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**AVALIAÇÃO DA DENSIDADE FOLICULAR E DE ESTRATÉGIAS  
PARA OTIMIZAR A OBTENÇÃO DE FOLÍCULOS PRÉ-ANTRAIS  
EQUINOS**

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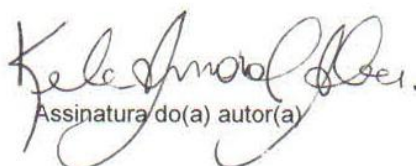
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KELE AMARAL ALVES

**AVALIAÇÃO DA DENSIDADE FOLICULAR E DE ESTRATÉGIAS  
PARA OTIMIZAR A OBTENÇÃO DE FOLÍCULOS PRÉ-ANTRAIS  
EQUINOS**

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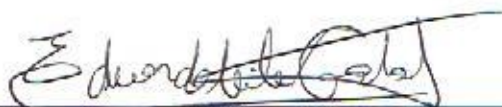
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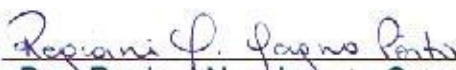
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## RESUMO

Estudos com folículos pré-antrais equinos são escassos e as características da dinâmica folicular pré-antral permanecem pouco elucidadas. A função ovariana (ex. foliculogênese) da égua possui diversas similaridades com a mulher e, baseado nestes aspectos, podemos considerá-la como modelo animal para estudos comparativos e técnicas que utilizam folículos pré-antrais na preservação da fertilidade feminina. O primeiro projeto teve a proposta de definir metodologias para agilizar o processo de avaliação histológica da população folicular pré-antral. No segundo projeto, avaliou-se as características da população folicular considerando o fator densidade folicular, determinando por meio de modelo matemático, o apropriado desenho experimental para estimar a densidade em fragmentos ovarianos. No terceiro projeto, analisou-se os efeitos da fase reprodutiva, ovário e estruturas ovarianas sobre a qualidade, classificação, densidade folicular e celular em fragmentos ovarianos. Os resultados permitiram estabelecer a melhor espessura de secção histológica e o número de fragmentos e de secções suficientes para estimar a densidade de folículos pré-antrais equinos. Adicionalmente, observou-se uma baixa densidade folicular associada a alta heterogeneidade dentre e entre os animais. Além disso, determinou-se a fase de diestro com a presença de corpo lúteo ativo como o melhor cenário para a coleta de fragmentos ovarianos com maior densidade folicular e celular. Finalmente, dos resultados originados deste trabalho, pode-se concluir: (i) obteve-se avanço no conhecimento da dinâmica folicular pré-antral equina; (ii) o conceito da égua como modelo animal para estudos de desafios reprodutivos na mulher foi reforçado; e (iii) ocorreu a geração de informações básicas para futuras pesquisas com biotecnologias que visam preservar ou implementar a capacidade reprodutiva como a criopreservação, cultivo *in vitro* e transplantes de tecido ovariano.

**Palavras- chave:** biópsia ovariana, densidade folicular, égua, folículos pré-antrais

## ABSTRACT

Studies with equine preantral follicles are scarce and the follicular dynamics features remain unclear. The mare's ovarian function (e.g., folliculogenesis) has similarities with woman's cycle and based on these aspects, this species can be considered an animal model for comparative studies and techniques with preantral follicles to preserve female fertility. The purpose of the first project was to determine methodologies that might speed up the process of histological evaluation of preantral follicle population. On the second project, we evaluated the follicular population features considering the follicular density factor by means of a mathematical model, to determine the appropriate experimental design to estimate the follicular density in ovarian fragments. The third project, analyzed the effects of reproductive phases, ovary, and ovarian structures on the quality, classification, follicular and cellular densities in ovarian fragments. The results allowed an establishment of the best thickness of histological sections and the ideal number of fragments and sections to estimate the equine preantral follicle density. Furthermore, it was observed a low follicular density associated with a high heterogeneity within and among animals. Moreover, we demonstrated the best scenario (diestrous phase with the presence of a functional corpus luteum) to collect ovarian fragments with higher follicular and cellular densities. In summary, this study demonstrated: (i) advance on the knowledge of equine follicular dynamic on the preantral phase; (ii) the concept of the mare as an animal model to study the reproductive challenges on woman; and (iii) provided basic information for future biotechnology research with the aim to preserve or improve the reproductive capability as cryopreservation, *in vitro* culture, and transplants of ovarian tissue.

**Keywords:** follicular density, mare, ovarian biopsy, preantral follicles

*Dedico este trabalho com amor e admiração:*

*Ao meu marido, Benner, pelo apoio, companheirismo e amor incondicionais.*

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*Cora Coralina  
Poetisa e escritora brasileira  
Cidade de Goiás - GO*

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**LISTA DE ABREVIATURAS**

AMH	Hormônio anti-Mulleriano
ANOVA	Análise de variância
ARTs	Assisted Reproductive Technologies
BMP-15	Proteína morfogenética óssea 15
BMP-4	Proteína morfogenética óssea 4
BMP-7	Proteína morfogenética óssea 7
BPU	Biopsy pick up method
CGP	Células germinativas primordiais
cm	Centímetros
cm <sup>2</sup>	Centímetros quadrados
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CV	Coeficiente de variação
EGF	Fator de crescimento epidermal
FAPEG	Fundação de Amparo à Pesquisa do Estado de Goiás
FGF-b	Fator de crescimento fibroblástico básico
FSH	Hormônio folículo estimulante
GDF-9	Fator de crescimento e diferenciação 9
ICSI	Injeção intracitoplasmática de esperma
ID	Identificação
IGF-1	Fator de crescimento semelhante a insulina
kg	Quilograma
KGF	Fator de crescimento de queratinócitos
KL	Kit ligante
LH	Hormônio luteinizante
LIF	Fator inibidor da leucemia
MHz	Megahertz
MIS	Substância inibidora Mulleriana
µm	Micrômetro
mm	Milímetro
mm <sup>2</sup>	Milímetros quadrados
MNF	Folículos morfologicamente normais

PAS	Ácido periódico-Schiff
SEM	Erro padrão da média
SIU	Southern Illinois University
TGF- $\alpha$	Fator de crescimento transformador alfa
TGF- $\beta$	Fator de crescimento transformador beta
TNF	Fator de necrose tumoral (TNF)
TZP	Processos citoplasmáticos transzonais
VEGF	Fator de crescimento endotelial vascular
ZP	Zona pelúcida

## CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

### 1 INTRODUÇÃO

A equideocultura para diversos fins é difundida em todo planeta e a eficiência reprodutiva destes animais tem grande importância econômica. Esta espécie foi considerada por muito tempo como a de menor fertilidade entre as espécies domésticas, o que pode ser atribuído às características de seleção e problemas relacionados ao manejo reprodutivo. Diante deste fato, há uma busca constante para aprimorar técnicas reprodutivas que garantem um melhor aproveitamento destes animais associado ao melhoramento genético.

Dentre as técnicas utilizadas destacam-se a inseminação artificial, a transferência de embriões e a produção *in vitro*. A transferência de embriões é a biotecnologia mais difundida após a inseminação artificial, pois proporciona um melhor aproveitamento de éguas de alto valor zootécnico durante a estação reprodutiva (ARRUDA et al., 2001). Porém, existem entraves à produtividade da TE, no que se refere a eficiência dos tratamentos de superovulação nos equinos quando comparado a outras espécies domésticas, visto que o seu menor desempenho aumenta potencialmente os custos do programa (LIRA et al., 2009).

Para aperfeiçoar as técnicas reprodutivas equinas é necessário esclarecer os mecanismos relacionados à foliculogênese ovariana, os fatores intrafoliculares envolvidos e a participação de células do estroma ovariano no processo reprodutivo. O interesse de grupos de pesquisa nos fatores que regulam a foliculogênese aumentou o número de publicações nas últimas décadas relacionadas ao desenvolvimento de metodologias como o isolamento e cultivo *in vitro* de folículos pré-antrais (EPPIG & SCHROEDER, 1989; SILVA-SANTOS et al., 2011). Neste sentido, a potencial fertilidade de fêmeas mamíferas pode ser mensurada por meio do número e qualidade dos ovócitos inclusos em folículos pré-antrais (SANTOS et al., 2006; STANSFIELD et al., 2011; ALVES et al., 2012).

Outro parâmetro importante, a densidade folicular, pode ser utilizada como um indicador da reserva de gametas disponíveis para utilização ao longo da vida reprodutiva (SCHUBERT et al., 2005; CHAMBERS et al., 2010). Além disso, estes indicadores devem ser considerados no desenvolvimento de biotecnologias

de reprodução assistida (biópsia ovariana, criopreservação, transplante e cultivo *in vitro*), visando manter ou melhorar a fertilidade animal.

Uma via segura e prática para a coleta de tecido ovariano, sem prejudicar a função reprodutiva, é por meio da biópsia ovariana guiada por ultrassonografia (AERTS et al., 2008; HAAG et al., 2013a). O uso da técnica de biópsia possibilitou a realização de diversos estudos com aplicações clínicas nas áreas da reprodução animal ou humana (humanos: SCHMIDT et al., 2003; VANACKER et al., 2013; bovinos: AERTS et al., 2005; JORSSEN et al., 2014; equinos: HAAG et al., 2013b,c). No entanto, até o momento nos equinos, limitado número de pesquisas têm sido reportadas em relação à população de folículos pré-antrais quando comparado com outras espécies (DRIANCOURT et al., 1982; SZLACHTA & TISCHNER, 2000; HAAG et al., 2013a,b,c).

Outro atributo importante para o estudo das características foliculares pré-antrais é a consideração da égua como modelo animal para estudar a dinâmica folicular da mulher, visto que existem diversas similaridades entre a dinâmica folicular e função ovariana destas fêmeas assim como no declínio da fertilidade decorrente da idade (CARNEVALE, 2008; BAERWALD, 2009; GINTHER, 2012; HAAG, 2013a). O uso de animais de produção como modelos favorece a pesquisa biomédica, pois pode ocasionar em maior eficiência reprodutiva humana e animal (GASTAL et al., 2011).

## **1.1 FOLÍCULOS PRÉ-ANTRAIS – CARACTERIZAÇÃO, ATIVAÇÃO E DESENVOLVIMENTO**

Os folículos ovarianos são constituídos pela associação do ovócito a células foliculares no parênquima ovariano e representam o potencial reprodutivo das fêmeas de cada espécie. Para que os mesmos originem novos indivíduos, é necessário que ocorram de forma dinâmica e ordenada uma sequência de eventos como: a formação dos folículos durante a fase fetal, a ativação dos mesmos na puberdade e o perfeito desenvolvimento e sinergismo com fatores extrínsecos ao ovário, para maximizar as chances de fertilização do ovócito (BINELLI et al., 2009).

A ovogênese é caracterizada pela produção de ovogônias oriundas das células germinativas primordiais (CGP). No embrião, as células germinativas primordiais migram para as gônadas em desenvolvimento, perdem suas características de motilidade e sofrem extensiva proliferação celular e redistribuição das organelas citoplasmáticas transformando-se em ovogônias (SADEU et al., 2006). Uma vez formadas, as ovogônias entram em meiose e diferenciam-se em ovócitos.

Coincidentemente ao início da primeira divisão meiótica, os folículos primordiais emergem do final do cordão distal e consistem em um ovócito circundado por uma camada de células somáticas escamosas denominadas a seguir de células da granulosa. Este cordão de células somáticas tem duas origens: células epiteliais superficiais em migração descendente no ovário e células derivadas do mesonéfron migrando ascendentemente através do ovário (VAN DEN HURK & ZHAO, 2005). As células da granulosa assim como as do estroma ovariano são indispensáveis para o crescimento ovocitário, diferenciação, meiose, maturação citoplasmática e controle da atividade transcricional dentro do ovócito, através da liberação de fatores estimulatórios e produção de hormônios (Figura 1; PALMA et al, 2012).

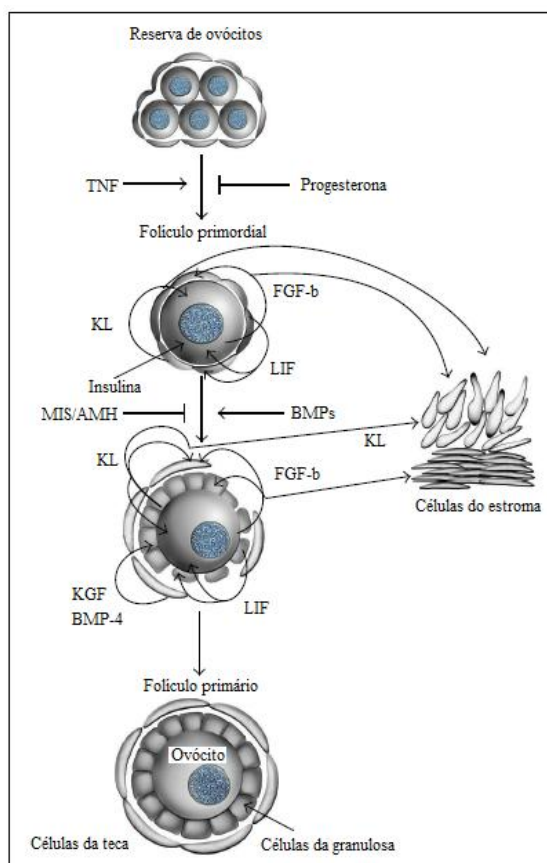


FIGURA 1 - Interações celulares na ativação de folículos pré-antrais. Proposta para interações celulares e o efeito de fatores de crescimento produzidos pelo ovócito, células da granulosa e células da teca no desenvolvimento folicular. Fator de necrose tumoral (TNF), Kit ligante (KL), fator de crescimento fibroblástico básico (FGF-b), fator inibidor da leucemia (LIF), proteína morfogenética óssea 4 (BMP-4), fator de crescimento de queratinócitos (KGF), substância inibidora Mulleriana (MIS) ou hormônio anti-Mulleriano (AMH).  
Fonte: Adaptado de PALMA et al., (2012)

A função do folículo ovariano é proporcionar um ambiente ideal para a manutenção da viabilidade, ativação, crescimento e maturação do ovócito (GONÇALVES et al., 2001). Os mecanismos que controlam a ativação folicular permanecem pouco elucidados, principalmente em animais de interesse zootécnico (BINELLI et al., 2009). Entretanto, acredita-se que diversos fatores de crescimento produzidos pelas células foliculares como a ativina, EGF (fator de crescimento epidermal), FGF (fator de crescimento fibroblástico), TGF- $\alpha$  (fator de

crescimento transformante - alfa), TGF- $\beta$  (fator de crescimento transformante - beta) e IGF-1 (fator de crescimento semelhante a insulina) atuam modulando a ação das gonadotrofinas FSH e LH, controlando a foliculogênese, esteroidogênese e atresia folicular (LEITÃO et al., 2009). Por sua vez, a proliferação e diferenciação das células da granulosa é influenciada pelo ovócito com a secreção de mediadores parácrinos, e através de extensões destas células na zona pelúcida, denominadas de processos citoplasmáticos transzonais (TZP) onde junções do tipo “gap” permitem transporte bidirecional de moléculas reguladoras (Figura 2).

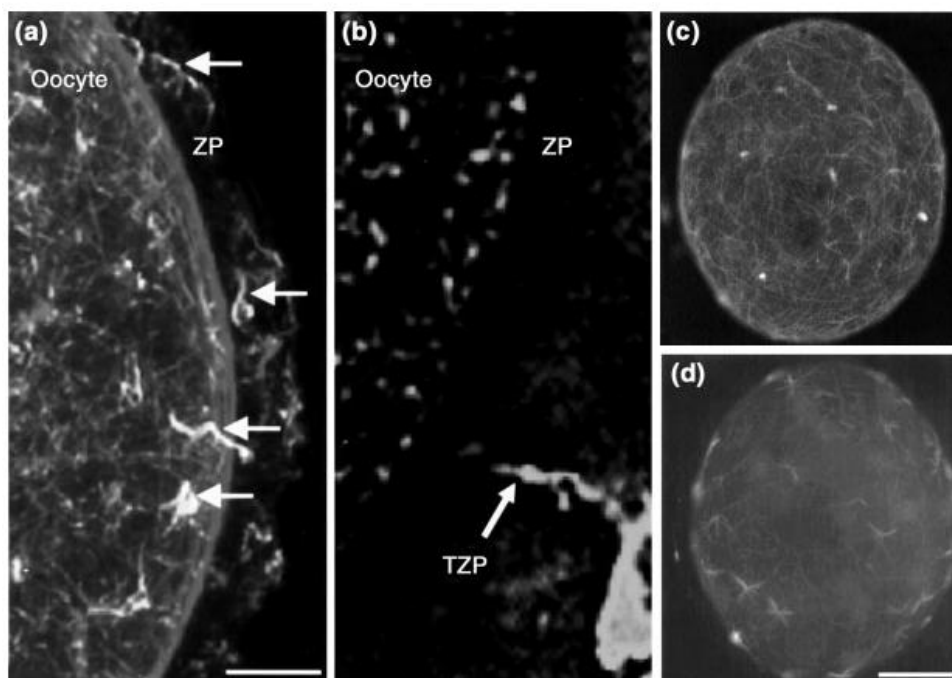


FIGURA 2 - Microscopia confocal de ovócitos isolados de folículos pré-antrais de camundongos. Nota-se a densa rede de microtúbulos (a,c,d) contendo processos citoplasmáticos transzonais (TZPs) derivados de células foliculares (seta); (b) ZP: zona pelúcida. Barras: (a,b - 5  $\mu$ m; c,d - 20  $\mu$ m).

Fonte: Adaptado de ALBERTINI et al. (2001)

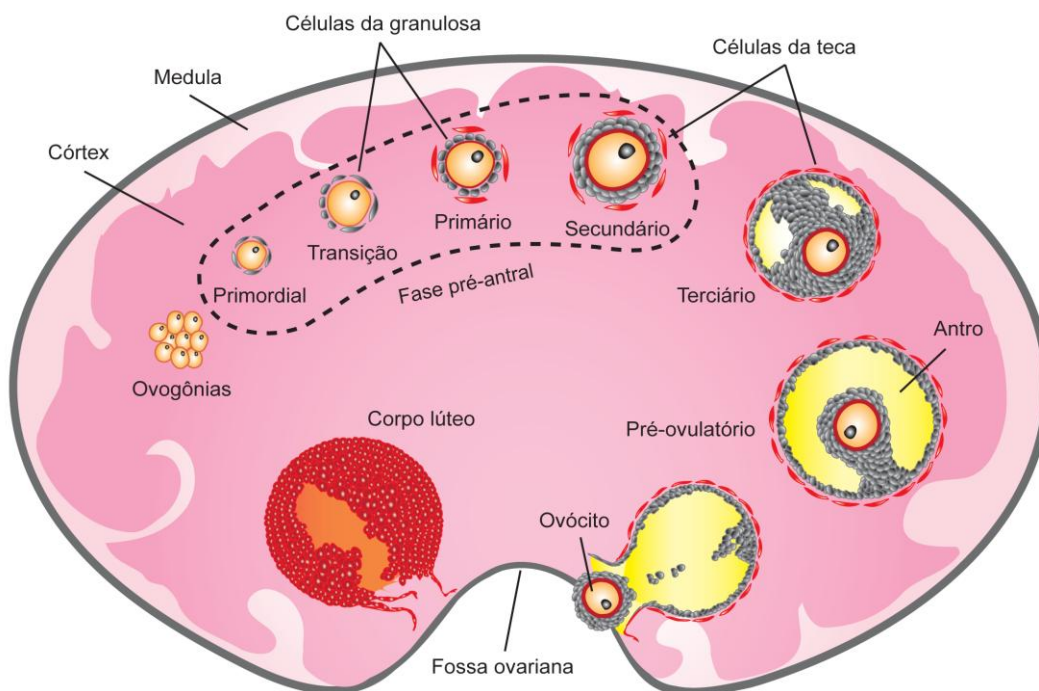
A classificação dos folículos ocorre de acordo com o grau de evolução folicular resultante do crescimento do ovócito e proliferação das células da granulosa. A diferença dos diâmetros foliculares entre os estádios de

desenvolvimento se torna progressivamente maior a cada estágio, indicando que o crescimento folicular é um processo contínuo, porém não linear (GRIFFIN et al., 2006).

Os folículos primordiais constituem o grupo de estruturas não ativadas (quiescentes) e estão representadas por ovócitos circundados por uma camada de células da granulosa de formato pavimentoso (Figura 3). O número destes folículos diminui ao longo da vida reprodutiva por apoptose, necrose ou atresia folicular. A partir do momento da ativação destes folículos, as células da granulosa modificam a sua forma de pavimentosa para cubóide e nesta fase os folículos são denominados de transição (Figura 3).

Os folículos primários possuem uma camada de células da granulosa, todas em formato cubóide ao redor do ovócito (Figura 3). Nestes, as mitocôndrias estão em número crescente, refletindo o aumento natural da atividade metabólica (SONGSASEN & WILDT, 2007). A proliferação das células da granulosa dos folículos primários leva à formação de várias camadas ao redor do ovócito, formando o folículo secundário (Figura 3; RODGERS & IRVING-RODGERS, 2010). Nestes folículos, em estágios mais avançados, as fibras de tecido conjuntivo organizam-se paralelamente e a zona pelúcida pode ser identificada (GONÇALVES et al., 2001).

Os folículos antrais contêm uma área preenchida por fluido folicular, denominada antro, e compreendem os folículos terciários (subordinados e dominantes) e pré-ovulatórios (Figura 3). Durante o seu desenvolvimento, a produção de fluido folicular é intensificada pelo aumento da vascularização e permeabilidade dos vasos sanguíneos, os quais estão fortemente relacionados com o aumento do diâmetro folicular (VAN DEN HURK & ZHAO, 2005). Este fluido pode servir como fonte de substâncias regulatórias derivadas do sangue e de secreções das células foliculares, ou seja, gonadotrofinas, esteróides, fatores de crescimento, enzimas e lipoproteínas (ALVES et al., 2013).



Kele Amaral Alves, 2014

**FIGURA 3 -** Representação morfológica do ovário equino. A estrutura ovariana peculiar da espécie apresenta as regiões cortical (interna) e medular (externa) invertidas com zonas de projeção da medula em direção a córtex. Os folículos estão dispersos no córtex ovariano e protegidos por um denso e vascularizado tecido conjuntivo (medular). Outra particularidade é a presença da fossa ovariana, região onde ocorre a ovulação. A classificação folicular desde a fase de formação a partir das ovogônias, segue as seguintes etapas: fase pré-antral - folículos primordiais (ativação), transição, primários e secundários (desenvolvimento); fase antral - folículos terciários (recrutamento e seleção), folículo pré-ovulatório - ovulação (que ocorre somente na fossa ovariana) e formação do corpo lúteo.

## 1.2 MANIPULAÇÃO E UTILIZAÇÃO DE FOLÍCULOS PRÉ-ANTRAIS EM BIOTÉCNICAS DE REPRODUÇÃO ASSISTIDA

Milhares de folículos pré-antrais podem ser obtidos de um único ovário de acordo com a espécie (Tabela 1). No entanto, é necessário o desenvolvimento de técnicas eficientes para o isolamento, conservação, manipulação e cultivo *in vitro* desses ovócitos após sua obtenção.

Sistemas de isolamento foram reportados e basicamente consistem na dissociação dos folículos pré-antrais dos demais componentes do parênquima ovariano, utilizando-se instrumentos mecânicos (ALVES et al., 2012) associados ou não a protocolos enzimáticos (KERONG et al., 2007).

A criopreservação é uma excelente alternativa para estocagem de um grande número de ovócitos, porém, a viabilidade folicular pode ser alterada pelo processo de criopreservação (AMORIM et al., 2011), uma vez que as células, em geral, são sensíveis aos crioprotetores usados nas etapas de exposição, congelamento e descongelamento. Em bovinos, a utilização de ovócitos imaturos (vesícula germinativa) possibilitou maior resistência à crioinjúria e melhores taxas de clivagem e de produção *in vitro* de blastocistos quando comparados ao maturados (metáfase II) (ZHOU et al., 2010).

Apesar de estudos apontarem diferentes taxas de viabilidade após a criopreservação de folículos pré-antrais (CARVALHO et al., 2011), o número absoluto remanescente e viável destas estruturas, após o congelamento e descongelamento, representa uma importante população de células que poderão ser armazenadas e, futuramente, cultivadas *in vitro* ou direcionadas para o desenvolvimento e complemento de outras biotecnologias.

Sistemas *in vitro* de cultivo folicular são modelos experimentais projetados para avaliar o desenvolvimento e o metabolismo de folículos, livres da influência sistêmica (HARTSHORNE, 1997). Dessa forma, é possível avaliar os efeitos de diversos fatores sobre o desenvolvimento folicular (FORTUNE, 2003). Dados da Tabela 2 ressaltam a complexidade desta linha de pesquisa.

TABELA 1 - Número de folículos pré-antrais obtidos por ovário de acordo com a espécie

Espécie	Nº folículos pré-antrais estimados por ovário Média (variação)	Autores
Bovinos <sup>†</sup>	89.577 (8.010 - 94.301)	SILVA-SANTOS et al., (2011)
Caninos	18.100 (6.200 - 42.000)	ALVES et al., (2012)
Caprinos	994 (70 - 2.974)	RODRIGUES et al., (1998)
<i>Cebus apella</i> (Macaco-prego)	53.035 (18.550 - 12.1050)	DOMINGUES et al., (2004)
Humanos <sup>§</sup>	29.020 (2.700-79.600)	GOUGEON & CHAINY (1987)
<i>Loxodonta africana</i> (Elefante africano)	197.561	STANSFIELD et al., (2011)
Ovinos <sup>£</sup>	16.627 (7.333 - 44.633)	AMORIM et al., (2000)
Suínos	131.938 (67.599 - 291.898)	ALVES et al., (2012)

<sup>†</sup> *Bos taurus taurus* - Vacas da raça Aberdeen Angus.

<sup>§</sup> Mulheres com idade entre 19 e 46 anos.

<sup>£</sup> Fêmeas não gestantes.

Nos últimos anos, estudos reportaram a produção de embriões e progênes por meio do cultivo de folículos pré-antrais em diversas espécies (suínos: WU et al., 2001; caprinos: MAGALHÃES-PADILHA et al., 2012; camundongos: WANG et al., 2011). Entretanto, estudos com folículos pré-antrais em equinos são escassos e têm reportado um baixo número de estruturas por secção histológica ou por mg de estroma ovariano, quando comparado a outras espécies (DRIANCOURT et al., 1982; HAAG et al., 2013). Por esta razão, estudos básicos com folículos pré-antrais equinos são necessários para retratar as características da população folicular e assim, aplicar este conhecimento no desenvolvimento reprodutivo da espécie e de outras na qual a égua possa servir como modelo animal (GASTAL et al., 2011).

TABELA 2 - Influência de hormônios e fatores de crescimento sobre o desenvolvimento e diferenciação de folículos pré-antrais cultivados *in vitro* em diferentes espécies

Classe folicular	Estímulo Positivo	Estímulo Negativo
Primordial - Primário	IGF-I e II (p); GDF-9 (r); Insulina (r, b, p); KL (r); BMP-15 (r)	IGF-I (b)
Primário - Secundário	FSH (r, p); BMP-15 (o); FGF-b (b); TGF- $\beta$ (r); Ativina (r); Andrógenos (p, b)	Estradiol (r)
Secundário - Terciário	FSH (r, p, b); GDF-9 (r, p, o); BMP-15 (o); FGF-b (b); IGF-I (r, b); LH (r); Estradiol (b); TGF- $\beta$ (r); Ativina (r, b)	FSH (r, b); IGF-I (b); TGF- $\beta$ (b); Ativina (r)

Espécie: (b) bovino, (p) primatas, (r) roedores, (o) ovino.

IGF-I e II: Fator de crescimento semelhante à insulina; GDF-9: Fator de crescimento e diferenciação 9; KL: Kit ligante; BMP-15: Proteína morfogenética óssea 15; FGF-b: Fator de crescimento fibroblástico básico; TGF- $\beta$ : Fator de crescimento transformante- $\beta$ .

Fonte: Adaptado de FORTUNE (2003)

A avaliação histológica é uma excelente ferramenta para avaliar características (morfometria, distribuição, qualidade, número e densidade) de uma população de folículos pré-antrais. Estudos com várias espécies utilizaram a avaliação histológica com a finalidade de estudar tais características no tecido ovariano em diversas circunstâncias (humanos: GOUGEON e CHAINY, 1987; SCHMIDT et al., 2003; equinos: DRIANCOURT et al., 1982; HAAG et al., 2013; suínos: ALVES et al., 2012; primatas: DOMINGUES et al., 2004; bovinos: LUCCI et al., 2002; SILVA-SANTOS et al., 2011). Porém, as metodologias histológicas demandam tempo e são trabalhosas (CORTVRINDT & SMITZ, 2001) e novas abordagens técnicas devem ser definidas trazendo agilidade e padronização.

### 1.3 BIÓPSIA OVARIANA

A coleta de tecido ovariano possibilita o acesso a população de folículos pré-antrais seja para estudos avaliativos ou para preservação da fertilidade feminina. Porém, a coleta pode ser difícil para algumas espécies pois as únicas formas de obtenção de tecido são através de abatedouros ou após a ovariectomia (HAAG et al., 2013a) as quais constituem métodos definitivos e não preservam a continuidade reprodutiva da fêmea. Este problema é amplificado na espécie equina devido a escassez ou ausência de abatedouros registrados em vários países. Nos Estados Unidos, o governo americano determinou o fechamento dos abatedouros desde 2007 e no Brasil existem apenas três em funcionamento.

Desta forma, a técnica de biópsia ovariana foi desenvolvida para possibilitar a coleta das amostras e acessar a reserva de folículos sem prejudicar a continuidade dos ciclos reprodutivos da fêmea (AERTS et al., 2005). Assim, técnicas de isolamento folicular desenvolvidas para diversas espécies (bovina: FIGUEIREDO et al., 1993; ovina: AMORIM et al., 2000; equina: HAAG et al., 2013b), podem ser adotadas após a obtenção dos fragmentos.

As primeiras aplicações da biópsia ovariana foram feitas em mulheres no intuito de pesquisar os efeitos da idade sobre a reserva de folículos pré-antrais (LASS et al., 1997). Em seguida, o crescente interesse na preservação da fertilidade estimulou estudos para avaliar a densidade e a distribuição folicular (SCHMIDT et al., 2003; SOLEIMANI et al., 2006) e a criopreservação de tecido ovariano (AMORIM et al., 2009).

Com a formação de bancos de fragmentos ovarianos congelados, a atenção científica voltou-se para a utilização prática destes fragmentos, ou seja, a restauração da atividade ovariana por meio de transplantes, reportando resultados satisfatórios desde o retorno ao crescimento folicular até o nascimento de bebês (DONNEZ et al., 2006; ROUX et al., 2010; DOLMANS et al., 2013). Estes estudos demonstraram como a coleta de tecido ovariano por meio do procedimento de biópsia criou novas perspectivas de pesquisa e de evolução as biotécnicas reprodutivas.

A biópsia ovariana foi adaptada para grandes animais (vacas e éguas) com o uso da guia de aspiração folicular e uma agulha de biópsia alongada (60 cm) acoplada ao equipamento de ultrassonografia (Figura 4). Desta forma, a coleta do tecido pode ser realizada via transvaginal e a preparação do animal envolve etapas como: contenção do animal de forma segura e confortável para segurança do operador, e limpeza das áreas anal e vaginal com a finalidade de se evitar a contaminação da agulha com agentes infecciosos que poderiam ser conduzidos para dentro da vagina e ovários. Além disso, a analgesia, sedação e antibióticoterapia são realizadas para garantir a coleta do material dentro das normas descritas nos códigos de ética e bem estar animal (AERTS et al., 2005; HAAG et al., 2013a).

Enquanto um operador principal insere a guia de biópsia e por via transretal aproxima o ovário do fundo vaginal, um auxiliar insere a agulha na guia e espera o momento certo para inseri-la no ovário dispará-la. Quando o operador localiza um bom local de coleta e posicionar a agulha dentro do córtex ovariano, o mesmo avisa ao ajudante para disparar a agulha e retirá-la com a amostra dentro do compartimento.

A biópsia do córtex ovariano bovino foi realizada inicialmente para reportar a presença de folículos pré-antrais nos fragmentos, testando a repetibilidade, a segurança da técnica e a taxa de sucesso (68%; razão do número de procedimentos realizados pelo número de fragmentos ovarianos obtidos) (AERTS et al., 2005).

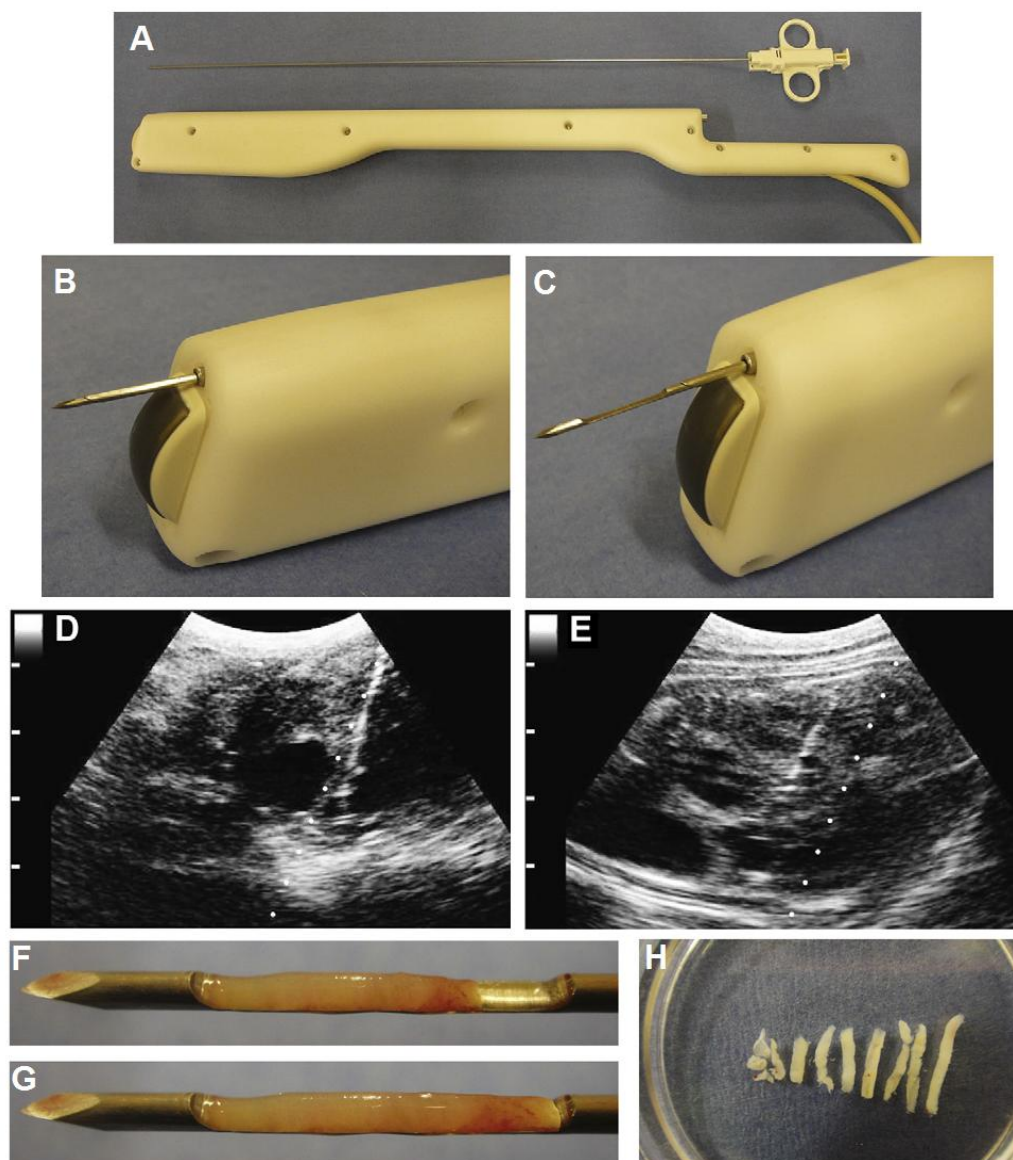


FIGURA 4 - Imagens demonstrando o dispositivo utilizado para biópsia ovariana equina. (A) Guia acoplada ao transdutor e agulha de biópsia ovariana; (B) agulha preparada para entrar no ovário; (C) agulha com o “notch” aberto pronto para coleta do tecido ovariano; (D) imagem ultrassonográfica do ovário sendo puncionado pela agulha de biópsia; (E) região ovariana após a coleta tecidual; (F,G) fragmentos ovarianos coletados e (H) dispostos em placa de petri. Fonte: Adaptado de HAAG et al., (2013a)

Em equinos, por meio da biópsia ovariana, um número satisfatório de folículos pré-antrais foi obtido (Tabela 3) e submetidos ao isolamento mecânico ou

análise histológica para validação da biotécnica. Repetibilidade, alta porcentagem de folículos normais (88%) e satisfatória taxa de sucesso (72%) foram reportados. Além disso, a técnica demonstrou ser segura, pois as éguas submetidas ao procedimento continuaram ciclando e avaliações ultrassonográficas posteriores não demonstraram traumas significativos ao parênquima ovariano (HAAG et al., 2013a).

A padronização da técnica de biópsia ovariana para a espécie equina representa um grande avanço nos estudos da foliculogênese além de estimular e possibilitar a pesquisa com cultivo *in vitro*, criopreservação e transplantes de tecido ovariano.

TABELA 3 - Número e viabilidade (%) de folículos pré-antrais equinos, obtidos por procedimento de biópsia ovariana, de acordo com a idade

Égua	Idade	Nº folículos pré-antrais	Nº folículos pré-antrais por mg de tecido ovariano	Viabilidade (%)
E	5	45	1,1	90,9
P	5	42	1,3	86,4
I	7	28	0,9	75,0
F	14	19	1,1	88,9
H	14	17	0,4	72,7
R	16	5	0,2	50,0

Fonte: Adaptado de HAAG et al., (2013a)

#### 1.4 A IMPORTÂNCIA DO USO DE MODELOS ANIMAIS

A Organização Mundial de Saúde considera a infertilidade como um problema de saúde pública onde o acesso ao diversos tipos de tratamento (curativos, preventivos ou de reprodução assistida) representa um grande desafio ao meio científico (BOIVIN et al., 2007). A paternidade pode ser considerada um dos principais objetivos da vida adulta e a dificuldade em obtê-la pode ocasionar desordens como a ansiedade e a depressão (FASSINO et al., 2002). Excluindo problemas clínicos e de má formação, a fertilidade feminina também pode ser

comprometida pela idade ou por tratamentos quimioterápicos os quais tendem a prejudicar as células ovarianas (MALTARIS et al., 2007).

Atualmente, muitas mulheres tendem a planejar suas gestações para mais tarde quando estiverem com a vida financeira e/ou profissional estáveis. A partir dos 35 anos começa um declínio das taxas de gestação (MENKEN et al., 1986) e aumento nas taxas de abortos espontâneos (SMITH & BUYALOS, 1996). A depleção acelerada do número de folículos ocorre após os 38 anos, sendo um processo contínuo até a menopausa, onde poucos ou nenhum folículo está disponível (GINTHER, 2012). Vários estudos reportaram correlação negativa entre a densidade da população folicular, em fragmentos de biópsia ovariana, e o avanço da idade (Figura 5; FADDY & GOSDEN, 1996; SCHEFFER et al., 1999; GLEICHER et al., 2011).

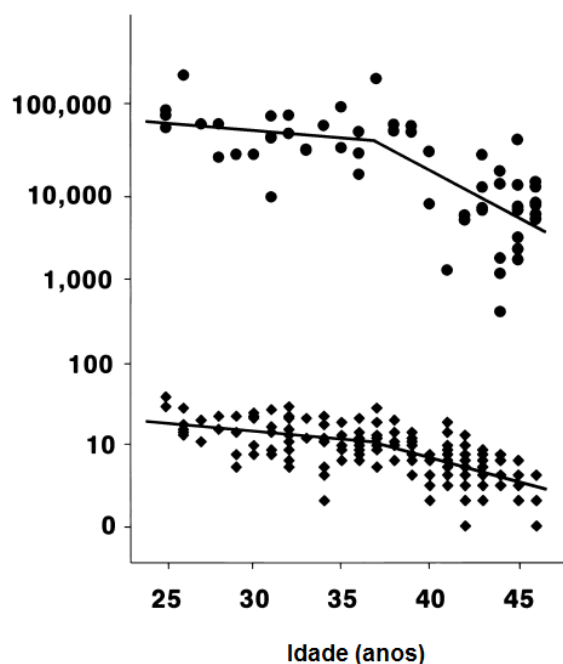


FIGURA 5 - Relação entre idade e número de folículos primordiais (curva superior - círculo) avaliados por histologia em mulheres (n = 56). Relação entre idade e número de folículos antrais (curva inferior - losango) no início da fase folicular em mulheres cíclicas (n = 162). Fonte: Adaptado de SCHEFFER et al. (1999)

A taxa de mortalidade de mulheres com câncer vem diminuindo a cada ano graças ao avanço no diagnóstico precoce e tratamentos (SIEGEL et al., 2013). Porém, os tratamentos adotados podem trazer efeitos colaterais tardios como desordens endócrinas e a falha ovariana prematura, resultando em infertilidade (NOYES et al., 2013). O risco de comprometimento da fertilidade aumenta com a idade da paciente e o tipo de tratamento adotado (DONNEZ et al., 2006). A biópsia ovariana acompanhada de biotécnicas de reprodução assistida (criopreservação, cultivo *in vitro* e transplante) pode superar estes desafios na reprodução humana. Vários estudos comprovaram a eficiência destas técnicas seja na viabilidade de folículos isolados de pacientes jovens ou na criopreservação e transplante dos fragmentos ovarianos (ROUX et al., 2010; DOLMANS et al., 2013; REVELLI et al., 2013).

Fatores intrínsecos a preservação e ao desenvolvimento folicular continuam desconhecidos e os métodos de criopreservação e cultivo devem ser continuamente aperfeiçoados. Para tanto é necessário o uso dos fragmentos de biópsia no desenvolvimento de projetos de pesquisa, porém a baixa disponibilidade de tecido ovariano humano, além de barreiras éticas, prejudicam o desenvolvimento de projetos na área (FRANSOLET et al., 2014). Dessa forma, a alternativa ideal para estudos comparativos da população folicular e de novos procedimentos é por meio do uso de modelos animais.

Roedores de laboratório tem sido o modelo animal mais popular na pesquisa biomédica, porém estes animais oferecem poucas vantagens para o estudo da dinâmica ovariana de humanos, pois além de serem poliovulatórios, possuem ciclos ovarianos curtos com fase luteal presente apenas se a fêmea estiver gestante (IRELAND et al., 2008). Os animais de produção são a alternativa neste contexto, por diversas razões: (1) a pesquisa pode ter dupla aptidão e beneficiar tanto a espécie humana como a animal; (2) o tamanho dos animais facilita o acesso aos órgãos reprodutivos de forma menos invasiva e possibilita a repetibilidade e controle experimental; (3) por fim, o custo é menor, desde que os estudos possam ser realizados em unidades de produção, com grande número de animais em diversas idades (SEIDEL, 2009).

Considerando as similaridades entre a foliculogênese antral, níveis hormonais circulantes (GINTHER et al., 2004; GINTHER et al., 2005) e a

depleção folicular inerente ao envelhecimento (HAAG et al., 2013a), a égua pode ser considerada um modelo animal para estudo da dinâmica da população folicular em mulheres. Ao oposto de outros animais de produção, as éguas são mantidas até sua idade senil (>20 anos) como animais de companhia ou de trabalho, o que possibilita o estudo comparativo de envelhecimento ovariano. Esta espécie normalmente fica estabulada próxima a seres humanos, compartilhando de muitos fatores ambientais que podem afetar a reprodução e a fertilidade (CARNEVALE, 2008). O trato reprodutivo das éguas pode ser facilmente acessado por palpação via retal e permite a aplicação da técnica de biópsia para coletar fragmentos ovarianos com padrão similar ao realizado em mulheres (HAAG et al., 2013a).

## **1.5 OBJETIVOS**

### **1.5.1 Gerais**

Avaliar por métodos histológicos fragmentos de ovário equino quanto ao número, a densidade, a classificação e a morfologia de folículos pré-antrais coletados em abatedouros ou por biópsia ovariana e utilizar os aspectos inerentes da espécie como modelo animal para realização de estudos relativos à população de folículos pré-antrais em éguas comparando com mulheres.

### **1.5.2 Específicos**

- Avaliar o efeito de diferentes espessuras de secção histológica em fragmentos ovarianos equinos com o propósito de determinar o número, distribuição, qualidade e densidade folicular; além de descrever as características morfométricas de folículos, ovócitos, núcleo dos ovócitos e de células da granulosa em cada classe folicular (Capítulo 2).

- Utilizar a égua como modelo animal para investigar os efeitos da idade sobre a densidade folicular. Além disso, estabelecer um modelo matemático para determinar o número necessário de fragmentos ovarianos e de secções

histológicas para estimar a densidade folicular em fragmentos de biópsia (Capítulo 3).

- Analisar a influência das fases reprodutivas e das estruturas ovarianas na qualidade, classificação, número e densidade de folículos pré-antrais equinos e na densidade de células do estroma ovariano coletados por procedimento de biópsia. Em adição, considerando a égua como modelo animal, avaliar o potencial dos resultados para aplicação em medicina reprodutiva humana (Capítulo 4).

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**CAPÍTULO 2 – Número e Densidade de Folículos Pré-Antrais Equinos em Diferentes Espessuras de Secção Histológica**

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**Number and Density of Equine Preantral Follicles in Different Ovarian Histological Section Thicknesses**

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**ABSTRACT**

Regardless of species, advances in preantral follicle culture and cryopreservation and transplant of ovarian tissue techniques are dependent upon the number and density of preantral follicles in the ovary. This study tested the effect of different histological section thicknesses on number, classification, and density of equine preantral follicles. An ovarian fragment was obtained from 5- to 10-year-old mares ( $n = 14$ ) after slaughter and each fragment was submitted to three histological section thickness treatments: 3, 5, and 7  $\mu\text{m}$ . The area ( $\text{cm}^2$ ) of each ovarian fragment was measured and the sections evaluated by light microscopy. The percentage of morphologically normal follicles (89%) was similar ( $P > 0.05$ ) among primordial, transitional, and primary follicles and also among histological section thicknesses. A higher ( $P < 0.05$ ) number of preantral follicles per histological section was seen in the 7- $\mu\text{m}$  ( $8.0 \pm 2.2$ ) than in the 3- $\mu\text{m}$  ( $3.4 \pm 0.7$ ) treatment. Furthermore, a linear regression analysis demonstrated that the number of preantral follicles increased ( $P < 0.05$ ) when a thicker section treatment was used. However, no association ( $P > 0.05$ ) between follicular density and treatment was observed. The mean number of preantral follicles per fragment ( $45.3 \pm 18.8$ ) and the follicular density ( $3.0 \pm 0.5$  follicles per  $\text{cm}^2$ ) were different ( $P < 0.05$ ) among mares. In conclusion, this study on equine preantral follicles demonstrated that: (1) a 7- $\mu\text{m}$  histological section thickness might be recommended because it allowed identification of a greater number of preantral follicles per sample, (2) a large individual variation in follicle population and density was detected regardless of histological section thickness, and (3) mares have a low number and density of preantral follicles when compared to those reported for other species.

*Keywords:* Equine ovary; Histology; Mare; Preantral follicles

## 1. Introduction

The ovarian reserve of preantral follicles and follicular density are important indicators of the reproductive capacity of a species. Moreover, this information can contribute to the development of reproductive techniques (e.g., ovarian biopsy, ovarian tissue cryopreservation, transplantation, and in vitro follicle culture) to maintain or improve fertility in mammals.

Although recent studies in horses have reported improvements in maturation and fertilization rates (after intracytoplasmic sperm injection [ICSI]) of oocytes originated from immature and mature follicles [1-4], the assisted reproductive technologies (ARTs) have been developed slowly when compared to those used with other species [3,5]. One of the causes for the slow progress of ARTs in horses has been the lack of consistent in vivo methods to harvest a large number of immature oocytes [6]. In the horse, distinct from other species, this process requires vigorous flushing and scraping of the follicle wall using ultrasonography [3]. As an alternative method to overcome this limitation, and considering that preantral follicles are of great abundance in the ovary, studies in our laboratory recently validated the use of a transvaginal, ultrasound-guided ovarian Biopsy Pick-Up (BPU) method to harvest preantral follicles in mares [7-9].

At this moment, a limited number of studies have reported on equine preantral follicle population; these reports have suggested the presence of a low number of preantral follicles in the ovary when compared with other species [7-9,10,11]. Therefore, studies on equine preantral follicle population and density are necessary to facilitate the development of ARTs using immature oocytes enclosed in preantral follicles.

Histological evaluation has been used to determine the features of preantral follicle population, classification, stages of development, and morphology, as well as follicular density [12]. However, this methodology is time-consuming and requires the development of technical approaches to overcome this problem [13]. The histological section thickness can influence the evaluation of preantral follicle population. Studies in this area used different section thicknesses (e.g., 3  $\mu\text{m}$ : mice [14]; 5  $\mu\text{m}$ : ovine [15], equine [16]; 6  $\mu\text{m}$ : ovine [17]; 7  $\mu\text{m}$ : caprine [18], bovine, [19]; or 10  $\mu\text{m}$ : women [20], equine [8-10]). These studies demonstrated the lack of a standardization for histological section thickness preparations even within the same species (e.g., ovine [21,22]). To our knowledge, there is no comparative study on the use of different histological section

thicknesses for evaluation of preantral follicles in any species. Moreover, assessment of preantral follicular density has not been reported in mares.

The purpose of this study was to assess the effect of different histological section thicknesses (3, 5, and 7  $\mu\text{m}$ ) for evaluation of equine ovarian fragments to determine: (1) the number, density, and morphology (normal, abnormal, and follicular class) of equine preantral follicles; (2) the diameter of follicles, oocytes, and oocyte nuclei; and (3) the number of granulosa cells per follicle class.

## **2. Materials and methods**

### *2.1. Ovaries*

Ovaries from 14 adult mixed-breed mares (5 to 10 years old) were harvested at an equine slaughterhouse. One fragment (15  $\times$  15  $\times$  1 mm) of a single ovary from each mare was collected and added to fixative Bouin's solution, transported to the laboratory, and after 24 hours placed in 70% ethanol until histological processing. During tissue collection, ovarian fragments with corpus luteum and antral follicles were avoided.

### *2.2. Histological processing and treatments*

After standard histological preparation, the samples were cut on a microtome with different section thicknesses (treatments) of 3, 5, or 7  $\mu\text{m}$ , placed on glass slides, and stained with Periodic Acid-Schiff (PAS) and hematoxylin. Regardless of the section thickness, every 10th histological section was evaluated to insure that each follicle was counted only once. After five mounted sections (one section per slide), the section thickness was changed to the next (i.e., 3, 5, and 7  $\mu\text{m}$ ). An average of 15 slides per ovarian fragment was evaluated.

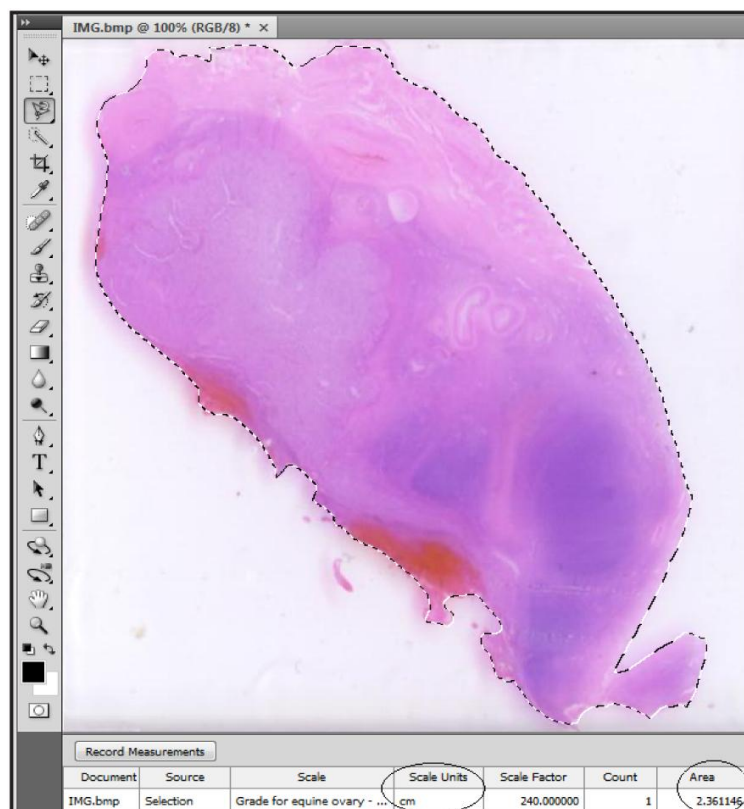
### *2.3. Microscopy and end points*

The slides were analyzed using light microscopy (Nikon, Japan) at magnification  $\times$ 400 and an image capture system (Leica Imaging Software, Wetzlar, Germany). The following end points were evaluated: number of follicles and follicle density per section thickness, per fragment, and per follicle class; follicle morphology; number of granulosa cells per follicle class; and diameters of follicles, oocytes, and oocyte nuclei. Follicle, oocyte, and oocyte nucleus diameters, along with number of granulosa

cells, were measured only in morphologically normal follicles. The average of two perpendicular measurements from the outer layer of granulosa cells was used as a measure of follicle diameter. The measurements were made only on follicles with a visible oocyte nucleus. All evaluations and measurements were performed by a single operator.

#### 2.4. Density of preantral follicles

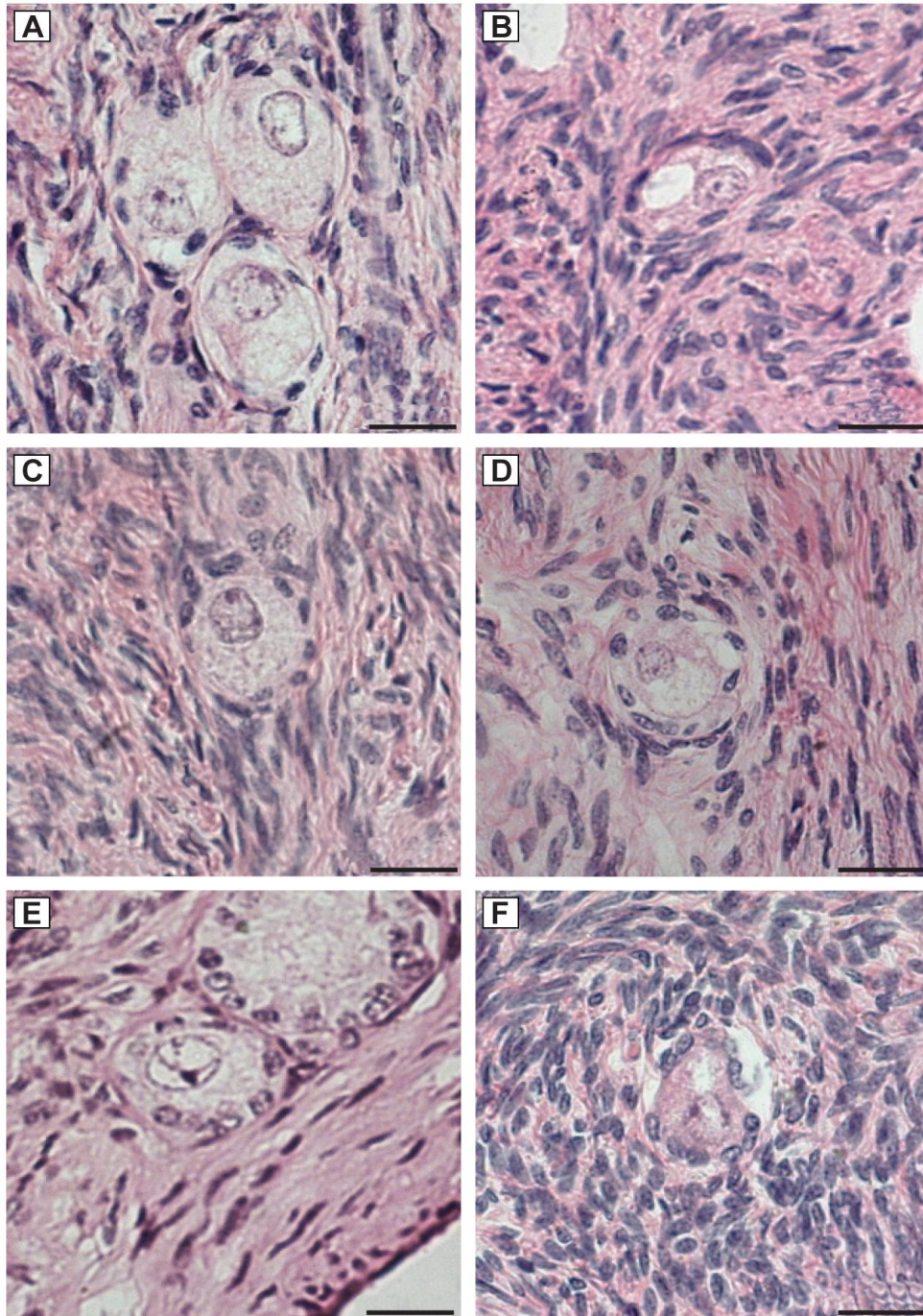
An image of every histological ovarian section was obtained using a photo editing program (Adobe Photoshop CS4, San Jose, USA) and the area's measurement ( $\text{cm}^2$ ) was recorded after scale calibration (Fig. 1). Therefore, follicle density was determined by the following formula: follicular density = number of preantral follicles/area of the ovarian section ( $\text{cm}^2$ ).



**Figure 1.** Equine ovary histological section image scanned on a photo editing program. The border of the section was delimited (dashed line) with a marker tool and the area was measured after scale calibration (black circle).

### *2.5. Morphology of preantral follicles*

Morphology evaluation was performed and the follicles were classified [23] as normal when the oocyte nucleus and an intact oocyte was present surrounded by granulosa cells that were well organized in one or more layers (Fig. 2A, C, E). Degenerated follicles (abnormal) were defined as those with a retracted cytoplasm or disorganized granulosa cell layers detached from the basement membrane and an oocyte with pyknotic nucleus (Fig. 2B, D, F). Moreover, preantral follicles were quantified and classified according to stage of development [24], i.e. primordial (oocyte surrounded by one layer of flattened granulosa cells and ellipsoid shape), transitional (one layer of flattened and cuboidal granulosa cells with ellipsoid shape), primary (one layer of cuboidal granulosa cells around the oocyte with spherical aspect), and secondary (oocyte with zona pellucida surrounded by two or more layers of cuboidal granulosa cells) follicles. All follicles with an intact oocyte, granulosa cell layer, and basement membrane were included to determine the preantral follicle population.



**Figure 2.** Histological illustration of equine preantral follicle morphology. (A) Normal and (B) abnormal primordial follicle; (C) normal and (D) abnormal transitional follicle; (E) normal and (F) abnormal primary follicle. Sections were stained with periodic acid-Schiff (PAS) and hematoxylin. Bars = 20  $\mu\text{m}$ .

### 2.6. Statistical analyses

All statistical analyses were performed using R statistical software version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Data for end points that

were not normally distributed were transformed to natural logarithms or square root. Total number of preantral follicles per section and fragment, follicular density (i.e., preantral follicles per  $\text{cm}^2$ ), number of preantral follicles within the same follicle class among section treatments, and number of granulosa cells per follicle class were compared by Kruskal-Wallis test. Follicle, oocyte, and nucleus diameters were compared among follicular classes using one-way ANOVA. The variables mare and histological section thickness were considered factors and submitted to a two-way ANOVA to verify main effects and interaction on the number of preantral follicles recorded. Proportion of abnormal follicles was analyzed by chi-square test. Number of preantral follicles and follicular density according to section thickness treatments was evaluated using linear regression analysis and Spearman correlation coefficients. Data are presented as mean  $\pm$  SEM, unless otherwise stated. A probability of  $P < 0.05$  indicated that a difference was significant, and  $P > 0.05$  and  $\leq 0.1$  indicated that a difference approached significance.

### **3. Results**

A total of 635 preantral follicles were analyzed in this study, and 21%, 30%, and 49% were seen in the 3-, 5-, and 7- $\mu\text{m}$  section thickness treatments, respectively. The mean number of preantral follicles observed per histological section using each section treatment is shown for each follicle class (Table 1). A higher ( $P < 0.05$ ) number of preantral follicles was seen in the 7- $\mu\text{m}$  treatment when compared with the 3- $\mu\text{m}$  treatment. However, the mean number of preantral follicles per section was similar ( $P > 0.05$ ) between 3- and 5- $\mu\text{m}$ , and 5- and 7- $\mu\text{m}$  treatments. Additionally, the thicker sections (5- and 7- $\mu\text{m}$ ) had a greater ( $P < 0.05$ ) number of transitional and secondary follicles.

**Table 1**

Mean ( $\pm$  SEM) number of equine preantral follicles according to class within different section thicknesses.

Follicle class	Section thicknesses		
	3 $\mu$ m	5 $\mu$ m	7 $\mu$ m
Primordial (n = 390)	2.4 $\pm$ 0.5 <sup>a,A</sup>	2.8 $\pm$ 0.6 <sup>a,A</sup>	4.7 $\pm$ 1.3 <sup>a,A</sup>
Transitional (n = 161)	0.6 $\pm$ 0.2 <sup>a,B</sup>	1.5 $\pm$ 0.3 <sup>b,A</sup>	2.0 $\pm$ 0.6 <sup>b,B</sup>
Primary (n = 63)	0.2 $\pm$ 0.1 <sup>a,B</sup>	0.4 $\pm$ 0.1 <sup>a,B</sup>	0.9 $\pm$ 0.2 <sup>a,B,C</sup>
Secondary (n = 21)	0.1 $\pm$ 0.0 <sup>a,C</sup>	0.2 $\pm$ 0.1 <sup>b,B</sup>	0.3 $\pm$ 0.1 <sup>b,C</sup>
Total (n = 635)	3.4 $\pm$ 0.7 <sup>a</sup>	5.0 $\pm$ 1.1 <sup>a,b</sup>	8.0 $\pm$ 2.2 <sup>b</sup>

<sup>a,b</sup> Within a row, values without a common superscript differed ( $P < 0.05$ ).

<sup>A,B,C</sup> Within a column, values without a common superscript differed among follicle classes ( $P < 0.05$ ).

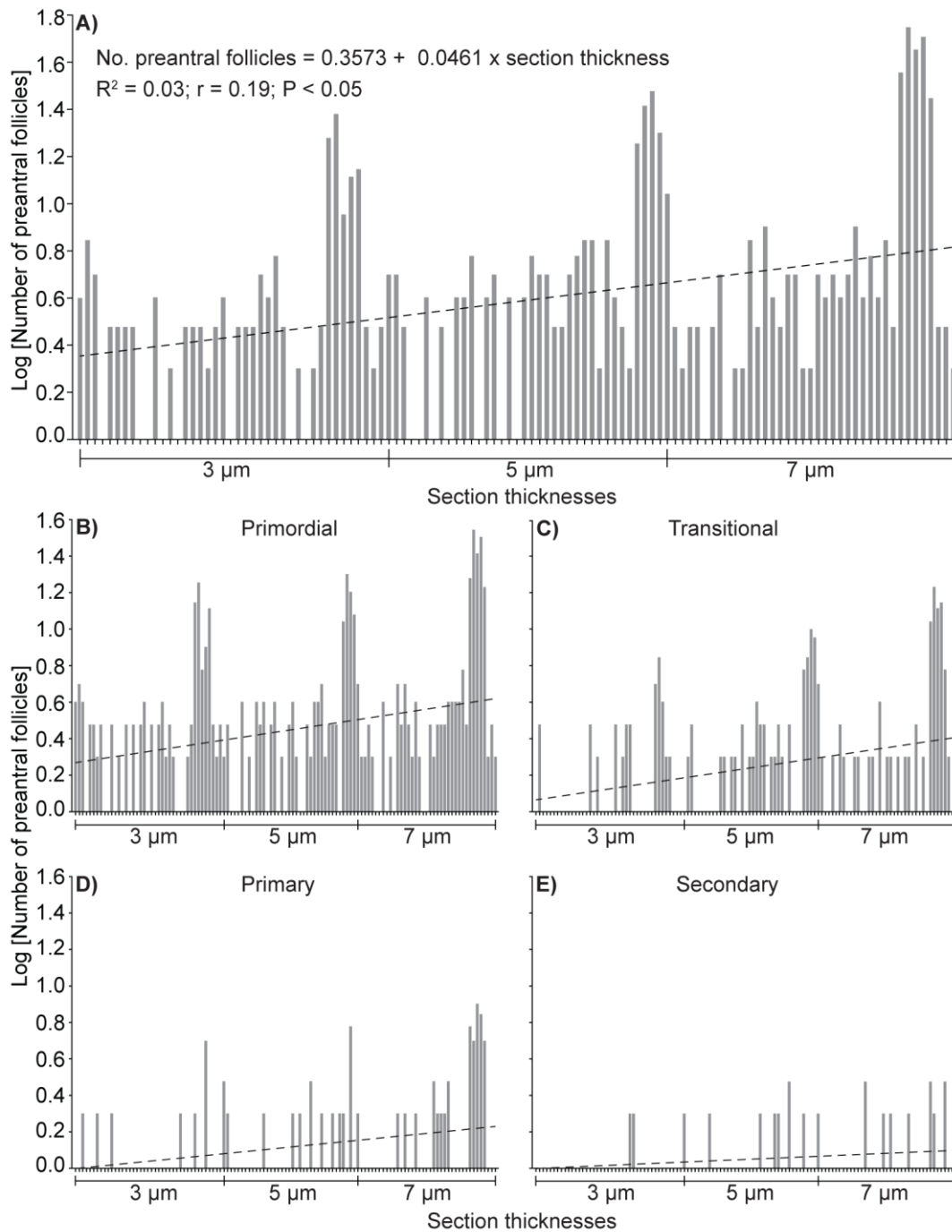
A mean of 45.3 preantral follicles per ovarian sample [range, 5 to 273 follicles; mean coefficient of variation (CV) = 155%] was recorded per mare (Table 2). Data initially grouped regardless of treatment (section thickness) after a two-way ANOVA using the mare and treatments as factors did not indicate the influence of individuals on the overall results. A linear regression analysis demonstrated that the estimated (log transformed) number of preantral follicles increased ( $P < 0.05$ ) when a thicker section treatment was used (Fig. 3A). Furthermore, a positive correlation ( $P < 0.05$ ) was observed for the number of preantral follicles per section with an increase of the section thickness. The same pattern was observed when the follicles were distributed on the respective developmental classes (Fig. 3B, C, D, and E). Effects of histological section thicknesses (3-, 5-, and 7- $\mu$ m) on the percentage of equine preantral follicles within representative individuals are shown (Fig. 4).

**Table 2**Mean ( $\pm$  SEM) number and follicular density of equine preantral follicles.

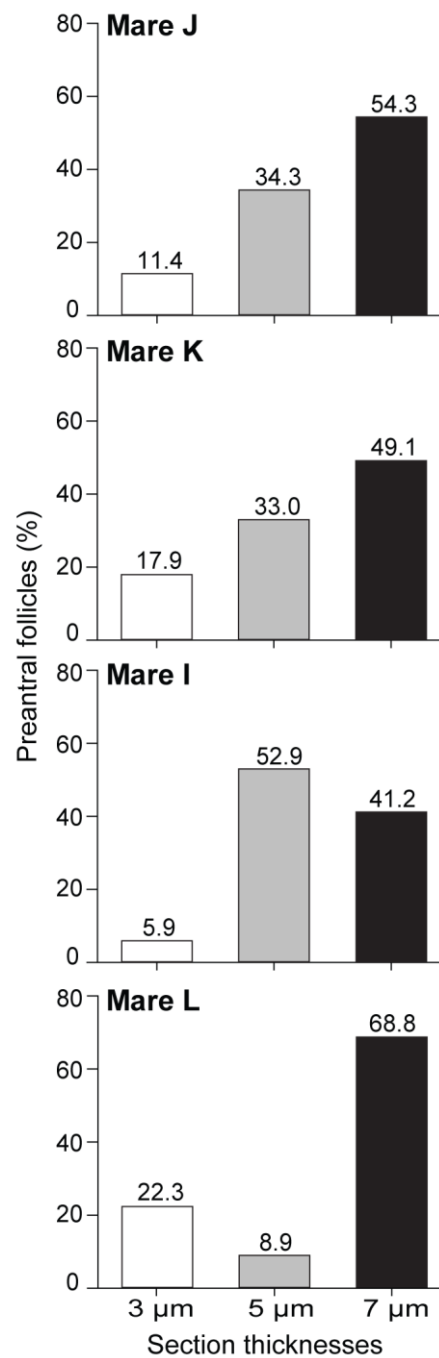
Mare	No. of preantral follicles per sample <sup>†</sup>	No. of preantral follicles per section <sup>§</sup>	Histological section area (cm <sup>2</sup> )	Follicular density/cm <sup>2</sup> <sup>‡</sup> (range)
A	24	2.6 $\pm$ 0.6 <sup>a</sup>	1.7 $\pm$ 0.0 <sup>a,c,d</sup>	1.5 $\pm$ 0.3 <sup>a,e</sup> (0-3.3)
B	19	1.3 $\pm$ 0.3 <sup>a</sup>	1.7 $\pm$ 0.0 <sup>a,c,d</sup>	0.7 $\pm$ 0.2 <sup>a</sup> (0-2.1)
C	8	2.6 $\pm$ 0.3 <sup>a,b</sup>	1.7 $\pm$ 0.2 <sup>a,c,d</sup>	1.5 $\pm$ 0.1 <sup>a,d,e</sup> (1.4-1.7)
D	14	2.0 $\pm$ 0.7 <sup>a</sup>	2.5 $\pm$ 0.1 <sup>b</sup>	0.8 $\pm$ 0.3 <sup>a</sup> (0-2.2)
E	19	2.3 $\pm$ 0.9 <sup>a</sup>	1.8 $\pm$ 0.1 <sup>a,c,d</sup>	1.2 $\pm$ 0.4 <sup>a,e</sup> (0-3.2)
F	23	2.0 $\pm$ 0.4 <sup>a</sup>	2.0 $\pm$ 0.1 <sup>a,b,d</sup>	1.0 $\pm$ 0.2 <sup>a,e</sup> (0-2.2)
G	37	2.6 $\pm$ 0.3 <sup>a</sup>	2.2 $\pm$ 0.2 <sup>a,b</sup>	1.1 $\pm$ 0.1 <sup>a,e</sup> (0-2.6)
H	23	3.2 $\pm$ 0.4 <sup>b</sup>	2.4 $\pm$ 0.0 <sup>b</sup>	1.3 $\pm$ 0.1 <sup>a,e</sup> (0.8-2.2)
I	34	4.2 $\pm$ 0.7 <sup>b</sup>	1.4 $\pm$ 0.0 <sup>c,e</sup>	3.0 $\pm$ 0.4 <sup>d</sup> (0.7-4.9)
J	35	2.5 $\pm$ 0.5 <sup>a</sup>	1.9 $\pm$ 0.0 <sup>a,b,d</sup>	1.3 $\pm$ 0.2 <sup>a,c</sup> (0-3.1)
K	273	27.3 $\pm$ 4.4 <sup>c</sup>	1.6 $\pm$ 0.0 <sup>c,d</sup>	17.0 $\pm$ 2.2 <sup>b</sup> (8.4-31.3)
L	112	22.4 $\pm$ 7.5 <sup>c</sup>	1.6 $\pm$ 0.0 <sup>a,c</sup>	14.0 $\pm$ 4.3 <sup>b</sup> (5.7-29.4)
M	5	1.6 $\pm$ 0.3 <sup>a</sup>	1.2 $\pm$ 0.0 <sup>c,e</sup>	1.3 $\pm$ 0.2 <sup>a,d</sup> (0.8-1.7)
N	9	2.2 $\pm$ 0.6 <sup>a,b</sup>	1.0 $\pm$ 0.0 <sup>e</sup>	2.2 $\pm$ 0.4 <sup>c,d,e</sup> (1.1-3.3)
Overall mean	45.3 $\pm$ 18.8	5.4 $\pm$ 0.8	1.8 $\pm$ 0.0	3.0 $\pm$ 0.5

<sup>a,b,c,d,e</sup> Within a column, values without a common superscript differed ( $P < 0.05$ ).

<sup>†</sup> Only one ovarian fragment was used per animal, and cut using 3-, 5-, and 7- $\mu$ m section thicknesses. <sup>§</sup> Number of preantral follicles per section was not influenced by the area of the histological sample. <sup>‡</sup> Section thicknesses had no effect on follicular density.



**Figure 3.** Number of preantral follicles (A) within different histological section thicknesses (3, 5, and 7  $\mu\text{m}$ ) and (B-E) in each follicular class. A simple linear regression analysis is represented by the equation and the dashed line for (B) primordial [No. primordial =  $0.4825 + (0.5781 \times \text{section thickness})$ ,  $R^2 = 0.02$ ,  $r = 0.16$ ,  $P < 0.07$ ]; (C) transitional [No. transitional =  $-0.3376 + (0.3475 \times \text{section thickness})$ ,  $R^2 = 0.03$ ,  $r = 0.21$ ,  $P < 0.05$ ], (D) primary [No. primary =  $1.4429 + (-0.1477 \times \text{section thickness})$ ,  $R^2 = 0.02$ ,  $r = 0.17$ ,  $P < 0.05$ ], and (E) secondary [No. secondary =  $-0.0588 + (0.0483 \times \text{section thickness})$ ,  $R^2 = 0.02$ ,  $r = 0.17$ ,  $P < 0.06$ ].

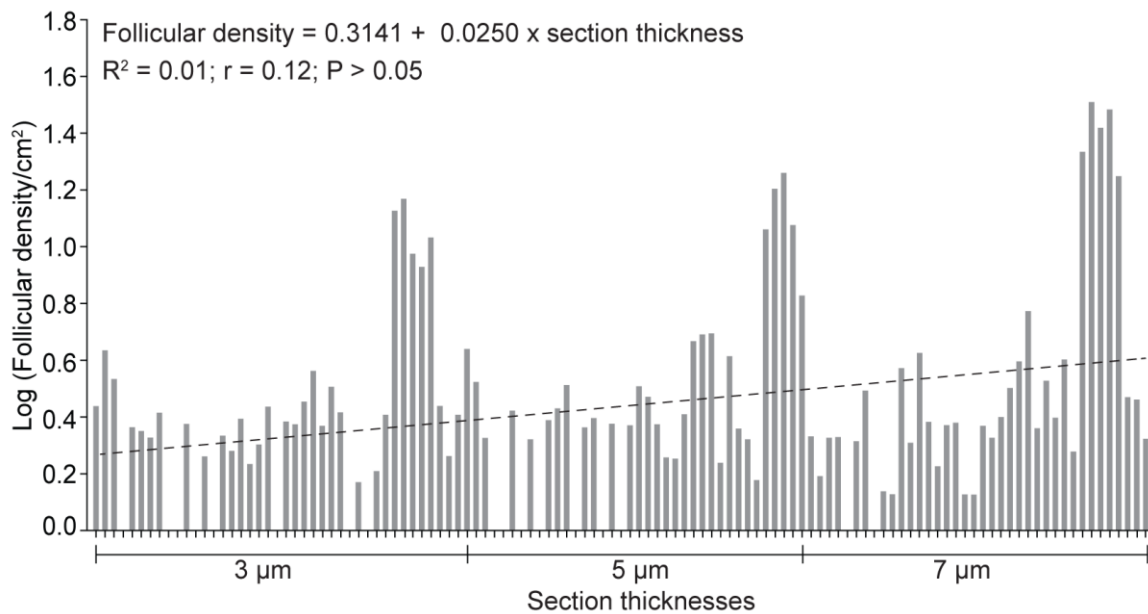


**Figure 4.** Effect of histological section thicknesses (3, 5, and 7 μm) on the percentage of equine preantral follicles within representative individuals.

The overall mean area of the histological section analyzed was  $1.8 \pm 0.0 \text{ cm}^2$ , with a mean of 5.4 preantral follicles observed per section (range, 1.3 to 27.3 follicles; CV = 146%). Because the sample size collected was standardized, the total number of preantral follicles recorded per section was not influenced by the area of the histological sample, as

confirmed by a two-way ANOVA (mare and histological section area as factors) and Spearman correlation analysis ( $r = -0.01$ ;  $P > 0.05$ ; data not shown).

The evaluated sections ( $n = 117$ ) had a mean density of 3 follicles per  $\text{cm}^2$  (range, 0 to 31;  $\text{CV} = 176\%$ ). In addition, as observed for the number of preantral follicles per sample and per section, heterogeneity of follicular density was also seen among mares ( $P < 0.05$ ; Table 2). No association ( $P > 0.05$ ) between estimated (log transformed) follicular density and treatments was observed, according to the linear regression analysis (Fig. 5). Furthermore, a high variation of preantral follicle density was seen both within and among each section thickness treatment.



**Figure 5.** Equine preantral follicle density (log transformed;  $\text{cm}^2$ ) within different histological section thicknesses (3, 5, and 7  $\mu\text{m}$ ).

An overall preantral follicle morphological normality rate of 89% was observed (86.9% primordial, 92% transitional, and 86.7% primary follicles), and the proportion of normal follicles was similar ( $P > 0.05$ ) among treatments (data not shown). The distribution of follicle population was 61.4% primordial, 25.4% transitional, 9.9% primary, and 3.3% secondary. The morphometric parameters of each follicular class are shown (Table 3). Overall, the follicle, oocyte, and oocyte nucleus diameters, along with the mean number of granulosa cells per follicle, increased ( $P < 0.05$ ) with follicle

development. Secondary follicles were not measured because of the low number of structures (n = 2) with visible oocyte nucleus.

**Table 3**

Mean ( $\pm$  SEM) follicle, oocyte, and nucleus diameters, and number of granulosa cells per section.

Follicle class	Diameter ( $\mu\text{m}$ ) <sup>†</sup>			No. of granulosa cells per follicle section <sup>†</sup>
	Follicle	Oocyte	Oocyte nucleus	
Primordial (n = 53)	32.8 $\pm$ 0.5 <sup>a</sup> (19-41) <sup>‡</sup>	26.0 $\pm$ 0.4 <sup>a</sup> (16-35)	13.5 $\pm$ 0.2 <sup>a</sup> (9-18)	6.2 $\pm$ 0.1 <sup>a</sup> (4-8)
Transitional (n = 46)	36.3 $\pm$ 0.4 <sup>b</sup> (29-42)	27.8 $\pm$ 0.4 <sup>b</sup> (23-33)	13.4 $\pm$ 0.2 <sup>a</sup> (10-16)	9.6 $\pm$ 0.2 <sup>b</sup> (7-13)
Primary (n = 13)	43.0 $\pm$ 0.7 <sup>c</sup> (39-48)	31.9 $\pm$ 0.7 <sup>c</sup> (27-36)	16.0 $\pm$ 0.3 <sup>b</sup> (13-18)	15.4 $\pm$ 0.6 <sup>c</sup> (12-19)

<sup>a,b,c</sup> Within a column, values without a common superscript differed ( $P < 0.05$ ).

<sup>†</sup> End point evaluated only in normal preantral follicles.

<sup>‡</sup> Represents range of the end points.

#### 4. Discussion

To our knowledge, this is the first study to evaluate the effect of ovarian histological section thicknesses on the number, density, and morphology of preantral follicles in any species. Moreover, this is the first study to report on density of preantral follicles in mares.

Although preantral follicles were visualized in all histological section thicknesses (3, 5, and 7  $\mu\text{m}$ ), a greater number of follicles was observed in the 7- $\mu\text{m}$  section thickness. Previous studies on equine ovarian tissue have used section thicknesses of 5  $\mu\text{m}$  [16,25], 7  $\mu\text{m}$  [6], and 10  $\mu\text{m}$  [8,9,11] to evaluate preantral follicles. However, no study has reported the efficiency of different histological section thicknesses on equine preantral follicle population and density under the same experimental conditions. The larger number of follicles observed in the 7- $\mu\text{m}$  section thickness might have occurred

because of the greater depth of the sample (5 slides  $\times$  10 sections  $\times$  7  $\mu\text{m}$  = 350  $\mu\text{m}$ ). The thicker sections contained more material and might be the best approach to use for estimating the number of preantral follicles. Although the 3- $\mu\text{m}$  section thickness could have facilitated the evaluation of follicular morphology (normal vs. abnormal), no difference was detected among treatments. Therefore, besides allowing detection of more follicles, the 7- $\mu\text{m}$  treatment was also efficient in terms of evaluating follicular morphology. Furthermore, the overall rate of morphological normal follicles (89%) was similar to that reported in previous studies in mares [7-9]. In addition, the proportion of preantral follicle classes and their morphometric characteristics were also similar to those in the aforementioned studies.

A progressive increase in follicle and oocyte diameters was observed with follicle development. Furthermore, as expected, oocyte nucleus diameter and number of granulosa cells in a cross-section plane also increased as the follicular classes advanced; these were novel results for the equine species. These findings together indicate the normal growth and development of preantral follicles and the gradual expansion of the follicular compartment, which have been associated with the increase of granulosa cell number in other species [26,27]. Another new finding was the percentage increase among the follicular classes (primordial to primary) for the diameter of follicles (31%), oocytes (22%), and oocyte nuclei (18%), and the number of granulosa cells (148%). These findings agree with the concept that the oocyte has an important role in follicular organization and possibly controls the proliferation and differentiation of granulosa cells [28].

A high variation in the number of preantral follicles per ovarian sample (5-273 follicles) and follicular density (0-31 follicles per  $\text{cm}^2$ ) among mares and within the same section treatments was detected. Several studies, regardless of species, have reported a large variation in the number of preantral follicles per ovary (human: 2,700 - 79,600 [20], swine: 67,599 - 291,898 [29], bovine: 59,798 - 78,820 [30], ovine: 7,333 - 44,633 [21]). In addition, similarly to our novel finding in mares, a high heterogeneity in density of preantral follicles per  $\text{mm}^2$  has been reported for several species (buffalo: 6 - 35 [31], squirrel monkeys: 0.01 - 303 [32], women: 0.6 - 159 [33], sheep: 0 - 52 [22]).

An earlier study [34] demonstrated that equine follicle population varies from 5,600 to 75,000, which is considered low when compared to other domestic species (i.e., swine: 185,000 - 779,000 [35], bovine 10,043 - 253,000 [36]). The low number and density of preantral follicles in the equine species might be because of the potential heterogeneous

distribution of these structures within the ovary [11] regulated by genes and factors that control survival, proliferation, colonization, and inclusion of primordial germ cells in follicular structures [37]. Variation in the number of preantral follicles may also be affected by age (equine [7], bovine [38], human [20,39]), and breed (bovine [36], ovine [40]).

Preantral follicles classified as primordial were the most frequently (61%) observed in this work, which agrees with our recent observations [7-9]. Studies in other species have reported similar results for the frequency of primordial follicles (humans: 87.8% [41], feline: 87% [42], primate: 73.3% [43]), characterizing this follicular stage as the main ovarian reserve of preantral follicles.

In summary, this study on equine preantral follicles demonstrated that: (1) a 7- $\mu$ m histological section thickness might be recommended because it allowed identification of a greater number of preantral follicles per sample, (2) a large individual variation in follicle population and density was detected regardless of histological section thickness, and (3) mares have a low number and density of preantral follicles when compared to those reported for other species.

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### **CAPÍTULO 3 – A Égua como Modelo Animal para Densidade de Folículos Pré-antrais e Idade Reprodutiva na Mulher**

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#### **The Mare Model for Preantral Follicle Density and Reproductive Aging in Women**

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**Abstract**

The ovarian biopsy technique is currently the most practical way to harvest preantral follicles and the mare has several features that could make it as an important animal model for women. The aims of this study were to evaluate: (i) preantral follicle density in ovarian biopsy fragments within and among mares; (ii) the influence of aging on density and quality of preantral follicles, and (iii) determine the minimum number of ovarian fragments and histological sections to estimate the equine follicular density. Mares were divided into three groups according to age: young, adult, and old. The biopsy pick-up method was used to collect the ovarian fragments and the samples were prepared for classical histology. Overall, 336 preantral follicles were recorded (9.9 follicles per ovarian fragment). Mean follicular density was 3.7 follicles per  $\text{cm}^2$  and differed ( $P < 0.05$ ) among animals and ovarian fragments from the same animal. Moreover, high variation among histological sections was also observed. The follicular density was different ( $P < 0.05$ ) among young (6.5), adult (4.1), and old mares (0.6). Similarly, the proportion of morphologically normal follicles reduced ( $P < 0.05$ ) between the youngest (97%) and oldest (84%) age groups. The Monte Carlo simulations results showed higher ( $P < 0.05$ ) detection of the follicular density (above 90%) in two experimental designs with 65 histological sections and 3 or 4 ovarian fragments. In conclusion, the results demonstrated that (i) the follicular density differed among ovarian fragments, within and among animals; (ii) the aging influenced negatively the density and rates of morphologically normal follicles; and (iii) a specific number of ovarian fragments and histological sections are required to estimate the follicular density on equine ovarian tissue.

**Keywords:** Preantral follicle; Follicular density; Ovarian biopsy

## Introduction

The functional potential of the ovary is usually associated with the number and quality of oocytes enclosed in follicular structures [1]. Research interest in the factors regulating early folliculogenesis has been highlighted due the development of methodologies for ovarian tissue collection, follicular isolation, and in vitro culture [2,3]. The woman fertility can be impaired by clinical conditions, chemotherapy, or age advance. Stored preantral follicles may contribute for new assisted reproductive technologies to overcome infertility challenges in clinical medicine [4].

Cryopreservation and culture methods for preantral follicles need to be constantly improved. However, the low availability of human ovarian tissue hinder the development of experiments on this area [5]. Thereby, the alternative for comparative studies of follicular population and new procedures is by the use of animal models.

The mare has several features that could be considered as an important animal model for comparative studies of preantral follicle population in biopsy fragments in women due the similarities as hormonal concentration, follicular waves, age-related decline in fertility, and preantral follicular heterogeneity [6,7,3,8].

The decline of fertility in women and mares could be assigned at the ovarian failure associated with poor oocyte quality [6]. Studies in women [9], mares [10,8], and sheep [5] have indicated that preantral follicles are not homogeneously distributed within the ovarian cortex. Furthermore, the number and quality of these structures are directly influenced by aging on several species (primates: [11]; women: [12]; mares: [3]).

The ovarian biopsy technique is currently the most practical way to harvest preantral follicles without jeopardizing reproductive function. Ovarian biopsy fragments from women, cows, and mares have a high variability on the number of preantral follicles [13,2,10], and information about follicular density is critical to determine the quantity of fragments during biopsy procedures. Therefore, studies are needed to define methodologies for quantification of preantral follicle density on histological sections from ovarian fragments and to evaluate the effect of aging on the density and morphology of preantral follicles.

The aims of this study were to evaluate: (i) preantral follicle density in ovarian biopsy fragments of the same mare and among animals, (ii) the influence of aging on density and quality of preantral follicles, and (iii) determine the minimum number of

ovarian fragments and histological sections required to estimate with higher probability the equine follicular density.

## **Materials and Methods**

### **1. Mares**

All experimental procedures were performed according to the United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training. The research protocol (#11-007) was approved by the Institutional Animal Care and Use Committee of Southern Illinois University. Light horse mares (n = 10) were separated in three groups according to age: young (5 to 6 years old; n = 3), adult (7 to 10; n = 4), and old (11 to 16; n = 3) and kept on pasture and orchard grass/alfalfa mixed hay, supplemented with ration and minerals. The ovaries and uterus of all mares were scanned daily by using a transrectal ultrasound scanner (Aloka SSD-900, Aloka Co., LTD., Wallingford, CT, USA) equipped with a 5 to 10 MHz linear array transducer (Aloka UST-5821-7.5). Mares were selected when the ovaries presented a sufficient available space for needle entry (i.e., lack of large preovulatory follicles or corpora lutea) and a previous history of successful biopsy procedures. No hormonal treatments were administered during the experimental period.

### **2. Ovarian tissue collection**

Ovarian fragments were obtained via the Biopsy Pick-up (BPU) method [3]. Immediately after each procedure, biopsy fragments (between 3 and 4 per mare) were fixed in Bouin's solution for 2 hours and then kept in 70% alcohol until histological preparation.

### **3. Histological processing and density of preantral follicles**

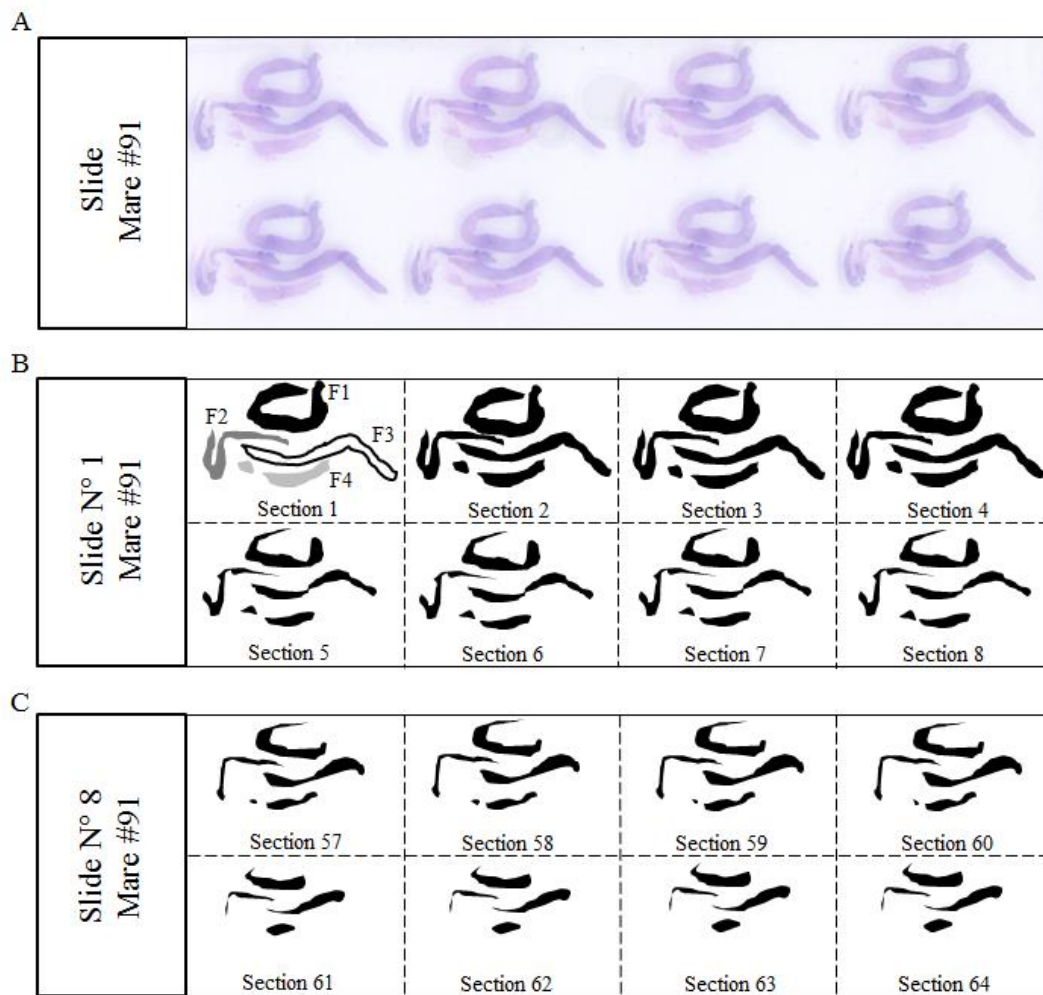
Ovarian fragments of each mare were embedded together in paraffin wax and totally cut in serial sections (10  $\mu$ m). The number of histological sections made per ovarian fragment range between 38 and 79. Samples were stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin. Each ovarian fragment was randomly numbered for analysis according to the position on the histological slide (Figure 1A, B, C). The perimeter of each histological section was delimited with a photo editing program (Adobe

Photoshop CS4, USA) and the area's measurement was recorded. The follicular density was determined by the following formula:

$$\text{Follicular density} = \text{Number of preantral follicles} / \text{Area of the histological ovarian section (cm}^2\text{)}$$

#### 4. Microscopy and end points

The ovarian histological sections were analyzed using a light microscope (Nikon, Tokyo, Japan) at magnification  $\times 400$  to examine the total number of follicles in each ovarian section, follicle class, and morphology. The follicles were classified according to their developmental stage [14] into primordial (one layer of flattened granulosa cells around the oocyte), transitional (flattened and cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte), and secondary (two or more layers of cuboidal granulosa cells around the oocyte). Only preantral follicles with oocyte nucleus visualized were counted and morphologically classified as normal (follicle containing an intact oocyte and granulosa cells well-organized without pyknotic nucleus) or abnormal (oocyte with pyknotic nucleus, retracted cytoplasm or disorganized granulosa cell layers [10]). All evaluations and measurements were performed by a single operator.



**Figure 1.** (A) Histological sections of ovarian fragments scanned on a photo editing program. (B) Schematic illustration of the first slide from mare 91 showing histological sections of ovarian fragments (F1, F2, F3, and F4) randomly numbered and (C) the eighth slide represents the subsequent histological sections of ovarian fragments. Area (cm<sup>2</sup>) of each ovarian fragment was measured within each histological section regardless of the size.

## 5. Statistical analyses

All statistical analysis was carried out using software programming environment R version 3.0.2 (R Foundation for Statistical Computing., Vienna, Austria). Data that were not normally distributed were submitted to logarithmic transformation. Follicular density among ovarian fragments, mares, and age groups were compared by Kruskal-Wallis test. Proportion of morphologically normal follicles and follicle class distribution per age group were analyzed by Fisher's exact test.

### 5.1 Monte Carlo simulations

To perform the simulations, it was hypothesized that the logarithmically transformed preantral follicle density followed a normal distribution. The variance components were calculated to determine the contribution of ovarian fragments and histological sections (random factors) on the total variance of follicular density.

Monte Carlo simulations was applied on the follicular density to determine the best combination of ovarian fragments (2, 3, or 4) and histological sections per fragment (10, 20, 40, or 65) to estimate the equine preantral follicle density. The estimation of the variance components for each combination of ovarian fragments and histological sections were used to generate 2,000 simulations per combined data set.

The probability of detecting the follicular density according the combination of ovarian fragments and histological sections was calculated (i.e., power of the test). Moreover, fixed factors (i.e., the effect of a treatment on follicular density that should be detected [5]) were defined at 50%, 30%, and 10% and added to the analysis. Data are presented as mean  $\pm$  SEM and the results were considered significantly different when  $P < 0.05$ .

## Results

A total of 34 ovarian fragments were obtained by the biopsy pick-up method (mean, 3.4 fragments per mare) and 2083 histological sections were analyzed. Overall, 336 preantral follicles were recorded with a mean of  $9.9 \pm 2.5$  follicles per ovarian fragment (range, 0-59). A larger proportion of primordial follicles (64.6%) followed by transitional (28.9%), primary (6.2%), and secondary (0.3%) was seen. The rate of morphologically normal follicles was 95% and no difference ( $P > 0.05$ ) was observed among ovarian fragments.

A mean follicular density of 3.7 follicles per  $\text{cm}^2$  was detected and this differed ( $P < 0.05$ ) among mares and ovarian fragments ( $P < 0.05$ ) of the same animal (Table 1). Considering the age group, it was observed a higher ( $P < 0.05$ ) variability on the follicular density for the adult animals when compared with young and old groups.

**Table 1.** Mean ( $\pm$  SEM) density of equine preantral follicles within different ovarian fragments of the same animal.

Age group (years)	Mare ID	Follicular density per ovarian fragment (number of preantral follicles/cm <sup>2</sup> )				Total per animal
		Fragment 1 <sup>†</sup> (n = 10)	Fragment 2 (n = 10)	Fragment 3 (n = 10)	Fragment 4 (n = 4)	
Young ( $\leq 6$ )	8	0.0 $\pm$ 0.0 <sup>a</sup>	0.0 $\pm$ 0.0 <sup>a</sup>	18.8 $\pm$ 4.4 <sup>b</sup>	-	6.3 $\pm$ 1.7 <sup>BE</sup>
	119	3.1 $\pm$ 1.4 <sup>a</sup>	17.4 $\pm$ 4.7 <sup>c</sup>	0.0 $\pm$ 0.0 <sup>b</sup>	5.4 $\pm$ 1.1 <sup>c</sup>	6.1 $\pm$ 1.2 <sup>BE</sup>
	124	19.1 $\pm$ 2.7 <sup>a</sup>	0.0 $\pm$ 0.0 <sup>b</sup>	1.9 $\pm$ 1.2 <sup>b</sup>	-	7.0 $\pm$ 1.1 <sup>B</sup>
Adult (7 – 10)	91	6.3 $\pm$ 1.6 <sup>a</sup>	1.4 $\pm$ 1.1 <sup>b</sup>	19.0 $\pm$ 2.6 <sup>c</sup>	4.1 $\pm$ 1.7 <sup>ab</sup>	7.7 $\pm$ 1.0 <sup>B</sup>
	116	1.9 $\pm$ 1.0 <sup>ab</sup>	0.0 $\pm$ 0.0 <sup>a</sup>	0.0 $\pm$ 0.0 <sup>a</sup>	3.2 $\pm$ 1.1 <sup>b</sup>	1.2 $\pm$ 0.4 <sup>AD</sup>
	121	10.5 $\pm$ 1.9 <sup>a</sup>	1.1 $\pm$ 0.8 <sup>b</sup>	0.0 $\pm$ 0.0 <sup>b</sup>	0.0 $\pm$ 0.0 <sup>b</sup>	3.2 $\pm$ 0.6 <sup>CDE</sup>
	122	6.0 $\pm$ 1.7 <sup>a</sup>	5.4 $\pm$ 1.1 <sup>a</sup>	1.6 $\pm$ 0.8 <sup>b</sup>	-	4.3 $\pm$ 0.7 <sup>BE</sup>
Old (11 – 16)	2	0.0 $\pm$ 0.0 <sup>a</sup>	0.7 $\pm$ 0.7 <sup>a</sup>	0.4 $\pm$ 0.3 <sup>a</sup>	-	0.4 $\pm$ 0.2 <sup>AC</sup>
	123	0.0 $\pm$ 0.0 <sup>a</sup>	0.5 $\pm$ 0.3 <sup>ab</sup>	1.4 $\pm$ 0.6 <sup>b</sup>	-	0.6 $\pm$ 0.2 <sup>AD</sup>
	126	0.6 $\pm$ 0.4 <sup>a</sup>	0.9 $\pm$ 0.5 <sup>a</sup>	1.0 $\pm$ 0.5 <sup>a</sup>	-	0.8 $\pm$ 0.2 <sup>AD</sup>
Overall mean						3.7 $\pm$ 0.2

<sup>abc</sup> Within a row, values without a common superscript differed ( $P < 0.05$ ).

<sup>ABCDEF</sup> Within a column, values without a common superscript differed ( $P < 0.05$ ).

<sup>†</sup> Ovarian fragments obtained with the biopsy method were from random areas in the ovary and not sequential. The number indicates the position on the slides (Figure 1).

A follicular density reduction ( $P < 0.05$ ) was observed with the advance of age (Table 2). In addition, the proportion of morphologically normal follicles reduced ( $P < 0.05$ ) considerably between the youngest and oldest groups of age (Table 2). The ratio of preantral follicle number by histological section analyzed had a large range (1:2 - 1:113) among mares and inside the age groups (Table 3). Additionally, regardless of the age group a high variability of follicular density was observed among serial sections of the same fragment within the same animal (Figures 2A, B, C).

**Table 2.** Mean ( $\pm$  SEM) density of equine preantral follicles and morphology per age group.

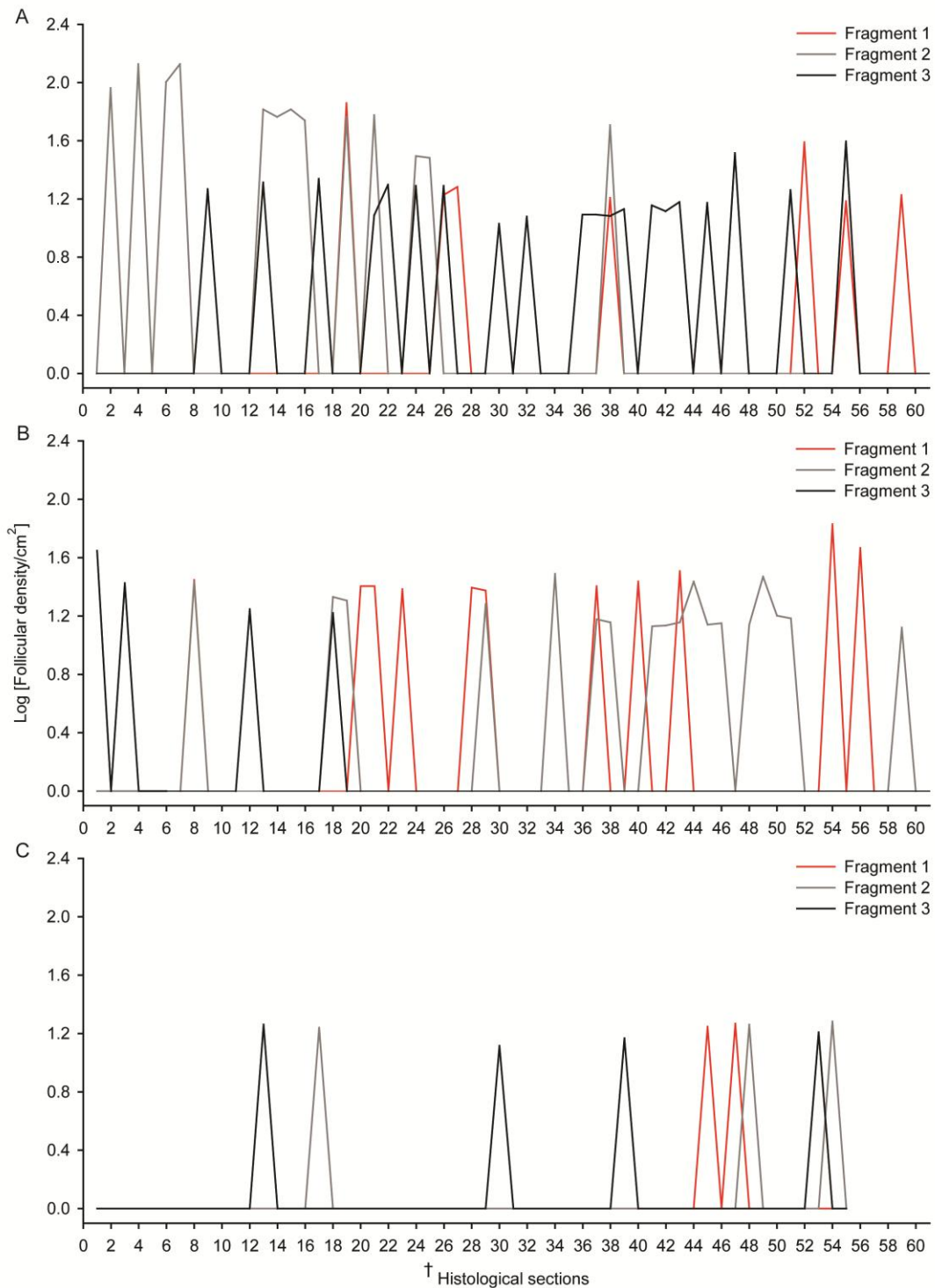
Age group (years)	Mare (n)	Preantral follicles (n)	Follicular density/cm <sup>2</sup>	MNF (%)
Young ( $\leq 6$ )	3	141	$6.5 \pm 0.7^a$	97.1 <sup>a</sup>
Adult (7 – 10)	4	176	$4.1 \pm 0.3^b$	94.3 <sup>ab</sup>
Old (11 – 16)	3	19	$0.6 \pm 0.1^c$	84.2 <sup>b</sup>
			Mean:	94.9

MNF: morphologically normal follicles.

<sup>abc</sup> Within a column, values without a common superscript differed ( $P < 0.05$ ).

**Table 3.** Number of preantral follicles recorded, histological sections analyzed, and ratio of follicles by histological section.

Age group (years)	Mare ID	Preantral follicles (n)	Histological sections analyzed (n)	Ratio
Young ( $\leq 6$ )	8	23	116	1:5.0
	119	55	235	1:4.2
	124	63	174	1:2.7
Adult (7 – 10)	91	79	260	1:3.2
	116	11	254	1:23.0
	121	47	286	1:6.0
	122	39	189	1:4.8
Old (11 – 16)	2	2	227	1:113.5
	123	8	177	1:22.1
	126	9	165	1:18.3
Total		336	2083	1:6.1



**Figure 2. Density of preantral follicles in histological sections of biopsy ovarian fragments from three representative mares.** A high variability of follicular density was observed among serial sections of the same fragment and among fragments within the same animal regardless of the age groups (A - young; B - adult; and C - old). † Each interval of axis “x” represents one histological section evaluated.

Based on the analysis of variance components, the histological sections contributed for the greatest variability (77%) on the total variance of follicular density when compared to the ovarian fragment which accounted for only 23% (Table 4).

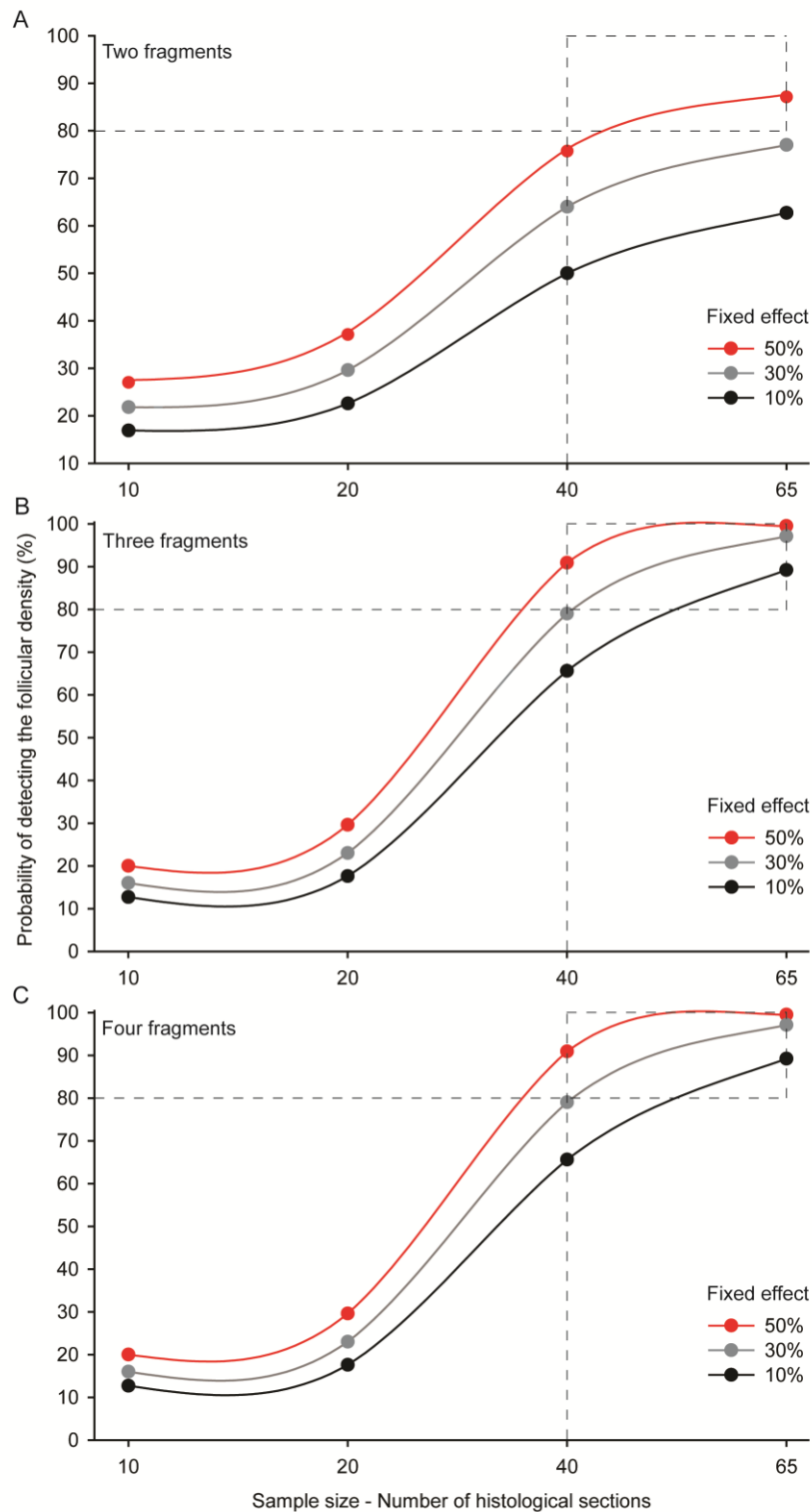
Monte Carlo simulations using different combinations of ovarian fragments and number of histological sections per fragment are shown (Figure 3). The probability to detect the follicular density (power of the test) with 10 or 20 histological sections regardless the number of fragments (2, 3, or 4) was less than 30% (Figures 3A, B, C). The combinations with 40 histological sections and 3 or 4 fragments presented more than 80% of follicular density detection with a fixed effect above 30% (Figures 3B, C).

In general, two experimental designs analyzing 65 histological sections with 3 or 4 fragments (Figures 3B, C) with fixed effects set at 30% and 50% generated a probability of detecting a precise follicular density above 90%. Moreover, the probability of detecting the follicular density using the same combination with fixed effect set at 10% ranged from 80 and 89% (Figures 3B, C).

**Table 4.** Variance components of the effects (ovarian fragment and histological section) for equine preantral follicular density.

Random Effects	n	Variance component	Standard deviation	Proportion of variance <sup>†</sup>
Ovarian fragments	34	0.05	0.23	22.7 %
Histological sections	2083	0.17	0.41	77.3 %

<sup>†</sup> The contribution of ovarian fragments and histological sections on the total variance of follicular density.



**Figure 3. Monte Carlo simulations for the probability of detecting the equine follicular density according to the number [two (A), three (B), and four (C)] of ovarian fragments analyzed. Dashed square represents the probability (power of test) above 80%.**

## Discussion

This study reported the equine preantral follicle density in ovarian biopsy fragments and the effect of aging on density and quality. Moreover, the results were used to develop an experimental design to overcome the high follicular heterogeneity observed on ovarian fragments. The follicular density is an important tool for application on ovarian biopsy technique, to indicate the number of fragments required with a sufficient quantity of preantral follicles that could be used on biotechnologies such as cryopreservation and transplantation procedures.

This study evaluated 2,083 histological sections from 34 equine ovarian fragments in which was observed a mean follicular density of 3.7 preantral follicles per  $\text{cm}^2$  (i.e., 0.037 follicles per  $\text{mm}^2$ ). In a preliminary work [8] we found a similar follicular density (3.0 follicles per  $\text{cm}^2$ ) using ovarian fragments obtained from slaughterhouse ovaries. Higher follicular density rates ( $\text{mm}^2$ ) have been reported from ovarian fragments in others species (buffalo fetuses – 35.8, [15]; mice – 10.5, [16]; women – 53.4, [17]).

Overall, the rate of morphologically normal follicles reported in this study was high (95%) and this indicates that the biopsy procedure does not compromise the preantral follicles and that their viability could be maintained for further evaluation such as in vitro follicle culture procedures [18].

A reduction on follicular density and on the rate of morphologically normal follicles was observed as the age advances. Reduced fertility with maternal aging has been well documented in humans [19] and animals (cow: [20]; monkey: [11]; mare: [6]). The primordial follicle population represents the reservoir of potential viable oocytes available to the reproducing female. This population suffers considerable decreases over the postnatal lifespan culminating on ovarian senescence at old age [21]. In women, a study [22] suggested that the follicular density is in constant decrease and the age accounts for 84% of the variation in preantral follicle number. Other studies indicated that genetic, stochastic, and environmental factors can contribute for ovarian follicular population decrease [23, 24]. These results indicate that the mare can be a good animal model to study ovarian senescence in women at the level of preantral (this study) and antral follicles [6, 25, 7].

A high level of follicular density heterogeneity was observed among mares, within ovarian fragments and histological sections of the same animal. Moreover, the

greatest proportion of this variance for preantral follicular density occurred on histological sections (77%) as reported for ewes [5] in which the major source of variability (90%) was due to the sections. The heterogeneous follicular density on the ovarian stroma has been previously reported in different species (buffalo fetuses: [15]; equine: [8]; women: [26]). The dispersion of follicular structures and the parenchymal heterogeneity previously observed might be associated with the cell migration into the gonadal ridges during the ovary embryonic formation [27].

Several methods to estimate ovarian follicular density have been used before, such as histological analysis [28], stereological techniques [29], fluorescent probes [1], and mathematical models [13]. The present study used a number of ovarian fragments (1 to 5 per mare) that is usually collected during an ovarian biopsy procedure [3]. Based on this number, the Monte Carlo simulations showed that in order to increase the probability to detect the equine follicular density above 80%, it is necessary to use at least 3-4 ovarian fragments combined with 65 histological sections analyzed per fragment, regardless of the fixed effect (10%, 30%, or 50%). This fact could be associated to the high follicular density variability detected among sections.

The Monte Carlo simulations has been tested in ewes [5] and the authors reported that the histological evaluation combined with a mathematical simulation could generate an experimental design model that considers the physiological follicular heterogeneity and provides important data to define the number of ovarian fragments and histological sections that should be analyzed for adequate follicular quantification. However, it is important to emphasize that the mentioned model is species-specific, and should be calculated according to the variation of follicular density among and within individuals of the same species.

In summary, our results showed that the equine follicular density differed among animals and within ovarian fragments of the same animal. The follicular density and rates of morphologically normal follicles were negatively influenced by aging. Moreover, independent of follicular heterogeneity, 3 to 4 ovarian fragments combined with 65 histological sections are required to estimate the follicular density on equine ovarian tissue.

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### **Author Contributions**

Conceived and designed the experiments: KAA BGA MOG MLG ELG. Performed the experiments: KAA BGA GDAG KTH ELG. Analyzed the data: KAA BGA GDAG MOG JRF ELG. Wrote the paper: KAA BGA GDAG MOG JRF MLG ELG.

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## **CAPÍTULO 4 – A Égua como Modelo para Acessar as Características da Dinâmica de Folículos Pré-antrais em Fragmentos Obtidos por Biópsia Ovariana**

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### **The Mare as a Model for Assessing the Dynamic Features of Preantral Follicles in Ovarian Biopsy Fragments**

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**Abstract**

Ovarian biopsy fragments allow the performance of studies with clinical applications in the field of female fertility considering mares as an animal model. Thus, the influence of reproductive phase (anestrous vs diestrous) and ovarian structures (antral follicles and corpus luteum) on the quality, class distribution, number and density of preantral follicles, and stromal cell density were investigated. Ovarian fragments (n = 142) were harvested by biopsy pick-up procedures from 19 mares and submitted to histological analysis. The mean preantral follicle density was  $2.7 \pm 0.1$  follicles/cm<sup>2</sup> and differed among anestrous ( $2.1 \pm 0.1$ ) and diestrous ( $3.5 \pm 0.1$ ). The ovarian stromal cell density was higher on diestrous and a positive correlation of stromal cell density with the number and density of preantral follicles was observed. The mean area (mm<sup>2</sup>) of ovarian structures increased on diestrous and had a positive correlation with number of preantral follicles, follicular density and stromal cell density. Biopsy fragments collected from ovaries with a corpus luteum had a higher follicular density, stromal cell density, and proportion of normal follicles. In conclusion, our results showed: (1) the diestrous phase influenced positively the preantral follicle quality, class distribution, follicular density and stromal cell density; (2) the area of ovarian structures was positively correlated with the follicular density and stromal cell density; and (3) the presence of an active corpus luteum had a positive effect on the quality of preantral follicles, follicular density and stromal cell density. In addition, we demonstrated herein the perspective of a potential ideal scenario to obtain ovarian fragments richer in preantral follicles number and quality during biopsy procedures which can be used in women to increase the chances to collect fragments with better quality.

## Introduction

Studies with human ovaries face ethical barriers and limited availability of material for research which does not happen with domestic animals. Animal models can offer the ability to conduct controlled experiments that would be proscribed in women (Kaplan 2004). Considering the similarities between women and mares relative on the dynamics of the reproductive cycles (Ginther *et al.* 2004, Ginther *et al.* 2005, Carnevale 2008, Baerwald 2009, Gastal *et al.* 2011, Ginther 2012), age-associated changes in fertility (Haag *et al.* 2013a) and the physiological preantral follicle heterogeneity (Haag *et al.* 2013b), the mare can be considered an important animal model to supply information about the follicular population in ovarian biopsy fragments.

The woman and mare are monovular species (Gastal 2009) and although the seasonality does not occur in women, this characteristic in an animal model allows to evaluate which reproductive factors may be operating when cyclic hormonal patterns are not occurring (Carnevale 2008). In mares, the changes on ovarian structures (antral follicles and corpus luteum) are well defined during different reproductive phases based on ultrasonography exam (Gastal *et al.* 2004, Ginther 2014). Studies with in vitro culture reported that ovarian structures produce hormones and growth factors that can stimulate the development and viability of preantral follicles (Al-Gubory & Martinet 1987, Skinner 2005, Gupta *et al.* 2007, Yang & Fortune 2007, Robinson *et al.* 2009).

The preantral follicle population represents the finite and available reserve of gametes that can be recruited during the reproductive lifespan of mammalian. Ovarian tissue collected by biopsy procedures allows the performance of many studies with clinical applications in the field of female fertility preservation and has been used to several species (women: Lass *et al.* 1997, Schmidt *et al.* 2003, Kwok & Johnson 2012, Vanacker *et al.* 2013, cows: Aerts *et al.* 2005, Jorssen *et al.* 2014, and mares: Haag *et al.* 2013b,c). This safe method ensures the collection of preantral follicles without jeopardizing the reproductive life (Haag *et al.* 2013a). However, there is a lack of reports to establish guidelines to identify which is the most suitable moment to perform a biopsy procedure that allows the collection of ovarian tissue with better quality e.g. higher follicular density associated with greater stromal cell density.

In addition, the follicular heterogeneity observed on ovarian fragments of different species (women: Schmidt *et al.* 2003, cows: Aerts *et al.* 2008, ewes: Fransolet *et*

*al.* 2014, and mares: Haag *et al.* 2013a) can impair their use on assisted reproductive technologies (ARTs) as in vitro culture, cryopreservation, and transplantation due to a lack of methods to detect the number and viability of preantral follicles at the moment of the procedures (Soleimani *et al.* 2006, Chambers *et al.* 2010, Fransolet *et al.* 2014).

Moreover, the equine parenchyma ovary contains considerable area of stroma and some studies have reported that the stromal cell density is an important indicator of tissue integrity and also play a role on follicular development (Gilchrist *et al.* 2004, Knight & Glistler 2006, Gerritse *et al.* 2011). Thus, the stromal cell density is an important parameter to be considered for maintaining the functionality of preantral follicles in biopsy fragments.

The aim of this study was to assess the influence of reproductive phase (anestrous and diestrous) and ovarian structures as antral follicles and corpus luteum on the: (1) quality, class distribution, number and density of preantral follicles, and (2) stromal cell density in ovarian fragments harvested by biopsy procedure. In addition, evaluate the results to consider the mare as a potential comparative model for dynamic features of preantral follicle population in ovarian fragments.

## **Materials and methods**

### ***Mares***

All experimental procedures were performed according to the United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training. The research protocol (#11-007) was approved by the Institutional Animal Care and Use Committee of Southern Illinois University. The study was carried out during the winter (December to March) and spring seasons (April to June) in the northern hemisphere. Light horse mares (n = 10) weighing between 400 and 600 Kg and 5 to 11 years old were used. No hormonal treatments were administered during the experimental period.

### ***Area of ovarian structures***

The ovaries and uterus of all mares were scanned daily from using a transrectal ultrasound scanner (Aloka SSD-900, Aloka Co., LTD., Wallingford, CT, USA) equipped with a 5 to 10 MHz linear array transducer (Aloka UST-5821-7.5). At the moment of the

biopsy procedure, the ovarian structures were recorded as small follicles (follicles between 6 to 26 mm of diameter), preovulatory follicles ( $\geq 36$  mm) and corpus luteum ( $\geq 30$  mm, days 4 to 12 of estrous cycle). The average of height and width of the ovarian structures at the maximal length was used to measure the structures diameter (Gastal *et al.* 1997). The area ( $\text{mm}^2$ ) of each ovarian structure was determined by the following formula:

$$\text{Area} = \pi \times (D/2)^2$$

Where:  $\pi = 3.14$ ; D = diameter (mm).

### ***Ovarian tissue collection***

Biopsies fragments (n = 3 to 4) were collected per each ovary (left and right) separately via the biopsy pick-up technique (Haag *et al.* 2013a). The procedures were performed during the winter (anestrous phase; follicles  $\leq 20$  mm and absence of corpus luteum) and spring seasons (diestrous phase, days 4 to 12 of estrous cycle, presence of a corpus luteum and compatible uterine echotexture). The same mares were used in both seasons.

### ***Histological processing and follicular density***

Ovarian fragments were fixed in Bouin's solution for 2 hours and then kept in 70% alcohol until histological preparation. The fragments were embedded in paraffin wax and totally cut in serial sections ( $7 \mu\text{m}$ ). Every section was mounted and stained with acid Schiff (PAS) and hematoxylin. The perimeter of histological sections was delimited with a photo editing program (Adobe Photoshop CS4, San Jose, USA) and the area's measurement ( $\text{cm}^2$ ) was recorded. Therefore, the follicular density was determined by the following formula:

Follicular density = number of preantral follicles/area of the ovarian section ( $\text{cm}^2$ ).

### ***Microscopy and end points***

Histological sections were analyzed on a light microscope (Nikon E200, Tokyo, Japan) at magnification  $\times 400$  and an image capture system (LEICA Imaging Software, Wetzlar, Germany). The following end points were recorded: number of preantral follicles and follicular density per ovarian fragment, follicular class distribution, follicle morphology (normal and abnormal), and ovarian stromal cells density. The

follicles were classified according to their developmental stage into primordial, transitional, primary and secondary.

### ***Morphology of preantral follicles***

Only preantral follicles with visualized oocyte nucleus were counted and morphologically classified as normal (follicle containing an intact oocyte and oocyte nucleus surrounded by granulosa cells well organized in one or more layers) or abnormal (follicles with a retracted cytoplasm or disorganized granulosa cells layers detached from the basement membrane and oocyte with pyknotic nucleus (Silva *et al.* 2000).

### ***Ovarian stromal cells density***

Ovarian stromal cells density was evaluated as described by Commin *et al.*, (2012) with some modifications. Briefly, a total of 10% of histological sections for each ovarian fragment were analyzed. Four random fields ( $50 \times 50 \mu\text{m} = 2,500 \mu\text{m}^2$ ) were selected and the stromal cell nuclei were counted to calculate the mean of stromal cells density per ovarian fragment. All evaluations and measurements were performed by a single operator.

### ***Statistical analyses***

All statistical analyses were performed using R statistical software version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Data for end points that were not normally distributed were transformed (base 10 logarithmic). The number of preantral follicles and follicular density per ovarian fragment within the same reproductive phase (anestrous or diestrous) and side ovary (left or right) were compared by Kruskal-Wallis test and among reproductive phase and side ovary by Wilcoxon-Mann-Whitney test. The follicular class distribution and the percentage of normal preantral follicles among reproductive phases were analyzed by chi-square test. Wilcoxon-Mann-Whitney test was used to compare the stromal cells density and area of the ovarian structures among the reproductive phases and side ovary. In addition, this test was used to analyze the number of preantral follicles, follicular density, and stromal cells density in biopsy fragments harvested from ovaries with antral follicles and corpus luteum. Correlation between the number of preantral follicles and follicular density with stromal cells density, and among the number of preantral follicles, follicular density and stromal cells density with the area

of ovarian structures were estimated by Spearman correlation analysis. Data are presented as mean  $\pm$  SEM, unless otherwise stated. A probability of  $P < 0.05$  indicated that a difference was significant, and  $P > 0.05$  and  $\leq 0.1$  indicated that a difference approached significance.

## Results

A total of 142 ovarian fragments were collected by the biopsy pick-up method (mean, 3.7 fragments per mare in each reproductive phase) and 13,462 histological sections were evaluated. Overall, 1,493 preantral follicles were recorded with a mean of  $10.5 \pm 1.7$  follicles per ovarian fragment [range, 0 to 165; mean coefficient of variation (CV) = 195%].

The mean follicular density was  $2.7 \pm 0.1$  follicles per  $\text{cm}^2$  (range, 0 to 39; CV = 214%) and differed ( $P < 0.05$ ) among mares (Table 1). This parameter showed variation within the same mare on different reproductive phases and in the overall mean it was observed a higher ( $P < 0.05$ ) follicular density in diestrous when compared with anestrus. Data of follicular density related to the ovary side (left and right) on different reproductive phases are shown (Table 2). Regardless of reproductive phase the overall mean follicular density was greater ( $P < 0.05$ ) on the right ovary. Furthermore, there was an increasing ( $P < 0.05$ ) of overall mean density rates on diestrous phase for both ovary sides.

**Table 1** Mean ( $\pm$  SEM) density of equine preantral follicles in ovarian biopsy fragments collected during anestrus and diestrus phases.

Mare	Follicular density (number of preantral follicles/cm <sup>2</sup> )		
	Anestrus (n = 73) <sup>†</sup>	Diestrus (n = 69)	Total (n = 142)
1	0.6 $\pm$ 0.1 <sup>aA</sup>	1.5 $\pm$ 0.2 <sup>bA</sup>	1.0 $\pm$ 0.1 <sup>AC</sup>
2	0.8 $\pm$ 0.1 <sup>aAB</sup>	1.0 $\pm$ 0.1 <sup>bAB</sup>	0.9 $\pm$ 0.1 <sup>AC</sup>
5	0.6 $\pm$ 0.1 <sup>aA</sup>	0.7 $\pm$ 0.3 <sup>aB</sup>	0.7 $\pm$ 0.1 <sup>A</sup>
8	17.3 $\pm$ 2.3 <sup>aD</sup>	16.0 $\pm$ 0.9 <sup>bD</sup>	16.4 $\pm$ 1.0 <sup>B</sup>
9	1.5 $\pm$ 0.2 <sup>aAB</sup>	1.1 $\pm$ 0.2 <sup>aAB</sup>	1.3 $\pm$ 0.1 <sup>AC</sup>
10	0.7 $\pm$ 0.1 <sup>AB</sup>	-	0.7 $\pm$ 0.1 <sup>AC</sup>
11	0.7 $\pm$ 0.1 <sup>aAB</sup>	0.5 $\pm$ 0.2 <sup>aB</sup>	0.7 $\pm$ 0.1 <sup>A</sup>
13	2.6 $\pm$ 0.3 <sup>aCE</sup>	3.0 $\pm$ 0.4 <sup>aC</sup>	2.7 $\pm$ 0.2 <sup>CD</sup>
21	3.1 $\pm$ 0.3 <sup>aC</sup>	3.8 $\pm$ 0.4 <sup>aC</sup>	3.5 $\pm$ 0.2 <sup>D</sup>
32	1.3 $\pm$ 0.2 <sup>aBE</sup>	0.9 $\pm$ 0.2 <sup>bAB</sup>	1.1 $\pm$ 0.1 <sup>AC</sup>
Overall mean	2.1 $\pm$ 0.1 <sup>a</sup>	3.5 $\pm$ 0.1 <sup>b</sup>	2.7 $\pm$ 0.1

<sup>†</sup> Total number of ovarian fragments collected via ovarian biopsy pick-up method.

<sup>a,b</sup> Within a row, values without a common superscript differed ( $P < 0.05$ ).

<sup>A,B,C,D,E</sup> Within a column, values without a common superscript differed ( $P < 0.05$ ).

Mare #10 did not participate on the diestrus phase of the experiment.

**Table 2** Mean ( $\pm$  SEM) density of equine preantral follicles in ovarian biopsy fragments collected from left and right ovaries in anestrus and diestrus phases.

Mare	Follicular density (number of preantral follicles/cm <sup>2</sup> )			
	Anestrus		Diestrus	
	Left ovary (n = 37) <sup>†</sup>	Right ovary (n = 36)	Left ovary (n = 36)	Right ovary (n = 33)
1	0.5 $\pm$ 0.1 <sup>aAC</sup>	0.7 $\pm$ 0.2 <sup>aA</sup>	1.2 $\pm$ 0.2 <sup>aA</sup>	1.8 $\pm$ 0.3 <sup>bAD</sup>
2	0.9 $\pm$ 0.1 <sup>aAC</sup>	0.8 $\pm$ 0.2 <sup>aAC</sup>	1.0 $\pm$ 0.2 <sup>aA</sup>	1.1 $\pm$ 0.2 <sup>aAB</sup>
5	1.0 $\pm$ 0.2 <sup>aAC</sup>	0.2 $\pm$ 0.1 <sup>bA</sup>	1.4 $\pm$ 0.5 <sup>aA</sup>	0.04 $\pm$ 0.0 <sup>bB</sup>
8	10.9 $\pm$ 1.5 <sup>aBD</sup>	24.6 $\pm$ 4.6 <sup>aD</sup>	16.4 $\pm$ 1.7 <sup>aC</sup>	15.7 $\pm$ 1.1 <sup>aC</sup>
9	0.3 $\pm$ 0.1 <sup>aA</sup>	3.3 $\pm$ 0.5 <sup>bB</sup>	0.5 $\pm$ 0.2 <sup>aA</sup>	1.7 $\pm$ 0.3 <sup>bAD</sup>
10	0.9 $\pm$ 0.2 <sup>aAC</sup>	0.5 $\pm$ 0.1 <sup>aAC</sup>	-	-
11	0.7 $\pm$ 0.2 <sup>aAC</sup>	0.7 $\pm$ 0.2 <sup>aAC</sup>	0.3 $\pm$ 0.2 <sup>aA</sup>	0.8 $\pm$ 0.3 <sup>aAB</sup>
13	3.0 $\pm$ 0.4 <sup>aD</sup>	2.3 $\pm$ 0.4 <sup>bBC</sup>	4.2 $\pm$ 0.7 <sup>aB</sup>	1.8 $\pm$ 0.4 <sup>bAD</sup>
21	4.6 $\pm$ 0.5 <sup>aB</sup>	1.4 $\pm$ 0.3 <sup>bAB</sup>	4.1 $\pm$ 0.7 <sup>aB</sup>	3.7 $\pm$ 0.6 <sup>aD</sup>
32	1.3 $\pm$ 0.2 <sup>aC</sup>	1.4 $\pm$ 0.3 <sup>aAB</sup>	0.1 $\pm$ 0.0 <sup>aA</sup>	2.3 $\pm$ 0.6 <sup>bA</sup>
Overall mean	1.9 $\pm$ 0.1 <sup>W</sup>	2.3 $\pm$ 0.2 <sup>X</sup>	3.0 $\pm$ 0.2 <sup>Y</sup>	3.9 $\pm$ 0.2 <sup>Z</sup>

<sup>†</sup> Total number of ovarian fragments collected via ovarian biopsy pick-up method.

<sup>a,b</sup> Within a row and same reproductive phase (anestrus or diestrus), values without a common superscript differed ( $P < 0.05$ ).

<sup>A,B,C,D</sup> Within a column, values without a common superscript differed ( $P < 0.05$ ).

<sup>W,X,Y,Z</sup> Within a row, regardless of the reproductive phase and ovary side values without a common superscript differed ( $P < 0.05$ ).

Mare #10 did not participate on the diestrus phase of the experiment.

The mean number of preantral follicles recorded per ovarian fragment during the anestrus and diestrus phases are shown (Table 3). The overall mean was similar among reproductive phases ( $P > 0.05$ ) and among mares within the same phase a higher variability was observed ( $P < 0.05$ ). Regardless of reproductive phase, no difference ( $P > 0.05$ ) was observed for the number of preantral follicles between the left and right ovaries,

but over again there was heterogeneity among animals and ovaries of the same animal (Table 4).

**Table 3** Mean ( $\pm$  SEM) number of equine preantral follicles in ovarian biopsy fragments collected during anestrus and diestrus phases.

Mare	Number of preantral follicles per ovarian fragment		
	Anestrus (n = 73) <sup>†</sup>	Diestrus (n = 69)	Total (n = 142)
1	2.7 $\pm$ 1.0 <sup>aA</sup>	9.1 $\pm$ 5.3 <sup>aACE</sup>	5.7 $\pm$ 2.6 <sup>AE</sup>
2	4.7 $\pm$ 2.9 <sup>aAC</sup>	4.7 $\pm$ 1.2 <sup>aAE</sup>	4.7 $\pm$ 1.5 <sup>ACE</sup>
5	2.7 $\pm$ 0.9 <sup>aA</sup>	1.7 $\pm$ 0.9 <sup>aB</sup>	2.2 $\pm$ 0.6 <sup>AE</sup>
8	31.0 $\pm$ 2.4 <sup>aB</sup>	60.8 $\pm$ 19.6 <sup>aD</sup>	50.9 $\pm$ 13.4 <sup>D</sup>
9	7.2 $\pm$ 3.5 <sup>aAD</sup>	4.6 $\pm$ 1.8 <sup>aAE</sup>	5.8 $\pm$ 1.9 <sup>E</sup>
10	4.1 $\pm$ 1.2 <sup>AD</sup>	-	4.1 $\pm$ 1.2 <sup>AE</sup>
11	2.3 $\pm$ 0.8 <sup>aA</sup>	1.2 $\pm$ 0.8 <sup>aB</sup>	1.8 $\pm$ 0.5 <sup>A</sup>
13	14.2 $\pm$ 5.6 <sup>aBCD</sup>	8.6 $\pm$ 3.8 <sup>aE</sup>	11.4 $\pm$ 3.3 <sup>BCE</sup>
21	22.3 $\pm$ 10.3 <sup>aBD</sup>	16.0 $\pm$ 4.5 <sup>aCE</sup>	18.7 $\pm$ 4.9 <sup>B</sup>
32	8.2 $\pm$ 3.4 <sup>aAD</sup>	3.2 $\pm$ 1.9 <sup>bAB</sup>	5.7 $\pm$ 2.0 <sup>AE</sup>
Overall mean	8.5 $\pm$ 1.4 <sup>a</sup>	12.6 $\pm$ 3.1 <sup>a</sup>	10.5 $\pm$ 1.7

<sup>†</sup> Total number of ovarian fragments collected via ovarian biopsy pick-up method.

<sup>a,b</sup> Within a row, values without a common superscript differed ( $P < 0.05$ ).

<sup>A,B,C,D,E</sup> Within a column, values without a common superscript differed ( $P < 0.05$ ).

Mare #10 did not participate on the diestrus phase of the experiment.

**Table 4** Mean ( $\pm$  SEM) number of equine preantral follicles in ovarian biopsy fragments collected from left and right ovaries in anestrus and diestrus phases.

Mare	Number of preantral follicles per ovarian fragment			
	Anestrus		Diestrus	
	Left ovary (n = 37) <sup>†</sup>	Right ovary (n = 36)	Left ovary (n = 36)	Right ovary (n = 33)
1	2.5 $\pm$ 1.8	3.0 $\pm$ 1.2	5.2 $\pm$ 2.5	14.3 $\pm$ 12.8
2	6.7 $\pm$ 6.0	2.7 $\pm$ 0.6	5.0 $\pm$ 1.8	4.5 $\pm$ 1.8
5	4.2 $\pm$ 1.6	1.2 $\pm$ 0.6	2.7 $\pm$ 1.6	0.3 $\pm$ 0.3
8	29.5 $\pm$ 5.5	32.5 $\pm$ 1.5	44.0 $\pm$ 26.4	77.7 $\pm$ 30.1
9	1.7 $\pm$ 0.7	14.6 $\pm$ 6.4	1.7 $\pm$ 0.2	7.5 $\pm$ 3.2
10	3.7 $\pm$ 1.3	4.5 $\pm$ 2.2	-	-
11	2.2 $\pm$ 1.2	2.5 $\pm$ 1.2	0.7 $\pm$ 0.4	2.0 $\pm$ 2.0
13	17.5 $\pm$ 10.2	11.0 $\pm$ 5.9	11.2 $\pm$ 7.6	6.0 $\pm$ 2.2
21	34.6 $\pm$ 18.2	10.0 $\pm$ 7.5	12.0 $\pm$ 5.2	20.0 $\pm$ 7.5
32	9.2 $\pm$ 4.9	7.2 $\pm$ 5.5	1.2 $\pm$ 1.2	5.2 $\pm$ 3.6
Overall mean <sup>§</sup>	9.5 $\pm$ 2.4	7.4 $\pm$ 1.6	9.3 $\pm$ 3.5	16.1 $\pm$ 5.4

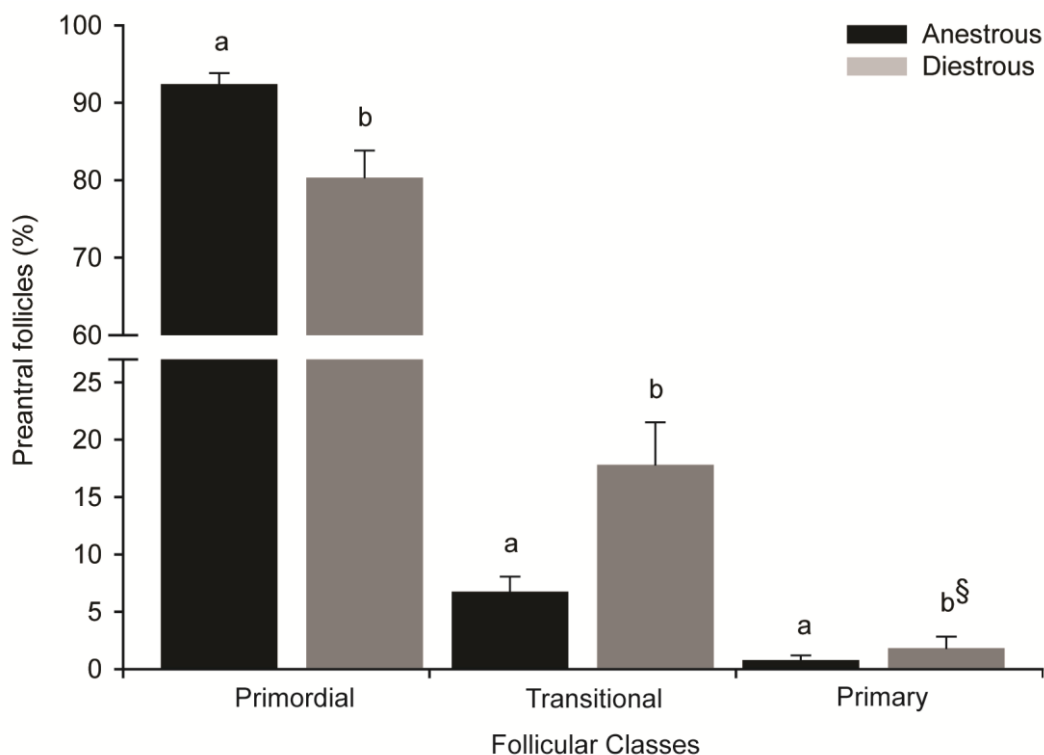
<sup>†</sup> Total number of ovarian fragments collected via ovarian biopsy pick-up method.

<sup>§</sup> No difference ( $P > 0.05$ ) was detected between ovaries within each reproductive phase.

Comparisons between ovaries and among mares within the same ovary and reproductive phase were not done because of the low number of observations (range, 2 to 4 fragments per ovary).

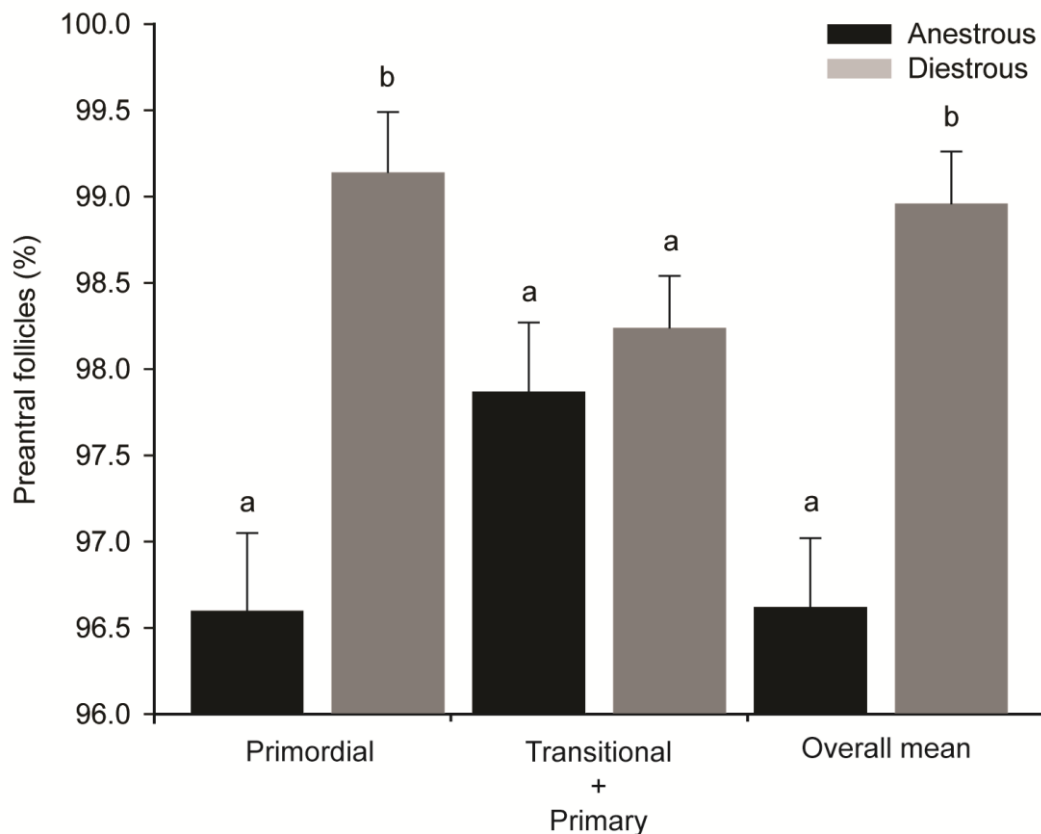
Mare #10 did not participate on the diestrus phase of the experiment.

The distribution of preantral follicle population was 85.3% primordial, 13.1% transitional, 1.4% primary, and 0.13% secondary. The percentage of primordial follicles was higher ( $P < 0.05$ ) on anestrus compared to the diestrus. In contrast, the growing follicles (transitional,  $P < 0.05$ ; and primary,  $P \leq 0.09$ ) was higher on diestrus phase (Figure 1).



**Figure 1** Mean ( $\pm$  SEM) percentage of equine preantral follicles according to class distribution in ovarian biopsy fragments collected during anestrous and diestrous phases. <sup>a,b</sup> Within the same follicle class, values without a common letter differed ( $P < 0.05$ ). <sup>§</sup>Tended to differ from transitional follicles during the same phase ( $P \leq 0.09$ ).

The overall percentage of normal preantral follicles differed ( $P < 0.05$ ) among anestrous (96%) and diestrous phases (98%) (Fig. 2). The percentage of normal primordial follicles was greater ( $P < 0.05$ ) during the diestrous phase (99%), however, this pattern was not observed ( $P > 0.05$ ) for transitional and primary follicles [these follicular categories were analyzed together due the low number of primary follicles ( $n = 5$ ) observed in anestrous phase for the follicular morphology].



**Figure 2** Mean ( $\pm$  SEM) percentage of morphologically normal equine preantral follicles according to class distribution in ovarian biopsy fragments collected during anestrous and diestrous phases. <sup>a,b</sup> Within the same follicle class and overall mean, values without a common letter differed ( $P < 0.05$ ). Because of the low number of primary follicles ( $n = 5$ ) observed in the anestrous phase, transitional and primary follicles classes were combined.

The percentage of normal preantral follicles in biopsy fragments collected during anestrous and diestrous phases according to the presence of different ovarian structures are shown (Table 5). Within the same ovarian structures (follicles  $\leq 26$  mm) a greater ( $P < 0.05$ ) percentage of normal follicles was observed during the diestrous phase. Moreover, on the diestrous phase the percentage of normality tended to be higher ( $P \leq 0.09$ ) in fragments collected from ovaries with a corpus luteum compared to ovaries with preovulatory follicles ( $\geq 36$  mm). Overall, a higher ( $P < 0.05$ ) preantral follicle normality rate was observed in biopsy fragments harvested from ovaries with the presence of a corpus luteum.

**Table 5** Percentage of morphologically normal equine preantral follicles in biopsy fragments collected during anestrus and diestrus phases, and presence of different ovarian structures (antral follicles and corpus luteum).

Ovarian structures	Normal preantral follicles (%)		
	Anestrus	Diestrus	Overall
‡	(n = 582) <sup>†</sup>	(n = 796)	(n = 1,378)
Follicles ≤ 26 mm	96.6 <sup>a</sup> (562/582)	98.8 <sup>bAB</sup> (352/356)	97.4 <sup>A</sup> (914/938)
Preovulatory follicle	-	97.9 <sup>A</sup> (96/98)	97.9 <sup>A</sup> (96/98)
Corpus luteum	-	99.7 <sup>B§</sup> (341/342)	99.7 <sup>B</sup> (341/342)

‡ Follicles ≤ 26 mm: 6 to 26 mm of diameter; Preovulatory follicle: ≥ 36 mm; and Corpus luteum: ≥ 30 mm, days 4 to 12 of estrous cycle.

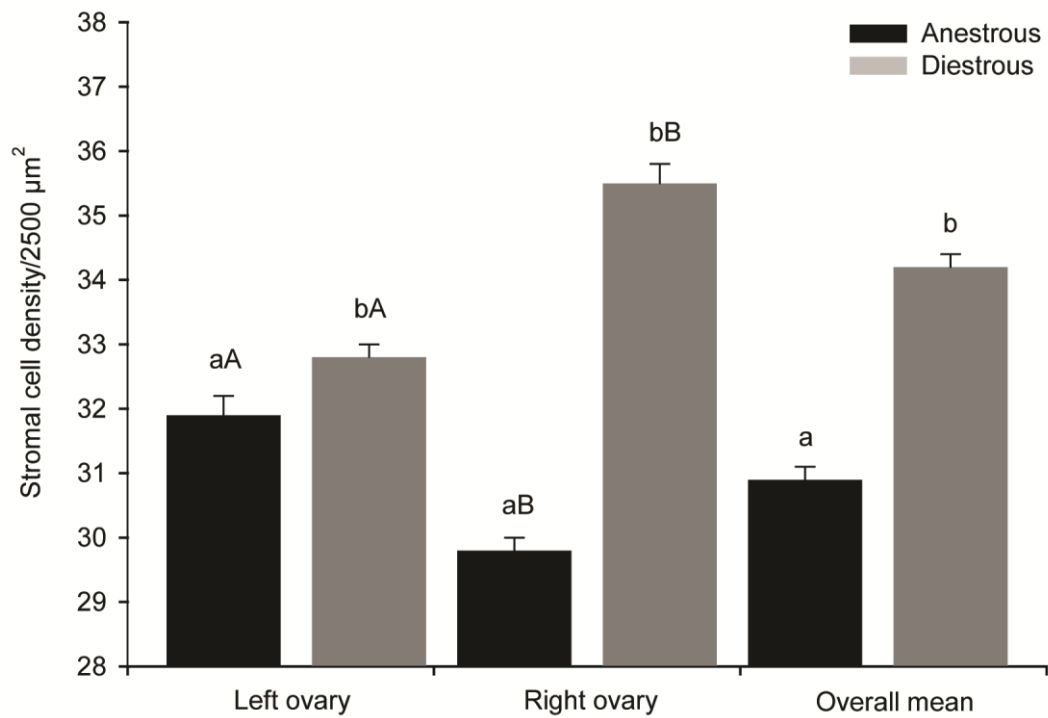
† Total number of preantral follicles evaluated.

<sup>a,b</sup> Within a row, values without a common superscript differed ( $P < 0.05$ ).

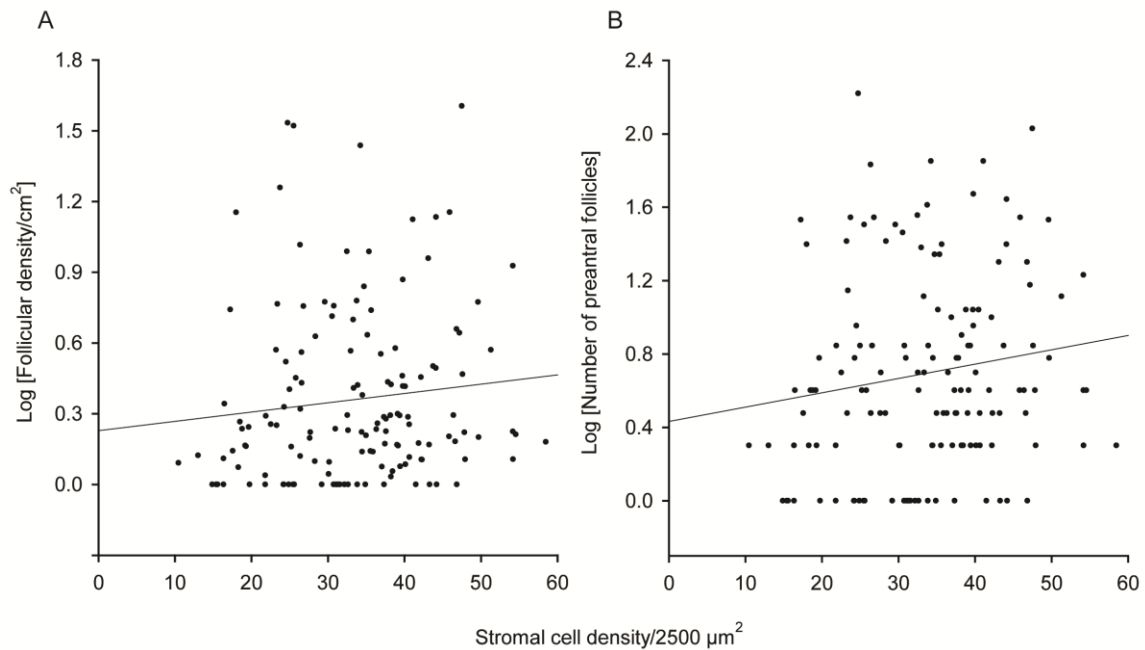
<sup>A,B</sup> Within a column, values without a common superscript differed ( $P < 0.05$ ).

§ Tended to differ from the preovulatory follicle ( $P \leq 0.09$ ).

The mean stromal cell density (cells/2,500  $\mu\text{m}^2$ ) of the ovarian fragments was  $32.0 \pm 0.1$  (range, 6 to 64 cells; CV = 37%). The cell density increased ( $P < 0.05$ ) on diestrus ( $34.2 \pm 0.1$ ) when compared to anestrus ( $30.9 \pm 0.1$ ) phase, regardless to the ovary side (Fig. 3). In addition, comparing the ovary sides within the same reproductive phase, we observed that the stromal cell density was higher ( $P < 0.05$ ) for the left during the anestrus and higher ( $P < 0.05$ ) for the right during diestrus phase. Positive correlations of stromal cell density with follicular density (tended to differ;  $P < 0.07$ ) and number of preantral follicles ( $P < 0.05$ ) per ovarian fragment were observed (Fig. 4A, B).

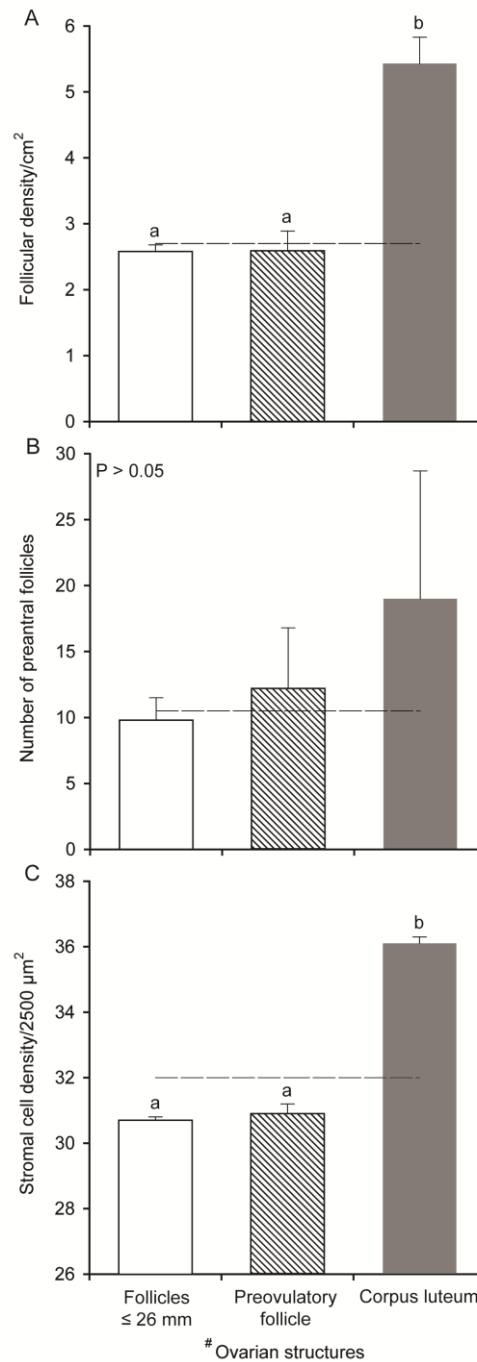


**Figure 3** Mean ( $\pm$  SEM) density of equine ovarian stromal cell in biopsy fragments collected from left and right ovaries during anestrus and diestrus phases. <sup>a,b</sup> Within the same ovarian side and overall mean, values without a common letter differed ( $P < 0.05$ ). <sup>A,B</sup> Within the same reproductive phase, left and right ovary values without a common letter differed ( $P < 0.05$ ).



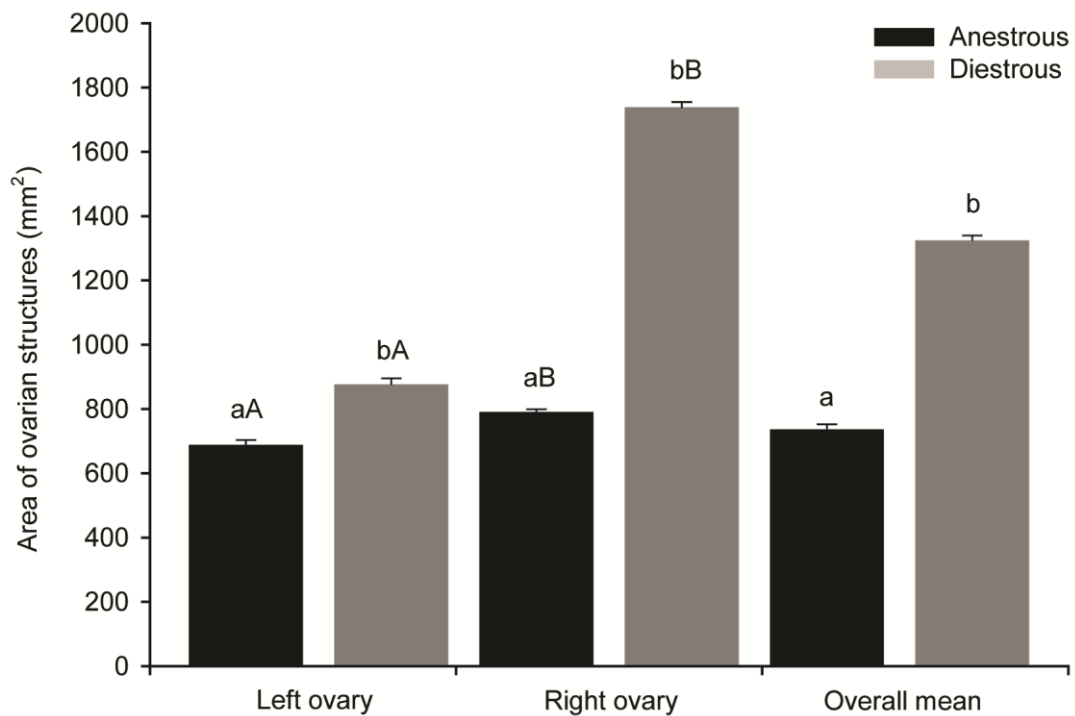
**Figure 4** Correlation between (A) follicular density and stromal cell density, and (B) number of preantral follicles and stromal cell density. The association among variables (black line) was evaluated by Spearman correlation coefficient [(A):  $r = 0.15$ ,  $P \leq 0.07$ ; (B):  $r = 0.16$ ,  $P < 0.05$ ]. Each point on the chart represents one ovarian fragment evaluated ( $n = 142$ ).

The follicular density, number of preantral follicles, and stromal cell density evaluated according to ovarian structures are shown (Fig. 5). Biopsy fragments harvested from ovaries with corpus luteum showed a greater ( $P < 0.05$ ) mean follicular density ( $5.4 \pm 0.4$ ) when compared with fragments from ovaries with preovulatory follicles ( $2.5 \pm 0.3$ ) or follicles  $\leq 26$  mm ( $2.5 \pm 0.1$ ; Fig. 5A). Nevertheless, the mean number of preantral follicles recorded was similar ( $P > 0.05$ ) among fragments from ovaries with all types of structures (follicles  $\leq 26$  mm:  $9.8 \pm 1.7$ ; preovulatory follicle:  $12.2 \pm 4.6$ ; and corpus luteum:  $19.0 \pm 9.7$ ; Fig. 5B). The mean stromal cell density was similar ( $P > 0.05$ ) among ovaries with follicles  $\leq 26$  mm ( $30.7 \pm 0.1$ ) and preovulatory follicles ( $30.9 \pm 0.3$ ) and differed ( $P < 0.05$ ) from ovaries with corpus luteum ( $36.1 \pm 0.2$ ; Fig. 5C).

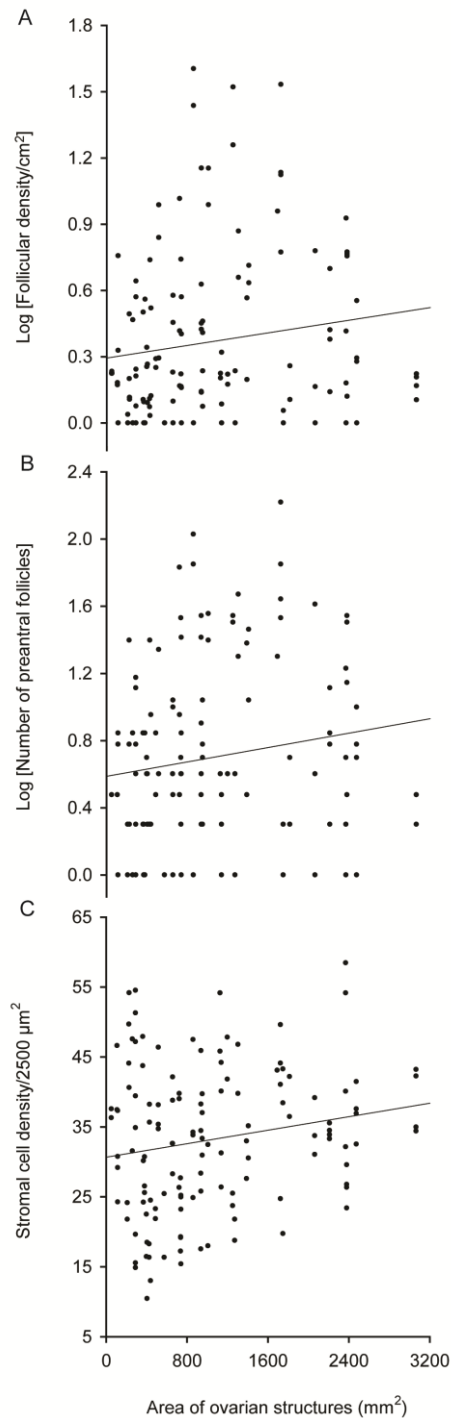


**Figure 5** Mean ( $\pm$  SEM) (A) follicular density, (B) number of preantral follicles, and (C) ovarian stromal cell density in biopsy fragments collected from ovaries with antral follicles and corpus luteum. Dashed line represents the average for (A) follicular density, (B) number of preantral follicles, and (C) stromal cell density. <sup>a,b</sup> Within the same parameter evaluated, values without a common letter differed ( $P < 0.05$ ). <sup>#</sup> Follicles  $\leq 26$  mm: 6 to 26 mm in diameter; Preovulatory follicle:  $\geq 36$  mm; and Corpus luteum:  $\geq 30$  mm, days 4 to 12 of estrous cycle.

In addition, the overall mean area ( $\text{mm}^2$ ) of ovarian structures was greater ( $P < 0.05$ ) on diestrous phase and the right ovary showed a greater area ( $P < 0.05$ ) of ovarian structures when compared to the left ovary regardless the reproductive phase (Fig. 6). A positive correlations ( $P < 0.05$ ) was observed between the area of ovarian structures and the follicular density, number of preantral follicles, and stromal cell density per ovarian fragment (Figure 7A, B and C).



**Figure 6** Mean ( $\pm$  SEM) area ( $\text{mm}^2$ ) of equine ovarian structures (antral follicles and corpus luteum) during the biopsy pick-up procedures performed during anestrus and diestrous phases. <sup>a,b</sup> Within the same ovarian side and overall mean, values without a common letter differed ( $P < 0.05$ ). <sup>A,B</sup> Within the same reproductive phase, left and right ovary values without a common letter differed ( $P < 0.05$ ).



**Figure 7** Correlation coefficients between (A) follicular density, (B) number of preantral follicles, and (C) ovarian stromal cell density with area of ovarian structures (antral follicles and corpus luteum). The association among variables (black line) was evaluated by Spearman correlation [(A):  $r = 0.21$ , (B):  $r = 0.21$ , (C):  $r = 0.10$ ;  $P < 0.05$ ]. Each point on the chart represents one ovarian fragment evaluated.

## Discussion

To our knowledge, this study reports for the first time in mares the influence of reproductive phases (anestrous and diestrous), side ovary (left and right), and the ovarian structures (antral follicles and corpus luteum) on the number of preantral follicles, follicular density, and ovarian stromal cell density from the ovarian biopsy fragments. In addition, due to the reproductive similarities between mares and women (Carnevale 2008, Ginther 2012) studies with assisted reproductive procedures in mares (animal model) provide relevant information that can be applied on clinical human reproduction.

The mean follicular density was 2.7 follicles/cm<sup>2</sup> and we observed an increase of 66% on density during diestrous when compared to anestrous phase. A high variation in the follicular density among and within the same animals was detected on reproductive phases (anestrous: 0-32; diestrous: 0-39 follicles/cm<sup>2</sup>). Furthermore, the right (69%) and left (57%) ovaries showed an increase of follicular density rates on diestrous with a high follicular heterogeneity.

Several studies reported the physiological follicular heterogeneity on ovarian fragments harvested from biopsy procedures (women: Schmidt *et al.* 2003, Rice *et al.* 2008, cow: Aerts *et al.* 2008, mare: Haag *et al.* 2013a,b,c) and slaughterhouse ovaries (cow: Silva-Santos *et al.* 2011, ewe: Fransolet *et al.* 2014). These results suggest that the preantral follicles are unevenly distributed among left and right ovaries of the same animal and the follicular density may be different among them. This is an individual characteristic and useful to decide the best ovary to perform the ovarian biopsy procedure. The reproductive phase can clearly affect the reserve of preantral follicles or antral follicular population on different species (mare: Driancourt *et al.* 1983, women: Qu *et al.* 2000, camel: Abdoon 2001, buffalo: Gupta *et al.* 2007, cow: Gendelman *et al.* 2010). One of the biggest challenges for experts in animal or human reproduction is to obtain ovarian biopsy fragments with greater density of preantral follicles for use in ARTs and these results can guide them regarding to the best reproductive phase to realize the tissue collection.

The overall mean number of equine preantral follicles in biopsies fragments was similar among ovaries (left and right) and reproductive phases (anestrous and diestrous). The results do not reflect the real potential of biopsy procedure because of two factors: the biopsies were performed in different spots of ovarian stroma recovering fragments with different sizes and the heterogeneity of follicular population (Lass *et al.*

1997). On the other hand, the follicular density uses an established measure unity (area) which equalizes all samples providing a more accurate evaluation of the follicular population in biopsy fragments that could be useful in the fields of ovarian tissue cryopreservation or transplantation.

Primordial follicles constitute the sole and critical reserve for all further follicle recruitment (Forabosco & Sforza 2007). A higher proportion of primordial follicles was observed on anestrus, however, the proportions of growing follicles (transitional and primary) were greater (primary tended to differ) during the diestrus. Moreover, the overall percentage of normal follicles was higher on diestrus (99%).

This fact could be explained by the process of activation (Fortune 2003) that is increased due the stimulatory factors as steroids (estradiol and progesterone), gonadotropins (FSH and LH), and growth factors (epidermal growth factor - EGF, transforming growth factor beta - TGF- $\beta$ , and vascular endothelial growth factor - VEGF) which are elevated during the breeding season (Ginther *et al.* 2003, Donadeu & Watson 2007). Studies *in vitro* showed the benefits of these factors on the development and viability of preantral follicles (Roy & Treacy 1993, Eppig & O'Brien 1996, Cushman *et al.* 2001, Celestino *et al.* 2011, Araujo *et al.* 2014).

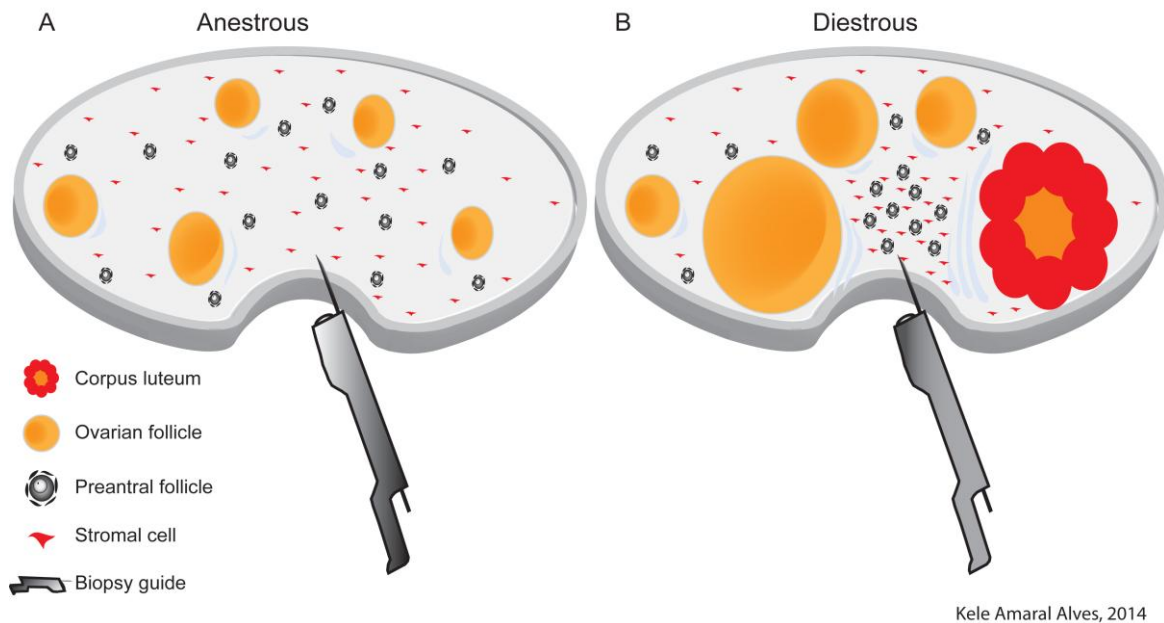
Diestrus phase showed a higher ovarian stromal cell density for both ovary sides when compared with anestrus. Overall, we observed an increase of 10% and a positive correlation of stromal cell density with the number and density of preantral follicles. Stromal cell density (in number of cells per  $\mu\text{m}^2$ ) in mares was different when compared to bitches (1.7-fold higher, Commin *et al.* 2012), or goats and ewes (8.5-fold lower, Faustino *et al.* 2010). The stromal cells are essential for the development and survive of grafted isolated follicles and have an important role in co-culture systems (Tingen *et al.* 2011, Dath *et al.* 2011). Furthermore, theca cells are derived from stromal cells and together produce steroids (androgens) and growth factors (TGF- $\beta$ , EGF, bone morphogenetic protein 7 - BMP-7) that are engaged on the follicular activation and viability (Palermo 2007, Orisaka *et al.* 2009, Young & McNeilly 2010, Qiu *et al.* 2013). Once the stromal cell and follicular density has an important role in female fertility, the association of these factors should be considered to define the most suitable ovarian biopsy fragments to be applied on ARTs.

In our study, the biopsies were performed in different reproductive conditions (anestrus and diestrus). In order to explain the influence of ovarian structures on the

number of preantral follicles and follicular and stromal cell density, we analyzed the data in two ways: (1) total area of ovarian structures (small follicles, preovulatory follicle, and corpus luteum if present), and (2) type of ovarian structures at the moment of biopsy procedure. Regarding the first aspect the overall mean area of ovarian structures increased over 79% on diestrous. In addition, the area of structures had a positive correlation with the number of preantral follicles, follicular density and stromal cell density and the right ovary showed a superior percentage area of 27% (anestrous) and 119% (diestrous) when compared to the left side. Moreover, biopsy fragments collected from ovaries with a corpus luteum showed: a 2-fold higher follicular density, an increasing of 15% on stromal cell density, and greater proportion of normal preantral follicles.

Studies reported the influence of corpus luteum on number of preantral follicles in different species (bovine: Gao *et al.* 2001, bubaline: Gupta *et al.* 2007, ovine: Al-Gubory & Martinet 1987). The primary function of the corpus luteum is the production of progesterone (Auletta & Flint 1987) and prostaglandin F<sub>2</sub> $\alpha$  (Niswender *et al.* 2000) and these hormones can promote better quality of preantral follicles (Kezele & Skinner 2003). However, none of them are related with the quantity of preantral follicles.

To our knowledge, there are no studies that described the association among the number of preantral follicles, follicular density, and stromal cell density with the area of ovarian structures for any species. This fact allowed us to propose a hypothesis that support the linked results observed in our study. During anestrous phase there is a low follicular activity and small follicles are present in the ovarian stroma. In contrast, on diestrous phase multiple structures as small, medium and preovulatory follicles and corpus luteum can be present in the same ovary. This fact, indicate that denser structures as corpus luteum associated or not to larger antral follicles can exert a greater pressure on the ovarian parenchyma and causes a consequently clustering of preantral follicles and stromal cell per unit area. At the anestrous the absence of large structures allow lower compression of ovarian tissue and possible dispersion of preantral follicle population (Fig. 8).



**Figure 8** Model proposing the difference of follicular density, number of preantral follicles, and stromal cell density among anestrus (A) and diestrus (B) phases. The increase of the area of ovarian structures during the diestrus due the presence of large preovulatory follicle and corpus luteum may increase the ovarian compression and consequently clustering of preantral follicles and stromal cell per unit area in ovarian fragments harvested by biopsy procedure.

In summary, our results showed that: (1) the diestrus phase influenced positively the quality of preantral follicles, class distribution, follicular density and stromal cell density; (2) the area of ovarian structures modified the stromal cell density and dynamics of preantral follicles regarding the number and follicular density; and (3) the corpus luteum has a positive effect on the quality of preantral follicles, follicular density and stromal cell density. In addition, we demonstrated the perspective of a possible ideal scenario for ovarian biopsy procedures which can be used in women to increase the chances of collection the ovarian fragments with better quality, i.e. higher follicular and stromal cell density. These findings reinforce the concept of the mare as a potential animal model to provide comparative insights about preantral follicle population.

#### **Declaration of interested**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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## CAPÍTULO 5 – CONSIDERAÇÕES FINAIS

As experiências obtidas durante o período de doutoramento trouxeram grande realização pessoal como a evolução do modo de agir e pensar científico, aumentando os horizontes no sentido da percepção e capacidade de análise de resultados. Neste âmbito, veio a tona a importância de um desenho experimental prévio com o desenvolvimento de hipóteses e objetivos claros, pois iniciar esta jornada com os caminhos e intenções traçadas tornou o processo ágil e seguro. Além disso, a pesquisa básica constitui uma base sólida de informações para aplicação em futuros procedimentos e estudos mais ambiciosos e tecnicados. O estabelecimento de parcerias acadêmicas como as realizadas durante o doutorado sanduíche no exterior proporcionou crescimento profissional e valiosas lições a serem carregadas por toda a vida.

Capítulo 2 - Durante o período de execução do primeiro projeto, a partir de ovários provenientes de abatedouro no Brasil, as frustrações eram frequentes em relação ao baixo número de folículos pré-antrais encontrados nas secções histológicas. Porém, avaliando os resultados de outra maneira, considerando a área das secções histológicas, foi possível calcular pela primeira vez a densidade folicular em fragmentos ovarianos equinos, além de observar a alta heterogeneidade fisiológica da população de folículos pré-antrais nas éguas. Fato semelhante aos resultados reportados para densidade folicular em outras espécies, inclusive na mulher. Além disso, também foi determinado pela primeira vez, o intervalo de secção mais apropriado para a realização de procedimentos histológicos no tecido ovariano equino. Devido a particular morfologia ovariana equina, onde a região medular está invertida, é altamente vascularizada e recobre o ovário com uma densa camada de tecido conjuntivo, pesquisas reportaram que as técnicas histológicas convencionais eram inadequadas para a avaliação do tecido ovariano.

Capítulo 3 - No segundo experimento, foi executada a técnica de biópsia ovariana, guiada por ultrassom, e avaliou-se a densidade folicular nos fragmentos coletados. Os resultados demonstraram uma densidade, distribuição de classes e morfologia folicular similares as do primeiro experimento, ou seja, alta variabilidade entre secções do mesmo fragmento, entre fragmentos do

mesmo animal e entre animais. Além disso, a utilização de um modelo matemático possibilitou a definição do desenho experimental (número de fragmentos ovarianos vs. número de secções necessárias por fragmento) para estimar a densidade folicular equina em fragmentos de biópsia e que poderá ser adotado no delineamento experimental de espécies onde a disponibilidade de material é escasso ou de difícil acesso (ex. Mulher).

Capítulo 4 - Os resultados do terceiro experimento demonstraram uma forte ligação entre as variáveis avaliadas, de tal modo que, estimulou a construção de uma hipótese que esclarece a dinâmica da densidade, qualidade e da classificação dos folículos pré-antrais nos ovários do mesmo animal, em diferentes fases do ciclo reprodutivo e na presença de estruturas ovarianas (folículos antrais e corpo lúteo). Portanto, estes resultados podem guiar os procedimentos de biópsia ovariana quanto ao melhor cenário (ovário a ser coletado, fase reprodutiva e estrutura ovariana presente) para a obtenção de fragmentos ovarianos com melhor qualidade (alta densidade folicular e celular) para aplicação clínica animal e medicina reprodutiva humana.

Todas as conclusões obtidas destes experimentos já estão servindo de base para estudos com novas técnicas de criopreservação e transplante de tecido ovariano.