção *in vitro* de embriões e o potencial destes em gerarem gestações a termo.

## **3. Revisão de literatura: Fisiologia da reprodução e produção *in vitro* de embriões em fêmeas Nelore pré-púberes**

### **3.1 Puberdade e seus mecanismos de controle**

A puberdade em fêmeas, é definida como a ativação do eixo hipotalâmico-hipofisário-gonadal (HHG) que leva a maturação do ovário e, conseqüentemente, à ovulação (Sisk e Foster, 2004). Além disso, é necessário o desenvolvimento e crescimento do animal a fim de permitir o estabelecimento e manutenção da prenhez (Roberts et al., 2017). Assim, a puberdade envolve uma série de eventos biológicos complexos, que levam a maturação das características sexuais para que então, seja obtida uma plena capacidade reprodutiva. Para isso, a fêmea precisa completar seu crescimento para atingir uma anatomia e tamanho de pelve ideal, e haver mineralização esquelética, para que possa emprenhar e gerar descendentes (Duittoz et al., 2016). O processo de maturação do eixo HHG inicia-se antes do nascimento, passa por alterações durante a fase pré-púbere e

finaliza-se após a puberdade (Kinder et al. 1995).

Em bovinos, segundo a teoria do gonadostato (Frisch e Revelle, 1970), durante a fase pré-púbere, existe maior sensibilidade dos neurônios de GnRH para o feedback negativo do estradiol produzido pelos folículos ovarianos. Animais pré-púberes ovariectomizados, sem reposição de estradiol, mostraram aumento dos pulsos de GnRH e LH semelhante ao que ocorre em animais adultos (Foster e Hileman, 2015), mostrando a regulação do estradiol sobre o hipotálamo. No entanto, os neurônios hipotalâmicos não possuem receptores de estradiol  $\alpha$ , o que revela que a inibição não ocorre por ação direta do estradiol (Lehman et al., 1993). Apesar do feedback negativo do estradiol sobre a liberação de GnRH durante a fase pré-púbere, Staigmiller et al. (1979) demonstraram que bezerras de 3 a 5 meses de idade tratadas com estradiol apresentaram picos de LH, mostrando uma possível ativação temporária do eixo hipotalâmico-hipofisário. Já que os neurônios hipotálâmicos não possuem o receptor de estradiol  $\alpha$ , acredita-se que a função do estradiol na inibição da liberação de GnRH seja mediada por neurônios de Kisspeptina (Dubois et al., 2016), os quais possuem receptores para estradiol e atuam na modulação da liberação de GnRH (Qiu et al., 2016). Utilizando roedores como modelo, foi demonstrado que ao se deletar os receptores de estradiol nos neurônios de kisspeptina, acelerou-se o início da puberdade revelando-se que o estradiol regularia negativamente a liberação de GnRH e, consequentemente, de LH através dos neurônios de kisspeptina (Cheong et al., 2015). Esta inibição ocorreria até o momento da maturação final do eixo HHG e, então, a liberação do GnRH na presença de estradiol, levaria ao aumento na pulsatilidade de LH. Assim, o LH poderia ser utilizado como um preditor da puberdade já que ocorre seu aumento 50 dias antes do início da puberdade (Day et al., 1987).

A nutrição é um fator importante quando se trata de puberdade, sendo necessário desenvolvimento e peso corporal mínimo do animal para que ocorra a maturação final do eixo HHG. Alguns estudos mostraram que o aumento da ingestão de nutrientes por fêmeas pré-púberes pode acelerar o início da puberdade em novilhas de corte (Cardoso et al., 2014a, b; Allen et al., 2017). Isto porque esses regimes alimentares que levam a altas taxas de ganho de peso, são acompanhados por aumento de adiposidade e, consequentemente,



de alguns hormônios como a leptina, fator de crescimento semelhante a insulina 1 (IGF1) e insulina (Cardoso et al., 2014b). A leptina é conhecida por modular a liberação de GnRH e estar envolvida com o processo de puberdade (Zieba et al., 2005). Acredita-se que a leptina não atue diretamente nos neurônios de GnRH, uma vez que estes não contêm receptores para esta proteína. Logo, a leptina atuaria na liberação de GnRH por meio de outros neurônios como os do neuropeptídeoY / peptídeo relacionado ao agouti (NPY/AgRP) e os neurônios da proopiomelanocortina/ e da transcrição regulada pela cocaína e anfetamina (POMC/CART; Ratra e Elias, 2014). Estes neurônios estão localizados no núcleo arqueado, junto com neurônios de kisspeptina, podendo este, também, ser modulado por fatores nutricionais (Ratra e Elias, 2014). O NPY e AgRP são peptídeos conhecidos por inibirem a liberação de GnRH, possuindo importância na regulação do GnRH durante a fase pré-púbere (Cardoso et al., 2018). Com o aumento do peso corporal do animal, observou-se diminuição nos transcritos de NPY e AgRP no núcleo arqueado, diminuição das concentrações de NPY no fluido cerebrospinal coletado no terceiro ventrículo (Cardoso et al., 2014a) e redução da ação dos neurônios de NPY nos neurônios de GnRH (Alves et al., 2015). Entretanto, a POMC/CART está relacionada com aumento da liberação de GnRH e foram mais expressas em novilhas que tiveram acelerada taxa de ganho de peso (Allen et al., 2012).

Outros fatores que podem influenciar a idade a puberdade é o ambiente e a genética. A estação do ano que os animais nascem podem influenciar a idade a puberdade (Kenny et al., 2018). Foi observado que novilhas nascidas no outono atingiram a puberdade mais jovens do as novilhas nascidas na primavera (Schillo et al., 1992). O fotoperíodo pode ser a principal razão para o clima influenciar na idade à puberdade em bovinos. Neste caso, sugere-se que a melatonina estaria envolvida na estimulação de sinais neuroendócrinos que influenciariam a secreção de LH (Schillo et al., 1992). Já no caso da genética, a idade a puberdade possui uma herdabilidade moderada variando entre 0,16 - 0,57 (Martinez-Velazquez et al., 2003). No entanto, estudos de genômica ampla tem identificado múltiplos genes associados com puberdade em fêmeas (Fortes et al., 2016) e dessa maneira poderiam ser utilizados em estratégias de seleção em programas de melhoramento genético. As fêmeas zebuínas (*Bos taurus indicus*), geralmente, possuem puberdade mais tardia do que

as fêmeas taurinas (*Bos taurus taurus*), como observado por Rodrigues et al. (2002) que avaliou novilhas desses dois genótipos sob as mesmas condições de manejo e ambiente. Assim, são desejadas as estratégias para diminuir a idade à puberdade em zebuínos ou para possibilitar a utilização desses animais pré-púberes na PIVE afim de diminuir o intervalo entre gerações, já que a puberdade mais tardia destes pode desfavorecer o melhoramento genético.

### **3.2 Endocrinologia da reprodução em fêmeas bovinas púberes vs pré-púberes**

Em fêmeas púberes, o crescimento folicular ovariano, a esteroidogênese e a ovulação são controlados pelo eixo hipotalâmico-hipofisário-gonadal no qual o hormônio liberador de gonadotrofinas (GnRH) controla a liberação dos hormônios hipofisários hormônio luteinizante (LH) e hormônio folículo estimulante (FSH), que agem nas gônadas estimulando a produção de esteroides como a progesterona (P4) e o 17 $\beta$  estradiol. Estes, por sua vez, agem por mecanismo de retroalimentação (*feedback*) no hipotálamo e na hipófise, regulando a liberação do GnRH, FSH e LH de maneira positiva ou negativa (revisado por Smith et al., 2018). Os esteroides sexuais, além de atuarem no controle do eixo hipotalâmico-hipofisário-gonadal, participam no controle da deposição de gordura (Connelly et al., 2015), desenvolvimento muscular (revisado por Smith et al., 2018) e ósseo do animal (Connelly et al., 2015; Michael et al., 2005; Oursler et al., 1991). Como verificado por McMillan et al. (2007), a deficiência de hormônios esteroides aumentam a remodelação óssea e, conseqüentemente, a perda óssea. Isso pode ser observado em mulheres no período de menopausa em que há baixas concentrações plasmáticas de estradiol, aumentando a reabsorção óssea (Wang et al., 2015; McMillan et al., 2007). O estradiol atua através da inibição da apoptose de osteócitos (Tomkinson et al., 1997), aumentando a produção de TGF (fator de crescimento tumoral) e inibindo a ação dos osteoclastos (Oursler et al., 1991). Assim, o estradiol produzido pelos folículos ovarianos desempenha papel crucial, não só no desenvolvimento reprodutivo, mas também no desenvolvimento corporal do animal.

A atividade do eixo hipotalâmico-hipofisário-gonadal já se inicia durante o período

embrionário, com o GnRH sendo produzido e liberado, estimulando a expressão das gonadotrofinas que estimulam a síntese de esteroides nas gônadas fetais (Duittoz et al., 2016). Após o nascimento, existe o estímulo para a liberação de FSH e de LH na corrente sanguínea de animais pré-púberes, já que se observa a presença desses hormônios em animais com 1 a 3 meses de idade (Evans *et al*, 1994, bezerras Hereford, *Bos taurus taurus*). Deste modo, esses hormônios estimulam o desenvolvimento folicular e as fêmeas pré-púberes apresentam ondas foliculares semelhantes às que ocorrem em vacas púberes (Evans et al., 1994), porém sem ovulação.

Em ovelhas, a quantidade e o padrão de crescimento de folículos antrais foram correlacionados positivamente às variações dos níveis de FSH e LH circulantes, do nascimento à primeira ovulação, e verificaram-se dois aumentos nas concentrações séricas de FSH e LH, sendo o primeiro aos dois a três meses de idade e o segundo aos quatro a cinco meses (Mahdia e Khallili, 2008). Bezerras Hereford tiveram maior desenvolvimento reprodutivo, com ovários apresentando um rápido crescimento de 1 aos 3,5 meses de idade, folículos atingindo maiores diâmetros dos 2 aos 3,5 meses e número de folículos  $\geq 3$ mm de diâmetro tendendo a aumentar de 1,5 a 3,5 meses de idade (Honaramooz et al., 2004) se comparados às demais idades na fase pré-púbere. Esse aumento quantitativo e do crescimento folicular em bezerras, provavelmente, reflete as maiores concentrações de FSH e LH que parecem estar presentes em bezerras mestiças Hereford entre os 3 a 5 meses de idade (Honaramooz et al., 1999). Corroborando estas informações, Rawlings et al. (2003) relataram que bezerras holandesas apresentaram maiores concentrações hormonais nos primeiros meses de vida e, posteriormente, aos 8,5 e 15 meses de idade, já antecedendo a puberdade.

### **3.3 Gonadotrofinas**

Os hormônios LH e FSH são glicoproteínas heterodímeras sintetizadas e secretadas pelas células gonadotróficas na adenohipófise, contêm uma subunidade  $\alpha$  em comum e uma subunidade  $\beta$  hormônio específica (Brown e McNeilly, 1999). Estes hormônios atuam

na regulação do crescimento e diferenciação das gônadas, na esteroidogênese e na gametogênese (Kumar e Matzuk, 2000). Apesar do FSH e do LH serem sintetizados pelo mesmo tipo celular, estes são produzidos dependendo da frequência e amplitude de pulsos de GnRH (Duittoz et al., 2016) e são secretados de maneira diferenciada, sendo o LH secretado de maneira pulsátil e o FSH secretado de maneira constitutiva (Farnworth, 1995).

O FSH estimula a proliferação e a diferenciação das células da granulosa e induz a formação do antro. Já o LH é crítico não só para a ovulação e a luteinização do folículo dominante, mas também para o crescimento e o desenvolvimento de um folículo dominante (Luo et al., 2011). O LH age nas células da teca estimulando a expressão de enzimas esteroidogênicas essenciais à produção dos andrógenos (Ivell et al., 2000). Nas células da granulosa, o LH regula a ação de diversos genes que atuam na esteroidogênese, sinalização autócrina e parácrina, transdução de sinal intracelular, regulação do citoesqueleto e da apoptose (Sasson et al., 2004).

Estas gonadotrofinas apresentam grande importância nos estágios mais avançados da foliculogênese. No entanto, já foi observada a ligação do FSH em células da granulosa de folículos com apenas uma única camada de células (Richards e Midgley, 1976). Argumentou-se, porém, que nestas fases iniciais, a ligação do FSH ao seu receptor ainda não permite a ativação do sistema adenilato ciclase, e por isso, podem ser não-funcionais (Wandji et al., 1992). Entretanto, detectou-se efeito do FSH no crescimento de folículos pré-antrais e antrais pequenos produzidos *in vitro* (Itoh et al., 2002) e *in vivo* (Tanaka et al., 2001), sugerindo um papel para este hormônio em folículos no início do desenvolvimento. Além disso, Barnett et al. (2006) observaram em camundongas, que a mutação de genes que controlam a expressão das gonadotrofinas e seus receptores, afeta não só a ovulação e a formação de corpo lúteo, mas também o processo de formação de folículos primordiais, o crescimento e a atresia folicular.

O efeito das gonadotrofinas, principalmente do FSH, é mediado pelas células da granulosa. Foi descrito que o tratamento com FSH durante as primeiras 6 horas de maturação *in vitro* (MIV) melhorou a competência ovocitária e permitiu a obtenção de mais de 45% de blastocistos, demonstrando a importância do FSH para o desenvolvimento

da competência ovocitária (Ali et al. 2005). Corroborando, Franciosi et al. (2015) demonstraram que o FSH está envolvido na aquisição da competência ovocitária, pois este regula a tradução dos RNAm maternos acumulados no ovócito. Consequentemente, o acúmulo dessas proteínas sintetizadas no ovócito está associado ao aumento da taxa de fecundação e à melhora no desenvolvimento embrionário. Além disso, os receptores de FSH tiveram maior expressão em células do cumulus de complexos cumulus-ovócitos (CCOs) provenientes de folículos com maiores diâmetros foliculares (Caixeta et al., 2009). Os CCOs de folículos maiores foram positivamente correlacionados aos ovócitos mais competentes (Caixeta et al., 2009), reforçando o papel do FSH na competência ovocitária.

No ovócito, a ação do pico de LH resulta na retomada da meiose, expansão das células do cumulus e na ovulação. Este hormônio apresenta importante papel no desenvolvimento da competência ovocitária, pois ao se ligar ao receptor nas células da granulosa, induz a expressão de ampirregulina, epirregulina e betacelulina (família EGF; Ashkenazi et al. 2005). Os membros da família EGF induzem a expressão da prostaglandina endoperoxídeo sintase 2 (PTGS2), tanto nas células da granulosa como nas células do cumulus, o que leva a um aumento na produção de prostaglandina E2 (PGE2). Por sua vez, a PGE2 ligada ao receptor ativa a proteína quinase 14 ativada por mitogênio (MAPK14) e, consequentemente, estimula a produção de ampirregulina e epirregulina nas células do cumulus (Prochazka et al., 2011). Estes se ligam aos receptores de EGF e de maneira autócrina, estimulam a transcrição de genes envolvidos na retomada da meiose e na expansão das células do cumulus (Shimada et al. 2006).

Mesmo que o LH tenha sua ação principalmente no final da foliculogênese, este também atua conjuntamente com o FSH auxiliando na proliferação celular, diferenciação, produção de estrógeno e posterior maturação (Qvist et al., 1990). Liu et al. (2002) observaram que, após o cultivo de folículos pré-antrais com LH e FSH, houve maturação dos folículos pré-antrais, aquisição de competência meiótica, fecundação e posterior formação de blastocisto. Além disso, em folículos de camundongos, o cultivo *in vitro* de folículos pré-antrais com LH e FSH estimulou a formação de antro de forma mais eficiente do que com o FSH sozinho, após 12 dias de cultivo (Ola et al., 2008). Isso demonstra a

importância do LH, não só nas fases finais da foliculogênese, mas também para o desenvolvimento inicial dos folículos.

### **3.4 Competência dos ovócitos de fêmeas pré-púberes vs púberes**

Um ovócito competente pode ser definido como aquele capaz de ser fecundado e desenvolver-se em um embrião (Eppig et al., 2002). A comunicação bidirecional entre o ovócito e as células do cumulus é fundamental para a aquisição da competência ovocitária.

A produção *in vitro* de embriões (PIVE) tem grande importância por potencializar o uso da fêmea objetivando-se um maior ganho genético dos rebanhos. Considerando-se então, a inclusão de animais pré-púberes nesta biotécnica, os ganhos seriam ainda maiores, pois isto diminuiria o intervalo entre gerações, maximizando o progresso genético (Lohuis, 1995) e aumentando a eficiência de sistemas de produção de leite e de carne.

No entanto, existem muitas variações nos resultados quando se utilizam bezerras como doadoras de ovócitos devido às respostas nas taxas de produção de embriões que diferem de acordo com as raças e idades estudadas. Além disso, ovócitos de fêmeas pré-púberes são considerados menos competentes dos que os de fêmeas púberes (Presicce et al., 1997; Palma et al., 2001; Oropeza et al., 2004; Batista et al., 2016; Currin et al., 2017; Baldassare et al., 2018), resultando em menores taxas de embriões e prenhez (Revel et al., 1995; Bols et al. 1999; Taneja et al., 2000 e Kauffold et al., 2005).

Em estudos comparativos (Tabelas 1 e 2), fêmeas púberes apresentaram taxas de produção de embriões superiores aos de fêmeas pré-púberes (~30% em fêmeas púberes *versus* ~10% em bezerras de 5 e de 7-10 meses de idade; Presicce et al., 1997; Zaraza et al., 2010). Um dos motivos para a diferença nas taxas de produção de embriões pode ser devido a qualidade morfológica dos ovócitos. A avaliação morfológica é feita com base na seleção de ovócitos com citoplasma homogêneo, granulações finas e com mais de três camadas de células do cumulus ao seu redor (revisado por Labrecque e Sirard, 2013; Blondin e Sirard, 1995). Além disso, o diâmetro do ovócito também está relacionado ao seu potencial de se desenvolver a embrião (Abreu et al., 2018; Makita et al., 2015; Otoi et

al., 1997). Ou seja, ovócitos com diâmetro entre 120 a 130  $\mu\text{m}$  apresentam maior capacidade de desenvolver embriões do que aqueles menores que 120  $\mu\text{m}$  (Otoi et al., 1997). Porém, a competência do ovócito depende ainda de estados bioquímicos e moleculares que permitam que este seja fecundado, se desenvolva até o estágio de embrião e que a gestação deste chegue a termo (Leoni et al., 2015).

Tabela 1. Levantamento de dados da literatura referentes a produção *in vitro* de embriões a partir de ovócitos de fêmeas pré-púberes diversas (raças e idades).

Raça	idade	Taxa embrião le fêmeas pré-púberes (%)	Taxa de embrião controle (%)	Valor de P	Proporção pelo controle (%)	Referência
Nelore	3-4 meses	12.9	30.9	P<0,05	41.7	Batista et al., 2016
Nelore	4-7 meses	1.3	24.4	P>0,05	5.4	Zacarias et al., 2018
Simental	3 meses	18.9	32.3	P<0,01	56.6	Palma et al., 2001
Ovários de abatedouro	4-7 meses	22.5	41.3	P<0,05	54.48	Camargo et al., 2005
Ovários de abatedouro	9-14 meses	23.8	32.4	P>0,05	73.46	Camargo et al., 2005
Ovários de abatedouro	9 meses	12.1	-----		-----	Córdova et al. 2010
Ovários de abatedouro	9 meses	9.4	-----		-----	Córdova et al., 2011

Tabela 2. Levantamento de dados da literatura referentes a produção *in vitro* de embriões a partir de ovócitos de fêmeas da raça Holandesa pré-púberes.

Raça	idade	Taxa embrião de fêmeas pré-púberes (%)	Taxa de embrião controle (%)	Valor de P	Proporção pelo controle (%)	Referência
Holandês	< 3 meses	19.9	-----		-----	Baldassare et al., 2018
Holandês	<3 meses	12.8	-----		-----	Currin et al., 2017
Holandês	3-4 meses	17.1	-----		-----	Currin et al., 2017
Holandês	3-4 meses	2.9	4.3	P>0,05	67.4	Batista et al., 2016
Holandês	> 4 meses	9.5	-----		-----	Baldassare et al., 2018
Holandês	>4meses	21.8	-----		-----	Currin et al., 2017
Holandês	5 meses	0	18	P<0,05	0	Presicce et al., 1997
Holandês	6-7 meses	1	24	P<0,05	4.1	Oropeza et al., 2004
Holandês	7 meses	1.0	27	P<0,05	3.7	Presicce et al., 1997
Holandês	6-9 meses	23.3	25.9	P>0,05	89.9	Bernal-Ulloa et al., 2016
Holandês	9 meses	7.0	29	P<0,05	24.1	Presicce et al., 1997
Holandês	9-10 meses	9	24	P>0,05	37.5	Oropeza et al., 2004
Holandês	11 meses	24.0	41.0	P<0,05	58.53	Presicce et al., 1997
Holandês	11-12 meses	10	28	P>0,05	35.7	Oropeza et al., 2004
Holandês	14-15 meses	33	22	P>0,05	150.0	Oropeza et al., 2004



O mecanismo de aquisição de competência dos ovócitos envolve processos relacionados tanto à maturação nuclear quanto à citoplasmática. A maturação nuclear envolve a quebra da vesícula germinativa, condensação dos cromossomos, progressão para metáfase I e extrusão do primeiro corpúsculo polar (Leoni et al., 2015). Já a maturação citoplasmática engloba os eventos de redistribuição de organelas, mudanças nas atividades do fator promotor de maturação (MPF) e da proteína quinase ativada por mitógeno (MAPK), aumento dos estoques de Cálcio, capacidade de descondensar a cromatina do espermatozoide e acúmulo de RNAm materno (Ferreira et al., 2009).

No estudo de De Paz *et al.* (2001), os ovócitos de bezerras mostraram uma maior densidade de microvilosidades na sua superfície e um maior número de vesículas endocíticas do que nos ovócitos de vacas. Por outro lado, os ovócitos de vacas mostraram uma população mitocondrial superior do que a observada em ovócitos de bezerras, sendo isto de grande importância para o metabolismo destes, uma vez que se sabe que o ATP sintetizado na mitocôndria é relevante na síntese e fosforilação de proteínas fundamentais para a maturação do ovócito (Stojkovic et al., 2001). Corroborando, Salamone et al. (2001) encontraram que tanto o MPF, MAPK e os receptores de inositol 1, 4, 5 trifosfato (IP3R) são substancialmente inferiores em ovócitos de bezerras do que em ovócitos de fêmeas bovinas adultas. Isto sugere que, um dos fatores da menor competência dos ovócitos de bezerras estaria no âmbito do citoplasma, já que ovócitos de fêmeas púberes possuem maior quantidade de mitocôndrias e de fatores que propiciam a maturação e a fecundação.

Entretanto, evidências de que o núcleo seria o responsável pela menor competência dos ovócitos de animais pré-púberes também foram observadas por Ptak et al. (2006), que realizaram a transferência nuclear entre ovócitos de cordeiras e de ovelhas adultas. Estes autores verificaram que, mesmo os ovócitos com diâmetros semelhantes aos de animais adultos não foram capazes de produzir blastocistos, ainda que após a transferência para o citoplasma de fêmeas adultas, reforçando assim uma falta de competência nuclear. Além disso, estudos demonstraram que fêmeas pré-púberes tem menor quantidade de transcritos quando comparadas às fêmeas adultas (Hosoe et al., 2011; Romar et al., 2011; Dorji et al., 2012a; Dorji et al., 2012b), o que reforça a falta de competência nuclear dos ovócitos de

animas pré-púberes. No entanto, como não foram encontrados estudos sobre a regulação da transcrição em ovócitos de bezerras, não se pode afastar também a hipótese de que a menor quantidade de transcritos poderia ser devido a uma desregulação nos mecanismos de controles epigenéticos ou por alterações moleculares ainda não identificadas nos ovócitos dessa categoria animal.

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## Capítulo II

### **Changes in reproductive and biometric development occur faster in 2 to 5 months of age than in 8 to 11 months old Nelore calves (*Bos taurus indicus*)**

T.S. Kawamoto<sup>1</sup>, J.H.M. Viana<sup>2</sup>, O.A.C. Faria<sup>3</sup>, A.A.G. Fidelis<sup>3</sup>, M.M Franco<sup>2</sup>, R.A. Figueiredo<sup>2</sup>

<sup>1</sup> - UFU – Federal University of Uberlândia, R. Ceará - Umuarama, Uberlândia – MG.

<sup>2</sup> Brazilian Agricultural Research Corporation (EMBRAPA) Genetic Resources and Biotechnology, PqEB - Av. W3 Norte (Final) S/N - Asa Norte, Brasília.

<sup>3</sup> UnB – Brasília University, Brasília, DF.

Corresponding author: Ricardo A. Figueiredo - ricardo.a.figueiredo@embrapa.br

**Short title:** Reproductive and biometric changes in prepubertal

#### **Abstract**

The study of reproductive development during the prepubertal phase of Nelore breed females is important in order to understand their physiology and, thus, be able to propose their use as oocyte donors in IVEP programs. Therefore, the aim of this study was to evaluate the LH/FSH serum concentrations and the associations among some reproductive and biometric features during two prepubertal periods of Nelore calves. Therefore, 8 Nelore calves and 8 cyclic pubertal cows (control) were evaluated by B-mode transrectal ultrasonography every other week from 2 to 5 and 8 to 11 months of age. The number and size of antral follicles, as well as ovarian and uterine horn diameters were recorded. On ultrasound evaluation day, blood samples were collected for plasma FSH and LH

measurements. Calves were monthly weighted and 3D scanned to recover rump biometric data (width, length, ratio and area) on both age groups. The rump area was calculated by the trapezium area defined by reference points on the prominences of rump bones. Associations were analyzed using the Spearman's correlation method, speed of development by non-linear regression and the means by t-test or Mann-Whitney test. Calves from 2 to 5 months old showed more moderate or positive correlations between reproductive and biometric development features than the 8 to 11 months old females. Rump geometry changes were observed up to 5 months of age. Furthermore, changes in biometric and reproductive development occur faster in 2 to 5 months than in 8 to 11 months' age females. Plasma LH ( $1.56 \pm 0.30$  ng/ml) and FSH concentrations ( $0.325 \pm 0.02$  ng/ml) in 2 to 5 months old calves were similar ( $P > 0.05$ ) to those of the cyclic pubertal cows (LH:  $1.58 \pm 0.51$  ng/ml; FSH:  $0.304 \pm 0.02$  ng/ml). However, the 8 to 11 months old females had their reproductive development stabilized and their LH plasma concentrations ( $0.93 \pm 0.24$  ng/ml) were lower ( $P < 0.05$ ) and FSH ( $0.495 \pm 0.02$  ng/ml) were higher ( $P < 0.001$ ) than those from cows (LH:  $1.2 \pm 0.64$  ng/ml; FSH:  $0.363 \pm 0.02$  ng/ml). These findings highlight an endocrine metabolism of gonadotrophin in the first months of life similar to that occur in adult cows and the importance of this period for the reproductive and biometric development.

Keywords: Rump, Ovary, Uterine, prepubertal

### **Implications**

It was evaluated the reproductive and somatic development of 2 to 5 and 8 to 11 months old Nelore calves and the serum gonadotropins average values in these two moments. In general, there is a high correlation between somatic and reproductive development, except for antral follicle count (AFC). However, there are differences in serum gonadotropins values and calf development according to age during the prepubertal phase. These findings highlight the importance of this phase for the reproductive development that may be associated to fertility after puberty. On the other hand, it has shown that biometric development could not be used to predict AFC, which is one of the important features used to select oocyte and embryo donors under assisted reproductive technology programs.

## Introduction

Prepubertal bovine females are potential oocyte donors for the ART, aiming to increase animal breeding and the use of this animal category that seems to be growing as a commercial trend. This has become more feasible due to advances in genomics that have allowed the evaluation and selection of genetic superior animals since the birth (Jiang et al., 2019; Wang et al., 2019). Therefore, it becomes important the knowledge about reproductive and biometric development during the prepubertal phase and how they can influence the forward animal development, particularly in *Bos taurus indicus*, since most research studies are conducted in *Bos taurus taurus*. The animal breeding programs are used for selecting high genetic merit animals and the animal selection can be based on their morphological (Olasege et al., 2019) and reproductive characteristics (Terakado et al., 2015). In this context, the reproductive traits are mainly focused on the fertility (MacNeil *et al.*, 2006) and the morphological traits can be directed to the carcass patterns (beef cattle), reproductive and productive efficiency. Measurements such as withers height and depth, body length and rump measures are some of the morphological characteristics usually taken. In addition to reveal carcass traits, some measures such as the rump may be also closely related to the calving easy (Wall *et al.*, 2005; Cue *et al.*, 1990).

In pubertal females, follicular growth, steroidogenesis and ovulation are controlled by the hypothalamic-pituitary-gonadal axis where the gonadotropin releasing hormone (GnRH) controls the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which act on the gonads stimulating steroids as progesterone (P4) and 17 $\beta$  estradiol, as well as proteic hormones such as inhibin production (reviewed by Kinder *et al.*, 1995). Thus, sexual steroids are produced by the growth and development of ovarian follicles. Among other actions these hormones are involved in fat deposition (Connelly *et al.*, 2015), muscle (reviewed by Smith et al., 2018) and bone development (Oursler *et al.*, 1991; Michael *et al.*, 2005; Connelly *et al.*, 2015). Therefore, ovarian function and follicular development play a crucial role in the animal body development.

The animal's morphology measures that reveal its biometric development, most of the



time, are taken through metric tapes and hypometers (Heinrichs et al., 2007). It difficults the evaluation, since it needs direct contact to the animal leading to stress conditions, making the procedure slower and more subject to failures. In this context, the 3D technologies use can make those evaluations faster, more accurate, enable the evaluation of several measures simultaneously, and therefore it has become quite attractive (Marinello *et al.*, 2015).

The bovine body measurements evaluation by image analysis has been performed (Marinello *et al.*, 2015; Pezzuolo *et al.*, 2018) under accuracy levels greater than 95% considering the digital image analyzes compared to the manual measurements for predicting the live animal weight, for instance (Tasdemir *et al.*, 2011). Thus, the hypothesis is that there is a more accelerated reproductive and biometric development in younger prepubertal animals accompanied by increases in serum gonadotropins concentrations when compared to prepubertal animals at older ages. Therefore, the aim of this study was to investigate the gonadotropins serum values, the reproductive tract development and to find out how some biometric and reproductive aspects are correlated during two prepubertal moments in Nelore females.

## **Materials and Methods**

### **Experimental design**

The eight Nelore females used on this study were generated by the use of sex sorted female semen on a timed artificial insemination protocol and they were born in a narrow parturition window between November and December 2017. Then, the animals were submitted to weekly transrectal ultrasonography evaluations in both 2 to 5 months and in 8 to 11 months of age periods. The ovarian diameter, AFC, number of follicles < 4 mm, number of follicles > 4 mm, maximum follicular diameter and the uterine horn diameter were evaluated. In addition, blood samples were collected once a day for FSH and twice a day for LH plasma measurements, on the same day of the ultrasound evaluation. Furthermore, the females were weighted and rump biometric measurements were evaluated by a conventional metric scale (tape measures) or 3D images acquisition. All of these procedures were repeated on these animals at 8 to 11 months of age. Similar procedures were done in adult cycling

cows as control measurements. The reproductive parameters and the biometric data (rump measures) were correlated and it was evaluated if there were changes in the reproductive tract development speed, rump conformation and hormonal changes, according to the age. The body weight gain of calves was 0.57 kg/day, starting from an average body weight of 90.9 kg and ending the experimental period with 245 kg.

### Ultrasonographic evaluations

The animals were evaluated by B-mode ultrasonography (MyLab 30 VetGold, Esaote. Genova, Italy) coupled to a 5-7.5 MHz rectal probe. The evaluation was standardized for frequency (7.5 MHz), focus depth and gain adjustments. Ovarian diameter, AFC, number of follicles < 4 mm, number of follicles > 4 mm, maximum follicular diameter and uterine horn diameter were evaluated once a week during the two referred prepubertal periods. For the 2 to 5 months old female evaluations, a probe guide was used to enable transrectal examination and when they reached the age of 8 to 11 months, the exam was done also by transrectal ultrasonography guiding the probe by hand. After they reached 15 months of age on, these evaluations were repeated to identify the puberty onset by the corpus luteum presence on the ovaries.

### Blood samples and hormonal measurements

Blood samples for plasma LH and FSH measurements were taken on every ultrasound evaluation day. They were collected by jugular vein puncture using needles and EDTA vacuum tubes. Then the blood samples were placed on ice, centrifuged (3,000g for 15 min) and the plasma samples were stored at -20 °C. The LH and FSH analysis were performed by radioimmunoassay (RIA) as described by Bolt *et al.* (1990). The assay sensitivity was 0.126 ng/mL for LH and 0.390 ng/mL for FSH. The intra-assay coefficient of variation was < 11%. The hormonal measurements were performed by the laboratory of endocrinology at the Sao Paulo State University (UNESP. Araçatuba - SP).

### Rump area data

The three-dimensional rump area images acquisition was performed by structured infrared light scanning using a portable sensor (iSense™, 3D Systems, Rock Hill, SC, USA) connected to a tablet computer (iPad Air 2, Apple Inc., Cupertino, CA, USA). This computer was equipped with a real time scanning app (<https://itunes.apple.com/us/app/isense/id807510940>). The equipment nominal resolution at 0.5 m was 0.9 mm for the x/y axes and 1.0 mm for the z axis (depth). The points-cloud data was transformed in a geometric surface and stored as OBJ files. The 3D images were then edited using the open-source software MeshLab (SourceForge, USA) to delete unspecific scans from the nearby objects and to perform the measurements. As a validation method, manual metric measurements were taken by metric tape. There were no differences between the rump manual measurements and those measured by 3D images acquisition, so only the image measurements were used. On the rump: the ilium or the ischium width projection, the ilium and the ischium ratio, area  $([\text{ilium} + \text{ischium} * \text{rump length}] / 2)$  and the rump length were evaluated. For illustrating the animal growth aspects, the rump images were cut in a 3D back-view taken from the animals at 2, 5, 8, and 11 months of age. Trapeziums were drawn for showing the changes in the rump geometry. For drawing the trapeze, it was used as reference points of the iliac crest and the tuber ischiadicum. It was chosen the rump that represented the average of all measurements as the illustrating image.

### Statistical analysis

The LH/FSH and reproductive parameters were analyzed by T-test or Mann Whitney and presented as mean and standard deviation. The relation between reproductive and biometric data was done by Spearman's correlation. For analyzing the reproductive development speed was used non-linear regression analysis (Gompertz curve) and the validation this curve was tested by deviance, where the fitted curve was compared to a straight line. The Gompertz model equation was  $Z * \exp(-b * \exp(a * x))$ , where "Z" is the maximum data value, "b" is the integration constant and "a" corresponds to the acceleration

rate. This non-linear regression analysis (Gompertz curve) was also used for comparing rump geometry development at different ages. All statistical procedures were performed by GraphPad Prism 6 or R cran 3.6.1 softwares, at 5% significance level.

## Results

The average reproductive parameters (Table 1) between 2 to 5 and 8 to 11 months old calves were compared. The 2 to 5 months old calves had ovarian diameter, number of follicles larger than 4 mm, maximum follicular and uterine horn diameter smaller than when these calves reached at the age of 8 to 11 months (Table 1).

Table 1. Reproductive parameters and body weight in calves from 2 to 5 and in heifers from 8 to 11 months' old (mean±SD)

Parameter		2-5 months old calves	8-11 months old heifers	P value
Ovarian diameter (mm)		16.5 ± 2.4 <sup>a</sup>	18.1 ± 2.1 <sup>b</sup>	< 0.0001
Antral follicle count (n)		20.7 ± 6.7	22.0 ± 8.9	0.5412
Number of follicles >4mm		3.4 ± 1.6 <sup>a</sup>	4.9 ± 2.2 <sup>b</sup>	< 0.0001
Maximum follicular diameter (mm)		7.2 ± 1.5 <sup>a</sup>	7.9 ± 1.3 <sup>b</sup>	0.0001
Uterine diameter (mm)		8.4 ± 1.0 <sup>a</sup>	10.1 ± 1.0 <sup>b</sup>	< 0.0001
Body Weight (Kg)	Pi	90.9	211.6	
	Pf	139.4	245	
	Pm	118.8 ± 22.9 <sup>a</sup>	227 ± 18.7 <sup>b</sup>	< 0.0001

Pi: initial average body weight; Pf: final average body weight; Pm: average body weight.

Different letters in the same line are different. T-test and Mann-Whitney analysis, P<0.05.

Among reproductive parameters' correlation in 2 to 5 months old calves (Figure 1A), a low relation of the AFC and the follicles number smaller than 4.0 mm to the other parameters were observed, and there were a weak but significant correlation (p <0.05) only

to the ovaries' diameter. Considering the other reproductive parameters (ovarian diameter, number of follicles greater than 4.0 mm, maximum follicular diameter and uterine horn diameter), it was observed a moderate significant correlation ( $p < 0.05$ ) among them. As expected, a moderate to strong correlation was observed among all biometric parameters ( $p < 0.01$ ). However, when analyzing biometric and reproductive parameters, a moderate positive correlation was observed among the biometric development and the number of follicles  $> 4.0$  mm, maximum follicular diameter, uterine horn and ovarian diameters ( $p < 0.01$ ).

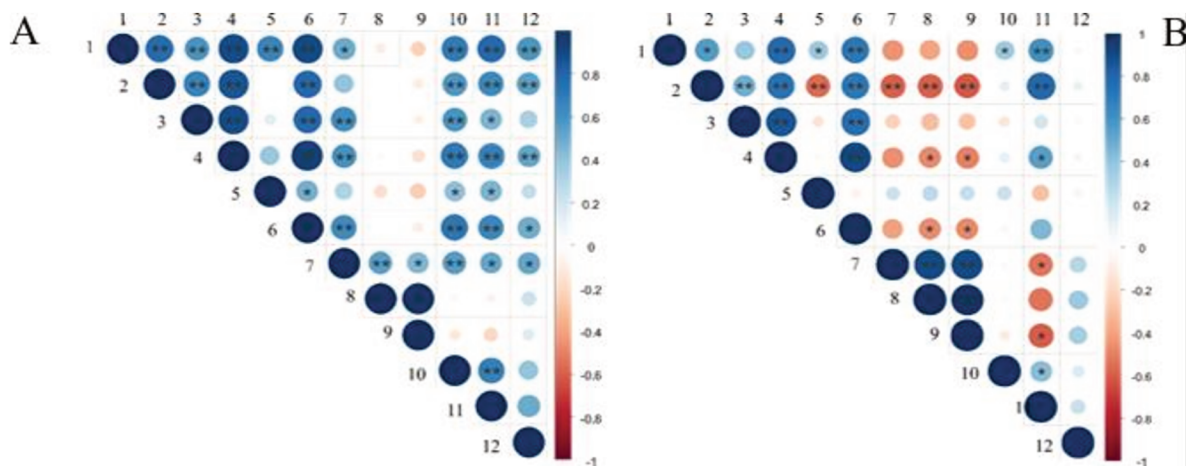


Fig. 1. A: Spearman's correlation between reproductive and biometric parameters in Nelore calves from 2 to 5 months of age. B: Spearman's correlation between reproductive and biometric parameters in Nelore calves from 8 to 11 months of age. 1. Length at ilium (cm), 2. Length in ischium (cm), 3. Rump length (cm), 4. Rump area (cm<sup>2</sup>), 5. Ratio between ilium's and ischium's length, 6. Body weight (Kg), 7. Ovarian diameter (mm), 8. Antral follicle count, 9. Number of follicles  $< 4.0$  mm, 10. Number of follicles greater than 4.0 mm, 11. Maximum follicular diameter (mm), 12. Uterine horn diameter (mm). \* $P < 0.05$  and \*\* $P < 0.01$ .

When the calves reached the age of 8 to 11 months, these relations changed (Figure

1B). Among the reproductive parameters, there were almost no correlation, and it just happened positive correlations between the AFC and the follicles smaller than 4.0 mm to the ovary's diameter ( $p < 0.01$ ). The maximum follicular diameter had a negative correlation to the ovary's diameter and the follicles smaller than 4.0 mm. Although the positive and strong correlations were maintained among most of the biometric parameters, the relation between the ilium/ischium ratio to the ischium became negative ( $p < 0.01$ ), showing a possible change in rump geometry. In addition, the biometric and reproductive data at these ages have shown an inversion pattern presenting a negative correlation of the ovarian diameter, the AFC and the follicles smaller than 4.0 mm to some rump measures. In this stage, the uterus' measures had no relation to the biometric data and only the maximum follicular diameter maintained a positive relation to the rump measurements.

The Gompertz model was used to analyze whether the observed correlation was due to a difference in the reproductive development speed in calves of different ages. Under this analysis, in 2 to 5 months old calves, it was observed the equations: Ovarian diameter ( $23.57 * \exp(-1.00983 * \exp(-0.33040 * x))$ ,  $r^2 = 0.44$ ), follicles larger than 4.0 mm ( $8 * \exp(-2.52892 * \exp(-0.33549 * x))$ ,  $r^2 = 0.23$ ), maximum follicular diameter ( $11.30 * \exp(-0.99893 * \exp(-0.24732 * x))$ ,  $r^2 = 0.21$ ) and uterine horn diameter ( $11.20 * \exp(-0.53403 * \exp(-0.19523 * x))$ ,  $r^2 = 0.16$ ). Thus, despite the low value of  $r^2$  (ranging from 0.2 to 0.4), due mainly to the large scattering of the data among the animals, it was possible to suggest a reproductive development rate ("a" value in Gompertz equation) for the calves at this early age (Figure 2A).

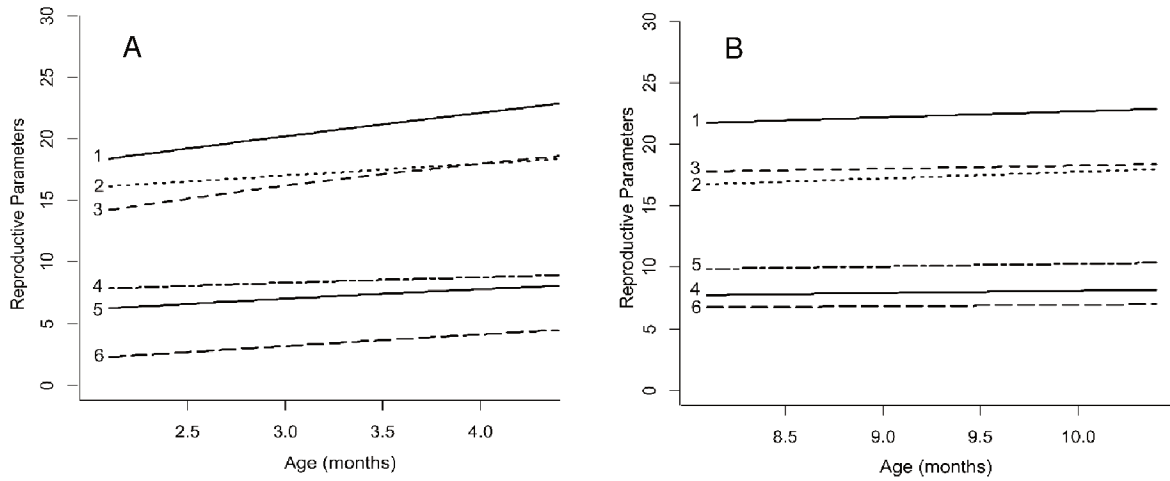


Fig. 2. Reproductive parameters non-linear regression curves. A: 2 to 5 months old calves. 1: antral follicle count ( $r^2 = 0.056$ ), 2: follicles smaller than 4.0 mm ( $r^2 = 0.014$ ), 3: ovarian diameter ( $r^2 = 0.442$ ), 4: maximum follicular diameter ( $r^2 = 0.207$ ), 5: uterine horn diameter ( $r^2 = 0.157$ ) and 6: ovarian follicles larger than 4.0 mm ( $r^2 = 0.232$ ). B: 8 to 11 months old females. 1: antral follicle count ( $r^2 = 0.001$ ), 2: follicles smaller than 4.0 mm ( $r^2 = 0.002$ ), 3: ovarian diameter ( $r^2 = 0.009$ ), 4: maximum follicular diameter ( $r^2 = 0.014$ ), 5: uterine horn diameter ( $r^2 = 0.024$ ) and 6: ovarian follicles larger than 4.0 mm ( $r^2 = 0.001$ ).

Therefore, in 2 to 5 months old calves the ovarian diameter and follicles larger than 4.0 mm presented a growth rate of 33%, and for the maximum follicular diameter and the uterine horn diameter it was 24% and 19%, respectively. To ensure that this curve was increasing, an analysis was performed to compare the curve to a straight line. Then it was found that all parameters, except follicles smaller than 4.0 mm ( $P > 0.05$ ), were different ( $P < 0.05$ ) from that straight line and it was presented as a growing trend (Table 2).

Table 2. Comparison among reproductive parameters non-linear regression curves (referred in Figure 3) to straight lines in 2 to 5 and 8 to 11 months old calves. F statistical with sum of squares (SQ) and P-value. When  $P > 0.05$ , the curves were not considered to be increasing.

Parameter	2 to 5 months of age			8 to 11 months of age		
	SQ	F	P-value	SQ	F	P-value
<b>Ovary diameter (mm)</b>	-238.9	74.6	<0.001	-4.3	0.9	0.3
<b>AFC (n)</b>	-244.0	5.6	0.02	-16.3	0.1	0.6
<b>Fol. &lt;4.0 mm (n)</b>	-61.114	1.300	0.2571	-19.8	0.2	0.6
<b>Fol. &gt;4.0 mm (n)</b>	-60.072	28.364	<0.001	-0.9	0.2	0.6
<b>Largest follicle diameter (mm)</b>	-42.315	24.526	<0.001	-2.4	1.5	0.2
<b>Uterine diameter (mm)</b>	-15.157	17.514	<0.001	-2.9	2.6	0.1

However, when the same parameters were observed on 8 to 11 months old females, it was observed that the  $r^2$  reached values below 0.01, which indicated that the data did not follow a growth trend line, being scattered to no apparent increasing variation (Figure 2B).

To validate this information, generated non-linear regression curves were also compared to straight lines, as it was done on the 2 to 5 months old animals. So, it was observed that all curves were equal to straight lines ( $P > 0.05$ ) showing that they did not have



an apparent reproductive development rate in this period (Table 2). Considering the rump geometry, it was observed the average of 1684.22 cm<sup>2</sup> for cyclic cows (control), and 514.22 cm<sup>2</sup> and 856.72 cm<sup>2</sup> on 2 to 5 or on 8 to 11 months old females, respectively. Besides calves from 2 to 5 months old calves had sharp geometry changes than at later ages that had more discreet development (Fig. 3A). The non-linear regression analysis confirmed the sharp changes in the 2 to 5 months old animals' rump geometry (Fig. 3B), that presented a development rate of 64% ( $711.40 * \exp(-2.05001 * \exp(-0.64280 * x))$ ;  $r^2 = 0.748$ ). For the 8 to 11 months old females (Fig. 3C), this developmental rate was 34% ( $1026.22 * \exp(-3.7862 * \exp(-0.3416 * x))$ ;  $r^2 = 0.303$ ).

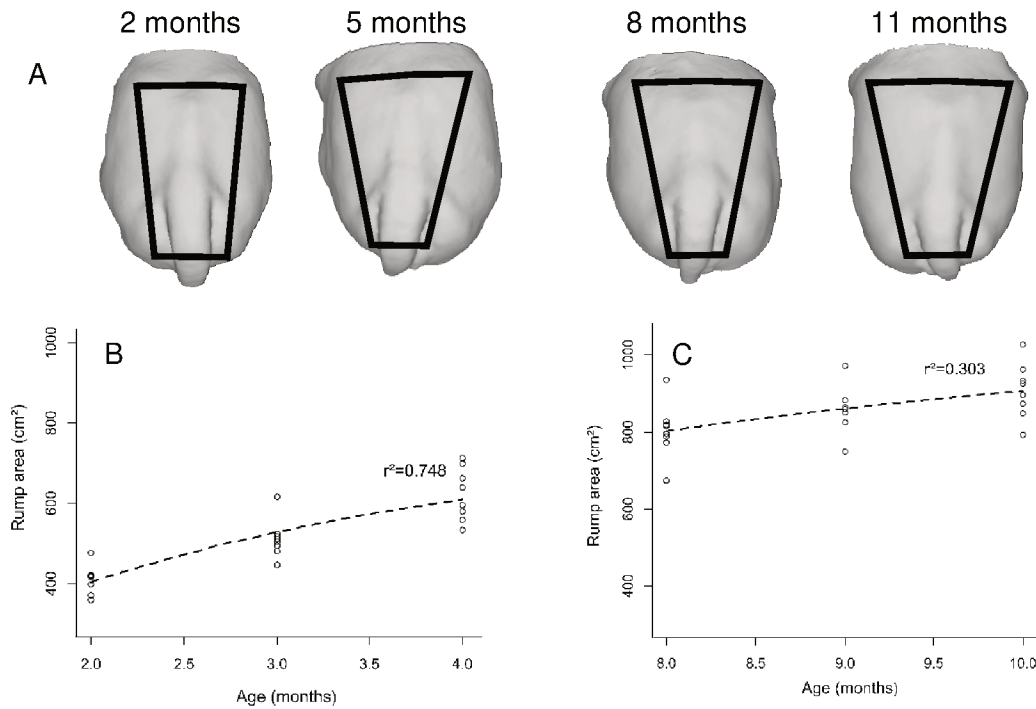


Fig. 3. Rump geometry changes. A: Back-view 3D images of Nelore females' rump at 2, 5, 8, and 11 months of age. Trapezoids show the changes in rump geometry. To build the trapeze, it was used as reference points the iliac crest and tuber ischiadicum. B: Non-linear regression curves with  $r^2$  value of rump geometry measures in 2 to 5 months old calves. C: Non-linear regression curves with  $r^2$  value of rump geometry measures in 8 to 11 months old females.

The LH and FSH plasma concentrations (figure 4) in 2 to 5 months old calves were similar ( $P > 0.05$ ) to those of the cyclic pubertal females. However, in 8 to 11 months old heifers' LH concentration was lower ( $P < 0.05$ ) and the FSH was higher ( $P < 0.001$ ) when they were compared to those from the control cows. These animals entered in the puberty phase on an average of 20.5 months (ranging from 17 to 24 months).

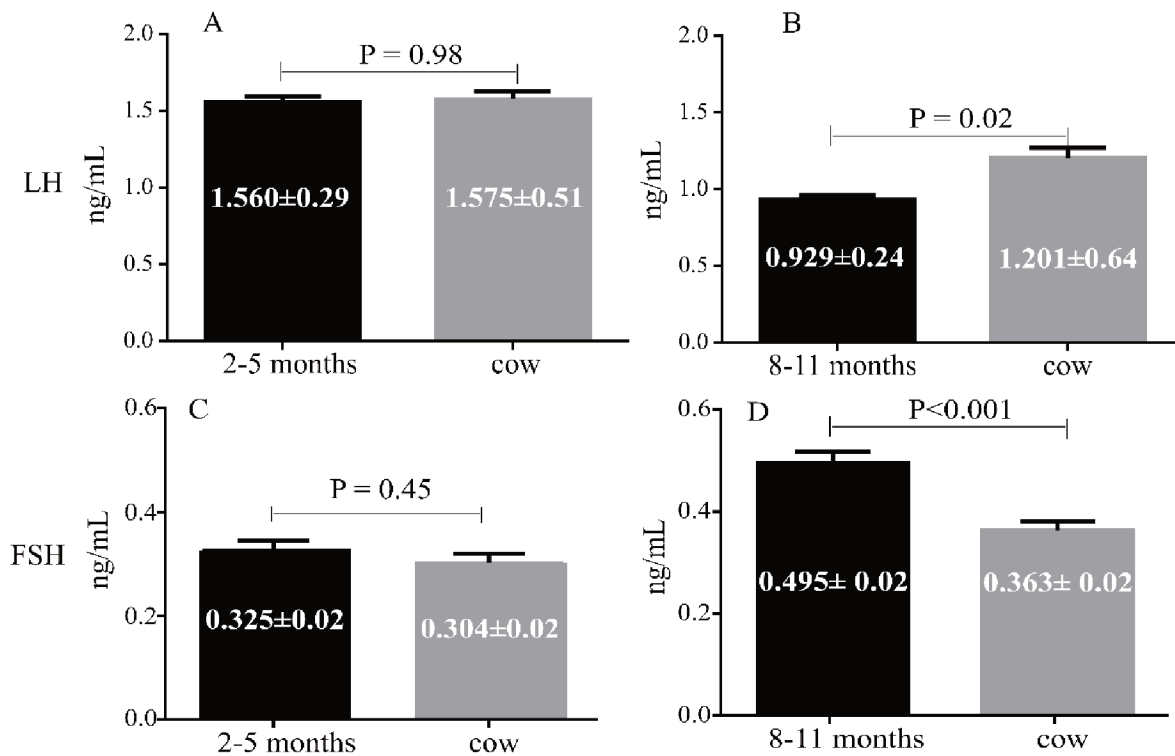


Fig. 4. A: Plasma LH concentrations (ng/mL) in Nelore calves (2 to 5 months old - black bar) and pubertal (gray bar) females. T-test,  $P < 0.05$ . B: Plasma LH concentrations (ng/mL) in Nelore heifers (8 to 11 months old - black bar) and pubertal (gray bar) females. T-test,  $P < 0.05$ . C: Plasma FSH concentrations (ng/mL) in Nelore calves (2 to 5 months old - black bar) and pubertal (gray bar) females. Mann-Whitney test,  $P < 0.05$ . D: Plasma FSH concentrations (ng/mL) in Nelore heifers (8 to 11 months old - black bar) and pubertal (gray bar) females. Mann-Whitney test,  $P < 0.05$ .

## Discussion

The main goal of this study was to evaluate the reproductive, biometric and hormonal patterns in prepubertal bovine females aiming a better understanding of these events on this peculiar developmental phase. The present results supported the hypothesis that the dynamics of reproductive and biometric development varies according to the prepubertal period. So, as observed in some correlation results, the calves had a positive and significant correlation between reproductive (ovarian diameter, number of follicles greater than 4.0 mm, maximum follicular diameter and uterine horn diameter) and biometric parameters (length at ilium, length in ischium, rump length, rump area, ratio between ilium's and ischium's length and body weight) in the first months of life (2 to 5 months of age). This correlation became nonexistent or negative with advancing of the age (8 to 11 months old animals) for all reproductive parameters when correlated with biometric ones, excepted for maximum follicular diameter that maintains a positive correlation with biometric parameters. This data revealed that the animals at a very early growth stage appears to have a faster reproductive development and we can hypothesize that they would have a higher serum estradiol available (it was not measured in this study) in relation to the body weight at this phase. This higher estradiol would act on uterine growth (Jdidi *et al.*, 2019) and on bone development (Connelly *et al.*, 2015), which would explain the existence of a positive correlation with rump measures. However, at later age, the reproductive tract growth seems to stabilize, while the biometric development continues to occur, that was indicated by none or negative correlation to the rump measures. In ewes was reported that 1 to 3.5 months old calves (Honaramooz *et al.*, 2004) or 2 to 3.5 months old (Bartlewski *et al.*, 2006) had a greater reproductive development, which were followed by a decline on these parameters, and then, they had a new increase occurring close to the puberty. Moreover, greater weight gains in the first three months of age were also reported to those sheep as well (Bartlewski *et al.*, 2006).

To verify this accelerated growth trend in 2 to 5 months old calves and more stabilized patterns in 8 to 11 months of age females, a nonlinear regression curve was established for the reproductive parameters of animals at different ages. These curves have shown that 2 to 5 months old calves had faster development than at later age (8 to 11 months old) and this

was confirmed by comparison of the curve to a straight line. Thus, the curves that were statistically different from a straight line indicated an increasing pattern, proving the existence of faster development growth in calves from 2 to 5 months, while in the 8 to 11 months old females there were no differences between the curve and a straight line. This shows that, at this later stage, heifers had a more stabilized reproductive development. This was observed in other studies which reported that, after the birth, there was an event called as “mini puberty” in sheep (Torres-Rovira *et al.*, 2016), bovine (Rawlings *et al.*, 2003; reviewed by Hernandez-Medrano *et al.*, 2012) and also in humans (reviewed by Kuiri-Hänninen *et al.*, 2014).

In the present study, rump development over the two periods was greater and had more marked change in its geometry at 2 to 5 months than at 8 to 11 months old. Some studies showed that the rump area was associated to pregnancy failure, anestrus, (Holm *et al.*, 2016) or dystocia (Holm *et al.*, 2014) on heifers, revealing the importance of understanding the rump measures, especially when we note that the early prepubertal phase is quite relevant in the conformational changes of the rump area. Thus, this work brings the innovation of studying the rump geometry that was possible due to the use of 3D technology. Then, the evaluations about correlation analysis and changes in rump geometry on 2 to 5 months' age calves, revealing a complex development mechanism, both reproductive and biometric, on those first months of life. It may be determinant for the future of the reproduction and to the animal growth. Studies described (Honaramooz *et al.*, 2004; Rawlings *et al.* 2003) that in the first months of life there is a faster development of the reproductive tract, later it reaches a plateau and then it turns to increase again near to the puberty. In addition, important hormonal changes occur at this early stage in cattle (Rawlings *et al.*, 2003) that needs to be further understood in Zebu cattle (*Bos taurus indicus*), since most of the reported works is performed mainly in Holstein (*Bos taurus taurus*) females. The marked differences in reproductive tract dynamics in different subspecies, breeds and managements make it difficult to extrapolate their patterns from one to another bovine herds, since animals of Nelore breed (*Bos taurus indicus*) show late puberty when compared to animals of Holstein breed (*Bos taurus taurus*).

Furthermore, our results showed the lack of correlation, at both Nelore ages' groups,

between AFC and the biometric parameters. Morotti *et al.* (2017) also described in Braford crossbred heifers (*indicus-taurus* 3/8 Nelore x 5/8 Hereford), that there was no correlation between the phenotypic characteristics and the AFC on these animals after weaning. It is a worth information, since the AFC is one of the parameters checked for selecting oocyte donor cows to IVEP. Therefore, animals could have been selected by the antral follicle population (Santa Cruz *et al.*, 2018), when this characteristic has no correlation to biometric development during the prepubertal phase, and it could imply on animal breeding delay. The magnitude of the AFC selection impact can be understood based on a 2015 data survey indicating that in Brazil it was produced 57.7% of the world's IVP embryos (Viana *et al.*, 2017) and that 20% of the born calves (average from 2005 to 2015) were produced by embryo transfer (Viana *et al.*, 2017).

The reproduction hormones analyzed in this study (FSH and LH) are produced by pituitary and they are important for acting on the antral follicles' growth, development and on steroid synthesis (reviewed by Kinder *et al.*, 1995). The growing follicles need LH to synthesize estradiol that, by its turn, acts in various tissues and causes negative feedback on FSH production (Ginther *et al.*, 1999). Our study revealed that 2 to 5 months old calves have a mean serum concentration of LH and FSH similar to that of adult cyclic cows.

Thus, based on the present results there would be a follicular growth (reaching diameters averages close to deviation), and serum LH in 2 to 5 months old calves possibly stimulated estradiol production (not measured in this study) that would act on the primary (ovary and uterine growth) and secondary (rump development) sexual characteristics development besides to induce negative FSH feedback similar as it happens on cyclic cows. In contrast, the 8 to 11 months old heifers had lower LH concentrations than those observed on adult females. It would lead to a lower follicle estradiol production and, consequently, they had increased FSH concentrations when compared to those on cows (Fig. 6 - Proposed model). Mauras *et al.* (1996) reviewed that in children, there is an increase in the amplitude of GnRH pulses that leads to an increase in FSH and LH, followed by an increase in the estradiol production that has led to an increase in the production of growth hormone (GH) and insulin-like growth (IGF-1). Moreover, Mauras *et al.* (1996) reviewed also that sex

steroids increase calcium absorption and so, can act in the mineralization of the skeleton which reinforces the model proposed in our study. Some other authors (Torres-Rovira et al., 2016; Rawlings et al., 2003; reviewed by Hernandez-Medrano et al., 2012) showed a brief hypothalamic-pituitary-gonadal axis activation soon after the birth and that once past the first months of life, there was a decreased serum gonadotropins' level. However, in this study hormonal measurement values were different to those reported for *Bos taurus taurus* cattle, on which they describe an increase in circulating FSH and LH levels from the two months of age on, then it decays, and it will rise again only near to the puberty (Rawlings *et al.*, 2003). Such difference in hormonal concentrations is acceptable since *Bos taurus taurus* cows had marked reproductive differences when compared to *Bos taurus indicus* like lower total follicle counts (Batista *et al.*, 2016; Sartori *et al.*, 2016), larger ovulatory follicle diameter (Sartori *et al.*, 2016) and earlier puberty. In addition, in adult cows, there was a difference in hormone circulating concentrations between the two subspecies, which *Bos taurus indicus* presented higher estradiol and lower FSH plasma concentration than those on *Bos taurus taurus* (Sartori *et al.*, 2016). These patterns influence the hypothalamic-pituitary-gonadal axis control and it is possible to observe such differences in hormone concentrations between the two subspecies, even for animals in the same age.



prepubertal period for the primary and secondary characteristics development that could be associated to the fertility after puberty. Such information could enable future studies to verify the influence of these characteristics in relation to other parameters, such as animal reproductive precocity and to evaluate the sustainability of including this animal category in some ART programs, as IVEP, for example.

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### **Declaration of interest**

The authors declare that there is no conflict of interest regarding the publication of this article.

### **Ethics statement**

All procedures were conducted in accordance and approved by Embrapa Ethics in Use of Animals Committee (Protocol CEUA #273/2017).

### **Software and data repository resources**

None of these data were deposited in an official repository.



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## Capítulo III

### **Features and developmental potential of oocytes collected from Non-stimulated Nelore (*Bos taurus indicus*) females in two prepubertal moments**

Taynan Stonoga Kawamoto<sup>1</sup>, João Henrique Moreira Viana<sup>2</sup>, Thais Preisser Pontelo<sup>3</sup>, Otávio Augusto Costa de Faria<sup>4</sup>, Andrei Antonioni Guedes Fidelis<sup>4</sup>, Margot Alves Nunes Dode<sup>2</sup>, Luna Nascimento Vargas<sup>1</sup>, Maurício Machaim Franco<sup>2</sup>, Ricardo Alaminio Figueiredo<sup>2</sup>

<sup>1</sup> - UFU – Federal University of Uberlândia, R. Ceará - Umuarama, Uberlândia – MG.

<sup>2</sup> Brazilian Agricultural Research Corporation (EMBRAPA) Genetic Resources and Biotechnology, PqEB - Av. W3 Norte (Final) S/N - Asa Norte, Brasília.

<sup>3</sup> UFLA – Federal University of Lavras, Av. Doutor Sylvio Menicucci, 1001, Lavras – MG.

<sup>4</sup> UnB – Brasília University, Brasília, DF.

Corresponding author: Ricardo A. Figueiredo - [ricardo.a.figueiredo@embrapa.br](mailto:ricardo.a.figueiredo@embrapa.br)

#### **Highlights**

- Prepubertal females can be used as oocyte donors in *in vitro* embryo production programs;
- There is difference in gene expression related to the epigenetic control of oocytes collected from prepubertal and adult females;
- The oocyte diameter of 8 to 11 months old females was similar to that on cyclic pubertal cows;
- Nelore females aged 8 to 11 months had oocyte competence similar to that on cyclic pubertal cows.

## Abstract

*In vitro* embryo production (IVEP) is a powerful tool for cattle herds genetic improvement. The inclusion of prepubertal females as oocyte donors on *in vitro* embryo production (IVEP) program is a strategy to shorten generation interval and to accelerate herds genetic gains. However, is reported that oocytes recovered from calves are less competent and generate fewer embryos than those obtained from adult cows. This study evaluated the features and the developmental capacity of oocytes obtained from Nelore females at 2 to 5 and at 8 to 11 months old. Eight calves underwent oocyte retrieval every other 15 days, from 2 to 5 months of age by laparoscopic ovum pick-up (OPU), and at 8 to 11 months old by transvaginal OPU. Adult cyclic cows were used as controls. The cumulus-oocyte complexes were used for IVEP. A subset of the oocytes was evaluated for morphology, diameter, and expression of genes related to chromatin compaction (HAT1, CREBBP, NCOA2, HDAC1, HDAC2 and HDAC3). Data were analyzed by ANOVA or Kruskal-Wallis test, depending on normality ( $P < 0.05$ ). Cyclic pubertal cows produced more ( $P < 0.05$ ) grade I (12.9% vs. 4.1% and 1.7%, respectively) and less grade III COC (30.1% vs. 44.5% and 49.0%, respectively) than the prepubertal animals of 2 to 5 and 8 to 11 months age. The oocyte diameter in older prepubertal females was similar to those in cows but greater than those in younger calves ( $124.8 \pm 8.5 \mu\text{m}$  and  $126.0 \pm 7.5 \mu\text{m}$  vs.  $121.3 \pm 7.5 \mu\text{m}$ , respectively,  $P < 0.05$ ). HDAC3 expression was downregulated ( $P < 0.05$ ) in prepubertal calves than cyclic pubertal cows, and no differences were found for the other genes. Blastocyst rates were similar between 8 to 11 months old and adult females (42.0% vs. 48.1%, respectively,  $P > 0.05$ ), but it was lower in 2 to 5 months old calves, when compared to their contemporaneous adult controls (31.0% vs. 71.6%, respectively,  $P < 0.05$ ). And when blastocysts rates were normalized to the proportion of their respective controls, it was lower in younger than in older prepubertal animals (43.7% vs. 78.7%, respectively,  $P < 0.05$ ). In summary, there was a progressive acquisition of oocyte developmental competence during the prepubertal period, and the IVEP results in 8 to 11 months old Nelore females were similar to those in cows. These results should be taken in account to decide whether is worthy to recover oocytes from early prepubertal calves.

Key words: LOPU, oocyte, gene expression, IVEP, embryo

## 1. Introduction

The *in vitro* embryo production (IVEP) allows the multiplication of genetically superior cattle favoring the genetic improvement. With the emergence of genomic technologies, the genetic superiority of the animal can be determined since its birth [1; 2]. Thus, can be interesting to include prepubertal females in IVEP programs, shortening the intergenerational interval and further accelerating genetic improvement [3], an alternative that seems to be growing as a commercial trend. This possibility could be even more attractive when working on Nelore breed (*Bos taurus indicus*), since the most Nelore cow have later puberty (around 24 months old [4]) than *Bos taurus taurus* (around 14 months of age [5]). So, the use of calves as oocyte donors could allow to decrease the reproduction costs as those animals could generate offsprings before the puberty age, consequently it accelerates the genetic gain. However, is related that prepubertal females have lower oocyte competence and embryo production [6-11] than pubertal animals.

Due to this low oocyte competence, hormonal treatments [10; 12; 13] and changes on *in vitro* culture media [14; 15] have been proposed. However, there is still a large variation in responses depending on breed [9], environment and age of the animal [10; 8], making it necessary to understand the reproductive physiology of those animals at different prepubertal phases. Embryo rates in prepubertal calves are described around 10 to 20% [8-11], while in pubertal cows range from 20 to 50% on average [8; 9; 16], depending on the laboratory protocol and on other factors. This lower oocyte competence observed in prepubertal female is probably due to cytoplasmic [17] and/or nuclear [18; 19] differences when compared to oocytes of adult cyclic cows.

Regarding those possible nuclear differences between prepubertal and pubertal females, Ptak et al. [19] observed no embryo production when they transferred the nucleus of prepubertal lamb oocyte to cytoplasm of pubertal females' oocyte sheep, showing that



there are differences between the nucleus of oocytes from prepubertal and pubertal sheep that compromise embryo production when subjected to micromanipulation. In addition, oocytes of prepubertal bovine females are known for having fewer transcripts than those of pubertal ones [20]. Since epigenetic factors control gene transcription, one of the possible causes of the lower transcript amount in calves' oocytes may be a change in the amount of epigenetic factors. Enzymes such as Histone Acetyltransferase 1 (HAT1), CREB Binding Protein (CREBBP) and Nuclear Receptor Coactivator 2 (NCOA2) are responsible for histone acetylation, favoring the occurrence of gene transcription. Histone Deacetylase 1 (HDAC1), Histone Deacetylase 2 (HDAC2) and Histone Deacetylase 3 (HDAC3) are enzymes responsible for histone deacetylation, that provides an environment in which transcription is unfavorable. Such enzymes are present in bovine oocytes and embryos [21] and appear to be related to the competence of bovine oocytes [22]. Thus, we hypothesized that oocyte competence could vary according to the animal age at two specific prepubertal phase periods. Therefore, the aim of the study was to evaluate the competence and chromatin compaction, through the embryo rates and gene expression of HAT1, CREBBP, NCOA2, HDAC1, HDAC2, and HDAC3, from Nelore female oocytes collected from 2 to 5 and 8 to 11 months of age.

## **2. Material and Methods**

This study was carried out at the Embrapa Genetic Resources and Biotechnology experimental farm in Brasilia, Federal District (DF), located at 15°52' and 15°56'S and 48°00' to 48°02'W, with altitude ranging from 1050 to 1250 m, under a tropical climate with dry winter. Animals were kept with their mothers, water available *ad libitum*, and fed corn silage, besides having pasture access (*Brachiaria decumbens*), and they were studied from March to May and from August to October 2018. This investigation was conducted in accordance to the Guiding of Care and Use of Research Animals of CEUA (Ethics Committee on Animal Use of the Embrapa Genetic Resources and Biotechnology, Brasilia, DF. Approved Protocol Number 273/2017).

## 2.1 Experimental design

Eight Nelore females used on this study were generated by the use of a sex sorted female semen on a timed artificial insemination (TAI) protocol and they were born in a narrow parturition period between December 2017 and January 2018. Then, the same animals were submitted to ovarian follicular aspirations when they were at 2 to 5 and 8 to 11 months old. From 2 to 5 months old, the calves had their follicles aspirated with a laparoscope system (LOPU) and from 8 to 11 months of age, by an ultrasound-guided transvaginal aspiration (OPU). Simultaneously to the prepubertal animals' follicular aspirations, adult cyclic cows had also their follicles aspirated (Control Group). The control cows used in the experiment were heifers considered pubertal, as they had regular estrous cycle with the presence of corpus luteum. The cumulus-oocyte complexes (COCs) recovered in both periods were then selected, classified for their morphological quality when 262 COCs were denuded and 807 COCs were submitted to *in vitro* embryo production. The denuded oocytes had their diameters measured having as limits the cytoplasmic membrane of the oocyte and they were stored at -80 °C until the qPCR analysis. The qPCR analyzes were performed for 4 pools of 15 immature oocytes. In addition, as a proof of concept of pregnancy, recipient cows received 15 and 14 embryos produced from oocytes of calves from 2 to 5 months and 8 to 11 months of age, respectively.

## 2.2 Oocyte recovery

The 2 to 5 months old calves, which have oocytes recovered by LOPU, were submitted to anesthetic procedures. The anesthetic protocol consisted of administrations of Xylazine Hydrochloride 2% (IM, Sedalex, Brazil), 1% Atropine Sulfate (UCB, Brazil) and Ketamine Hydrochloride 10% (IM; Dopalen, Ceva, Brazil). Lidocaine Hydrochloride 2.0% (SC, 5.0 mL, Bravet, Brazil) was also subcutaneous injected previously to the incision sites in the abdominal areas. So, a catheter with an insufflator (Inalar compact, NS group, Sao Paulo, Brazil) connected to its air valve was then introduced, which also served as an access

route for the laparoscope. Then, two more catheters were inserted, one for passing the anatomical caliper and the other for the aspiration system. The aspiration system consisted of a vacuum pump (59 mmHg negative pressure, WTA, Brazil) coupled to a disposable 21G needle. The ovaries were held with the aid of an anatomical caliper and the ovarian follicles were then aspirated. Fluid from aspirated follicles was collected in a conical tube containing aspiration liquid composed of 50 mL of phosphate buffered saline (PBS), 1% fetal bovine serum (FBS, Gibco, MA, USA) and 32 mg of heparin (Sigma Aldrich, St. Louis, MO, USA), at a temperature of approximately 37 °C. In 8 to 11 months old heifers and in cyclical pubertal cow, follicle aspiration was performed by a transvaginal ultrasound device (Mylab; Esaote) with a microconvex 7.5 MHz transducer. The ultrasound image helped to guide a 21G disposable needle coupled to an adapted aspiration system. The ovarian follicle aspiration procedure was performed as previously described [23]. Then the obtained COC's were immediately taken to the laboratory (beside to the corral) for the recovery, classification and IVEP procedures.

### 2.3 Morphology and oocyte diameter analysis

The COCs were morphologically classified as per the criterion established by Stojkovic et al. [24] as grade I (homogeneous cytoplasm oocyte and compact cumulus oophorus with several layers), grade II (homogeneous cytoplasm oocyte with small irregular pigmentation areas, fewer layers of cumulus cells than in grade I), grade III (heterogenous/vacuolated cytoplasm oocyte, less than three layers of cumulus or partially denuded cells), and grade IV (heterogeneous oocyte, denuded or expanded cumulus cells). For diameter analysis denuded oocytes were measured using the Motic Images Plus 2.0 software (Motic China Group Co. Ltd., Xiamen, China).

### 2.4 *In vitro* embryo production (IVEP)

For *in vitro* maturation (IVM), 25 to 30 oocytes were placed in 150 µl of maturation media drop. The IVM media consisted of TCM-199 Earl's salts (Gibco ®) supplemented

with 10% fetal bovine serum (FBS, Gibco ®), 0.01 IU/ml of follicle stimulating hormone (FSH), 0.1 mg/ml of L-glutamine, 0.075 mg/ml of amikacin, 0.1 µM of cysteamine, and 0.2 mM of sodium pyruvate. The IVM was performed during 22 h at 38.5 °C and 5% CO<sub>2</sub> in air. After IVM, COCs were transferred to a 150 µl drop of fertilization media, consisting of Tyrode's Albumin Lactate Pyruvate (TALP), supplemented with 0.5 mM of penicillamine, 0.25 mM of hypotaurine, 25 mM of epinephrine, and 10 mg/ml of heparin. Frozen semen from the same previously tested for IVEP bull was used for all groups and replicates. After thawing, the sperm were selected by Percoll gradient method [25] with 90% (400 µl) and 45% (400 µl) putted in 1.5 ml microcentrifuge tubes and it was centrifuged for 5 min at 9000 rpm. The resultant pellet was re-suspended with fertilization media in a final concentration of  $1 \times 10^6$  motile spermatozoa/mL. So, the oocytes were co-incubated with the sperm, for 18 to 20 h, at 38.5 °C and 5% CO<sub>2</sub> in air. The day of insemination was considered as day 0 (D0). Eighteen hours after insemination, the presumptive zygotes of all treatments were washed and transferred to a 150 µL drop of synthetic oviduct fluid media containing amino acids, citrate, and inositol (SOFaaci [Holm et al., 1998]), supplemented with bovine serum albumin, and it was incubated at 38.5 °C with 5% CO<sub>2</sub> for 7 days. Embryos were evaluated on Day 2 (D2) for cleavage, and on days 6 and 7 (D6 and D7) to determine the blastocyst rates.

## 2.5 RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was isolated using the RNeasy Plus MicroKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with minor modifications. The total RNA was used for cDNA synthesis using the GoScript Reverse Transcription System (Promega, Madison, USA) according to the manufacturer's instructions as well. Reactions were incubated at 70 °C for 5 minutes, 42 °C for 60 minutes, and 70 °C for 15 minutes.

## 2.6 Real-Time PCR (qPCR)

Real-time quantitative polymerase chain reactions (RT-qPCRs) were performed using the Fast SYBR Green Master Mix kit (Applied Biosystems). Each sample was analyzed

in triplicate and PCR specificities were determined by examining the melting curves and amplicon sizes on an agarose gel. Reactions were performed in a final volume of 25 $\mu$ L using template cDNA equivalent to 0.6 of an oocyte. The PCR conditions were 95 °C for 5 minutes followed by 50 cycles of denaturation at 95 °C for 10 seconds and then annealing and extension at 60 °C for 30 seconds. The gene names, primer sequences, amplicon sizes, and annealing temperatures are listed in Table1. The GAPDH and  $\beta$ -ACTIN were taken as the endogenous control gene. These genes were chosen because, in the literature, it appears that oocytes from prepubertal calves have less transcripts than pubertal cows. Therefore, the transcript analysis of histone acetylation/deacetylation may inform whether the smaller amount of transcripts is due to a difference in epigenetic transcripts between oocytes from calves and cows. The relative expression of each gene was calculated using the  $\Delta\Delta C_t$  method with efficiency correction by the Pfaffl method [26].

## 2.7 Statistical analysis

The oocyte diameters and blastocyst rates were tested for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett's test) and were evaluated by ANOVA. Calf embryo rates were standardized by their respective controls (calf embryo rate/control rate) in order to compare embryo rates between the 2 to 5 months and 8 to 11 months of age. Oocyte morphological quality is qualitative data and were evaluated by Kruskal-Wallis. The transcripts data were tested for normality and homoscedasticity and were analyzed by ANOVA or Kruskal-Wallis depending of normality data. Statistical significance was considered when  $P < 0.05$ .

Table 1: Primers for qPCR

Gene	Sequence	Fragment size (bp)	Annealing temperature (°C)	Accession number
GAPDH	F: GGC GTG AAC CAC GAG AAG TAT AA R: 5' CCC TCC ACG ATG CCA AAG T 3'	119	60	NM_001034034.2
ACTB	F: GGC ACC CAG CAC AAT GAA GAT CAA R: 5' ATC GTA CTC CTG CTT GCT GAT CCA 3	134	60	NM_173979.3
CREBBP	F: GTT CTC CAC TAC GAC ATC ATC R: CTT GTT GAC TCG GTC TTC C	150	60	NM_001164022.1
NCOA2	F: CCT GGG ATG GAC ATG ATT AAG R: TGG GTC GAA ACG AAG AGA	125	60	XM_027561151.1
HAT1	F: AAT TGA GAG ACT TTG TGC TTG TGA R: TTC AAT GAC ACG TCG ATA ATC TTC	392	60	NM_001034347.1
HDAC1	F: ATC GGT TAG GTT GCT TCA ATC TG R: GTT GTA TGG AAG CTC ATT AGG GA	188	60	NM_001037444.2
HDAC3	F: GAA GAG GCC ATT AGT GAA GAG R: TCA GTC CTG TCG TAG GTT AG	227	60	NM_001206243.1
HDAC2	F: TTC CTG GAA CAG GAG ACT TA R: ATC ACC AGA TAG GGA GTC TG	194	60	NM_001075146.1

F (forward); R (reverse); bp (base pair).

### 3. Results

The recovery rate of aspirated follicles in 2 to 5 months old calves was an average of 88.91%, while follicles of 8 to 11 months old heifers aspirated by transvaginal ultrasound had 56.73% oocyte recovery rate. A total of 1,817 oocytes were analyzed, which 362 oocytes were evaluated for diameter. The 8 to 11 months old heifers had oocyte diameter ( $124.75 \mu\text{m} \pm 8.45$ ) similar ( $P > 0.05$ ) to those of pubertal cow ( $126.04 \mu\text{m} \pm 7.46$ ) and were larger ( $P$

<0.05) than those of 2 to 5 months old calves ( $121.30 \mu\text{m} \pm 7.47$ ). Regarding morphological quality, it was observed that cyclic adult cows had higher grade I and II quality oocytes, while prepubertal animals had more grade III and IV oocytes (Table 2).

Table 2. Diameter (mean  $\pm$  SD) and grade of oocyte morphological quality (%) of cyclic cows and prepubertal females from 2 to 5 and 8 to 11 months of age

Treatment	Oocyte diameter mean $\pm$ SD (N)	Grade of oocyte quality n (%)			
		GI	GII	GIII	GIV
2-5 months old	121,30 $\pm$ 7,47 <sup>b</sup>	9	134	262	130
	(85)	(1,7) <sup>b</sup>	(25,0) <sup>a</sup>	(49,0) <sup>a</sup>	(24,3) <sup>b</sup>
8-11 months old	124,75 $\pm$ 8,45 <sup>a</sup>	16	49 (12,6) <sup>b</sup>	173	151
	(78)	(4,1) <sup>b</sup>		(44,5) <sup>a</sup>	(38,9) <sup>a</sup>
Cyclic cows (control)	126,04 $\pm$ 7,46 <sup>a</sup>	115	279	269	230
	(99)	(12,9) <sup>a</sup>	(31,2) <sup>a</sup>	(30,1) <sup>b</sup>	(25,7) <sup>ab</sup>

Different letters in the same column are different. ANOVA and Kruskal- Wallis test,  $P < 0.05$ .

The oocyte diameter difference reflected on the *in vitro* embryo production which at the first moment, pubertal cyclic cows had higher embryo production (71.6%;  $P < 0.05$ ) than 2 to 5 months old calves (31.0% - Table 3). In a second evaluation moment, heifers aged 8 to 11 months had similar embryo production (42%;  $P > 0.05$ ) to pubertal cyclic cows (48.1%; Table 4). Moreover, was observed that 2 to 5 and 8 to 11 months old females had blastocyst rates of 43.8% and 78.7%, respectively when compared and standardized to their respective adult controls.

Table 3. Cleaved and blastocyst production at day 6 (D6) and at day 7 (D7) on *in vitro* culture of oocytes collected from cyclic cows or 2 to 5 months old calves

<b>Treatment</b>	<b>N</b>	<b>Cleaved</b>	<b>Blastocyst D6</b>	<b>Blastocyst D7</b>
		<b>n (%)</b>	<b>n (%)</b>	<b>n (%)</b>
<b>Cows</b>	247	214 (86,6)	122(49,4) <sup>a</sup>	177 (71,6) <sup>a</sup>
<b>2 to 5 months old</b>	171	121(70,8)	27(15,8) <sup>b</sup>	53 (31,0) <sup>b</sup>

Different letters in the same column are different. ANOVA, P<0.05

Table 4. Cleaved and blastocyst production at day 6 (D6) and at day 7 (D7) on *in vitro* culture of oocytes collected form cyclic cows or 8 to11 months old prepubertal heifers

<b>Treatment</b>	<b>N</b>	<b>Cleaved</b>	<b>Blastocyst D6</b>	<b>Blastocyst D7</b>
		<b>n (%)</b>	<b>n (%)</b>	<b>n (%)</b>
<b>Cows</b>	270	193(71,5)	90(33,3)	130 (48,1)
<b>8 to 11 months old</b>	119	89 (74,8)	22(18,5)	50 (42,0)

Different letters in the same column are different. ANOVA, P<0.05



Table 5. Proportion of blastocyst rates of oocytes collected from prepubertal females when divided by the correspondent results of their respective cow controls.

Category	Total embryos (n)	D6/Control (%)	D7/Control (%)
<b>2 to 5 months old</b>	53	31.04	43.78 <sup>a</sup>
<b>8 to 11 months old</b>	50	59.63	78.77 <sup>b</sup>

Different letters in the same column are different. T-test,  $P < 0.05$

Regarding the expression of HAT1, HDAC1, HDAC2, HDAC3, CREBBP and NCOA2 genes, there was difference only for HDAC3 having less transcripts in immature oocytes of prepubertal females than on those in adult cows ( $P < 0.05$ ). The other genes had the same expression levels among prepubertal and pubertal animals' immature oocytes ( $P > 0.05$  - Figure 1).

As a proof of concept, 29 embryos produced from prepubertal females' oocytes were transferred to recipient cows. Considering the 15 transferred embryos originated from the oocytes of the 2 to 5 months old calves, 6 recipient cows were diagnosed pregnant at 30 days post embryo fertilization, there was one gestational loss at 60 days and then, 4 transferred embryos became offsprings. By the other hand, from the 14 transferred embryos originated from the oocytes of the 8 to 11 months old heifers' oocytes, two produced pregnancy diagnosed at 30 days, one pregnancy confirmed at 60 days, and one got birth.

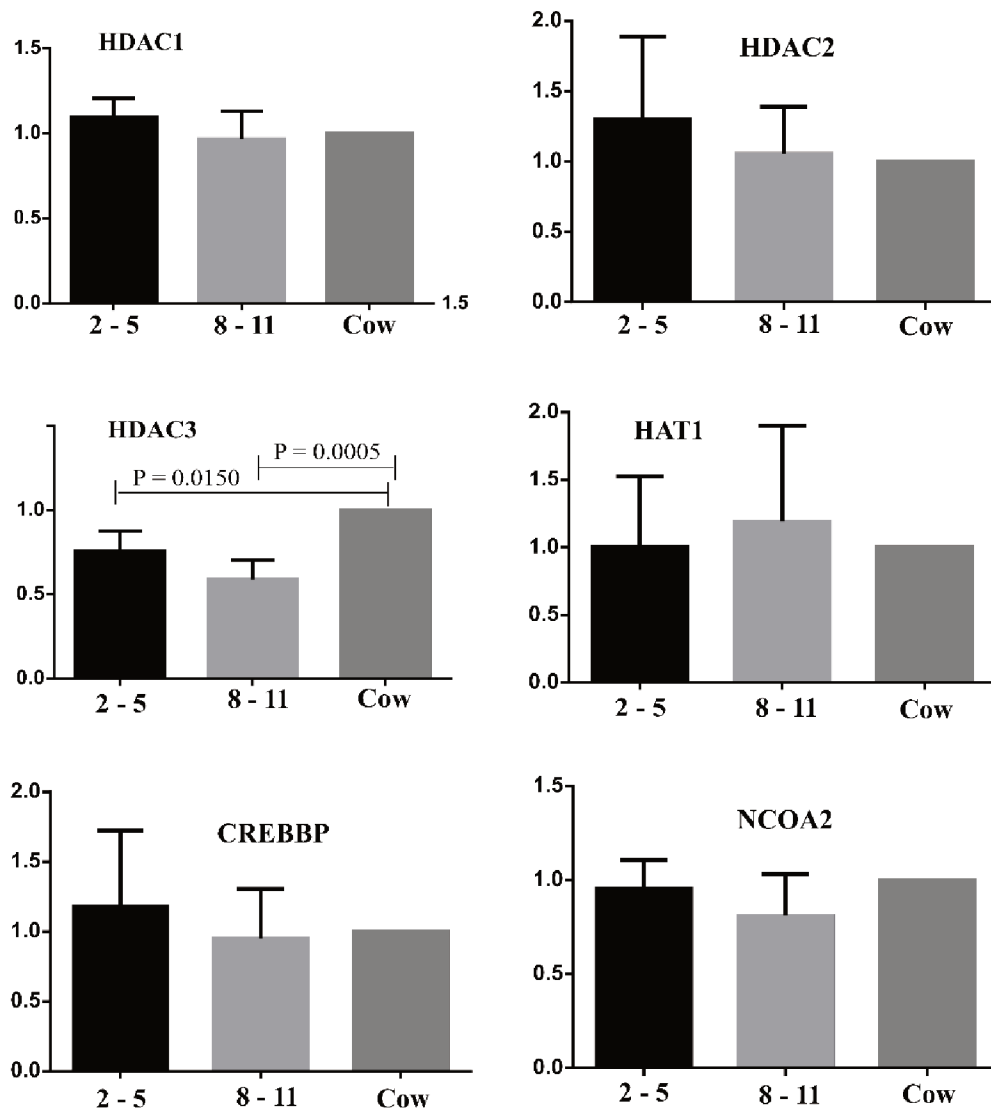


Figure 1. Gene expression of HDAC1, HDAC2, HDAC3, HAT1, CREBBP E NCOA2 in immature oocytes of 2 to 5 months old calves (black bar), 8 to 11 months old heifers (light gray bar), and cyclic adult cows (dark gray bar). The P value is informed in the figure when statistical difference was found.  $P < 0.05$ .

#### 4. Discussion

Oocytes are selected according to their morphological quality seeking to estimate their development capacity in IVEP. Considering the morphological quality in this study, it was observed that prepubertal females had fewer grade I oocytes and more grade III oocytes than those of adult cyclic cows. Oocytes with homogeneous brown color or with few granulated cytoplasm (Grades I and II) were found to have intermediate levels of ATP, greater first polar body extrusion capacity, and greater organelle organization after IVM similar to matured oocytes in vivo [27]. In contrast, pale oocytes (poorer quality) had low ATP levels with low polar body extrusion rate, and black or coarsely granulated cytoplasm oocytes (Grade IV) had the highest ATP concentrations which could indicate dysfunction in the oocyte energy consumption leading to lowest embryo production rates [27]. Corroborating, it was reported that there were higher cleavage and embryo rates produced by grade I and II and lower in grade III and IV oocytes [28; 29]. Regarding the oocyte diameter, we have found 8 to 11 months old heifers had the same oocyte diameter as the pubertal cows. Since the diameter were similar between 8 to 11 months old calves and adult cyclic cows, it is possible that the lower morphological quality observed in 8 to 11 months old calves when compared to the cows maybe due to the aspiration system methodology that was optimized for adult cyclic cows and it has been adapted for the 8 to 11 months old calves. Thus, 8 to 11 months old calves have the similar oocyte diameter than those on cows and then, it could be possible that they would have similar growth capacity, accumulation of organelles and messenger RNAs in their oocyte. This speculation could be deducted by the embryonic production results, since 8 to 11 months old heifers' blastocyst rates were similar to those in adult cows, and it was higher than those observed in 2 to 5 months of age calves.

The prepubertal bovine females' embryonic production data in the literature are quite varied, showing blastocyst rates from 0 to 20% [6-11], depending on the breed and on the animal age, for instance. When the data from the mentioned works above are taken to standardize the embryo production rates to the rates of their respective controls, it's observed that 5 to 12 months old Holstein females [6; 8; 14] presented a range from 0% to 89.9% of

embryo production. In 3 to 4 months old Nelore females [9] the rates were 41.7% and in 3 months old Simental females [7] it was 56.6% of embryo production. In this study, the 2 to 5 months old Nelore females had embryo production rates of 31% and on those of 8 to 11 months old it was 42%. Despite these good embryo rates, when the calves' embryo production rates were standardized to the cyclic cow rates, it was found that 2 to 5 months of age calves showed 43.7% and 8 to 11 months old females 78.77% of embryo rates in relation to their respective controls. This result highlights that calves from 8 to 11 months of age presented embryo production rates similar to those of adult cyclic cows even without exogenous hormones stimulation. Therefore, females aged 8 to 11 months had more competent oocytes than the 2 to 5 months old calves. A possible explanation for this difference in oocyte competence at the different ages prepubertal females would be that, the very younger females are in a more rapid phase of body and reproductive growth than the older prepubertal females (8 to 11 months old), causing its hormones to be directed preferentially to the animal growth rather than to the reproductive focus (unpublished data of this team). Mauras et al. [32] reviewed that in children, there is an increase in the production of estradiol that leads to an increase in the growth hormone (GH) production besides calcium absorption, and it makes the infant stage important for the child's development and growth.

Currently, some studies reported the importance of understanding histone acetylation level throughout the maturation process and a possible association of these proteins to the acquisition of oocyte competence [33-36]. In this study, we sought to analyze the chromatin compaction of prepubertal calves' oocytes. A higher amount of HDAC3 transcript was observed in cyclic pubertal females than in prepubertal females. HAT1, CREBBP and NCOA2 genes are involved with histone acetylation and HDAC1, HDAC2 and HDAC3 genes with the histone deacetylation. Histone acetylation is related to the state of open chromatin (euchromatin), which is able to transcription. Deacetylation is directly related to a closed chromatin (heterochromatin), thus repressing transcription. These mechanisms play a fundamental role in the preparation of oocyte for maturation, and the condensation of chromatin that is essential for the meiosis resumption [37]. Thus, the higher amount of HDAC3 transcripts observed in this study may suggest that cows' oocytes would be better

prepared for chromatin condensation that occurs during the first 8 hours of *in vitro* maturation. Thus, changes in chromatin configuration along the epigenetic events at the meiotic maturation beginning would promote gene silencing, occurring in parallel with the acquisition of oocyte competence [38-40]. In addition, HDAC3 has shown great importance in the maturation of the oocyte, being responsible for the meiosis arrest by repressing the expression of amphiregulin before the LH peak [41], and in embryogenesis since mutant mice for the HDAC3 gene had defects in gastrulation that led to embryonic death [42]. Oocytes from prepubertal females are known for showing fewer total transcripts than pubertal females [43-46]. Thus, one of the causes of this lower number of transcripts present in prepubertal oocytes could be due to some down or upregulation of epigenetic factors. However, in this study, no differences were identified in the other transcripts related to histone acetylation (HAT1, CREBBP and NCOA2), so, by analyzing these transcripts amounts it cannot be suggested that there is higher transcriptional activity in cyclic pubertal cows than in calves. This shows that other genes involved in epigenetic control, unlike those evaluated, could be differentially expressed in prepubertal female oocytes when compared to those of the cyclic cows.

Prepubertal females can be potential oocyte donors for IVEP, as it was shown on the results of the present study, that had a concept proof of 4 offsprings obtained from 2 to 5 months of age calves' oocytes and 1 birth from 8 to 11 months of age calves' oocytes.

In summary, the present study results suggest that 2 to 5 months old calves had less competent oocytes than 8 to 11 months old calves. In this context, there was a progressive acquisition of oocyte developmental competence during the prepubertal period, and IVEP results in the 8 to 11 months old Nelore calves were similar to those in adult cyclic cows. Thus, these results shown that the 8 to 11 months old calves could be used as oocyte donors without exogenous hormones stimulation and it could help to decide whether is worthy to recover oocytes from even earlier prepubertal calves (2 to 5 months old), for instance.

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## Capítulo IV – Considerações Finais

O uso de fêmeas pré-púberes pode contribuir para o aumento do ganho genético dos rebanhos, e possibilitar menores custos nos sistemas de produção. Assim, estudos para um melhor entendimento da fisiologia reprodutiva e sobre o emprego desta categoria animal em programas de PIVE são necessários. O presente estudo revelou a existência de mudanças mais intensas na fase pré-púbere de fêmeas Nelore (*Bos taurus indicus*), tanto na geometria da garupa (característica sexual secundária), quanto para uma maior velocidade de desenvolvimento dos parâmetros reprodutivos (características sexuais primárias) nos primeiros meses de vida (2 a 5 meses), quando comparados aos animais de 8 a 11 meses de idade. Além disso, o desenvolvimento mais intenso nas bezerras de 2 a 5 meses de idade foi acompanhado por concentrações de gonadotrofinas séricas (LH e FSH) semelhantes às observadas em animais adultos. Verificou-se ainda que as fêmeas de 8 a 11 meses de idade apresentaram competência ovocitária semelhante à das vacas adultas, com maior taxa de produção embrionária do que a observada nas bezerras mais jovens, sugerindo uma aquisição progressiva de desenvolvimento de competência dos ovócitos ao longo da fase pré-púbere. Uma possível especulação para isto, seria a de que as gonadotrofinas séricas (mesma concentração observada em fêmeas cíclicas), na fase de 2 a 5 meses de idade, poderiam contribuir para acentuar a produção de estradiol, e que este, nesta fase, atuaria mais intensamente no desenvolvimento corporal do animal do que na preparação de um ovócito mais competente. Isso confirmou a importância de se entender melhor o desenvolvimento reprodutivo da fêmea bovina durante esta fase. Assim, concluiu-se que as bezerras Nelore de 8 a 11 meses de idade, após terem passado por uma fase mais crítica de desenvolvimento reprodutivo e corporal (dos 2 aos 5 meses de idade), são potenciais doadoras de ovócitos para programas de PIVE, podendo-se obter destas, taxas de embriões semelhantes às de fêmeas adultas, mesmo na ausência de estimulação por hormônios exógenos.

