UNIVERSIDADE ESTADUAL PAULISTA - UNESP

INSTITUTO DE BIOCIÊNCIAS - CAMPUS BOTUCATU

ESTUDO DE ASSOCIAÇÃO GENÔMICA E PERFIL DE EXPRESSÃO GÊNICA DE FOLÍCULOS ANTRAIS EM NOVILHAS DAS RAÇAS NELORE E ANGUS, ASSOCIADAS AO FENÓTIPO DE ALTA E BAIXA POPULAÇÃO FOLICULAR

Por

MAURÍCIO GOMES FAVORETO

Orientador

CIRO MORAES BARROS

BOTUCATU

2015

ESTUDO DE ASSOCIAÇÃO GENÔMICA E PERFIL DE EXPRESSÃO GÊNICA DE FOLÍCULOS ANTRAIS EM NOVILHAS DAS RAÇAS NELORE E ANGUS, ASSOCIADAS AO FENÓTIPO DE ALTA E BAIXA POPULAÇÃO FOLICULAR

Por

MAURÍCIO GOMES FAVORETO

Orientador

CIRO MORAES BARROS

Tese Apresentada Programa de Pós Graduação do Instituto de Biociências de Botucatu - Departamento de Farmacologia, Universidade Estadual Paulista "JÚLIO DE MESQUITA FILHO" – UNESP - CAMPOS DE BOTUCATU Para Obtenção do Título de DOUTOR EM FARMACOLOGIA.

BOTUCATU

2015

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Favoreto, Maurício Gomes.
Estudo de associação genômica e perfil de expressão gênica de folículos antrais em novilhas das raças nelore e angus, associadas ao fenótipo de alta e baixa população folicular / Maurício Gomes Favoreto. - Botucatu, 2015

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Ciro Moraes Barros
Coorientador: Jose Buratini Junior
Capes: 21001006

1. Bovino - Reprodução. 2. Genômica. 3. Expressão gênica. 4. Nelore (Zebu). 5. Folículos.

Palavras-chave: Bovino; Expressão gênica ; Folículos; GWAS; Reprodução.

“À minha esposa Bárbara Loureiro e minha filha Isadora Loureiro Favoreto por tudo que representam em minha vida.”

“Aos meus pais Odilon Soares Favoreto e Maria da Penha Gomes Favoreto pela incansável luta para tornar meus sonhos realidade.”

“COM AMOR DEDICO”

AGRADECIMENTOS

Aos meus irmãos, Tatiana Gomes Favoreto e Willian Gomes Favoreto que apesar da distância estão sempre presentes com apoio e orações. Vocês também fazem parte desta conquista.

Aos meus avós José Favoreto, Elda Soares Favoreto, José Batista Gomes (*in memoriam*) e Rufina Vial Gomes (*in memoriam*) pelo exemplo de vida.

A todos meus familiares que participaram desta caminhada, o meu sincero obrigado.

Ao meu orientador Prof. Dr. Ciro Moraes Barros, exemplo de profissional, agradeço não somente pelo acolhimento em seu laboratório, mas também de minha esposa e minha filha. Agradeço por compartilhar um pouco de seu vasto conhecimento adquirido ao longo de tantos anos dedicados a pesquisa e finalmente, agradeço a total confiança e liberdade para realizar meus trabalhos.

Ao meu co-orientador Prof. Dr. José Buratini que contribuiu cientificamente na elaboração do projeto e por permitir livre acesso ao seu laboratório sempre que necessário.

Aos membros da banca pela disponibilidade e em especial ao Prof. Dr. Mario Binelli pela grande contribuição científica.

Aos meus amigos do famoso Lab. Ciro: Rafael, Ronaldo, Paty, Anthony, Mari, Rafaela, Eduardo Razza, Eduardo Lucaccin, Kaká e Janahi pela paciência, ajuda e companheirismo. Foram vários momentos felizes dentro e fora do Lab. que sentirei saudades.

À seção de Pós Graduação do Instituto de Biociências de Botucatu, professores e funcionários do Departamento de Farmacologia pela dedicação e atenção.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pela concessão da bolsa de Doutorado (Processo: 2011/17370-0) e pela concessão do auxílio de pesquisa (2011/50964-0), sem os quais não seria possível a realização destes trabalhos.

MUITO OBRIGADO!

SUMÁRIO

LISTA DE TABELAS

Tabelas **Páginas**

LISTA DE FIGURAS

Figuras **Páginas**

RESUMO

Resumo da Tese Apresentada ao Programa de Pós Graduação da Universidade Estadual Paulista "Julio de Mesquita Filho" Para Obtenção do Título de Doutor em Farmacologia

ESTUDO DE ASSOCIAÇÃO GENÔMICA E PERFIL DE EXPRESSÃO GÊNICA DE FOLÍCULOS ANTRAIS EM NOVILHAS DAS RAÇAS NELORE E ANGUS ASSOCIADAS AO FENÓTIPO ALTA E BAIXA POPULAÇÃO FOLICULAR

Maurício Gomes Favoreto

Março de 2015

Animais com alta população folicular (APF) antral possuem melhor desempenho reprodutivo quando comparados a animais de baixa população folicular (BPF). Essa variação no número de folículos antrais pode estar associada a diferentes perfis hormonais e de expressão de genes reguladores da ativação e crescimento folicular. Objetivou-se com o presente trabalho: 1) avaliar o perfil global de expressão gênica de folículos antrais em animais com APF e BPF nas raças Nelore (*Bos indicus*) e Aberdeen Angus (*Bos taurus*) e 2) identificar diferenças genotípicas através de um estudo de associação genômica (GWAS - *Genome Wide Association Study*) usando polimorfismos de nucleotídeo de sítio único (SNPs). Inicialmente foram avaliadas 155 novilhas da raça Nelore e 132 da raça Angus com idade e peso semelhantes. Os animais foram sincronizados e a contagem dos folículos realizada através de ultrassonografia 24 a 48 horas após o início do estro. Os grupos APF e BPF foram formados com base na média do número de folículos ± desvio padrão nas duas raças. Para avaliar o perfil global de expressão gênica usou-se a técnica do microarranjo. Um total de 15 novilhas Nelore (6 APF e 9 BPF) e 17 novilhas Angus (9 APF e 8 BPF) foram abatidas 12 a 24 horas após a ovulação e os ovários transportados ao laboratório. Foi realizado um *pool* de RNA extraído de três folículos (≤ 4 mm) individualmente (um *pool* por animal) dissecados do ovário. A análise dos dados procedeu-se no FlexArray 1.6.1.1 e os genes com "*fold change*" > 1,5 e P ≤ 0,05 foram considerados diferencialmente

expressos. Para o estudo de GWAS 72 novilhas da raça Nelore (32 APF e 40 BPF) e 48 da raça Angus (21APF e 27 BPF) foram selecionadas. O DNA foi extraído do sangue e/ou bulbo capilar e a genotipagem foi realizada com o chip 777 HD bovine Illumina®. Os SNPs foram submetidos ao controle de qualidade usando como critérios de exclusão: *call rate* (IDCR) ≤ 90%, MAF ≤ 0,02, *call rate* (SNPCR) ≤ 0,98 e HWE ≤ 1 x 10^{-5}. Após o controle de qualidade os SNPs foram submetidos ao teste Cochran-Armitage para associação genótipo/fenótipo nas duas raças. Os genes diferencialmente expressos e os genes associados aos SNPs foram submetidos à análise de enriquecimento funcional pela ferramenta online DAVID. A análise da expressão gênica revelou 1205, 311 e 315 (log2; *fold change* > 1,5) genes diferencialmente expressos entre raças, grupos e interação raça x grupo, respectivamente. Resultados das análises da expressão global dos genes apontaram diversos genes que estão mais expressos em folículos de novilhas Nelore associados à ativação e crescimento folicular, destacando-se genes do sistema IGF1. Entre os grupos, genes relacionados à via de sinalização MAPK (CACNA1S, NTRK1 e FGFR1) de proliferação celular foram mais expressos em novilhas de APF. No estudo de GWAS 181 e 201 SNPs tiveram associação genótipo/fenótipo (P ≤ 10E-3) nas raças Nelore e Angus, respectivamente. Um total de 97 genes foram associados aos 181 SNPs na raça Nelore e a análise funcional identificou genes da via ROBO-STLIT que podem estar envolvidos no controle da migração das células germinativas para o ovário. Na raça angus 57 genes foram associados aos 201 SNPs, destacando-se o gene Fibrilin 1 (FBN1) envolvidos na regulação de fatores de crescimento. Os resultados mostraram que existem diferenças no perfil de expressão gênica de folículos antrais entre as raças Nelore e Angus e entre os grupos de APF e BPF. Além disso, existem diferenças genotípicas entre os grupos APF e BPF em cada raça estudada.

Palavras Chave: Bovino; Expressão gênica; Folículos; GWAS; Reprodução.

ABSTRACT

Abstract of the Dissertation presented to the Pos Graduation Program of the Universidade Estadual Paulista "Julio de Mesquita Filho" as a requirement for the degree of doctor in Pharmacology

GENOMIC ASSOCIATION AND GENE EXPRESSION PROFILE IN ANTRAL FOLLICLES FROM NELORE AND ANGUS HEIFERS ASSOCIATED WITH THE HIGH AND LOW FOLLICLE COUNT PHENOTYPE

Maurício Gomes Favoreto

March of 2015

Animals with high antral follicle count (HFC) have a better reproductive performance when compared with animals with low follicle count (LFC). This variation in the number of antral follicles can be associated with different hormonal profiles and expression of genes regulating activation and follicular growth. The objective of the present work was: 1) to evaluate the global gene expression of antral follicles in animals with HFC and LFC in Nelore (*Bos indicus*) and Aberdeen Angus (*Bos taurus*) heifers and 2) to identify genotypic differences through a genomic wide association study (GWAS) using single nucleotide polymorphisms (SNP). Initially, 155 Nelore heifers and 132 Angus heifers with similar body weight and age were selected. The animals were synchronized and follicle count was done through ultrasound 24 to 48 h after estrus. The groups LFC and HFC were formed using the average of follicles ± standard deviation in both breeds. To evaluate the global gene expression profile we use a microarray chip. A total of 15 Nelore heifers (6 HFC and 9 LFC) and 17 Angus heifers (9 HFC and 8 LFC) were slaughter 12 to 24 h after ovulation and ovaries were transported to the laboratory. A pool of RNA from three follicles (< 4 mm) from each animal was used to the analysis using FlexArray 1.6.1.1 and the genes with fold change > 1.5 and $P \leq 0.05$ were considered differentially expressed. For the GWAS study 72 Nelore heifers (32 HFC and 40 LFC) and 48 Angus heifers

(21 HFC and 27 LFC) were selected. The DNA was extracted from blood and hair bulb and genotyping was done using the 777 HD Illumina chip. The SNPs were submitted to a quality control test using an exclusion criteria: *call rate* (IDCR) ≤ 90%, MAF ≤ 0.02, *call rate* (SNPCR) ≤ 0.98 and HWE ≤ 1 x 10^{-5}. After the quality control test the SNPs were submitted to the Cochran-Armitage test to the association of phenotype/genotype in both breeds. The genes differentially expressed and the genes associated with the SNPS were submitted to an enrichment analysis through the on line DAVID tool. The gene expression analysis revealed 1205, 311 and 315 (log2; *fold change* > 1.5) genes differentially expressed between breed, groups and interaction breed x group, respectively. Results from the global gene expression analysis showed several genes associated with follicle activation and growth over expressed in follicles from Nelore heifers, with highlights to the IGF1 system. Between groups, genes related to MAPK signaling (CACNA1S, NTRK1 and FGFR1) of cellular proliferation were over expressed in heifers from the HFC group. In the GWAS study 181 and 201 SNPs had a genotype/phenotype association (P ≤ 10E-3) in Nelore and Angus heifers, respectively. A total of 97 genes were associated to the 181 SNPs in the Nelore breed and the functional analysis identified genes in the ROBO-STLIT pathway that can be involved in the control of germ cell migration to the ovary. In the Angus heifers 57 genes were associated with the 201 SNPs, highlighting the Fribilin 1 (FBN1) gene, involved in regulation of growth factors. The results show differences in the gene expression profile in antral follicles from Nelore and Angus heifers and between HFC and LFC groups. Furthermore, there were genotypic differences between HFC and LFC groups within each breed studied.

Key words: Bovine; Gene Expression; Follicles; GWAS; Reproduction.

INTRODUÇÃO

O desempenho reprodutivo é um dos principais focos da pecuária, principalmente na espécie bovina. Visando obter uma maior exploração do potencial genético de fêmeas selecionadas para incremento do potencial produtivo, diversas biotécnicas de reprodução assistida, tais como a inseminação artificial (IA), transferência de embriões (TE), produção *in vitro* de embriões (PIV) e recentemente a clonagem, têm sido amplamente utilizadas. No entanto, visando maximizar a produção e melhorar os índices reprodutivos, pesquisadores e produtores têm voltado suas atenções não somente as biotécnicas de reprodução disponíveis, mas para características mensuráveis nas fêmeas bovinas. Dentre elas, a variação do número de folículos recrutados por onda folicular tem sido amplamente estudada recentemente.

Diversos trabalhos mostraram associação positiva entre número de folículos recrutados por onda folicular e fertilidade. Animais que possuam alta população folicular (APF) comparados com animais com média e baixa população folicular (BPF) apresentaram melhores desempenhos reprodutivos quando submetidos á técnicas de reprodução assistida (Ireland et al., 2007; 2009; Mossa et al., 2012). Este fenótipo de APF se torna ainda mais atrativo, pois, apesar de haver variação do número de folículos entre os animais de mesma raça, esta característica possui alta repetibilidade em um mesmo animal (Burns et al., 2005). Embora a ativação e o desenvolvimento de um grupo de folículos em uma onda folicular tenham sido amplamente estudados ao longo de décadas, e uma gama de proteínas e hormônios foram identificados como essenciais, a complexidade biológica destes eventos não estão completamente elucidadas.

Após a conclusão do projeto de sequenciamento do genoma bovino um enorme número de marcadores moleculares do tipo polimorfismo de nucleotídeo de sítio único (SNPs – *single*

nucleotide polymorphism) foram descobertos e estão sendo utilizados para pesquisas genômicas em bovinos. Estudos de associação genômica (GWAS – *Genome wide Association Study*) permitem identificar estas variações no DNA que estão correlacionadas com certa característica, apontando para a localização e identificação dos genes envolvidos com a expressão da característica fenotípica de interesse e, com isso, auxiliar a compreensão dos mecanismos biológicos (Ku et al., 2010). Uma das limitações dessa tecnologia é a necessidade da identificação dos SNPs ligados aos fenótipos de interesse para cada raça e em alguns casos dentro de uma população de uma mesma raça (Druet et al., 2010).

Baseado na premissa que a variação da população folicular antral entre indivíduos é determinada geneticamente, objetivou-se com o presente trabalho a identificação de diferenças genotípicas, através de um estudo de GWAS com polimorfismos de base única (SNPs), em novilhas das raças Nelore e Aberdeen Angus com o fenótipo de alta e baixa população folicular antral. Além disso, foi avaliado o perfil de expressão global de genes de folículos antrais através da técnica do microarranjo. Avanços na compreensão de mecanismos que envolvem a variação folicular de uma população podem resultar em novas estratégias para seleção de animais com maior fertilidade.

CAPÍTULO 1

1.1 REVISÃO DE LITERATURA

1.1 Foliculogênese

Na maioria dos mamíferos a foliculogênese inicia-se durante a vida fetal ou logo após o nascimento e pode ser definida como o processo de formação, crescimento e maturação folicular (Van Den Hurk & Zhao, 2005). Na espécie bovina a foliculogênese se inicia com o recrutamento de células da granulosa pelo ovário para formar os folículos primordiais ainda na vida embrionária, por volta de 90 a 120 dias de gestação (Tanaka et al., 2001; Burkhart et al., 2010; Yang & Fortune, 2008). Neste estágio o oócito se encontra com seu ciclo celular bloqueado na prófase 1 da meiose e somente após a ovulação haverá a retomada da meiose (Picton et al., 1998).

1.1.1 Morfologia e Estrutura do Folículo

Estruturalmente o folículo ovariano é composto por um oócito circundado por células somáticas (granulosa e tecais) e durante a ativação e crescimento folicular, sua morfologia é alterada, uma vez que o oócito cresce e as células circundantes se multiplicam e diferenciam (Lucci et al., 2001). De acordo com o grau de evolução e crescimento, os folículos podem ser classificados em pré-antrais (primordiais, primários e secundários) ou antrais (terciários e pré-ovulatórios) (Van Den Hurk & Zhao, 2005).

Os folículos primordiais são constituídos por um oócito quiescente, circundado por uma camada células da pré-granulosa pavimentosas de forma justaposta e sem junções específicas (Lucci et al., 2001). Essa classe de folículos permanece quiescente até a ativação, e depois entra

para o grupo de folículos em crescimento (primários, secundários e terciários) (Van Den Hurk & Zhao, 2005). Os mecanismos seletivos os quais alguns folículos começam a crescer e outros permanecem quiescentes ainda não estão completamente elucidados, porém estudos morfométricos sugerem que os folículos iniciam o crescimento baseados na ordem em que eles são formados (Hirshfield, 1991). Além disso, sabe-se que a ativação e desenvolvimento de folículos pré-antrais independe de estímulos gonadotróficos agudos e é regulado principalmente por fatores intraovarianos (Van Den Hurk & Zhao, 2005). Após a ativação, os folículos primordiais adquirem de forma gradual células da granulosa de morfologia cúbica e em seguida ocorre a transição para folículos primários.

Os folículos primários são formados por um oócito circundado por células da granulosa de formato cubóide dispostas em uma única camada (Lucci et al., 2001). Com a multiplicação das células da granulosa, uma nova camada de células é formada, dando origem ao grupo de folículos chamados secundários (Lucci et al., 2001). A intensa proliferação das células da granulosa leva a formação de uma cavidade repleta de liquido folicular, entre as camadas de células da granulosa, denominada antro, e a partir deste estágio os folículos passam a ser denominados terciários ou antrais. O fluido antral pode servir como uma importante fonte de substâncias regulatórias derivadas do sangue ou de secreções das células foliculares, i.e., gonadotrofinas, esteroides, fatores de crescimento, enzimas e lipoproteínas. Durante o desenvolvimento folicular, a produção de fluido antral é intensificada pelo aumento da vascularização folicular e permeabilidade dos vasos sanguíneos, os quais estão fortemente relacionados com o aumento do folículo antral (Van Den Hurk & Zhao, 2005).

1.1.2 Fatores Regulatórios Parácrinos e Autócrinos de Folículos Pré-antrais

Os fatores parácrinos e autócrinos envolvidos na ativação, diferenciação e crescimento dos folículos incluem proteínas e hormônios (Palma et al., 2012). Através de estudos com técnicas de cultivos *in vitro*, animais mutantes, inibidores específicos e abordagens de imuno-neutralizações, um grande número de fatores foram encontrados e descritos por serem importantes para ativação e desenvolvimento dos folículos. Entre os fatores, destacam-se: kit ligante (Fortune et al., 2010; Parrot & Skinner, 1999), Neurotrofinas (NTs) (Romero et al., 2002), Fator de Crescimento Vascular Endotelial (VEGF) (Kazele et al., 2005; Danforth et al., 2003), Fatores de Crescimento Transformantes (TGFβ) (Knight & Glister, 2006), Fator Inibidor de Leucemia (LIF) (Nilsson et al., 2002), Fatores de Crescimento Fibroblástico (FGFs) (Nilsson et al., 2001; Buratini et al., 2005; Machado et al., 2009), entre outros.

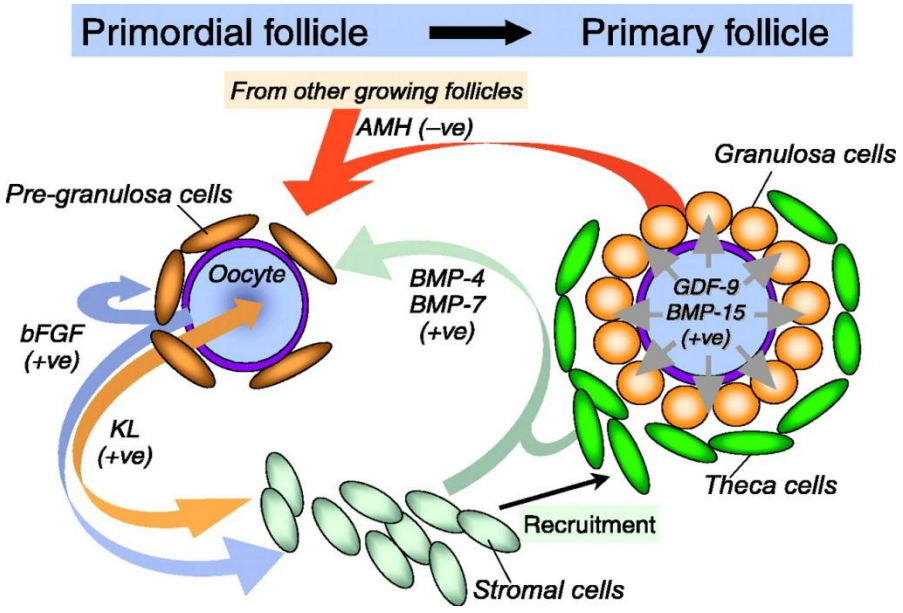


Figura 1.1. Potenciais interações de sinalizações (+ve, estimulatória; −ve, inibitória) envolvidas na transição de folículos primordiais para primários; kit ligante (KL) e fator de crescimento fibroblástico básico (bFGF) secretados pelas células da pré-granulosa e oócito

respectivamente, possuem efeitos estimulatórios no oócito e nas células da granulosa; eles também promovem o recrutamento no estroma/interstício das células da teca circundantes (Knight & Glister, 2006).

O Kit ligante é um fator produzido pelas células da granulosa e sua interação com seu receptor c-Kit podem ativar diferentes vias de sinalização (Celestino et al., 2009). Em um estudo *in vitro*, trabalhando com ovários de ratas e camundongos após o nascimento demonstrou-se que a via fosfatidilinositol 3 kinase (PI3K) no oócito é regulada pelo kit ligante e possui grande importância no desenvolvimento inicial do folículo (Reddy et al., 2005). Após o kit ligante ativar seu receptor ocorre fosforização da região intracelular, estimulando a atividade da PI3K o que leva a conversão de fosfatidilinositol bisfosfato (PIP2) a fosfatidilinositol trifosfato (PIP3). O PIP3 recruta e ativa a fosfatidilinositol - dependente da kinase 1, seguida pela ativação da molécula sinalizadora AKT que promove proliferação celular, sobrevivência e a síntese de proteínas e glicogênio (Jonh et al., 2008).

A superfamília dos TGFβ tem sido extensamente reportada na literatura como importantes peptídeos de regulação e desenvolvimento folicular (Knight & Glister, 2006). Dentre os TGFβs destacam-se: BMP-4 e BMP-7 expressos pelas células do estroma ovariano e/ou pelas células da teca; o GDF-9 e BMP-15 expressos pelos oócitos de mamíferos (Juengel & McNattty, 2005; Juengel et al., 2002; Nilsson & Skinner, 2003). Ambos GDF-9 e BMP-15 exercem seus efeitos bilógicos pela ligação a um receptor do tipo I, referido como um receptor ativina semelhante a kinase (ALK) e ao receptor de BMP do tipo II (Moore et al., 2003; Mazerbourg et al., 2004). A supressão do gene GDF-9 bloqueia o desenvolvimento a partir do folículo primário, diminui a proliferação das células da granulosa e causa crescimento anormal do oócito (Dong et

al., 1996), indicando que o GDF-9 possui papel importante no desenvolvimento folicular inicial. O outro fator derivado do oócito BMP-15 estimula a proliferação das células indiferenciadas da granulosa de forma FSH-independente (McNatty et al., 2000). Embora a expressão de GDF-9 e BMP-15 sejam importantes para fertilidade, há uma variação quanto ao requerimento destes fatores entre as espécies. Em camundongos a deleção específica do gene BMP-15 causa sub-fertilidade (Yan et al., 2001), no entanto, ovelhas homozigotas mutantes para inativação do gene BMP-15 são completamente inférteis (McNatty et al., 2005).

As proteínas BMP-4 e BMP-7 são expressas pelas células da teca e/ou células do estroma ovariano do folículo primário/secundário e possui papel funcional (Knight & Glister, 2006). O cultivo *in vitro* de ovários de ratas neonatal com BMP-4 aumentou o número de folículos pré-atrais em desenvolvimento (Nilsson & Skinner, 2003), e a administração intrabursal de BMP-7 em ratas *in vivo* aumentou o número de folículos pré-antrais e antrais (Lee et al., 2001) demonstrando a ação positiva destes fatores no desenvolvimento folicular.

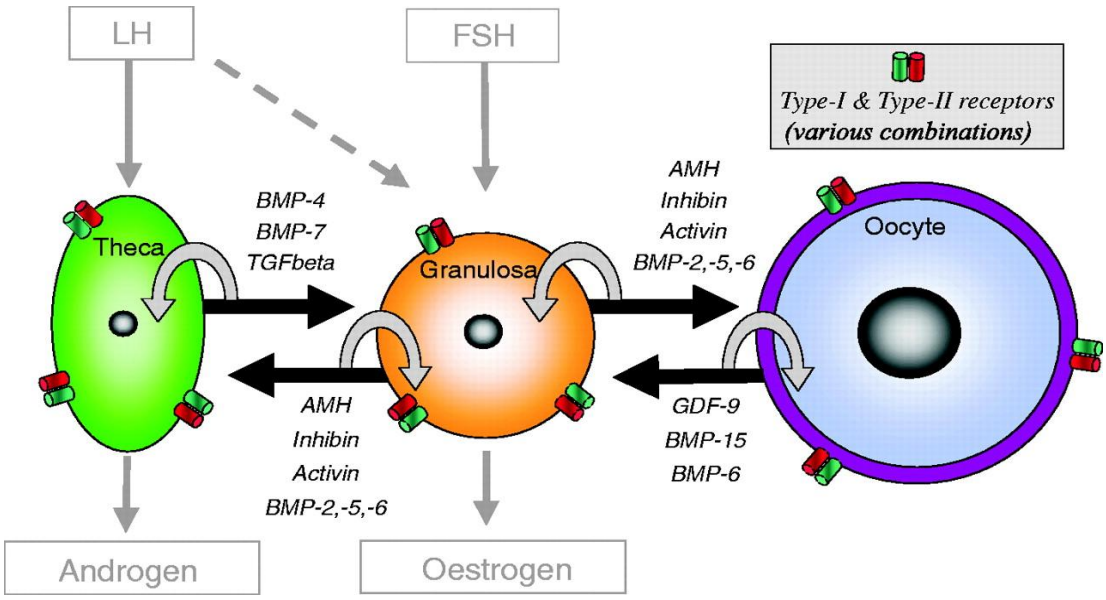


Figura 1.2. Membros da superfamília dos TGF-β fazem parte da crescente lista de ligantes extracelulares implicados na comunicação bidirecional entre as células da granulosa,

células da teca e oócito. Ambos os eventos de sinalizações autócrinas (setas de cor cinza) e parácrinas (setas de cor preta) são possíveis, dependendo da combinação apropriada de expressão dos receptores tipos I e II na superfície celular (Knight & Glister, 2006).

Existem evidências de que Neurotrofinas como o fator de crescimento do nervo (NGF) e seus receptores tipo tirosina-kinase (TrkA) são importantes na regulação da ativação folicular. Em Camundongos *Knockout* para o gene do NGF apresentaram um acentuado atraso no desenvolvimento folicular inicial, caracterizado pela redução do número de folículos primários e secundários por ovário (Romero et al., 2002). Outras dois tipos de Neurotrofinas (neutotropina 3 e 4 e BDNF) e seus receptores tipo tirosina-kinase (TrkB) também parecem ser muito atuante na sobrevivência dos oócitos de ratas neonatal com importância na manutenção de folículos primordiais (Paredes et al., 2004, Romero et al., 2002).

1.1.3 Desenvolvimento Antral dos Folículos

Durante a fase do ciclo estral e em intervalos de 7-10 dias, ocorre um súbito crescimento de pequenos folículos com 3 a 4 mm (onda de crescimento folicular), que pode ser detectada por ultrassonografia, caracterizando o desenvolvimento folicular antral (Guinter et al., 2003; Roche et al., 1998). As gonadotrofinas possivelmente não estão envolvidas no inicio do crescimento folicular, embora estudos tenham detectado a presença de RNA do receptor de FSH (FSHr) em folículos com uma ou duas camadas de células (Webb et al., 2003). No entanto, as fases finais de crescimento são controladas principalmente por mecanismos endócrinos, destacando-se as gonadotrofinas (FSH e o LH) e os hormônios esteroides (estradiol e progesterona; Webb et al., 2007).

O desenvolvimento de folículos antrais com 3 a 4 mm é um processo dependente de FSH (Austin et al., 2001) e a emergência da onda folicular é estimulada pelo aumento de sua concentração plasmática (Guinter et al., 2003). Esse grupo de folículos cresce a uma taxa similar durante dois ou três dias. Com 12 horas do pico de FSH, um grupo de três a cinco folículos maiores se destacam dos demais, e com a queda dos níveis de FSH plasmático, ocorre uma subsequente seleção de um folículo que se tornará dominante (Guinter et al., 2003; Beg & Guinter, 2001). Os fatores que regulam o recorrente aumento/diminuição nas concentrações de FSH durante o ciclo estral são originários do ovário, particularmente na fase de crescimento de folículos ≥ 5 mm (Gibons et al., 1997) e do folículo dominante (Guinter et al., 1996). A aromatase, uma enzima fundamental para síntese do estradiol, está presente em folículos com 4 mm, apesar disso, baixos níveis de estradiol estão presentes em folículos com 5 a 7 mm (Echternkamp et al., 1994; McNatty et al., 1984). Não se sabe se o estradiol em baixos níveis neste momento, entra na circulação em concentrações suficientes para levar a um efeito de *feedback* negativo no FSH após seu pico, durante seu declínio inicial (Guinter et al., 1996). Entretanto, um pouco antes ou no inicio da seleção de um folículo em novilhas holandesas, as concentrações de estradiol no fluido folicular começam a aumentar rapidamente no futuro folículo dominante (F1) e no primeiro folículo subordinado. Assim, tem sido sugerido que produtos esterodoigênicos (estradiol) e não-esterodoigênicos (inibina) produzidos pelo folículo dominante suprimem a secreção de FSH e/ou LH, prevenindo o crescimento dos folículos subordinados (Beg et al., 2002; Beg et al., 2001).

Ao final da fase de crescimento comum dos folículos, usualmente um folículo continua crescendo e se torna o F1 e os folículos remanescentes (subordinados) regridem. Portanto, o fim da fase comum de crescimento e a distinta diferença na taxa de crescimento entre os dois maiores

folículos é chamada de desvio folicular (Guinter et al., 1997). O fenômeno de desvio folicular tem sido atribuído ao aumento da sensitividade ou responsividade do futuro F1 ao FSH e ao hormônio luteinizante (LH) (Guinter et al., 1996). Em média, uma transiente elevação de LH se inicia antes do desvio e declina após o desvio (Bergfelt et al., 2000; Kulick et al., 2001). O requerimento de LH para o desenvolvimento do F1 após o desvio foi indicado pelo seu menor diâmetro quando os níveis de LH foi experimentalmente reduzido (Guinter et al., 1996), e a supressão de LH previne crescimento folicular além de 9 mm (Gong et al., 1995). Ainda, quando o F1 atinge aproximadamente 10 mm, ele adquire receptores de LH (LHr) nas células da granulosa e a capacidade ovulatória em resposta ao pico de LH (Sartori et al., 2001).

Apesar do processo de desenvolvimento folicular antral e de seleção do F1 serem semelhantes nas raças *Bos indicus* e *Bos taurus*, existem algumas diferenças principalmente em relação ao tamanho do folículo no momento do desvio folicular. Em raças *Bos taurus*, especialmente na raça holandesa, o desvio ocorre geralmente quando o folículo alcança o tamanho de 8,5 - 9,0 mm (Guinter et al., 1996; Sartori et al., 2001) enquanto que em novilhas nelore o desvio ocorre quando o maior folículo atinge 5 - 6 mm (Sartorelli et al., 2005; Gimenez et al., 2008). Embora o tamanho dos folículos seja diferente entre estes grupos genéticos, não existe diferença no momento em que ocorre o desvio no ciclo estral, ou seja, em torno de 2,3 - 2,8 dias após a ovulação para ambos (Sartori & Barros, 2011). Além disso, o F1 de novilhas *Bos indicus* (nelore) adquirem a capacidade ovulatória com menor diâmetro, com média de 7,6 mm (Gimenez et al., 2008), quando comparado com raças *Bos taurus* (holandesas) que adquirem sua capacidade quando o F1 atinge 10 mm (Sartori et al., 2001). Isso ocorre porque em vacas nelore as células da granulosa do F1 adquirem receptores de LH com um diâmetro menor quando comparados a vacas holandesas (Nogueira et al., 2007).

1.2 População Folicular e Fertilidade na Espécie Bovina

Nos últimos anos houve um crescente interesse em se estudar a correlação da quantidade de folículos antrais que emergem por onda folicular com a fertilidade da espécie bovina. Normalmente o número de folículos antrais por onda possui alta repetibilidade (0,84 - 0,9) em um mesmo animal (Burns et al., 2005) e permanece constante até o inicio da depleção das reservas foliculares do ovário, por volta de 8 a 10 anos na espécie bovina (Ireland et al., 2007). Em contraste, há uma grande variação na quantidade de folículos recrutados na população e em diferentes grupos genéticos (Burns et al., 2005). Assim, é possível classificar animais com alta (APF) e baixa população (BPF) folicular através da contagem de folículos maiores que 3 mm por ultrassonografia. Mossa et al., (2012) demonstrou em um recente estudo que vacas leiterias com alto número de folículos maiores que 3 mm, tiveram maiores taxas de prenhes na primeira inseminação, menor intervalo do parto até a concepção e menor número de inseminações comparando com animais com baixa contagem folicular.

A maior fertilidade nestes animais pode estar associada a diferenças nos perfis hormonais e à expressão de genes reguladores do recrutamento folicular (Ireland et al., 2007; Ireland et al., 2009; Mossa et al., 2010). Animais de BPF possuem menores níveis plasmáticos de progesterona (Jimenez-Krassel et al., 2009) e níveis baixos deste hormônio estão associados a maior taxa de mortalidade embrionária em bovinos (Stronge et al., 2005, McNeill et al., 2006). Além disso, os níveis de FSH e LH e abundancia de RNA mensageiro para genes marcadores de oócitos com baixa qualidade estão aumentados em animais de BPF (Ireland et al., 2007). Níveis elevados de FSH podem ser deletérios para os oócitos, pois causam um aumento na expressão gênica para aromatase nas células da granulosa e também de receptores para estrógeno nas células do

cúmulos, proporcionando níveis mais elevados de estradiol intrafolicular e consequentemente bloqueio na maturação oocitária (Ireland et al,. 2009).

Animais com APF possuem enorme potencial para realização das biotécnicas de reprodução, principalmente animais das raças *Bos indicus*. No Brasil o número de embriões produzidos *in vitro* aumenta a cada ano e isso pode ser explicado pela maioria de seu rebanho ser composto por raças *Bos indicus*, especialmente nelore. As raças zebuínas se caracterizam por possuírem maior número de folículos por onda quando comparado a raças taurinas. Enquanto raças taurinas possuem em torno de 18 - 24 folículos por onda (Ireland et al., 2007) as raças zebuínas podem ter o dobro (Buratini et al., 2000). Essa característica proporciona uma maior recuperação de oócitos obtidos por aspiração folicular, e consequentemente maior número de embriões produzidos nestas raças (Pontes et al., 2011). Animais com BPF também respondem menos aos protocolos de superestimulação e consequentemente produzem menor número de embriões. Ireland e colaboradores (2007) demonstraram que novilhas com BPF superestimuladas produziram menor número de embriões totais (4,7 $\pm$ 0,7) e de embriões transferíveis (3,8 $\pm$ 0,8) em relação às novilhas com ACF (10,6 $\pm$ 2,7 e 5,4 $\pm$ 1,3, embriões totais e transferíveis, respectivamente).

1.2 *Single Nucleotide Pymorphism* (SNPs) e Estudos de Associação Genômica (*GWAS – Genome Wide Association Study*)

A identificação e mapeamento dos genes que interferem na expressão de características de importância zootécnica e econômica, visando a melhor compreensão do controle genético de características complexas como as poligênicas, têm sido amplamente usadas atualmente como forma de seleção para indivíduos de uma população (Heyes et al., 2009). Os estudos de GWAS

tem caráter altamente inovador, pois permitem a identificação de associações estatísticas de centenas de locos genômico e características complexas aumentando o entendimento sobre importantes vias moleculares e biológicas. (Sccott et al., 2007). Essa abordagem se tornou amplamente viável com as tecnologias de genotipagem massal através do desenvolvimento de microarranjos de alta densidade para detecção de milhares de variações genômicas simultaneamente, sendo a forma de variação genômica mais comum os SNPs (Brookes, 1999).

O sequenciamento do DNA de uma espécie nada mais é que determinação da ordem linear das quatro bases nitrogenadas do genoma: adenina (A), Citosina (C), Guanina (G) e Timina (T). Os SNPs, por definição, são alterações individuais nessas bases e as mutações mais comuns são as transições, onde ocorrem trocas de uma purina por outra purina (A "! G) ou de uma pirimidina por outra pirimidina (C" ! T). Porém, menos frequentes podem ocorrer as transversões quando há troca que uma purina por uma pirimidina, ou vice-versa (C/T"! A/G; Caetano, 2009). Os SNPs ocorrem aproximadamente a cada 700 pares de base (bp) em *Bos taurus* e 300 bp em *Bos indicus*, proporcionando uma maior variação genética nessa espécie. Com um genoma de três bilhões de bp o *Bos indicus* pode ter até dez milhões de SNPs (Seidel, 2010). Embora essas variações constituam apenas uma pequena percentagem do genoma (1% em humanos), elas formam a base da biodiversidade ou da variabilidade individual em resposta aos estímulos ambientais (Ibeagha-awemu et al., 2008).

Normalmente, os SNPs são bialélicos, mas podem aparecer como tri ou tetra alélicos, porém, esses SNPs não são distribuídos homogeneamente no genoma. Na maioria das vezes os SNPs acontecem nas regiões não codificadoras do genoma, sendo raros nas regiões codificadoras (Kim & Misra, 2007). Mesmo que os SNPs encontrados nas regiões não codificadoras não causam alteração nas proteínas, eles servem como importantes marcadores genéticos para

estudos comparativos e de evolução. Quando os SNPs estão localizados nas regiões codificadoras do gene, eles podem afetar a transcrição, causando mudanças na produção das referidas proteínas, além de poder causar alterações na estrutura e função, levando ao desenvolvimento de doenças ou mudança na resposta à determinada droga ou toxina.

O principal método de sequenciamento utilizado para prospecção do SNP chip foi baseado na técnica do microarranjo, que consiste de uma lâmina onde são impressos milhares de oligonucleotídeos permitindo que eles sejam analisados ao mesmo tempo (Rapley & Harbron, 2004). Esse método baseia-se na incorporação de desoxinucleotídeos (dNTPs) e de didesoxinucleotídeos (ddNTPs) a uma fita de DNA que está sendo formada e utiliza como molde o DNA de interesse. Assim que os ddNTPs são adicionados à cadeia, a extensão é interrompida, pois os ddNTPs não possuem o grupo (OH) 3', necessário para ligação do próximo desoxinucleotídeo (dNTP). Os ddNTPs são marcados com fluoresncência, assim podem ser detectados e a sequência dos nucleotídeos identificada (Regitano et al., 2009). Para amenizar a possibilidade de erros por hibridização de um alelo com outro que não corresponda a 100% de sua sequencia, várias *probes* do mesmo fragmento são colocadas na lâmina (Rapley & Harbron, 2004).

Informações sobre um grande número de SNPs mapeados do genoma possui grande valor, pois eles podem funcionar como marcadores indiretos para SNPs funcionais. Assim, o SNP pode estar associado ao fenótipo diretamente, como é o caso de SNPs que alteram a regulação e/ou expressão gênica, ou indiretamente, quando está ligada a mutação casual por desequilíbrio de ligação (LD) (Collins et al., 1998). O LD é a medida do grau de associação não aleatória de dois alelos entre dois ou mais *locus* do genoma, formando blocos de haplótipos que segregam juntos na população. Em animais de produção o padrão de LD é gerado,

principalmente, pela seleção e endogamia, decrescendo devido a recombinações e mutações (Ardlie et al., 2002).

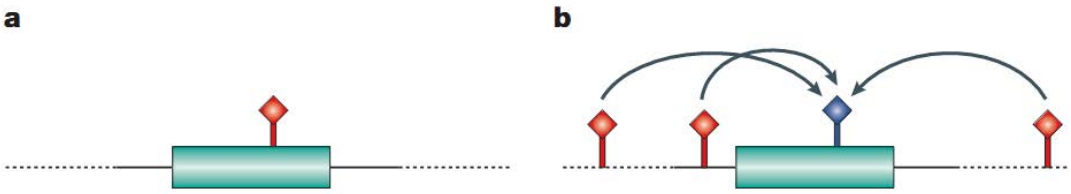


Figura 1.3. Apresentação dos métodos de associação direta (a) e indireta (b) para elucidar os genes envolvidos com a expressão fenotípica. Em (a) temos um caso onde o SNP (em vermelho) possui associação direta com o fenótipo. Em (b) os SNPs (vermelho) são escolhidos com base no LD para obter mais informações possíveis sobre outros SNPs. Neste caso o SNP (azul) é testado para associação indireta (Hirschhorn & Daly 2005).

1.3.1 Aplicação dos SNPs na Reprodução

Com o aumento do conhecimento do genoma humano e outras espécies de interesse zootécnico, tornou-se evidente que variações genéticas de SNPs são frequentes em hormônios e nos receptores do eixo hipotalâmico-hipofisario-gonadal e em diversos genes ligados a reprodução (Cargill et al., 1999; Gray et al., 2000, Taylor et al., 2001). Normalmente, a incidência desses polimorfismos em um único gene varia de 15 a 50% em uma população normal, indicando que tal variação genética não tem efeitos deletérios pronunciados na reprodução, pois se assim fosse, a evolução teria erradicado os indivíduos portadores dessas mutações. Entretanto, é provável que tais mudanças genéticas, em SNPs únicos ou em varias combinações, são capazes de modificar o *feedback* do sistema endócrino ou a ação dos

hormônios, resultando em diferentes performances reprodutivas, variando entre indivíduos totalmente funcionais a indivíduos inférteis.

Um exemplo de variação genética nos hormônios do sistema reprodutivo são as variações na cadeia β do LH, que altera as propriedades biológicas da molécula em humanos (Lamminen & Huhtaniemi, 2001). Uma variante mostra bioatividade *in vitr*o maior, mas tem uma meia vida na circulação menor quando comparada ao tipo nativo do hormônio. A expressão dessa variante pode resultar em alteração da esteroidogênese ovariana e atrasar a progressão da puberdade, dependendo do estado homo ou heterozigoto (Raivio et al., 1996). Além disso, estudos demonstraram que a expressão de mRNA da variante do LH é influenciada pelos polimorfismos na região promotora da subunidade β LH do gene. Isto resulta em maior atividade basal do promotor da subunidade β do gene LH e uma diferente resposta à estimulação hormonal comparando com o gene LH nativo (Jiang et al., 1999). Estes estudos demonstraram que os polimorfismos podem exercer seus efeitos por dois mecanismos. Um, por mudança das propriedades bioquímicas do produto do gene, ou por ação em nível de transcrição por mudança na atividade da região promotora. Visto que as gonadotrofinas FSH e LH têm um papel crucial na regulação das gônadas e que os genes para o FSH e o LH são altamente conservados por toda a evolução, é possível especular que uma mudança mínima no exon ou na região promotora desses genes pode trazer grandes conseqüências para a função gonadal.

Atualmente, em humanos, o teste mais promissor para predizer a resposta ovariana é o de SNPs para o receptor de FSH, o qual pode direcionar para a dose mais apropriada de FSH para cada individuo (Fauser et al., 2008). Mutações do FSHR estão associadas à amenorréia (Doherty et al., 2002; Meduri et al., 2003), e um SNP comum nesse gene (rs6166, que causa uma mudanca de asparagina (A) para a serina (S) no codon da posicao 680; p.S680N) está associado a uma

sensibilidade diferente à estimulação do FSH exógeno e endógeno (Perez Mayorga et al., 2000). Além disso, observações recentes sugeriram que o polimorfismo do hormônio anti-mulleriano e seu receptor (tipo II) também estão associados à sensibilidade ao FSH no ovário humano (Kevenaar et al., 2007). Mesmo que o progresso nesta área tem sido lento, os fatores genéticos podem ser utilizados como marcadores para a resposta ovariana. Polimorfismos de base única em genes envolvidos na biossíntese de esteróides e no eixo hipotalâmico-hipofisário-gonadal foram identificados em pacientes anovulatórios ou com síndrome do ovário policístico. Um SNP comum no gene da aromatase também está sendo estudado (Kin & Misra, 2007).

No bovino, dois SNPs semelhantes foram localizados nas posições 1506 e 1593 do exon 10 do FSH (Rahal et al., 2000). Nada se sabe sobre a frequência desses SNPs. Especula-se que os SNPs do receptor de FSH também estejam presentes em outras espécies. Para o SNP-29 na região promotora, um estudo que compara a sequência do promotor do gene FSHR de ratos, camundongos e ovinos (Sairam & Subbarayan, 1997) revelaram a presença de ambos A (rato e ovino) ou G (camundongo) comparando com G ou A no humano.

Um estudo de associação entre os SNPs para o gene FSHR (FSHR-278 e FSHR-320) e a resposta ao tratamento superovulatório em vacas da raça Holandesa Chinesa mostrou que a mutação G>A (AGGGACA to AGGAACA) na posição 278 teve um efeito significativo no numero total de oócitos coletados, sendo que animais homozigotos para a mutação tiveram um número significativamente maior de oócitos que os animais heterozigotos e os nativos. Animais homozigotos para a mutação também apresentaram um numero maior de embriões transferíveis. Entretanto, a homozigose teve um efeito deletério no numero de embriões degenerados, com os animais homozigotos para a mutação apresentando um número significativamente maior de embriões degenerados. Essas características foram replicadas em tratamentos de superovulação

realizados com um mês de intervalo. A mutação no lócus 320 não mostrou nenhuma associação com a resposta ao tratamento (Yang et al., 2010). Assim, o polimorfismo do FSHR pode ser um marcador eficaz em espécies de interesse zootécnico.

Feugang e colaboradores (2009) realizaram um estudo de GWAS multi-estágio em touros Holandeses. Na primeira fase foram analisados vinte touros de alta e baixa fertilidade, evidenciando quatro SNP significativamente associados à característica ($p \leq 10^{-4}$), e na segunda fase apenas esses SNP foram interrogados em uma população de 200 animais. Foram confirmados a associação significativa de apenas dois deles (rs29024867 e rs41257187; $p < 0.05$). Os resultados confirmaram a importância do gene da Integrina Beta 5 (IGB5) na fertilidade de bovinos, embora não tenha sido definido um papel específico para esse gene.

1.4 Referências Bibliográficas

Ardlie, K. G., L. Kruglyak and M. Seielstad (2002). "Patterns of linkage disequilibrium in the human genome." Nat Rev Genet **3**(4): 299-309.

Austin, E. J., M. Mihm, A. C. Evans, P. G. Knight, J. L. Ireland, J. J. Ireland and J. F. Roche (2001). "Alterations in intrafollicular regulatory factors and apoptosis during selection of follicles in the first follicular wave of the bovine estrous cycle." Biol Reprod **64**(3): 839-848.

Beg, M. A., D. R. Bergfelt, K. Kot and O. J. Ginther (2002). "Follicle selection in cattle: dynamics of follicular fluid factors during development of follicle dominance." Biol Reprod **66**(1): 120-126.

Beg, M. A., D. R. Bergfelt, K. Kot, M. C. Wiltbank and O. J. Ginther (2001). "Follicular-fluid factors and granulosa-cell gene expression associated with follicle deviation in cattle." Biol Reprod **64**(2): 432-441.

Beg, M. A. and O. J. Ginther (2006). "Follicle selection in cattle and horses: role of intrafollicular factors."Reproduction **132**(3): 365-377.

Bergfelt, D. R., E. L. Gastal and O. J. Ginther (2001). "Response of estradiol and inhibin to experimentally reduced luteinizing hormone during follicle deviation in mares." Biol Reprod **65**(2): 426-432.

Brookes, A. J (1999). "The essence of SNPs" Gene **(234)**: 177-186.

Buratini, J., V. F. Glapinski, I. C. Giometti, A. B. Teixeira, I. B. Costa, M. C. Avellar, C. M. Barros and C. A. Price (2005). "Expression of fibroblast growth factor-8 and its cognate receptors, fibroblast growth factor receptor (FGFR)-3c and-4, in fetal bovine preantral follicles." Mol Reprod Dev **70**(3): 255-261.

Buratini, J., C. A. Price, J. A. Visintin and G. A. Bó (2000). "Effects of dominant follicle aspiration and treatment with recombinant bovine somatotropin (BST) on ovarian follicular development in nelore (Bos indicus) heifers."Theriogenology **54**(3): 421-431.

Burkhart, M. N., J. L. Juengel, P. R. Smith, D. A. Heath, G. A. Perry, M. F. Smith and H. A. Garverick (2010). "Morphological development and characterization of aromatase and estrogen receptors alpha and beta in fetal ovaries of cattle from days 110 to 250." Anim Reprod Sci **117**(1-2): 43-54.

Burns, D. S., F. Jimenez-Krassel, J. L. Ireland, P. G. Knight and J. J. Ireland (2005). "Numbers of antral follicles during follicular waves in cattle: evidence for high variation among animals, very high repeatability in individuals, and an inverse association with serum follicle-stimulating hormone concentrations." Biol Reprod **73**(1): 54-62.

Caetano, A.R (2009). Marcadores SNP: conceitos básicos, aplicações no manejo e no melhoramento animal e perspectivas para o future. R. Bras. Zootec. **(38):** 64-71.

Cargill, M., D. Altshuler, J. Ireland, P. Sklar, K. Ardlie, N. Patil, N. Shaw, C. R. Lane, E. P. Lim, N. Kalyanaraman, J. Nemesh, L. Ziaugra, L. Friedland, A. Rolfe, J. Warrington, R. Lipshutz, G. Q. Daley and E. S. Lander (1999). "Characterization of single-nucleotide polymorphisms in coding regions of human genes." Nat Genet **22**(3): 231-238.

Celestino, J.J.H., Matos M.H.T., Saraiva M.V.A., Figueiredo J.R. (2009). "Regulation of ovarian folliculogenesis by Kit Ligand and the c-Kit system in mammals." Anim. Reprod., **6**(3): 431-439.

Collins, F. S., L. D. Brooks and A. Chakravarti (1998). "A DNA polymorphism discovery resource for research on human genetic variation." Genome Res **8**(12): 1229-1231.

Danforth, D. R., L. K. Arbogast, S. Ghosh, A. Dickerman, R. Rofagha and C. I. Friedman (2003). "Vascular endothelial growth factor stimulates preantral follicle growth in the rat ovary." Biol Reprod **68**(5): 1736-1741.

Doherty, E., P. Pakarinen, A. Tiitinen, A. Kiilavuori, I. Huhtaniemi, S. Forrest and K. Aittomäki (2002). "A Novel mutation in the FSH receptor inhibiting signal transduction and causing primary ovarian failure." J Clin Endocrinol Metab **87**(3): 1151-1155.

Dong, J., D. F. Albertini, K. Nishimori, T. R. Kumar, N. Lu and M. M. Matzuk (1996). "Growth differentiation factor-9 is required during early ovarian folliculogenesis." Nature **383** (6600): 531-535.

Druet, T., Schrooten, C., DE Roos, A. P (2010) "Imputation of genotypes from different single nucleotide polymorphism panels in dairy cattle". J Dairy Sci, **93** (11): 5443-54.

Echternkamp, S. E., H. J. Howard, A. J. Roberts, J. Grizzle and T. Wise (1994). "Relationships among concentrations of steroids, insulin-like growth factor-I, and insulin-like growth factor binding proteins in ovarian follicular fluid of beef cattle." Biol Reprod **51**(5): 971-981.

Fauser, B. C., K. Diedrich, P. Devroey and E. A. R. W. G. 2007 (2008). "Predictors of ovarian response: progress towards individualized treatment in ovulation induction and ovarian stimulation." Hum Reprod Update **14**(1): 1-14.

Feugang, J. M., Kaya, A., Page, G. P., Chen, L., Mehta, T., Hirani, K., Nazareth, L., Topper, E., Gibbs, R., Memili, E. (2009) "Two-stage genome-wide association study identifies integrin beta 5 as having potential role in bull fertility". **BMC Genomics, 10**: 176.

Fortune, J. E., M. Y. Yang and W. Muruvi (2010). "The earliest stages of follicular development: follicle formation and activation." Soc Reprod Fertil Suppl **67**: 203-216.

Gibbons, J. R., M. C. Wiltbank and O. J. Ginther (1997). "Functional interrelationships between follicles greater than 4 mm and the follicle-stimulating hormone surge in heifers." Biol Reprod **57**(5): 1066-1073.

Gimenes, L. U., M. F. Sá Filho, N. A. Carvalho, J. R. Torres-Júnior, A. H. Souza, E. H. Madureira, L. A. Trinca, E. S. Sartorelli, C. M. Barros, J. B. Carvalho, R. J. Mapletoft and P. S. Baruselli (2008). "Follicle deviation and ovulatory capacity in Bos indicus heifers." Theriogenology **69**(7): 852-858.

Ginther, O. J., M. A. Beg, K. Kot, C. Meira and D. R. Bergfelt (2003). "Associated and independent comparisons between the two largest follicles preceding follicle deviation in cattle." Biol Reprod **68**(2): 524-529.

Ginther, O. J., K. Kot, L. J. Kulick and M. C. Wiltbank (1997). "Emergence and deviation of follicles during the development of follicular waves in cattle." Theriogenology **48**(1): 75-87.

Ginther, O. J., M. C. Wiltbank, P. M. Fricke, J. R. Gibbons and K. Kot (1996). "Selection of the dominant follicle in cattle." Biol Reprod **55**(6): 1187-1194.

Gong, J. G., T. A. Bramley, C. G. Gutierrez, A. R. Peters and R. Webb (1995). "Effects of chronic treatment with a gonadotrophin-releasing hormone agonist on peripheral concentrations of FSH and LH, and ovarian function in heifers." J Reprod Fertil **105**(2): 263-270.

Gray, I. C., D. A. Campbell and N. K. Spurr (2000). "Single nucleotide polymorphisms as tools in human genetics." Hum Mol Genet **9**(16): 2403-2408.

Hayers, J. B., Bowman, P. J., Chamberlain, A. J., Goddard, M. E. (2009). "Invited review: Genomic selection in dairy cattle: Progress and challenges". J. Dairy Sci. **92**:433–443

Hirshfield, A. N. (1991). "Development of follicles in the mammalian ovary." Int Rev Cytol **124**: 43-101.

Hirschhorn, J. N.; Daly, M. J. (2005) "Genome-wide association studies for common diseases and complex traits". Nat Rev Genet, **6** (2): 95-108

Ibeagha-Awemu, E. M., P. Kgwatalala, A. E. Ibeagha and X. Zhao (2008). "A critical analysis of disease-associated DNA polymorphisms in the genes of cattle, goat, sheep, and pig." Mamm Genome **19**(4): 226-245.

Ireland, J. J., F. Ward, F. Jimenez-Krassel, J. L. Ireland, G. W. Smith, P. Lonergan and A. C. Evans (2007). "Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-quality embryos after ovarian stimulation in cattle." Hum Reprod**22**(6): 1687-1695.

Ireland, J. J., A. E. Zielak-Steciwko, F. Jimenez-Krassel, J. Folger, A. Bettegowda, D. Scheetz, S. Walsh, F. Mossa, P. G. Knight, G. W. Smith, P. Lonergan and A. C. Evans (2009). "Variation in the ovarian reserve is linked to alterations in intrafollicular estradiol production and ovarian biomarkers of follicular differentiation and oocyte quality in cattle." Biol Reprod **80**(5): 954-964.

Jiang, M., P. Pakarinen, F. P. Zhang, T. El-Hefnawy, P. Koskimies, K. Pettersson and I. Huhtaniemi (1999). "A common polymorphic allele of the human luteinizing hormone beta-subunit gene: additional mutations and differential function of the promoter sequence." Hum Mol Genet **8**(11): 2037-2046.

Jimenez-Krassel, F., J. K. Folger, J. L. Ireland, G. W. Smith, X. Hou, J. S. Davis, P. Lonergan, A. C. Evans and J. J. Ireland (2009). "Evidence that high variation in ovarian reserves of healthy young adults has a negative impact on the corpus luteum and endometrium during estrous cycles in cattle." Biol Reprod **80**(6): 1272-1281.

John, G. B., T. D. Gallardo, L. J. Shirley and D. H. Castrillon (2008). "Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth." Dev Biol **321**(1): 197-204.

Juengel, J. L., N. L. Hudson, D. A. Heath, P. Smith, K. L. Reader, S. B. Lawrence, A. R. O'Connell, M. P. Laitinen, M. Cranfield, N. P. Groome, O. Ritvos and K. P. McNatty (2002). "Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep." Biol Reprod **67**(6): 1777-1789.

Juengel, J. L. and K. P. McNatty (2005). "The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development." Hum Reprod Update **11**(2): 143-160.

Kevenaar, M. E., A. P. Themmen, J. S. Laven, B. Sonntag, S. L. Fong, A. G. Uitterlinden, F. H. de Jong, H. A. Pols, M. Simoni and J. A. Visser (2007). "Anti-Müllerian hormone and anti-Müllerian hormone type II receptor polymorphisms are associated with follicular phase estradiol levels in normo-ovulatory women." Hum Reprod **22**(6): 1547-1554.

Kezele, P. R., J. M. Ague, E. Nilsson and M. K. Skinner (2005). "Alterations in the ovarian transcriptome during primordial follicle assembly and development." Biol Reprod **72**(1): 241-255.

Kim, S. and A. Misra (2007). "SNP genotyping: technologies and biomedical applications." Annu Rev Biomed Eng **9**: 289-320.

Knight, P. G. and C. Glister (2006). "TGF-beta superfamily members and ovarian follicle development."Reproduction **132**(2): 191-206.

KU, C. S.; Loy, E.Y (2010). "The pursuit of genome-wide association studies: where are we now? Journal of Human Genetics" **55** :195-206.

Kulick, L. J., D. R. Bergfelt, K. Kot and O. J. Ginther (2001). "Follicle selection in cattle: follicle deviation and codominance within sequential waves." Biol Reprod **65**(3): 839-846.

Lamminen, T. and I. Huhtaniemi (2001). "A common genetic variant of luteinizing hormone; relation to normal and aberrant pituitary-gonadal function." Eur J Pharmacol **414**(1): 1-7.

Lee, W. S., F. Otsuka, R. K. Moore and S. Shimasaki (2001). "Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat." Biol Reprod **65**(4): 994-999.

Lucci, C. M., R. V. Silva, C. A. Carvalho, R. Figueiredo and N. Báo (2001). "Light microscopical and ultrastructural characterization of goat preantral follicles." Small Rumin Res **41**(1): 61-69.

Machado, M. F., V. M. Portela, C. A. Price, I. B. Costa, P. Ripamonte, R. L. Amorim and J. Buratini (2009). "Regulation and action of fibroblast growth factor 17 in bovine follicles." J Endocrinol **202**(3): 347-353.

Mazerbourg, S., C. Klein, J. Roh, N. Kaivo-Oja, D. G. Mottershead, O. Korchynskyi, O. Ritvos and A. J. Hsueh (2004). "Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5." Mol Endocrinol **18**(3): 653-665.

McNatty, K. P., A. E. Fidler, J. L. Juengel, L. D. Quirke, P. R. Smith, D. A. Heath, T. Lundy, A. O'Connell and D. J. Tisdall (2000). "Growth and paracrine factors regulating follicular formation and cellular function." Mol Cell Endocrinol **163**(1-2): 11-20.

McNatty, K. P., D. A. Heath, K. M. Henderson, S. Lun, P. R. Hurst, L. M. Ellis, G. W. Montgomery, L. Morrison and D. C. Thurley (1984). "Some aspects of thecal and granulosa cell function during follicular development in the bovine ovary." J Reprod Fertil **72**(1): 39-53.

McNatty, K. P., P. Smith, L. G. Moore, K. Reader, S. Lun, J. P. Hanrahan, N. P. Groome, M. Laitinen, O. Ritvos and J. L. Juengel (2005). "Oocyte-expressed genes affecting ovulation rate." Mol Cell Endocrinol **234**(1-2): 57-66.

McNeill, R. E., M. G. Diskin, J. M. Sreenan and D. G. Morris (2006). "Associations between milk progesterone concentration on different days and with embryo survival during the early luteal phase in dairy cows."Theriogenology **65**(7): 1435-1441.

Meduri, G., P. Touraine, I. Beau, O. Lahuna, A. Desroches, M. C. Vacher-Lavenu, F. Kuttenn and M. Misrahi (2003). "Delayed puberty and primary amenorrhea associated with a novel mutation of the human follicle-stimulating hormone receptor: clinical, histological, and molecular studies." J Clin Endocrinol Metab **88**(8): 3491-3498.

Moore, R. K., F. Otsuka and S. Shimasaki (2003). "Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells." J Biol Chem **278**(1): 304-310.

Mossa, F., F. Jimenez-Krassel, J. K. Folger, J. L. Ireland, G. W. Smith, P. Lonergan, A. C. Evans and J. J. Ireland (2010). "Evidence that high variation in antral follicle count during follicular waves is linked to alterations in ovarian androgen production in cattle." Reproduction **140**(5): 713-720.

Mossa, F., S. W. Walsh, S. T. Butler, D. P. Berry, F. Carter, P. Lonergan, G. W. Smith, J. J. Ireland and A. C. Evans (2012). "Low numbers of ovarian follicles ≥3 mm in diameter are associated with low fertility in dairy cows." J Dairy Sci **95**(5): 2355-2361.

Nilsson, E., J. A. Parrott and M. K. Skinner (2001). "Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis." Mol Cell Endocrinol **175**(1-2): 123-130.

Nilsson, E. E., P. Kezele and M. K. Skinner (2002). "Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries." Mol Cell Endocrinol **188**(1-2): 65-73.

Nilsson, E. E. and M. K. Skinner (2003). "Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development." Biol Reprod **69**(4): 1265-1272.

Nogueira, M. F., J. Buratini, C. A. Price, A. C. Castilho, M. G. Pinto and C. M. Barros (2007). "Expression of LH receptor mRNA splice variants in bovine granulosa cells: changes with follicle size and regulation by FSH in vitro."Mol Reprod Dev **74**(6): 680-686.

Palma, G. A., M. E. Argañaraz, A. D. Barrera, D. Rodler, A. Mutto and F. Sinowatz (2012). "Biology and biotechnology of follicle development." ScientificWorldJournal **2012**: 938138.

Paredes, A., C. Romero, G. A. Dissen, T. M. DeChiara, L. Reichardt, A. Cornea, S. R. Ojeda and B. Xu (2004). "TrkB receptors are required for follicular growth and oocyte survival in the mammalian ovary." Dev Biol **267**(2): 430-449.

Parrott, J. A. and M. K. Skinner (1999). "Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis." Endocrinology **140**(9): 4262-4271.

Perez Mayorga, M., J. Gromoll, H. M. Behre, C. Gassner, E. Nieschlag and M. Simoni (2000). "Ovarian response to follicle-stimulating hormone (FSH) stimulation depends on the FSH receptor genotype." J Clin Endocrinol Metab **85**(9): 3365-3369.

Picton, H., D. Briggs and R. Gosden (1998). "The molecular basis of oocyte growth and development." Mol Cell Endocrinol **145**(1-2): 27-37.

Pontes, J. H., F. A. Melo Sterza, A. C. Basso, C. R. Ferreira, B. V. Sanches, K. C. Rubin and M. M. Seneda (2011). "Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (Bos indicus) donors." Theriogenology **75**(9): 1640-1646.

Rahal, P., A. C. Latronico, M. B. Kohek, R. F. de Lucia, M. P. Milazzotto, M. B. Wheeler, J. B. Ferraz, J. P. Eler and J. F. Garcia (2000). "Polymorphisms in the bovine follicle-stimulating hormone receptor gene." Anim Genet**31**(4): 280-281.

Raivio, T., I. Huhtaniemi, R. Anttila, M. A. Siimes, L. Hagenäs, C. Nilsson, K. Pettersson and L. Dunkel (1996). "The role of luteinizing hormone-beta gene polymorphism in the onset and progression of puberty in healthy boys." J Clin Endocrinol Metab **81**(9): 3278-3282.

Reddy, P., L. Shen, C. Ren, K. Boman, E. Lundin, U. Ottander, P. Lindgren, Y. X. Liu, Q. Y. Sun and K. Liu (2005). "Activation of Akt (PKB) and suppression of FKHRL1 in mouse and rat oocytes by stem cell factor during follicular activation and development." Dev Biol **281**(2): 160-170.

Regitano, L.C.A., Méo-Niciura, S.C., Ibelli, A.M.G., Gouveia, J.J.S (2007). Protocolos de bilogia molecular aplicada à produção animal. EMBRAPA Pecuária Sudeste **(1)**: 66p.

Roche, J. F., Mihn, M., Diskin, M. G., Ireland, J. J. (1998). "A Review of Regulation of Follicle Growth in Cattle". J ANIM SCI **76**:16-29.

Romero, C., A. Paredes, G. A. Dissen and S. R. Ojeda (2002). "Nerve growth factor induces the expression of functional FSH receptors in newly formed follicles of the rat ovary." Endocrinology **143**(4): 1485-1494.

Sairam, M. R. and V. S. Subbarayan (1997). "Characterization of the 5' flanking region and potential control elements of the ovine follitropin receptor gene." Mol Reprod Dev **48**(4): 480-487.

Sartorelli, E. S., L. M. Carvalho, D. R. Bergfelt, O. J. Ginther and C. M. Barros (2005). "Morphological characterization of follicle deviation in Nelore (Bos indicus) heifers and cows." Theriogenology **63**(9): 2382-2394.

Sartori, R. and C. M. Barros (2011). "Reproductive cycles in Bos indicus cattle." Anim Reprod Sci **124**(3-4): 244-250.

Sartori, R., P. M. Fricke, J. C. Ferreira, O. J. Ginther and M. C. Wiltbank (2001). "Follicular deviation and acquisition of ovulatory capacity in bovine follicles." Biol Reprod **65**(5): 1403-1409.

Scott, L. J., K. L. Mohlke, L. L. Bonnycastle, C. J. Willer, Y. Li, W. L. Duren, M. R. Erdos, H. M. Stringham, P. S. Chines, A. U. Jackson, L. Prokunina-Olsson, C. J. Ding, A. J. Swift, N. Narisu, T. Hu, R. Pruim, R. Xiao, X. Y. Li, K. N. Conneely, N. L. Riebow, A. G. Sprau, M. Tong, P. P. White, K. N. Hetrick, M. W. Barnhart, C. W. Bark, J. L. Goldstein, L. Watkins, F. Xiang, J. Saramies, T. A. Buchanan, R. M. Watanabe, T. T. Valle, L. Kinnunen, G. R. Abecasis, E. W. Pugh, K. F. Doheny, R. N. Bergman, J. Tuomilehto, F. S. Collins, and M. Boehnke (2007). "A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants": Science (316): 1341-1345.

Seidel, G. E. (2010). "Brief introduction to whole-genome selection in cattle using single nucleotide polymorphisms." Reprod Fertil Dev **22**(1): 138-144.

Stronge, A. J., J. M. Sreenan, M. G. Diskin, J. F. Mee, D. A. Kenny and D. G. Morris (2005). "Post-insemination milk progesterone concentration and embryo survival in dairy cows." Theriogenology **64**(5): 1212-1224.

Tanaka, Y., K. Nakada, M. Moriyoshi and Y. Sawamukai (2001). "Appearance and number of follicles and change in the concentration of serum FSH in female bovine fetuses." Reproduction **121**(5): 777-782.

Taylor, J. G., E. H. Choi, C. B. Foster and S. J. Chanock (2001). "Using genetic variation to study human disease." Trends Mol Med **7**(11): 507-512.

van den Hurk, R. and J. Zhao (2005). "Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles." Theriogenology **63**(6): 1717-1751.

Webb, R. and B. K. Campbell (2007). "Development of the dominant follicle: mechanisms of selection and maintenance of oocyte quality." Soc Reprod Fertil Suppl **64**: 141-163.

Webb, R., B. Nicholas, J. G. Gong, B. K. Campbell, C. G. Gutierrez, H. A. Garverick and D. G. Armstrong (2003). "Mechanisms regulating follicular development and selection of the dominant follicle." Reprod Suppl **61**: 71-90.

Yan, C., P. Wang, J. DeMayo, F. J. DeMayo, J. A. Elvin, C. Carino, S. V. Prasad, S. S. Skinner, B. S. Dunbar, J. L. Dube, A. J. Celeste and M. M. Matzuk (2001). "Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function." Mol Endocrinol **15**(6): 854-866.

Yang, M. Y. and J. E. Fortune (2008). "The capacity of primordial follicles in fetal bovine ovaries to initiate growth in vitro develops during mid-gestation and is associated with meiotic arrest of oocytes." Biol Reprod **78**(6): 1153-1161.

Yang, W. C., S. J. Li, K. Q. Tang, G. H. Hua, C. Y. Zhang, J. N. Yu, L. Han and L. G. Yang (2010). "Polymorphisms in the 5' upstream region of the FSH receptor gene, and their association with superovulation traits in Chinese Holstein cows." Anim Reprod Sci **119**(3-4): 172-177.

CAPÍTULO 2

Follicle Gene Expression Profile of Nelore and Angus Heifers with Low and High Ovarian Follicle Count

Running Head:

Follicle Gene Expression Profile of Nelore and Angus Heifers with Low and High Ovarian Follicle Count

M.G. Favoreto[1]; B. Loureiro[2]; R.L. Ereno[1]; A.G. Pupulin[1]; V. Queiroz[1]; J. Buratini[3]; M. Binelli[4]; C.M. Barros[1].

[1]Department of Pharmacology, Institute of Biosciences, São Paulo State University (UNESP), Botucatu, Brazil;
[2]Department of Veterinary Medicine, Vila Velha University (UVV), Vila Velha, Brazil;
[3]Department of Physiology, Institute of Biosciences, São Paulo State University (UNESP), Botucatu, Brazil;
[4]Department of Animal Reproduction, São Paulo University, (USP), Pirassununga, Brazil;

[1]corresponding autor: barrosciro@gmail.com

ABSTRACT

The number of antral follicles counted by ultrasound (AFC) is associated with fertility in cattle. Cows with higher follicle count (HFC) have a higher reproductive performance compared to cows with lower follicle count (LFC). In this study we aimed to define the preantral follicle count by histology and to identify differentially expressed genes (DEG) through the microarray technology in Nelore and Angus heifers with HFC and LCF. Ovaries of each animal were scanned with an ultrasound device 12-24 hours after estrus. The groups were formed based on the average of the total follicles (≥ 3 mm) counted for each breed consistently ± standard deviation. For the histology analysis, preantral follicles were counted and classified under a stereo microscope and the density of follicles was determined using the handtool on the ImageJ software. Microarray analysis was performed on pools of three follicles dissected from the ovary (one pool per animal) from 15 Nelore (6 HFC and 9 LFC) and 17 Angus heifers (9 HFC and 8 LFC. Histological results showed greater primordial follicle density in Angus heifers ($P<0.02$) and a tendency of secondary follicles density to be higher ($P=0.09$) in Nellore heifers. Microarray data analysis revealed 1205 (782 could be annotated) DEG (log2; fold change >1.5) between follicles from Angus vs. Nelore heifers. Of the annotated genes, 316 were downregulated and 466 were upregulated. Among groups (LFC vs. HFC) the analysis revealed 311 DEG (188 could be annotated) with 142 downregulated and 46 upregulated from annotated genes. When comparing the interaction between breeds and groups 315 were DEG. Several genes that were upregulated in the Nelore were associated with follicle activation and development, including genes from IGF1 system. Among groups genes with MAPK pathway CACNA1S, NTRK1 and FGFR1 were higher expressed in HFC group when compared with LFC group. These genes have been associated with cell proliferation.

Key Words: Bovine; Gene Expression; Follicle; Reproduction

INTRODUCTION

The follicular development in bovines emerges in a wave-like pattern (Reviewed by Adams et al., 2008). The number of emerging follicles in each wave has a high variation among cows but is highly repeatable within individual (Burns et al., 2005; Ireland et al., 2007). Recent studies have associated the number of antral follicle count greater than 3mm counted by ultrasound with fertility in dairy cattle. Dairy cows with low follicle count had lower pregnancy rates at first service, longer interval from calving to conception and higher number of services received during the breeding season compared with cows with higher follicle count (Mossa et al., 2012). Furthermore, cows with HFC had increased number of total oocyte recovery by ultrasound-guide follicular aspiration (OPU) and, consequently, a greater number of total embryos produced by IVF (Ireland et al., 2007).

The number of embryos produced by IVF is increasing every year worldwide. In Brazil, this number is especially large due to the large population of Nelore cows (*Bos indicus*). Even thought there is only small differences in the characteristics of oestrus cycles and follicles waves between *Bos taurus* and *Bos indicus* animals (Sartori et al., 2010), the number of follicles recruited per wave is different between them (Sartori & Barros., 2011). Overall, *Bos taurus* animals have around 18 to 24 follicles per wave (Ireland et al., 2007), while *Bos indicus* have twice this amount (Buratini et al., 2000). The numbers of ocoytes collected in each ultradound-guide ovum pick up (OPU) is highly correlated with number of follicles recruited by wave (Tamassia et al., 2003). Thus, *Bos indicus* cows provide higher numbers of occytes obtained by

OPU and higher number of embryos produced by IVF when compared with *Bos Taurus* animals (Pontes et al., 2011).

How many follicles are recruited in a follicular wave and the mechanism that leads to the high variation in number of ACF within and between subspecies is not completed elucidated. Morphmetric studies suggest that the growth of follicles starts based on the order in which they are formed (Hirshfield, 1991). Moreover, it is known that the activation and development of preantral follicles is gonadotropin independent and is mainly regulated by intraovarian factors (McNatty et al., 2009). As the follicles continuous to growth, the late stages of development are controlled mainly by endocrine mechanisms, highlighting the gonadotropins especially FSH and LH (Webb et al., 2003; Webb et al., 2007). The aim of this study was to compare by histology the density of preantral follicles present in the ovary of Nelore (*Bos indicus*) and Angus (*Bos taurus*) heifers with high or low antral follicle count and to compared the global gene-expression patterns of follicles extracted from these animals.

MATERIAL AND METHODS

Experimental design

A group of a 155 Nelore and 132 Angus heifers with similar body weight and age (ranged between 22 to 28 months) were injected with two doses of PGF2α 11 days apart to initiate luteolysis and synchronize occurrence of ovulation. Ovaries of each animal were scanned with an ultrasound device (US; Mindray Vet DPS 2200, São Paulo, Brazil) equipped with a 7.5-MHz probe once 12-24 hours after estrus. Two separate perpendicular measurements were made on

each ovary individually to determine the total of follicles ≥ 3 mm for three consecutive estrous cycles. The groups were formed based on the average of the total follicles (≥ 3 mm) counted for each breed consistently ± standard deviation and classified in high (HFC) and low (LFC) follicle count. A total of 18 Nelore heifers were selected and allocated in the groups: 8 with HFC (≥ 40 follicles) and 10 with LHC (≤ 20 follicles). For Angus heifers 28 were selected and allocated into the groups: 15 with HFC (≥ 20 follicles) and 13 with LFC (≤ 10 follicles).

Table 2.1. Numbers of animals selected, total AFC average and groups AFC average for Nelore and Angus heifers with LFC and HFC.

	Nelore		Angus	
	HFC 40 ≥ Fol	LFC ≤ 20 Fol	HFC 20 ≥ Fol	LFC ≤ 10 Fol
Number of animals	8	10	15	13
Groups AFC average	52 ± 1.7	16 ± 1.54	27 ± 1.24	10 ± 1.41
Total AFC average	32 ± 3.1a		20 ± 2.6b	

Histology of the Ovary

All selected animals were synchronized again as described. Heifers that have shown estrus were slaughtered 24 hours after ovulation, confirmed by ultrasonography, and the ovaries collected and transported to the laboratory. The ovary containing the *corpus hemorragium* was used for histology measurements. It was sliced longitudinally in six parts and fixed in paraformaldehyde 10 % for 24 h and 70 % ethanol until processed. Two parts of the longitudinal slices were blocked in paraffin and ten consecutive ovarian sections, 8 µm thick, were obtained from each ovarian slice every 50 cuts. Sections were stained using eosin and hematoxylin.

Follicles were counted and classified under a stereo microscope with a 40 x objective, only follicles with a visible oocyte were counted. Follicles were classified according to their stage of development as primordial, primary or secondary (Lucci et al., 2001). The area of each section was measured using the handtool on the ImageJ software. Area measurements were expressed as square pixels and the density of each follicle classification was calculated as follicle/log10 [pixel^2]. For statistical analysis the data were submitted to ANOVA using the PROC General Linear Models (GLM) do SAS (SAS for Windows, version 9.2; SAS Institute, Inc., Cary, NC, USA).

Follicle Dissection and RNA extraction

A total of 5 follicles with 4 mm or less was dissected from the ovary contralateral to the *corpus hemorragium*. Total RNA was extracted from the whole follicle individually using an RNeasy Micro Kit (Qiagen, Valencia, CA-USA) according to the manufacturer's instructions. Concentration of the input RNA was determined by Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and RNA quality was determined by use of the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples that showed high RNA integrity (RIN $\geq$ 7) were used for the microarray hybridization and qPCR analysis.

Microarray Processing

Microarray analysis was performed on pools of three follicles (one pool per animal) from 15 Nelore (6 HFC and 9 LFC) and 17 Angus heifers (9 HFC and 8 LFC). To exclude dominant or atretic follicles, before microarray, all follicles were analyzed individually by quantitative real time polymerase chain reaction (RT-qPCR) for RNA expression of aromatase, FSHR and VEGF.

Biotin-labelled and fragmented cRNA was obtained using a 3' IVT Express kit (Affymetrix, Santa Clara, CA, USA) to perform hybridizations using a GeneChip Bovine Genome Array (Affymetrix). Briefly, 250 ng of total RNA were used to generate the cDNA by Expression 3' Amplification Two-Cycle cDNA Synthesis Kit in conjunction with GeneChip Eukaryotic Poly A RNA Control Kit. A double stranded cDNA products were purified using GeneChip Sample Cleanup Module and in vitro transcribed to generate a biotinylated complementary RNAs (cRNAs) with GeneChip Expression 3'Amplification IVT Labeling Kit. Next, 12µg of cRNA generated in the IVT reaction were fragmented using 5X Fragmentation Buffer and RNase-free water contained within the GeneChip Sample Cleanup Module. The fragmentation reaction was applied to generate fragments with 35-200 bases with a peak at approximately 100-200 bases. Twelve micrograms of fragmented cRNA were made up into hybridization cocktail in accordance with the Affymetrix manual and corresponding to a 100 format array. The hybridization cocktail was added to the appropriate array and hybridized for 16 h at 458°C. The array was washed and stained on the GeneChip Fluidics Station 450 and later scanned using the GeneChip Scanner 3000 (Affymetrix).

Transcriptome Data Analysis

Microarray data analyses for identification of differentially expressed genes (DEG) were performed using FlexArray 1.6.1.1 (Michal Blazejczyk, Mathieu Miron, Robert Nadon (2007) that uses an R program language and BioConductor with a friendly interface to users. The raw data was adjusted for background signal intensity and normalized across all genes chips using a Probe Logarithmic Intensity Error (PLIER). Statistical data was analyzed by linear model (LiMMA, Smyth, 2005). Genes showing fold change ≥ 1.5 or ≤ -1.5 and $P < 0.05$ were

considered as differentially expressed. Genes that expression was higher in follicles from Angus heifers when compared with Nelore heifers were considered to be upregulated. Genes that expression was lower in follicles from Angus heifers when compared to Nelore heifers were considered to be downregulated. For groups (HFC and LFC), genes that had higher expression on the LFC group were considered to be upregulated and genes that had lower expression on the LFC group were considered to be downregulated. DAVID (Database for Annotation, Visualization and Integrated Discovery; Dennis et al., 2003) was used to determine gene ontologies (GO) and functions in which differentially expressed genes were overrepresented at $P < 0.05$.

Validation of Microarray Results by Quantitative PCR Reaction

The validation of microarray results was performed using RT-qPCR. A total of eight genes that were differentially expressed between breeds (IFI44, CYP2C87, GBP1, GHR, PARP14, NTRK2, IGFBP3, TGFβ2) were selected based on functional analysis using DAVID. Total RNA (1μg) was incubated with DNAse I (Invitrogen®) and then reverse transcribed for cDNA with SuperScript III (Invitrogen) using Oligo-dT primer. Relative real-time RT-PCR analysis was performed with a StepOne Plus thermo cycler with Power Sybr Green PCR Master Mix (Applied Biosystems) with bovine-specific primers (Table 2). The PCR reactions were carried out in 25 μl volumes with 1μl of each sample and PCR cycling conditions were: 95°C for 10 min, 40 cycles at 95°C for 10 sec followed by annealing at 60°C for 1 minute. Reactions were optimized to provide maximum amplification efficiency for each gene (90-110%). Each sample was analyzed in duplicate, and the specificity of each PCR product was determined by melting curve analysis and amplicon size determination in agarose gels. To select the most stable

housekeeping gene for detailed analyses, were tested peptidylprolyl isomerase A (*PPIA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L15 (*RPL15*) gene expression and amplification profiles were compared using the geNorm applet for Microsoft Excel (medgen.ugent.be/genorm; (Ramakers et al., 2003). After analysis, the most stable housekeeping gene was *PPIA*. For the statistical analysis, CT values were subjected to ANOVA using the PROC GLM do SAS and significance was considered to be $P < 0.05$.

Table 2.2. Primers used for a microarray validation on real-time PCR.

Genes	Strand	Primer Sequence	Annealing Temperature (C^o)
IFI44	Forward	5'- GCTAGAGGCAGTTCACAGAAA - '3	54.7 ^{0}C
	Reverse	5'- GCAGAGAGGATCAGGACATCTA - 3'	55.4 ^{0}C
CYP2C87	Forward	5'- CCCAAGGGCACAGATATACTAAC - '3	54.8 ^{0}C
	Reverse	5'- AAGTGGCCAGGGTCAAATAC - 3'	55.1 ^{0}C
GBP1	Forward	5'- CCAGCACAGGAGGTATCATAAG - '3	54.7 ^{0}C
	Reverse	5'- CATGACCAACTATGTCCCAAGA - 3'	54.4 ^{0}C
GHR	Forward	5'- CCACTTCTCATTGGTGGAACT - '3	54.8 ^{0}C
	Reverse	5'- GTCGCTTACCTGGGCATAAA - 3'	55.1 ^{0}C
PARP14	Forward	5'- CAGACCAGACATAAATGGGAAGA - '3	54.3 ^{0}C
	Reverse	5'- CCTTTGGAGGAGGCACAATTA - 3'	54.9 ^{0}C
NTRK2	Forward	5'- CTGGTAGATGTGGGTGGTATTT - '3	54.2 ^{0}C
	Reverse	5'- CTGTGAGCTGTCTCTGCATT - 3'	54.9 ^{0}C
IGFPB3	Forward	5'- ACAGACACCCAGAACTTCTCCTC - '3	54.2 ^{0}C
	Reverse	5'- GCTTCCTGCCCTTGGA - 3'	54.9 ^{0}C
TGFβ2	Forward	5'- AGGCACGCTTTGCAGGTATT - '3	54.2 ^{0}C
	Reverse	5'- TAGCAGGAGATGTGGGCTCT - 3'	54.9 ^{0}C

RESULTS

Histology of the Ovary

Histological results from the ovary showed that Angus heifers had a greater density of preantral follicles comparing to the Nelore heifers ($P < 0.02$). Similar results were detected regarding primordial follicles, that presented a greater follicle density on Angus heifers when comparing with Nelore heifers, but there was no difference within groups. When groups were analyzed within breeds for Angus heifers the HFC group had a greater density than the LFC group ($P < 0.02$; Figure 1). There was no difference on primary follicles density within breeds or groups (Figure 2). The density of secondary follicles tended to be higher ($P = 0.09$) in Nellore heifers when compared with Angus heifers. Moreover, Nellore heifers with HFC had more secondary follicles comparing to LFC ($P < 0.01$; Figure 3).

Differential Expression Gene Profile

Venn diagram illustrating numbers of DEG is presented on Figure 4. Microarray data analysis revealed 1205 genes differentially expressed (log2; fold change > 1.5) between follicles from Angus and Nellore heifers. From those, 782 could be annotated. Of the annotated genes, expression of 316 genes were significantly lower on follicles from Angus heifers when compared with follicles from Nelore heifers (i.e. downregulated genes) and 466 genes had higher expression on follicles from Angus heifers when compared with follicles from Nelore heifers (i.e. upregulated genes). Among groups (LFC and HFC), 311 genes were differentially expressed and from those 188 could be annotated, with 142 genes significantly lower expressed on follicles from the LFC group (i.e downregulated genes) when compared with follicles from the HFC and

220 46 genes were significantly higher expressed on LFC (i.e upregulated genes) when compared
221 with follicles from the HFC group. With regards to the interaction effect between breeds and
222 groups a total of 315 genes were differentially expressed and from those 186 could be annotated.
223
224 ***Functional analysis***
225 Functional enrichment analysis was applied for the down and up regulated DEG for
226 breed, group and interaction effect in the integrate network with DAVID Functional annotation
227 tool. A total of 59 gene ontologies (GO) related to genes that were higher expressed in follicles
228 from Nelore heifers (i.e. downregulated genes) were significantly ($P < 0.05$) over-represented in
229 3 categories: biological process, cellular component and molecular function. Biological process
230 was the most representative category with osteoblast differentiation (BMP4, IGF1, IGFBP3 and
231 IGFBP5), cell proliferation (BMP4, SATB1, EMX2, IGF1, MAP7, EMPEP and FABP7) and
232 bone development (BMP4, COL13A1, IGF1, IGFBP3 and IGFBP5) being prominent (Table 3).
233 Moreover, biological adhesion and cell adhesion (CLDN16, COL4A3, MPZL2, TNXB, CD44,
234 PVRL2, STAB2, CLDN11, GPNMB, NTM and TGFβ2), fatty acid metabolic process (TYRP1,
235 TNXB, PTGES, ACACA, ALOX5 and GHR), regulation of cell death (BMP4, COL4A3,
236 NTRK1, BTC, NR4A2, TMEM102, IFG1, ZBTB16, IGFBP3 and TGFβ2) and transmembrane
237 receptor protein tyrosine kinase signaling pathway (TIAM1, NTRK1, NTRK2, GFRA2 and
238 GHR) were important functional processes that were higher expressed on follicles from Nelore
239 heifers when compared with follicles from Angus heifers. The higher expressed genes on
240 follicles from Angus heifers (i.e. upregulated genes) were over-represented in 46 GO. The most
241 representative GO category was biological processes with the organic acid catabolic process
242 (ACOX2, AMDHD1, PDPR, AASS, IDO1, NOS2, and PON3), carboxylic acid catabolic

process (ACOX2, AMDHD1, PDPR, AASS, IDO1, NOS2, and PON3) and negative regulation of homeostatic process (P2RX7, TERF2IP and IFI6) being most significant (Table 4).

Among the groups a total of 19 GO terms were significantly ($P < 0.05$) associated with genes that were higher expressed on follicles from HFC group (i.e. downregulated genes) with monosaccharide metabolic process (GPD2, ATF3, ENO2, NAGK and CACNA1A) the most significantly related to biological function. The higher expressed genes on LFC (i.e. upregulated genes) were over-represented only with two significant GO terms, regulation of JAK-STAT (HGS and EPO) related to biological process and cofactor binding (LOC788912, SDR39U1, PYGM, ALB and FDXR) related to molecular function.

Results of the Quantitative real-time PCR

The accuracy of microarray results was evaluated by RT-qPCR. A total of eight genes (IFI44, CYP2C87, GBP1, GHR, PARP14, NTRK2, IGFBP3, TGFβ2) that were differentially expressed between breeds based on microarray results were subjected to the analysis. For all eight genes, the fold change for Angus relative to Nelore heifers was in the same direction for qPCR as the fold change determined by microarray hybridization. The RT-qPCR validation confirmed differential expression levels for six genes ($P < 0.05$), one gene with tendency ($P = 0.07$) and one were no significant of the eight selected genes (Figure 5).

DISCUSSION

Activation and development of ovarian follicles require coordinated events that induce functional and morphological changes, leading to cell differentiation and oocyte development. DNA microarray technology permits the analysis of global gene expression profiles that are quite useful to understand the basis of cellular biology. Currently finds on this study leads our hypothesis that the follicle variation between *Bos indicus* and *Bos taurus* and in the same subspecies results in a different gene expression profile of the AFC.

In this study, we demonstrate through histological analysis of the ovaries a high variation between breeds on the preantral follicles count. Although Nelore heifers had a greater number of antral follicles ≥ 3mm when examined by ultrasound (Table 1), histological analysis of the ovarian cortex shown that Angus heifers had a greater preantral follicles density compared to Nelore heifers. The higher density of preantral follicle on Angus is associated with a higher number of primordial follicles density on this breed (Figure 1). However, the density of secondary follicles tended to be higher on Nelore heifers (Figure 3). In a study comparing follicular population through histology analysis between *Bos indicus* and *Bos indicus-taurus* cows, Silva-Santos et al. (2014) showed similar results with greater numbers of primordial follicles and lower numbers of secondary follicles on *Bos indicus-taurus* comparing to *Bos indicus*. This finds suggest the existence of different mechanisms during follicle activation, transition and growth on *Bos indicus* and *Bos taurus* animals. Moreover, it is possible that *Bos indicus* and *Bos taurus* animals are born with the same number of follicles in the ovary and due to the higher follicle recruitment though out their estrous cycles *Bos indicus* animals have a depleted reserve in comparison to *Bos taurus* animals.

290 Gene expression data from the present study supports the idea that patterns of follicles
291 gene expression are different between breeds and groups. For instance, analyses of the nominal
292 differentially expressed genes using DAVID bioinformatics resources identified a number of
293 functional categories that were different in follicles from Nelore compared to Angus heifers. Of
294 note, genes associate with osteoblast differentiation (BMP4, IGF1, IGFBP3 and IGFBP5), cell
295 proliferation (BMP4, SATB1, EMX2, IGF1, MAP7, EMPEP and FABP7) and bone
296 development (BMP4, COL13A1, IGF1, IGFBP3 and IGFBP5) are higher expressed on follicles
297 from Nelore heifers. Growth hormone and the IGF system composed by IGF1, IGF2 and their
298 receptors and Insulin grown factor binding proteins (IGFBPs) exert an important role on follicle
299 development (Silva et al., 2009). The IGF1 seems to play a key role on increasing the sensitivity
300 of small antral follicles (5 mm diameter in cattle) to gonadotropin responsiveness (Monget et al.,
301 2002). In cattle, *in vivo* dose-response experiments with GH treatment demonstrate that GH act
302 by rising circulating IGF1 and/or insulin levels increasing the numbers of health follicles with 2
303 – 5 mm (Gong et al., 1997). The IGF1 can also stimulate proliferation and differentiation on
304 granulose cells *in vitro* (Monniaux & Pisselet, 1992). Furthermore, a recently study in *zebrafish*
305 showed evidence that IGF1 also exert an important function on follicular activation by increasing
306 kit-ligant gene expression (Yao et al. 2014). The IGFBPs have been described to prolong the
307 half-life of the IGF1 and can either inhibit or stimulate the promotion of grown effects of the
308 IGFs on cell culture (Monget et al 2002). The higher expression of genes from the IGF system
309 (IGF1, IGFBP3 and 5) and GH receptor (GHR) can be contributing to greater AFC on Nelore
310 heifers.

311 Genes involved in transmembrane receptor protein tyrosine kinase signaling pathway are
312 also higher expressed in follicles from Nelore heifers including the Neurotrophic tyrosine kinase

receptor type 1 and 2 (NTKR1 and NTKR2). The Nerve Growth Factor (NGF), one of the neurotropins (NTs), and their receptor Tyrosine kinase receptor A (TrkA) have been demonstrated to exert important effects on assembling primordial follicles, promoting the early stages of follicle development (Wang et al., 2014). The NGF is a protein that is necessary for survival, maintenance and development of the neuronal population (Chaves et al., 2013). In the ovaries the NGF can act direct or indirect increasing the survivability of follicles. In caprines, preantral follicles cultivated *in vitro* with NGF, enclosed in fragments of ovaries demonstrated the greater survivability in a dose-dependent way (Chaves et al., 2010). The NGF also may act indirectly increasing follicular survival through the production of biologically active follicle-stimulating hormone receptors (FSHr) (Salas et al., 2006). Another gene higher expressed in Nelore, Bone Morphogenic Proteins 4 (BMP4), is a member of TGFβ-superfamily. The TGFβ-superfamily cytokines are directly involved in the follicle development in mammals (Knigh and Glister, 2006). The addition of BMP4 in the ovarian cortex cultivated *in vitro* promote the primordial to primary follicle transition in neonatal rat ovaries, moreover adding BMP4 neutralizing antybody to culture system promote a progressive loss of oocytes and follicles and progressive increase in cell apoptosis (Nilsson and Skinner, 2003).

When genes were compared between groups the membrane receptors of the MAPK pathway (Figure 6) CACNA1S, NTRK1 and FGFR1 were over expressed in HFC group. These genes have been associated with cell proliferation in ovarian cancer and mitosis of ovarian germ cells. The calcium channel CACNA1 have been studied in C. elegans on which its increase leads to an enhancement in seam cell proliferation through a decrease in POP1 expression. The POP1 gene acts negatively regulating cell proliferation. Furthermore, treatment of somatic gonad cells with CACNA1 RNA*i* lead to an increase in POP1 expression (LaBonty et al., 2014). This finds

suggest that CACNA1 is acting to increase cell proliferation and to maintain the undifferentiated status of the cells. Basic FGF (bFGF) plays diverse roles in cell proliferation and differention during embryo development. Specifically in the ovarian germ cell development, bFGF accelerates cell proliferation by binding with FGFR1 and also maintain its undifferentiated status (He et al., 2012; Lin et al., 2012). Those two genes along with NTRK1, which has been discussed above, are three out of the five membrane receptors in the cell proliferation part of the MAPK pathway. This is especially important since the receptors are the first molecules to be activated after binding of the ligand. Furthermore, TRPC4, a short transient receptor potential channel which forms a non-selective calcium-permeable cation channel, was higher expressed in follicles from the HFC group. This TRPC4 plays a major role in cell proliferation and death by controlling gene expression, progression through the cell cycle and DNA synthesis (Holmuhamedov et al., 2002). In the ovary, inhibition of TRPC4 lead to an anti-proliferative effect of adenocarcinoma cells (Zeng et al., 2013). The GO monosaccharide metabolic process was the GO with the most expressive P value. It had several genes (GPD2, ATF3, ENO2, NAGK and CACNA1A) that were higher expressed in follicles from the HFC group. These genes are involved in cell metabolism, especially glucose. In the ovary, studies have shown that ENO2 expression is higher during preantral and antral follicle development and lower after ovulation, being controlled by gonadotropins (Yoshioka et al., 2011).

The GO that had the higher percentage (16 %) of its genes higher expressed in follicles from HFC group was plasma membrane with 19 genes differentially expressed (TRPC4, CRYAB, GPR171, DRD5, ENPEP, MBP, CCR9, P2RY14, NTRK2, CD99L2, MC4R, SLC22A6, GPNMB, NT5E, TSHR, GFRA2, CACNA1A, HTR2A, FN1) followed by extracellular region with 13 % represented by 16 genes (CPA5, C3, NDP, CHI3L1, F9, BNBD-

9-LIKE, DEFB7, APOC4, SERAC1, RSPO3, CFH, LOC788983, FIBIN, FGF1, ADAMTS5, SPP1, FN1) higher expressed in follicles from the HFC group (Supplementary file 2) . These GO are cellular component and describes location where a gene product may act. Plasma membrane and the extracellular region are important for transport of carbohydrate, amino acid, protein and ions. Furthermore, these ontologies may be involved in ATP activity, cell cycle and division, protein targeting, regulation of transcription and other signaling pathways by facilitating passage of molecules through the membrane. Several of these genes can be found in the literature associated with ovarian cellular proliferation and follicular development. Expression of fibronectin 1 (FN1), a component of the extracellular matrix, was higher in granulose cells from pre-ovulatory follicles from Nellore cows superovulated with Follicle Stimulating Hormone (Dias et al., 2013). Here the follicles from the HFC group had a greater expression of FN1. Furthermore, TGFβ elicited an increase in FN at the protein and mRNA levels (Colman-Lemer et al., 1999). In this study, TGFβ2 was upregulated in the follicles from Nelore heifers, which overall have more follicles when compared to Angus or other breeds. The chemokine receptor CCR9 and its ligand (TECK) are associated with cellular resistance to apoptosis and migration. They are mostly expressed in cancers including ovary carcinoma, but they are also present in some healthy tissues including the ovaries. The peak expression of CCR9 and TECK are observed close to ovulation, being observed an 800-fold increase in mRNA expression (Zhou et al., 2005). Melanocortin-4 receptor (MC4R), increased in HFC heifers, is also associated with ovulation, pregnancy in MC4R deficient mice is rather rare and ovulation is decreased, with CL number reduced to zero while atretic and cystic follicles are increased (Sandrock et al., 2009). Thyroid-stimulating hormone receptor (TSHR), another gene that was increased in follicles from the HFC group, exist in the ovary and its level is controlled by gonadotropins through the cAMP

and steroid feedback (Sun et al., 2010). Expression of TSHR is involved in FSHR and LHR agonist recognition, through tyrosine-sulfatation (Sasaki et al., 2007). Lack of thyroid activity causes infertility which is also characterized by follicular arrest and disturbed ovulation (Abel et al., 2000; Ma et al., 2004; Hosoda et al., 2008).

Overall, results show that genes related to cellular metabolism and proliferation are over expressed in Nelore and HFC heifers and through histology we could observe a decrease in follicle reserve on these animals and an increase in secondary follicles. The different pattern of gene expression can be responsible for the higher recruitment and development of follicles on these animals. It is known that the follicle count characteristic is highly repeatable between estrus cycles and is inherited within generations. Our work shows genes that are important for this mechanism and can be used as molecular markers to select animals for assisted reproductive technologies.

CONCLUSION

Nelore heifers have a different pattern of gene expression regulating follicle recruitment and development. Furthermore, HFC heifers presented an increased expression of genes associated with cellular development and metabolism.

REFERENCE

Abel, MH, AN Wootton, V Wilkins, I Huhtaniemi, PG Knight, and HM Charlton 2000 The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. *Endocrinology* **141** 1795-1803.

Adams, GP, R Jaiswal, J Singh, and P Malhi 2008 Progress in understanding ovarian follicular dynamics in cattle. *Theriogenology* **69** 72-80.

Buratini, J, CA Price, JA Visintin, and GA Bó 2000 Effects of dominant follicle aspiration and treatment with recombinant bovine somatotropin (BST) on ovarian follicular development in nelore (Bos indicus) heifers. *Theriogenology* **54** 421-431.

Burns, DS, F Jimenez-Krassel, JL Ireland, PG Knight, and JJ Ireland 2005 Numbers of antral follicles during follicular waves in cattle: evidence for high variation among animals, very high repeatability in individuals, and an inverse association with serum follicle-stimulating hormone concentrations. *Biol Reprod* **73** 54-62.

Chaves, RN, AM Alves, AB Duarte, VR Araújo, JJ Celestino, MH Matos, CA Lopes, CC Campello, KP Name, SN Báo, and JR Figueiredo 2010 Nerve growth factor promotes the survival of goat preantral follicles cultured in vitro. *Cells Tissues Organs***192** 272-282.

Chaves, RN, AM Alves, LF Lima, HM Matos, AP Rodrigues, and JR Figueiredo 2013 Role of nerve growth factor (NGF) and its receptors in folliculogenesis. *Zygote* **21** 187-197.

Colman-Lerner, A, ML Fischman, GM Lanuza, DM Bissell, AR Kornblihtt, and JL Barañao 1999 Evidence for a role of the alternatively spliced ED-I sequence of fibronectin during ovarian follicular development. *Endocrinology* **140** 2541-2548.

Dennis, G, BT Sherman, DA Hosack, J Yang, W Gao, HC Lane, and RA Lempicki 2003 DAVID: Database for Annotation, Visualization, and Integrated Discovery.*Genome Biol* **4** P3.

Dias, FC, MI Khan, MA Sirard, GP Adams, and J Singh 2013 Differential gene expression of granulosa cells after ovarian superstimulation in beef cattle. *Reproduction* **146** 181-191.

Gong, JG, G Baxter, TA Bramley, and R Webb 1997 Enhancement of ovarian follicle development in heifers by treatment with recombinant bovine somatotrophin: a dose-response study. *J Reprod Fertil* **110** 91-97.

He, B, J Lin, J Li, Y Mi, W Zeng, and C Zhang 2012 Basic fibroblast growth factor suppresses meiosis and promotes mitosis of ovarian germ cells in embryonic chickens.*Gen Comp Endocrinol* **176** 173-181.

Hirshfield, AN 1991 Development of follicles in the mammalian ovary. *Int Rev Cytol* **124**43-101.

Holmuhamedov, E, L Lewis, M Bienengraeber, M Holmuhamedova, A Jahangir, and A Terzic 2002 Suppression of human tumor cell proliferation through mitochondrial targeting. *FASEB J* **16** 1010-1016.

Hosoda, Y, N Sasaki, and T Agui 2008 Female infertility in grt mice is caused by thyroid hormone deficiency, not by insufficient TPST2 activity in the reproductive organs. *J Vet Med Sci* **70** 1043-1049.

Ireland, JJ, F Ward, F Jimenez-Krassel, JL Ireland, GW Smith, P Lonergan, and AC Evans 2007 Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-quality embryos after ovarian stimulation in cattle. *Hum Reprod* **22** 1687-1695.

Knight, PG, and C Glister 2006 TGF-beta superfamily members and ovarian follicle development. *Reproduction* **132** 191-206.

LaBonty, M, C Szmygiel, LE Byrnes, S Hughes, A Woollard, and EJ Cram 2014 CACN-1/Cactin plays a role in Wnt signaling in C. elegans. *PLoS One* **9** e101945.

Lin, J, Y Jia, W Zeng, Y Mi, and C Zhang 2012 Basic FGF promotes proliferation of ovarian granulosa cells in the laying chickens via FGFR1 and PKC pathway. *Reprod Domest Anim* **47** 135-142.

Lucci, CM, RV Silva, CA Carvalho, R Figueiredo, and N Báo 2001 Light microscopical and ultrastructural characterization of goat preantral follicles. *Small Rumin Res* **41** 61-69.

Ma, X, Y Dong, MM Matzuk, and TR Kumar 2004 Targeted disruption of luteinizing hormone beta-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. *Proc Natl Acad Sci U S A* **101** 17294-17299.

McNatty, KP, DA Heath, T Lundy, AE Fidler, L Quirke, A O'Connell, P Smith, N Groome, and DJ Tisdall 1999 Control of early ovarian follicular development. *J Reprod Fertil Suppl* **54** 3-16.

Monget, P, S Fabre, P Mulsant, F Lecerf, JM Elsen, S Mazerbourg, C Pisselet, and D Monniaux 2002 Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. *Domest Anim Endocrinol* **23** 139-154.

Monniaux, D, and C Pisselet 1992 Control of proliferation and differentiation of ovine granulosa cells by insulin-like growth factor-I and follicle-stimulating hormone in vitro.*Biol Reprod* **46** 109-119.

Mossa, F, SW Walsh, ST Butler, DP Berry, F Carter, P Lonergan, GW Smith, JJ Ireland, and AC Evans 2012 Low numbers of ovarian follicles ≥3 mm in diameter are associated with low fertility in dairy cows. *J Dairy Sci* **95** 2355-2361.

Nilsson, EE, and MK Skinner 2003 Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biol Reprod* **69**1265-1272.

Pontes, JH, FA Melo Sterza, AC Basso, CR Ferreira, BV Sanches, KC Rubin, and MM Seneda 2011a Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (Bos indicus) donors.*Theriogenology* **75** 1640-1646.

Pontes, JH, FA Melo Sterza, AC Basso, CR Ferreira, BV Sanches, KC Rubin, and MM Seneda 2011b Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (Bos indicus) donors.*Theriogenology* **75** 1640-1646.

Ramakers, C, JM Ruijter, RH Deprez, and AF Moorman 2003 Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* **339** 62-66.

Salas, C, M Julio-Pieper, M Valladares, R Pommer, M Vega, C Mastronardi, B Kerr, SR Ojeda, HE Lara, and C Romero 2006 Nerve growth factor-dependent activation of trkA receptors in the human ovary results in synthesis of follicle-stimulating hormone receptors and estrogen secretion. *J Clin Endocrinol Metab* **91** 2396-2403.

Sandrock, M, A Schulz, C Merkwitz, T Schöneberg, K Spanel-Borowski, and A Ricken2009 Reduction in corpora lutea number in obese melanocortin-4-receptor-deficient mice. *Reprod Biol Endocrinol* **7** 24.

Sartori, R, and CM Barros 2011 Reproductive cycles in Bos indicus cattle. *Anim Reprod Sci***124** 244-250.

Sartori, R, MR Bastos, PS Baruselli, LU Gimenes, RL Ereno, and CM Barros 2010 Physiological differences and implications to reproductive management of Bos taurus and Bos indicus cattle in a tropical environment. *Soc Reprod Fertil Suppl* **67** 357-375.

Sasaki, N, Y Hosoda, A Nagata, M Ding, JM Cheng, T Miyamoto, S Okano, A Asano, I Miyoshi, and T Agui 2007 A mutation in Tpst2 encoding tyrosylprotein sulfotransferase causes dwarfism associated with hypothyroidism. *Mol Endocrinol* **21**1713-1721.

Silva, JR, JR Figueiredo, and R van den Hurk 2009 Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis.*Theriogenology* **71** 1193-1208.

Silva-Santos, KC, GM Santos, LS Siloto, JT Santos, ER Oliveira, FZ Machado, CO Rosa, and MM Seneda 2014 The correlation between the number of antral follicles and ovarian reserves (preantral follicles) in purebred Bos indicus and Bos taurus cows.*Anim Reprod Sci* **151** 119-125.

Smyth, G. 2005 Limma: linear models for microarray data. In 'Bioinformatics and computacional biology solutions using r and Bioconductor'. (Eds V.C.R Gentleman; S Dudoit; R Irizarry and W Huber) Springer: New Work 397-420

Sun, SC, PJ Hsu, FJ Wu, SH Li, CH Lu, and CW Luo 2010 Thyrostimulin, but not thyroid-stimulating hormone (TSH), acts as a paracrine regulator to activate the TSH receptor in mammalian ovary. *J Biol Chem* **285** 3758-3765.

Tamassia, M, Y Heyman, Y Lavergne, C Richard, V Gelin, JP Renard, and S Chastant-Maillard 2003 Evidence of oocyte donor cow effect over oocyte production and embryo development in vitro. *Reproduction* **126** 629-637.

Wang, Y, N Liang, G Yao, H Tian, Y Zhai, Y Yin, and F Sun 2014 Knockdown of TrkA in cumulus oocyte complexes (COCs) inhibits EGF-induced cumulus expansion by down-regulation of IL-6. *Mol Cell Endocrinol* **382** 804-813.

Webb, R, and BK Campbell 2007 Development of the dominant follicle: mechanisms of selection and maintenance of oocyte quality. *Soc Reprod Fertil Suppl* **64** 141-163.

Webb, R, B Nicholas, JG Gong, BK Campbell, CG Gutierrez, HA Garverick, and DG Armstrong 2003 Mechanisms regulating follicular development and selection of the dominant follicle. *Reprod Suppl* **61** 71-90.

Yao, K, SW Lau, and W Ge 2014 Differential regulation of Kit ligand A expression in the ovary by IGF-I via different pathways. *Mol Endocrinol* **28** 138-150.

Yoshioka, N, N Takahashi, W Tarumi, MT Itoh, and B Ishizuka 2011a Gonadotropins up-regulate the expression of enolase 2, but not enolase 1, in the rat ovary. *Endocr J***58** 941-948.

Yoshioka, N, N Takahashi, W Tarumi, MT Itoh, and B Ishizuka 2011b Gonadotropins up-regulate the expression of enolase 2, but not enolase 1, in the rat ovary. *Endocr J***58** 941-948.

Zeng, B, C Yuan, X Yang, SL Atkin, and SZ Xu 2013 TRPC channels and their splice variants are essential for promoting human ovarian cancer cell proliferation and tumorigenesis. *Curr Cancer Drug Targets* **13** 103-116.

Zhou, C, J Wu, J Borillo, L Torres, J McMahon, Y Bao, and YH Lou 2005 Transient expression of CC chemokine TECK in the ovary during ovulation: its potential role in ovulation. *Am J Reprod Immunol* **53** 238-248.

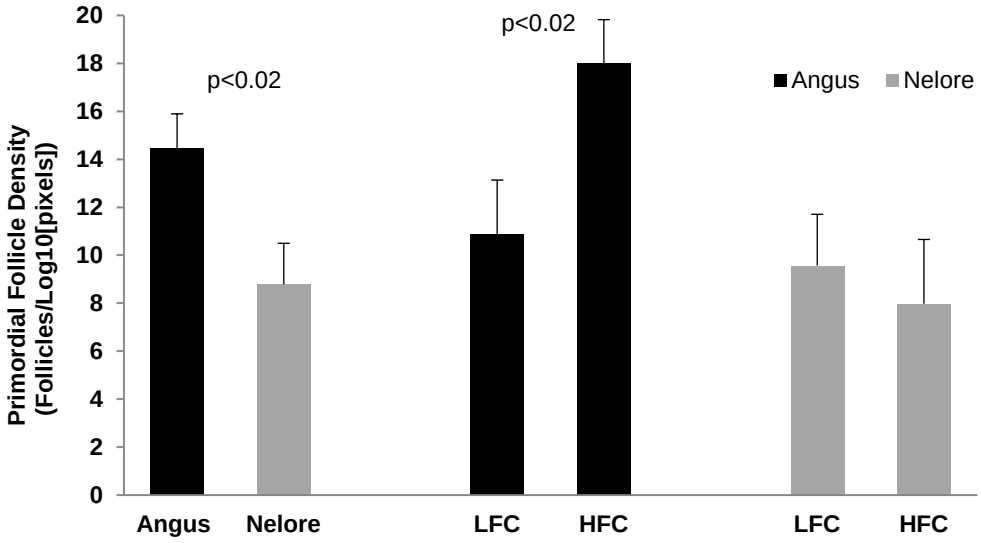


Figura 2.1. Primordial follicle density (Follicles/Log10[pixels]). Black bars represent Angus heifers and gray bars represent Nelore heifers with low follicle count (LFC) and high follicle count (HFC).

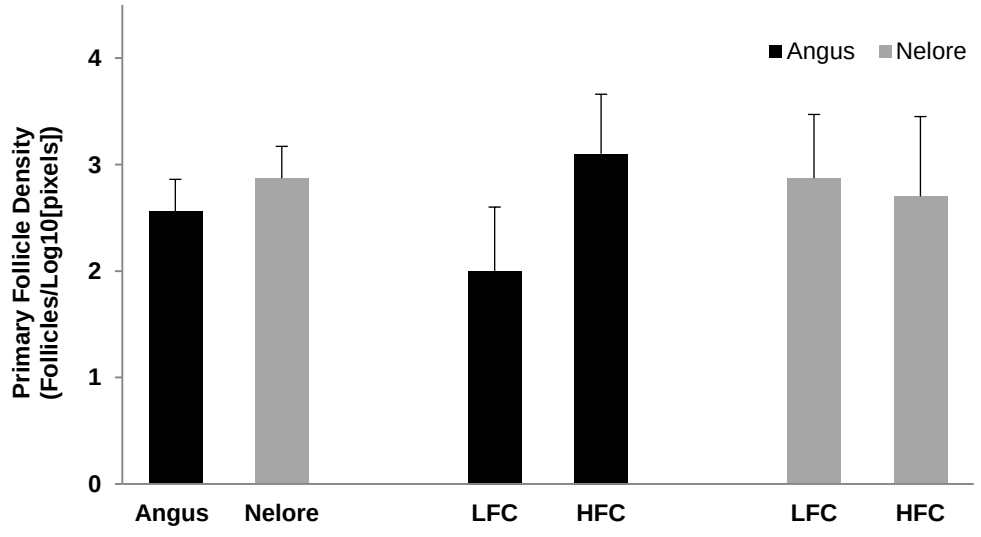


Figura 2.2. Primary follicle density (Follicles/Log10[pixels]). Black bars represent Angus heifers and gray bars represent Nelore heifers with low follicle count (LFC) and high follicle count (HFC).

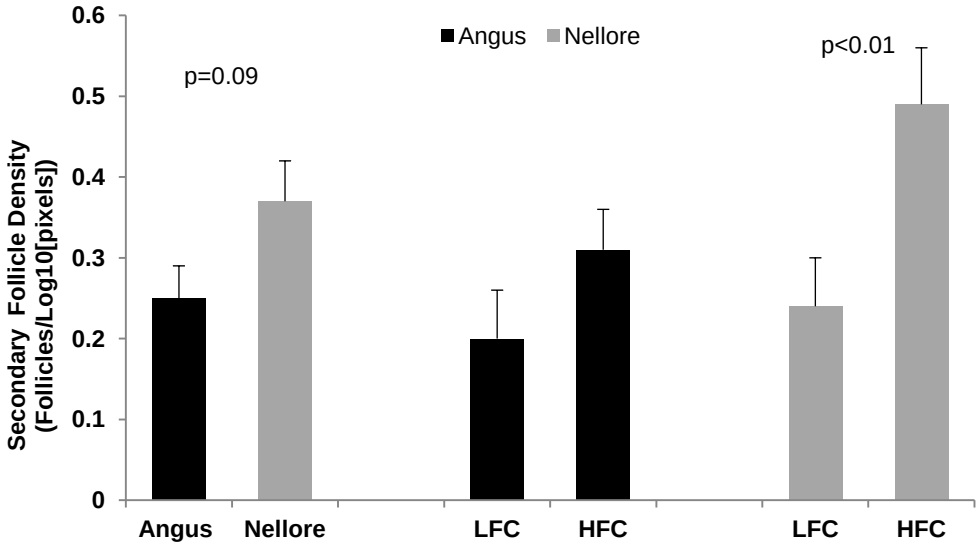


Figura 2.3. Secondary follicle density (Follicles/Log10[pixels]). Black bars represent Angus heifers and gray bars represent Nelore heifers with low follicle count (LFC) and high follicle count (HFC).

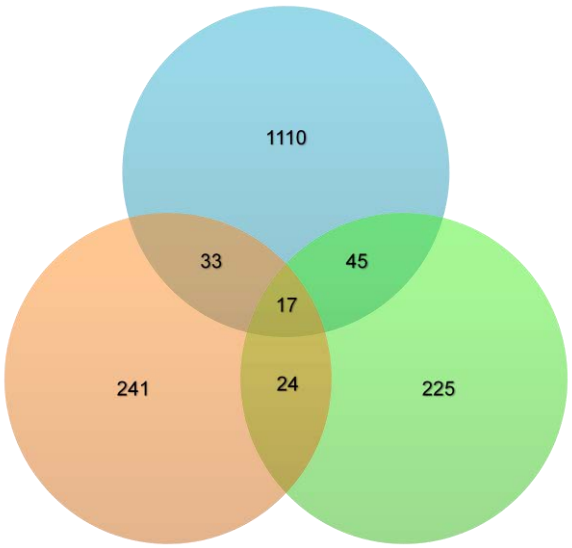


Figura 2.4. Venn diagram of the differentially expressed between breeds (blue - 1205 genes, 701 and 504 up- and downregulated, comparing Angus vs Nelore heifers), groups (green - 311 genes, 77 and 234 genes up- and downregulated comparing LFC with HFC) and

interaction effect between breeds and groups (red - 315 genes). Intersections indicate common genes).

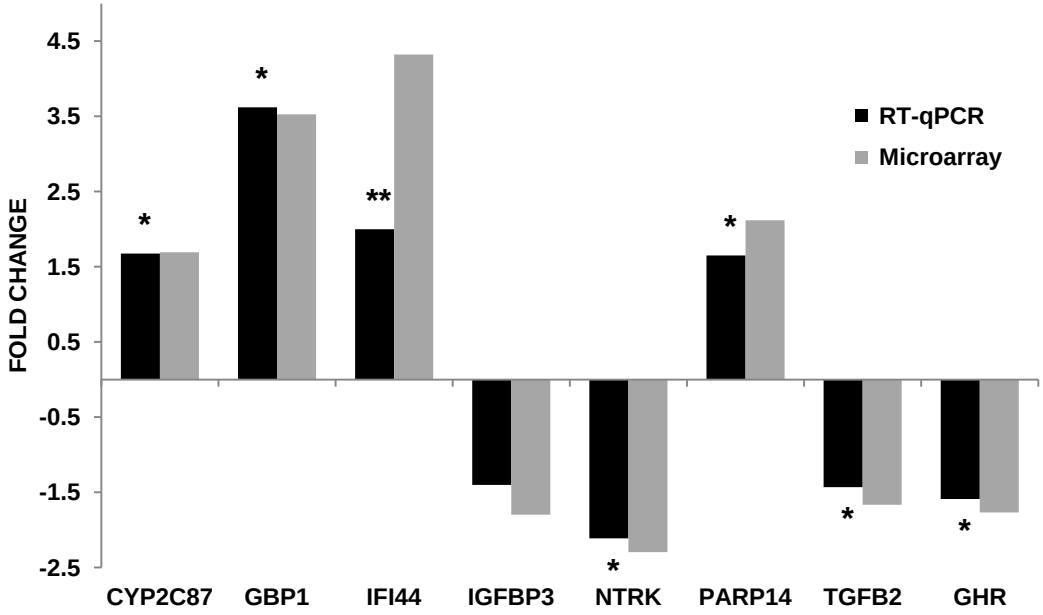


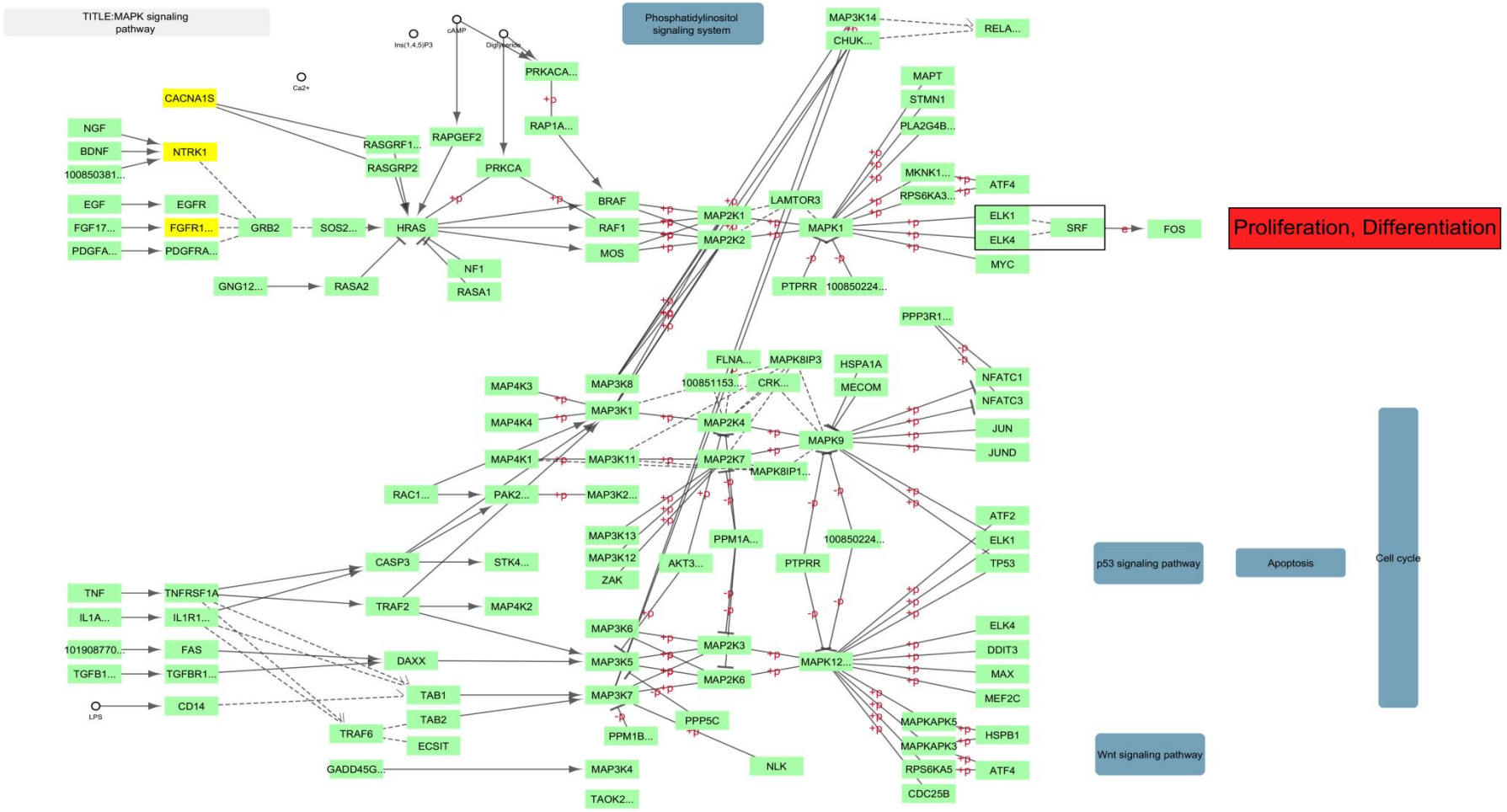
Figura 2.5. Quantification (log2 of the fold change) of the mRMA profile of follicles on selected DEG between breeds on microarray results. Gray bars represent the differential level of expression of transcripts detected in the microarray experiment, while black bars represent the differential level of expression of the same transcripts obtained by real-time PCR. (*Values represent P < 0.05 and **value represent P < 0.1 for real time PCR).

Table 2.3. Top 10 Gene Ontology terms related to biological process of the genes over expressed in Nelor heifers (i.e. down-regulated) comparing Angus vs. Nelore heifers.

Category	GO ~ Term	Count	%	PValue
GOTERM_BP_FAT	GO:0001649 ~ osteoblast differentiation	4	1.6	0.003068383
GOTERM_BP_FAT	GO:0008283 ~ cell proliferation	7	2.8	0.006192001
GOTERM_BP_FAT	GO:0060348 ~ bone development	5	2	0.008399566
GOTERM_BP_FAT	GO:0001503 ~ ossification	5	2	0.008399566
GOTERM_BP_FAT	GO:0001501 ~ skeletal system development	7	2.8	0.008965672
GOTERM_BP_FAT	GO:0048660 ~ regulation of smooth muscle cell proliferation	3	1.2	0.00918216
GOTERM_BP_FAT	GO:0042417 ~ dopamine metabolic process	3	1.2	0.00918216
GOTERM_BP_FAT	GO:0030182 ~ neuron differentiation	7	2.8	0.009320967
GOTERM_BP_FAT	GO:0007281 ~ germ cell development	4	1.6	0.013842831
GOTERM_BP_FAT	GO:0022610 ~ biological adhesion	11	4.4	0.015950268

Table 2.4. Top 10 Gene Ontology terms related to biological process of the genes over expressed in Angus heifers (i.e. up-regulated) comparing Angus vs. Nelore heifers.

Category	GO ~ Term	Count	%	PValue
GOTERM_BP_FAT	GO:0016054 ~ organic acid catabolic process	7	1.9	0.002211484
GOTERM_BP_FAT	GO:0046395 ~ carboxylic acid catabolic process	7	1.9	0.002211484
GOTERM_BP_FAT	GO:0032845 ~ negative regulation of homeostatic process	3	0.8	0.009851153
GOTERM_BP_FAT	GO:0006952 ~ defense response	12	3.2	0.011946464
GOTERM_BP_FAT	GO:0009063 ~ cellular amino acid catabolic process	5	1.3	0.01214098
GOTERM_BP_FAT	GO:0006955 ~ immune response	14	3.8	0.01511071
GOTERM_BP_FAT	GO:0050708 ~ regulation of protein secretion	4	1.0	0.016106832
GOTERM_BP_FAT	GO:0006508 ~ proteolysis	23	6.2	0.020616151
GOTERM_BP_FAT	GO:0009310 ~ amine catabolic process	5	1.3	0.020988679
GOTERM_BP_FAT	GO:0003013 ~ circulatory system process	6	1.6	0.021683946


TITLE:MAPK signaling pathway
Phosphatidylinositol signaling system
cAMP
Ins(1,4,5)P3
Diglyceride
Ca2+
CACNA1S
NGF
BDNF
100850381...
NTRK1
EGF
EGFR
FGF17...
FGFR1...
PDGFA...
PDGFRA...
GRB2
SOS2...
HRAS
GNG12...
RASA2
NF1
RASA1
RASGRF1...
RASGRP2
RAPGEF2
PRKACA...
RAP1A...
PRKCA
BRAF
RAF1
MOS
MAP2K1
MAP2K2
LAMTOR3
MAPK1
PTPRR
100850224...
MAP3K14
CHUK...
RELA...
MAPT
STMN1
PLA2G4B...
MKNK1...
RPS6KA3...
ATF4
ELK1
ELK4
MYC
SRF
FOS
Proliferation, Differentiation
PPP3R1...
NFATC1
NFATC3
JUN
JUND
FLNA...
MAPK8IP3
HSPA1A
MECOM
100851153...
CRK...
MAP4K3
MAP4K4
MAP4K1
MAP3K8
MAP3K1
MAP3K11
MAP2K4
MAP2K7
MAPK9
MAPK8IP1...
RAC1...
PAK2...
MAP3K2...
MAP3K13
MAP3K12
ZAK
PPM1A...
AKT3...
PTPRR
100850224...
ATF2
ELK1
TP53
CASP3
STK4...
TRAF2
MAP4K2
TNF
TNFRSF1A
IL1A...
IL1R1...
101908770...
FAS
TGFB1...
TGFBR1...
LPS
CD14
DAXX
TAB1
TAB2
TRAF6
ECSIT
GADD45G...
MAP3K6
MAP3K5
MAP3K7
MAP2K3
MAP2K6
PPP5C
PPM1B...
NLK
MAP3K4
TAOK2...
MAPK12...
ELK4
DDIT3
MAX
MEF2C
MAPKAPK5
MAPKAPK3
RPS6KA5
CDC25B
HSPB1
ATF4
p53 signaling pathway
Apoptosis
Cell cycle
Wnt signaling pathway

5

Figura 2.6. David pathway interaction network analysis. Genes involved in MAPK – signaling, which were differentially expressed between the LFC compared to HFC are presented. The network displays yellow node that represent genes higher expressed in the HFC group compared to LFC. Relationships between molecules are indicated by solid (direct) or dashed (indirect) connecting lines.

CAPÍTULO 3

Genome-Wide Associations Study of Nelore and Angus Heifers With Low and High Ovarian Follicle Count

Running Head:

Genome-Wide Associations Study of Nelore and Angus Heifers With Low and High Ovarian Follicle Count

M.G. Favoreto[1]; B. Loureiro[2]; R.L. Ereno[1]; A.G. Pupulin[1]; J. Buratini[3]; C.M. Barros[1].

[1]Department of Pharmacology, Institute of Biosciences, São Paulo State University (UNESP), Botucatu, Brazil;

[2]Department of Veterinary Medicine, Vila Velha University (UVV), Vila Velha, Brazil;

[3]Department of Physiology, Institute of Biosciences, São Paulo State University (UNESP), Botucatu, Brazil;

[1]corresponding autor: barrosciro@gmail.com

ABSTRACT

Performing a genome-wide association study (GWAS) may improve the understanding of antral follicle variation counted by ultrasound in bovines. The aimed of this study was to performe a GWAS on Nelore and Angus heifers with high (HFC) and low (LFC) antral follicle count. A total of 72 Nelore heifers (32 HFC and 40 LFC) and 48 Angus heifers (21 HFC and 27 LFC) were selected and the DNA was extracted from blood and hair bulb. Genotyping was done using the 777 HD Illumina chip. The SNPs were submitted to a quality control test using exclusion criteria: *call rate* (IDCR) ≤ 90%, MAF ≤ 0.02, *call rate* (SNPCR) ≤ 0.98 and HWE ≤ 1 x 10^{-5}. After the quality control test the SNPs were submitted to the Cochran-Armitage test to the association of phenotype/genotype in both breeds. The genes associated with the SNPs were submitted to an enrichment analysis through the on line DAVID tool. The GWAS showed 181 and 201 SNPs with genotype/phenotype association (P ≤ 10E-3) in Nelore and Angus heifers, respectively. A total of 97 genes were associated to the 181 SNPs in the Nelore breed and the functional analysis identified genes in the ROBO-STLIT pathway that can be involved in the control of germ cell migration to the ovary. In the Angus heifers 57 genes were associated with the 201 SNPs, highlighting the Fribilin 1 (FBN1) gene, involved in regulation of growth factors.

Key Words: Bovine; GWAS; Follicle; Reproduction

INTRODUCTION

Reproductive efficiency in cattle is an important element of the animal production systems. Recent studies have associated the number of antral follicle count (AFC) greater than 3 mm counted by ultrasound with fertility in dairy cattle. Dairy cows with low follicle count have lower pregnancy rates at first service, longer interval from calving to conception and higher number of services received during the breeding season compared with cows with higher follicle count (HFC; Mossa et al., 2012). Furthermore, cows with HFC have increased number of total oocyte recovery by ultrasound-guide follicular aspiration (OPU) and, consequently, a greater number of total embryos produced by IVF (Ireland et al., 2007).

In the last years the use of genomic information is being widely used as strategy to improve selection of phenotypes such as increased milk production (Chamberlain et al., 2012), fertility (Peñagaricano et al., 2011; Huang et al., 2010) and other economic traits of interest in bovines. Genome-wide association study combined with new techniques of SNP prospection allowed the identification of a subset of markers that can explain important portions on the variation of these characteristics. An alternative approach to finding SNPs associated with a phenotype of interest is a candidate gene approach where individual genes are selected as candidate based on their known function (Naukkarinem et al., 2010)

Identify genomic markers and possible genes, responsible for a genetic variation in Nelore and Angus with HFC and LFC can improve the understanding of biological pathways involved in AFC phenotype. The objective of this study was to perform a GWAS study in Nelore and Angus heifers with HFC and LFC using the high-density SNPs array to identify genetic variants and possible genes associated with these phenotypes.

MATERIAL AND METHODS

Animals Selection and Phenotypic Data

A group of a 155 Nelore and 132 Angus heifers with similar age (ranged to 22 and 28 months) and body weight were injected with two doses of PGF2α 11 days apart to initiate luteolysis and synchronize occurrence of ovulation. Ovaries of each animal were scanned with an ultrasound device (US; Mindray Vet DPS 2200, São Paulo, Brazil) equipped with a 7.5-MHz probe one day after ovulation for three consecutive estrous cycles to determine the groups high (HFC) and low (LFC) follicle count. The groups were formed based on the average of the total follicles (≥ 3 mm) counted for each breed consistently ± standard deviation (Table 3.1). A total of 72 Nelore heifers were selected and allocated in the groups: 32 with HFC (40 ≥ follicles) and 40 with LHC (20 ≤ follicles). For Angus heifers 48 were selected and allocated into the groups: 21 with HFC (20 ≥ follicles) and 27 with LFC (≤ 10 follicles).

Table 3.1. Numbers of animals, follicle average count and standard deviation for Nelore and Angus heifers with HFC and LFC.

	Nelore		Angus	
	HFC 40 ≥ Fol	LFC ≤ 20 Fol	HFC 20 ≥ Fol	LFC ≤ 10 Fol
Number of animals	43	35	22	27
Fol average count	49	15	25	6
Standard deviation	9.3	3.9	5.1	2.3

Sample and DNA extraction

For all animals selected, samples of blood from the caudal vein or capillary bulb from the tail hair were collected for DNA extraction. For the blood samples the DNA was extracted using

the MiniPrep kit (Axygen bioscience), whereas the capillary bulb was extracted by NucleoSpin Tissue kit (Macherey-Nagel) according to manufacturer's instructions protocols. After extraction, the quality of the DNA samples was assessed by determining the ratio A260/280 in a biophotometer. The samples were accepted when values remained between 1.8 and 2.0 and the DNA was quantified and diluted to a concentration ranged from 50 ng/µL to 150 ng/µL for subsequent genotyping.

Genotyping and SNPs Quality Control

Genotyping was performed by DEOXI BIOTECNOLGIA LTDA, Araçatuba, São Paulo, Brazil using BovineHD 770K BeadChip (Infinium BeadChip, Illumina, San Diego, CA) according to manufacturer's instructions protocol. Genotypes were obtained in Illumina A/B allele format and used to represent a covariate value at each locus coded as 0, 1 or 2, representing the number of B alleles. The initial data analysis and visualization was performed by GenomeStudio Data Analysis Software (ILLUMINA, 2013). For GWAS, SNPs were subjected to a quality control in which only autossomal SNPs with known genomic coordinate were considered. Samples with Call rate (IDCR) lower than 90% were removed from the study. SNPs were removed if they present minor allele frequency (MAF) ≤ 0.02, Call rate (SNPCR) ≤ 0.98 and P-value for Fisher's exact test for Hardy-Weindberg Equilibrium (HWE) $\leq 1 \times 10^{-5}$. After filtering, a total of 538.575 SNPs were utilized on the GWAS study.

GWAS Test and Gene Enrichment Analysis

The Cochran-Armitage test was used to genomic association between HFC and LFC for Nelore and Angus heifers. The Cochran-Armiatge (CA) trend test is commonly used as a

genotype-based test for candidate gene association. Moreover, the CA test utilizes a set of scores that can be obtained as an efficient score test for a logistic regression (Clark et al., 2011). The test was used for comparisons type case-control study (GenABEL package) using the LFC as control group in both subspecies. Significant SNPs were mapped to corresponding or nearby genes through linkage disequilibrium using the PLINK software (Bush et al., 2009; Bohmanova et al., 2010). However, there are not LD patterns reported for Nelore breed, we consider only the genes with $r^2 > 0.8$ from significant SNP for functional analysis. The genes associated with SNPs direct or indirect through linkage disequilibrium were submitted to the functional enrichment analyses on DAVID (Database for Annotation, Visualization and Integrated Discovery; Dennis et al., 2003) to determine gene ontologies (GO), functions and pathways in which genes were overrepresented at $P < 0.05$.

RESULTS

After quality control a total of 538.575 SNPs distributed in 29 chromosomes were used to test the association study relating phenotype and genotype. The profiles of p values (in terms of –log[p]) of all tested SNPs for Nelore and Angus heifers are showed in Figures 3.1 and 3.2. A total of 181 SNPs for Nelore and 201 SNPs for Angus heifers had a moderate association ($p \leq$ 10E-3) comparing the groups HFC *vs* LFC.

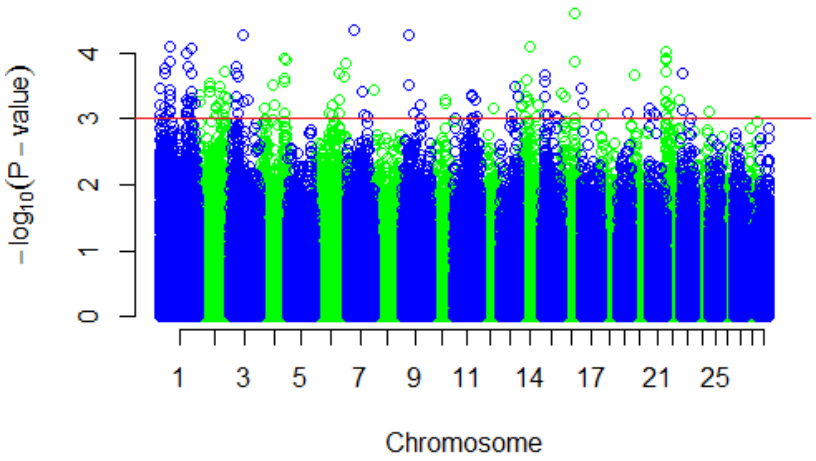


Figura 3.1. Genome-wide association analysis for HFC comparing with LFC in Nelore heifers. The Manhattan plot demonstrates the results of association after correction for population structure. The horizontal red line indicates the whole-genome with moderate significance threshold [-log (p ≤ 10E-3)].

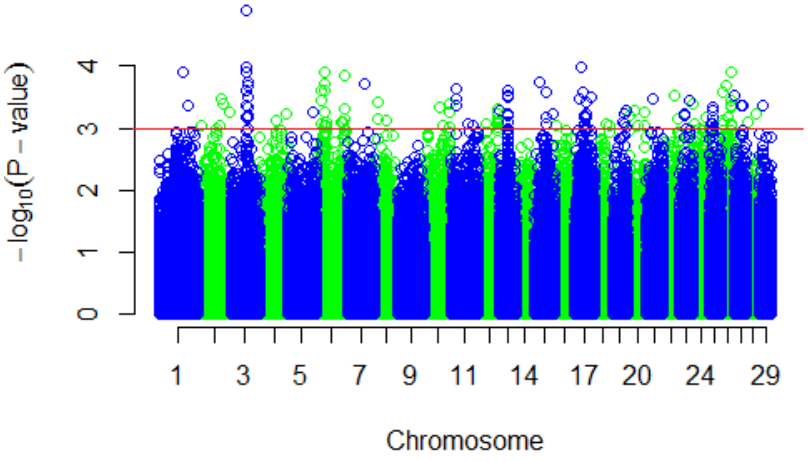


Figura 3.2. Genome-wide association analysis for HFC comparing with LFC in Angus heifers. The Manhattan plot demonstrates the results of association after correction for population structure. The horizontal red line indicates the whole-genome with moderate significance threshold [-log (p ≤ 10E-3)].

147 The 181 SNPs from Nelore heifers were mapped in 23 different chromosomes and the
148 most significant SNPs ($p \leq 9.8E-4$) are present on chromosomes 1, 3, 7, 14, 16 and 22 (Table
149 3.2). For Angus heifers, all 201 were mapped in 29 chromosomes and the most significant SNP
150 ($p = 1.24E-4$) is present on chromosome 3 (Table 3.2).
151
152 **Table 3.2. Description of the most significant SNPs for Nelore e Angus heifers**

Chromosome	SNPs		P (Nelore)	P (Angus)
	Nelore	Angus		
1	rs136289764		8.11E-04	
1	rs109443367		8.47E-04	
3	rs43704025	rs43338364	5.39E-04	1.24E-04
7	rs110807077		4.53E-04	
9	rs136692332		5.39E-04	
14	rs132707253		8.01E-04	
14	rs41730052		8.01E-04	
14	rs110253276		8.01E-04	
16	rs109100442		2.47E-04	
22	rs133905094		9.38E-04	

153 SNPs Identification: http://www.ncbi.nlm.nih.gov/pubmed
154
155 The GWAS showed a total of 97 different genes that were associated directly or indirectly
156 through LD ($r2 > 0.8$) to all 181 SNPs from Nelore heifers (Table 3.3). Functional enrichment
157 analysis was applied for all 97 genes associated in the integrate network with DAVID Functional
158 annotation tool. A total of 5 gene ontologies (GO) were significantly over-represented in 3
159 categories: biological process, cellular component and molecular function (Table 3.3). The most
160 significant in biological processes were cell motion (ROBO1, PRKDC, DCDC2, DNAH7 and
161 SLIT3), cell molity (ROBO1, PRKDC, DCDC2 and DNAH7) and localization of cell (ROBO1,
162 PRKDC, DCDC2 and DNAH7). For Angus heifers 52 genes (Table 3.4) were associated to all
163 201 SNPs and functional enrichment analysis showed 18 significantly GO ($p < 0.05$) over-
164 representing the three categories. The two most significant biological processes were kidney

development (PKHD1, FBN1, and NID1) and urogenital system development (PKHD1, FBN1 and NID1).

Table 3.3. Gene Ontology terms related to biological process, cellular component and molecular function of the genes associated directly or indirectly with SNPs in the HFC from Nelore and Angus heifers.

	Category	GO ~ Term	Count	%	P Value
Nelore	GOTERM_BP_FAT	GO:0006928 ~ cell motion	5	11.4	0.0247963
	GOTERM_BP_FAT	GO:0048870 ~ cell motility	4	9.1	0.0353441
	GOTERM_BP_FAT	GO:0051674 ~ localization of cell	4	9.1	0.0353441
	GOTERM_CC_FAT	GO:0044459 ~ plasma membrane part	11	25.0	0.0191278
	GOTERM_CC_FAT	GO:0016010 ~ dystrophin-associated glycoprotein complex	2	4.5	0.037901
Angus	GOTERM_BP_FAT	GO:0001822 ~ kidney development	3	8.3	0.016709545
	GOTERM_BP_FAT	GO:0001655 ~ urogenital system development	3	8.3	0.021581307
	GOTERM_CC_FAT	GO:0043228 ~ non-membrane-bounded organelle	12	33.3	0.00622552
	GOTERM_CC_FAT	GO:0043232 ~ intracellular non-membrane-bounded organelle	12	33.3	0.00622552
	GOTERM_CC_FAT	GO:0005604 ~ basement membrane	3	8.3	0.010070639
	GOTERM_CC_FAT	GO:0044420 ~ extracellular matrix part	3	8.3	0.021725277
	GOTERM_CC_FAT	GO:0044430 ~ cytoskeletal part	6	16.7	0.034533053
	GOTERM_CC_FAT	GO:0005730 ~ nucleolus	5	13.9	0.044721609
	GOTERM_CC_FAT	GO:0005856 ~ cytoskeleton	7	19.4	0.046153395
	GOTERM_MF_FAT	GO:0004532 ~ exoribonuclease activity	2	5.6	0.025735265
	GOTERM_MF_FAT	GO:0016896 ~ exoribonuclease activity, producing 5'-phosphomonoesters	2	5.6	0.025735265
	GOTERM_MF_FAT	GO:0003779 ~ actin binding	4	11.1	0.026608078
	GOTERM_MF_FAT	GO:0005516 ~ calmodulin binding	3	8.3	0.031688284
	GOTERM_MF_FAT	GO:0003774 ~ motor activity	3	8.3	0.032524241
	GOTERM_MF_FAT	GO:0016796 ~ exonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 5'-phosphomonoesters	2	5.6	0.039327703
	GOTERM_MF_FAT	GO:0046872 ~ metal ion binding	14	38.9	0.041380647
	GOTERM_MF_FAT	GO:0043169 ~ cation binding	14	38.9	0.044509348
	GOTERM_MF_FAT	GO:0043167 ~ ion binding	14	38.9	0.049846183

DISCUSSION

The present study showed for the first time a genomic association between antral follicle variation in Nelore and Angus heifers. However, the sample size used in this study is greatly smaller than GWAS practiced in humans, especially involving diseases, which often exceeds 10.000 individuals (Sun, 2012). Thus, caution must be taken from results because the size of the populations analyzed can be a limiting factor of the power for detecting significant associations and some SNPs might be a false positive.

The GWAS across all the 29 autosomal chromosomes showed no genomic region associated with antral follicle count variation in Nelore and Angus heifers which are illustrated in the Manhattan plots (figures 3.1 and 3.2) where no high peak is present. The medium/low peaks observed in the figures can be attributed to two factors: many SNPs have a low effect on phenotype and; the variable antral follicle variation in bovines has their regulation controlled by many genes. No suggestive or significant SNPs were strongly associated with HFC in heifers from both breeds. However, the GWAS identified a number of SNPs with moderate association and candidate genes in animals with HFC in Nelore and Angus heifers.

The analyses of the nominal associated genes using DAVID bioinformatics resources identified a number of functional categories that were different in HFC group comparing with LFC group in Nelore and Angus heifers. Of note, cell motion (ROBO1, PRKDC, DCDC2, DNAH7 and SLIT3), cell molity (ROBO1, PRKDC, DCDC2 and DNAH7) and localization of cell (ROBO1, PRKDC, DCDC2 and DNAH7) were the GO related with biological process in Nelore heifers. The Roundabout (ROBO) transmembrane proteins constitute a conserved family of receptors including ROBO1, ROBO2, ROBO3 and ROBO4, which together with their

198 repellent ligand SLIT (SLIT1, SLIT2 and SLIT3), have important roles in regulation of axon
199 guidance decisions (Devine & Key, 2008). However, there is evidence that SPLI/ROBO pathway
200 act in cellular processes outside the nervous system (Wong et al., 2002). In a study with human
201 adult ovary, the expression of SLIT2, SLIT3 and ROBO2 was increased during luteal phase and
202 was negatively regulated by hCG and cortisol. Furthermore, blocking the SLIT-ROBO activity
203 reduce apoptosis and increase migration in luteal cells (Dickinson et al., 2008). In ovarian
204 development, the SLIT-ROBO pathway could be involved in the control of germ cell migration.
205 The SLIT2, SLIT3, ROBO1, ROBO2 and ROBO4 expression have been demonstrated in ovine
206 fetal ovary at days 50, 60, 70 and 80 of gestation, period that occur follicle formation. Moreover,
207 at the time of increased SLIT-ROBO expression there was a significant reduction in the
208 proliferation of oocytes in the developing ovary (Dickinson et al., 2010). Although this GWAS
209 study find markers that were associated with ROBO1 (BovineHD0100007707) and SLIT3
210 (BovineHD2000000133) genes in Nelore with HFC, the influence of SLIT-ROBO pathway in
211 the follicle formation on this breed still needs confirmation though other functional experiments,
212 nevertheless it opens a new field of study on how and if the SLIT-ROBO pathway is influencing
213 de AFC in Nelore heifers.

214 With regards to Angus heifers two GO terms related to biological process, kidney
215 development (PKHD1, FBN1, and NID1) and urogenital system development (PKHD1, FBN1
216 and NID1) were significantly associated. Fibrillins (FBN1, 2 and 3) are glycoproteins that are
217 present in connective tissues (Zhang et al. 1995) and form the backbone structure of small
218 diameter called microfibrils (Sakai et al., 1991). Besides that, Fibrillins have regulatory functions
219 by binding and sequestering growth factors. Fibrillin contributes to the extracellular regulation of
220 endogenous TGFβ activity by providing a structural platform that controls the diffusion, storage,

presentation, and release of the ligands (Ramirez and Sakai, 2010). In addition, they also interact with the prodomains of bone morphogenic proteins (BMPs) and grown and differentiation factors (GDFs; Sangle et al., 2008), that are members of the TGFβ superfamily described as important in the follicle development and function of the ovary (Knight & Glister, 2006; Dong et al., 1996; McNatty et al., 2000; Yan et al., 2001). Taken together, the importance of TGFβ superfamily controlling folliculogenesis and the concepts of how the Fibrilins regulates TGFβ and BMP signaling, the fibrilin may perform an important role in the ovary, including folliculogenesis.

Understanding how the candidate genes act modeling the phenotype is a big challenge, further complicated by the fact that environment can influence the phenotype. However, the functional information generated on the present GWAS, prioritized the investigation of biological processes and may contribute to future research aiming at the validation of those genes and elucidation the mechanisms involved on phenotype. Moreover, the data generated could also be available in banks for futures meta-analyzes, where combining with other studies can increase the sample size and the power to detect significant associations.

REFERENCE

Bohmanova, J, M Sargolzaei, and FS Schenkel 2010 Characteristics of linkage disequilibrium in North American Holsteins. *BMC Genomics* **11** 421.

Bush, WS, G Chen, ES Torstenson, and MD Ritchie 2009 LD-spline: mapping SNPs on genotyping platforms to genomic regions using patterns of linkage disequilibrium. *BioData Min***2** 7.

Chamberlain, AJ, BJ Hayes, K Savin, S Bolormaa, HC McPartlan, PJ Bowman, C Van der Jagt, S MacEachern, and ME Goddard 2012 Validation of single nucleotide polymorphisms associated with milk production traits in dairy cattle. *J Dairy Sci* **95** 864-875.

Clarke, GM, CA Anderson, FH Pettersson, LR Cardon, AP Morris, and KT Zondervan 2011 Basic statistical analysis in genetic case-control studies. *Nat Protoc* **6** 121-133.

Dennis, G, BT Sherman, DA Hosack, J Yang, W Gao, HC Lane, and RA Lempicki 2003 DAVID: Database for Annotation, Visualization, and Integrated Discovery.*Genome Biol* **4** P3.

Devine, CA, and B Key 2008 Robo-Slit interactions regulate longitudinal axon pathfinding in the embryonic vertebrate brain. *Dev Biol* **313** 371-383.

Dickinson, RE, L Hryhorskyj, H Tremewan, K Hogg, AA Thomson, AS McNeilly, and WC Duncan 2010 Involvement of the SLIT/ROBO pathway in follicle development in the fetal ovary. *Reproduction* **139** 395-407.

Dickinson, RE, M Myers, and WC Duncan 2008 Novel regulated expression of the SLIT/ROBO pathway in the ovary: possible role during luteolysis in women. *Endocrinology* **149** 5024-5034.

Dong, J, DF Albertini, K Nishimori, TR Kumar, N Lu, and MM Matzuk 1996 Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383** 531-535.

Huang, W, BW Kirkpatrick, GJ Rosa, and H Khatib 2010 A genome-wide association study using selective DNA pooling identifies candidate markers for fertility in Holstein cattle. *Anim Genet* **41** 570-578.

Ireland, JJ, F Ward, F Jimenez-Krassel, JL Ireland, GW Smith, P Lonergan, and AC Evans2007 Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-quality embryos after ovarian stimulation in cattle. *Hum Reprod* **22** 1687-1695.

Knight, PG, and C Glister 2006 TGF-beta superfamily members and ovarian follicle development.*Reproduction* **132** 191-206.

McNatty, KP, AE Fidler, JL Juengel, LD Quirke, PR Smith, DA Heath, T Lundy, A O'Connell, and DJ Tisdall 2000 Growth and paracrine factors regulating follicular formation and cellular function. *Mol Cell Endocrinol* **163** 11-20.

Mossa, F, SW Walsh, ST Butler, DP Berry, F Carter, P Lonergan, GW Smith, JJ Ireland, and AC Evans 2012 Low numbers of ovarian follicles ≥3 mm in diameter are associated with low fertility in dairy cows. *J Dairy Sci* **95** 2355-2361.

Naukkarinen, J, I Surakka, KH Pietiläinen, A Rissanen, V Salomaa, S Ripatti, H Yki-Järvinen, CM van Duijn, HE Wichmann, J Kaprio, MR Taskinen, L Peltonen, and

E Consortium 2010 Use of genome-wide expression data to mine the "Gray Zone" of GWA studies leads to novel candidate obesity genes. *PLoS Genet* **6** e1000976.

Peñagaricano, F, KA Weigel, and H Khatib 2012 Genome-wide association study identifies candidate markers for bull fertility in Holstein dairy cattle. *Anim Genet* **43 Suppl 1** 65-71.

Ramirez, F, and LY Sakai 2010 Biogenesis and function of fibrillin assemblies. *Cell Tissue Res* **339**71-82.

Sakai, LY, DR Keene, RW Glanville, and HP Bächinger 1991 Purification and partial characterization of fibrillin, a cysteine-rich structural component of connective tissue microfibrils. *J Biol Chem* **266** 14763-14770.

Sengle, G, NL Charbonneau, RN Ono, T Sasaki, J Alvarez, DR Keene, HP Bächinger, and LY Sakai 2008 Targeting of bone morphogenetic protein growth factor complexes to fibrillin. *J Biol Chem* **283** 13874-13888.

Sun, YV 2012 Integration of biological networks and pathways with genetic association studies.*Hum Genet* **131** 1677-1686.

Wong, K, HT Park, JY Wu, and Y Rao 2002 Slit proteins: molecular guidance cues for cells ranging from neurons to leukocytes. *Curr Opin Genet Dev* **12** 583-591.

Yan, C, P Wang, J DeMayo, FJ DeMayo, JA Elvin, C Carino, SV Prasad, SS Skinner, BS Dunbar, JL Dube, AJ Celeste, and MM Matzuk 2001 Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Mol Endocrinol* **15** 854-866.

Zhang, H, W Hu, and F Ramirez 1995 Developmental expression of fibrillin genes suggests heterogeneity of extracellular microfibrils. *J Cell Biol* **129** 1165-1176.

308 **Table 3.4. Associated SNPs with respective candidate genes for Nelore heifers.**

Gene	Chromosome	SNP	Gene	Chromosome	SNP	Gene	Chromosome	SNP
RSRC1	1	BTB-01568926	DC1I1	4	BovineHD0400003849	SGK3	14	Hapmap49131-BTA-34531
PLCH1	1	BovineHD0100031992	AGMO	4	BovineHD0400007019	PREX2	14	BovineHD1400009858
ANKUB1	1	BovineHD0100033727	A0JNG1	4	BovineHD0400001266	KCNB2	14	BTA-107899-no-rs
WWTR1	1	BovineHD0100033813	RERG	5	BovineHD0500027036	JPH1	14	BovineHD1400011391
AT1B3	1	BovineHD0100036157	BST1	6	BovineHD0600032820	SNTB1	14	BovineHD1400023903
EPHB1	1	BovineHD0100038454	NPNT	6	BovineHD0600005674	ZC3H12C	15	BovineHD1500005193
CEP63	1	BTB-00063883	A0JN38	6	BovineHD0600019557	F1N2Z9	15	BovineHD1500005842
APC13	1	BovineHD0100038705	EPHA5	6	BovineHD0600022781	E1BA24	15	BovineHD1500009535
ENTK	1	BovineHD0100005454	ART3	6	BovineHD0600025443	STIM1	15	BTA-114838-no-rs
C21orf63	1	BovineHD0100000686	11-Sep	6	BovineHD0600025874	LOC790886	16	BovineHD1600001673
ROBO1	1	BovineHD0100007707	FRAS1	6	BovineHD0600026271	RALGPS2	16	BovineHD1600017259
E1BJS9	1	BovineHD0100010534	SGCD	7	BovineHD0700020503	ANGL1	16	BovineHD1600017298
A5PKG1	1	BovineHD0100022104	HEM2	8	BovineHD0800031076	FBXW8	17	BovineHD1700017212
SMARCAL1	2	BovineHD0200030269	CLCN3	8	BovineHD0800000483	ADAMTS18	18	BovineHD1800001383
F1MTX0	2	BovineHD0200040258	TMEM2	8	BovineHD0800014467	F1MX91	18	ARS-BFGL-NGS-59215
KIAA1486	2	BovineHD0200033024	RIMS1	9	BovineHD0900002983	DOCK2	20	BovineHD2000000529
NMI	2	BovineHD0200013025	ZNF292	9	Hapmap54718-rs29022960	RNF180	20	BovineHD2000021261
MYO7B	2	BovineHD0200001364	EML5	10	BovineHD1000029437	FAM196B	20	BovineHD2000000555
IWS1	2	BovineHD0200001420	A6QLI2	10	BTA-114684-no-rs	SLIT3	20	BovineHD2000000133
DNAH7	2	BovineHD0200024162	A7YWN4	11	ARS-BFGL-NGS-10436	F1MJV4	20	BovineHD2000018883
ORC2	2	BovineHD0200025582	LBH	11	BovineHD1100019791	CCDC33	21	BovineHD2100021281
NRP2	2	BovineHD0200027119	FAM179A	11	BovineHD1100020297	LYZL4	22	BovineHD2200004477
PIK3R3	3	BTA-20822-no-rs	IFT172	11	BovineHD1100020629	F2Z4H7	22	BovineHD2200005456
NHRF3	3	BovineHD0300006834	Q3SZR7	11	BovineHD1100022452	AZI2	22	BovineHD2200000741
SYWM	3	BovineHD0300007564	HS1BP3	11	BovineHD4100009043	ZCWPW2	22	BovineHD4100015404
TBX15	3	BovineHD0300007604	XRN2	13	BovineHD1300011855	RBMS3	22	BTB-00830411
SPAG17	3	BovineHD0300007956	C20orf123	13	BovineHD1300021958	ATP2B2	22	ARS-BFGL-NGS-14331
MAN1A2	3	BovineHD0300035509	KIAA0146	14	BovineHD1400005988	EGFR	22	BovineHD2200000246

ATP1A1	3	BovineHD0300036028	PRKDC	14	BovineHD1400006081	DCDC2	23	BovineHD2300009744
Q2KI63	3	BovineHD0300002619	A6QLA9	14	BovineHD4100011332	TTC39C	24	BTB-01623856
ECHD2	3	BovineHD0300027093	A4IFV2	14	BovineHD4100011399	RB27B	24	BovineHD2400015606
AT1A2	3	BovineHD0300003116	TRIM55	14	BovineHD1400009278	CTNNA3	28	BTB-00980953
F1MY54	4	BovineHD0400033064						

309

310

311 **Table 3.5. Associated SNPs with respective candidate genes for Angus heifers.**

Gene	Chromosome	SNP	Gene	Chromosome	SNP	Gene	Chromosome	SNP
Q32KQ4	1	BovineHD0100028647	FBN1	10	BovineHD1000017884	SHANK1	18	ARS-BFGL-NGS-25117
CBS	1	BovineHD0100041801	PRKCH	10	BovineHD1000021003	DNAH2	19	BovineHD1900008265
MYLK	1	BovineHD0100019384	FNTB	10	BovineHD1000022127	LEPREL4	19	BovineHD1900012163
A8E661	2	BovineHD0200026032	A8E641	11	BovineHD1100020762	PSA4	21	ARS-BFGL-NGS-24797
PALMD	3	BovineHD0300013319	MYO16	12	BovineHD1200025736	PTPRG	22	BovineHD2200011275
IFI44	3	BovineHD0300019648	RALGAPA2	13	BovineHD1300011643	ATG7	22	BovineHD2200016071
KAD5	3	BovineHD0300019991	KIZ	13	BovineHD1300011819	CRISP1	23	BovineHD2300005886
ACM2	4	BovineHD0400028468	XRN2	13	BovineHD1300011851	PKHD1	23	BovineHD2300006404
TNS3	4	BovineHD0400020885	A6QQD3	15	BTB-01820462	FAM59A	24	BovineHD2400006778
ADCY1	4	BovineHD0400021263	SYT9	15	BovineHD1500013002	TNFRSF11A	24	BovineHD2400017755
F19A5	5	BovineHD0500034919	A6QNL3	16	BovineHD1600012798	PARN	25	BovineHD2500003745
PPP2R2C	6	BovineHD0600029214	F262	16	BovineHD1600001371	LIPA	26	BTA-62081-no-rs
SLC2A9	6	BovineHD0600030984	CD045	17	BovineHD1700011280	SORCS1	26	BovineHD2600007572
WDR1	6	BovineHD0600031016	CUX2	17	BovineHD1700016280	RBM20	26	BovineHD2600008410
A7YWG6	6	BovineHD0600008622	RBM19	17	BovineHD1700017987	F1MX91	18	ARS-BFGL-NGS-59215
FRAS1	6	BovineHD0600026329	LRBA	17	BovineHD1700002101	DOCK2	20	BovineHD2000000529
EBF1	7	BovineHD0700021323	MYH14	18	ARS-BFGL-NGS-3584	RNF180	20	BovineHD2000021261
A1A4J5	8	ARS-BFGL-NGS-1787				FAM196B	20	BovineHD2000000555

CONCLUSÕES GERAIS

O estudo de expressão gênica através do microarranjo demonstrou que tanto as raças Nelore e Aberdeen Angus e os grupos alta e baixa população folicular apresentaram diferentes perfis de expressão global dos genes em folículos antrais. A raça Nelore destacou-se por apresentar maior expressão de genes relacionados à ativação e crescimento folicular, que podem estar contribuindo com o maior número de folículos antrais nessa raça. Isso foi evidenciado no estudo histológico para avaliação dos folículos pré-antrais, onde animais da raça Angus apresentaram maior densidade de folículos primordiais e animais da raça Nelore tenderam a apresentar maior densidade de folículos secundários, indicando que podem existir diferentes mecanismos de ativação e crescimento folicular e/ou atuação diferenciada de genes importantes para tais fatores. Em relação aos grupos, a via de sinalização relacionada à proliferação celular apresentou genes mais expressos em animais com alta população folicular o que também pode estar contribuindo para maior número de folículos nestes animais.

O estudo de associação genômica mostrou que existem diferenças genotípicas nos grupos de alta e baixa população folicular para ambas as raças. Apesar de não haver regiões genômicas com forte associação a este fenótipo no presente estudo, existem marcadores com moderada associação e genes candidatos que podem estar influenciando o número de folículos. Esta associação moderada se deve ao fato do número de animais usados serem bem inferior ao recomendado para este tipo de estudo e por ser uma característica poligênica. Apesar disso, os dados deste trabalho podem ficar a disposição em banco de dados e contribuir para futuras meta-análises e para outros estudos semelhantes, aumentando o numero de animais e consequentemente o poder da análise.

BIOGRAFIA

Maurício Gomes Favoreto nasceu na cidade de Itapemirim, Espírito Santo - Brasil, em 20 de dezembro 1978. Filho de Odilon Soares Favoreto e Maria da Penha Gomes Favoreto viveu sua vida em Muniz Freire - ES até ingressar no curso de Medicina Veterinária da Universidade Federal de Lavras em julho de 1998. Em 2001 ingressou no programa de bolsa de extensão rural oferecido pela Fundação de Amparo a Pesquisa de Minas Gerais – FAPEMIG sob a supervisão do Prof. Marcos Neves Pereira. Durante este período prestou assistência técnica a pequenos produtores de leite da região de Lavras – MG e participou de pesquisas na área de bovinocultura de leite. Graduou-se em julho de 2003 e ingressou no mestrado em Produção Animal em fevereiro de 2004 na Universidade Estadual do Norte Fluminense (UENF) sob a orientação do Prof. Alberto Magno Fernandes com término em 2006. Durante o período do mestrado estagiou durante o ano de 2005 na EMBRAPA gado de leite em Juiz de Fora – MG onde realizou sua pesquisa. No período de agosto 2008 a agosto 2010 atuou como research scientist no Animal Science Department da Univesity of Florida - USA sob a supervisão do Prof. José Eduardo Portela dos Santos, o qual desenvolveu pesquisas nas áreas de reprodução, nutrição e sanidade em bovinos de leite. Em Março de 2011 ingressou no curso de doutorado na Universidade Estadual Paulista (UNESP) em Botucatu – SP sob a supervisão do Prof. Ciro Moraes Barros desenvolvendo as pesquisas na área de biologia molecular e reprodução em bovinos.