PRISCILA VENDRAMINI SILVA

GENE EXPRESSION IN SOWS WITH HIGH AND LOW OVULATION RATE, HIGH AND LOW BIRTH WEIGHT LITTER AND THE EFFECT OF SOW CATABOLIC STATUS ON EMBRYONIC GENE EXPRESSION

Thesis presented to the Genetics and Breeding Graduate Program of the Universidade Federal de Viçosa, in partial fulfillment of the requirements for degree of *Doctor Scientiae.*

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Em uma gaiola dourada um pintassilgo vivia
Cantava numa toada, tão cheia de nostalgia
Que parecia seu canto, um ai dorido e queixoso
Era um suspiro, era um pranto
Pelo seu ninho saudoso.
Talvez buscasse o coitado,
Neste cantar esquecer a vida livre do prado
Desde a alvorada nascer.
Uma bondosa menina, cheia de amor e piedade,
À ave gentil, pequenina
Depressa deu liberdade.
Com a alma então à vibrar,
Numa expressão de alegria,
De quem vai ver o seu lar que a longo tempo não via,
O passarinho galante, batendo as asas voou,
E num gorjeio triunfante, no espaço, além recuou!

(Inah Glanzmann Vendramini, 92 anos)

À minha querida avó Inah

Dedico.

Biography

Priscila Vendramini Silva, daughter of Décio Evandro da Silva and Livia Vendramini, born on april 5th, 1982 in São Paulo, SP, Brazil. She began her studies at the Universidade Federal de Viçosa in 2002 obtaining her degree in Biochemistry in 2006.

In same year of 2006 she started her Master on in Genetics and Breeding Program at the same Institution under supervision of Prof Simone Guimarães. She finished her master in 2008, entitled Gene expression in pig ovary during the estrous cycle.

In August of 2008 she began the doctoral graduation program in Genetics and Breeding at Universidade Federal de Viçosa. During her doctoral program she spent 14 months at the Department of Agricultural Food and Nutritional Science, University of Alberta, Edmonton, Canada. It was there that she developed a large part of her doctoral research project. Ms. Silva completed her dissertation defense on September 11, 2012 to obtain the tittle of Doctor Scientiae in Genetics and Breeding.

Summary

ABSTRACT

SILVA, Priscila Vendramini; D.S., Universidade Federal de Viçosa, September, 2012. **Gene expression in sows with high and low ovulation rate, high and low birth weight litter and the effect of sow catabolic status on embryonic gene expression.** Adviser: Simone Eliza Facioni Guimarães. Co-Advisers: Paulo Sávio Lopes e Fabyano Fonseca e Silva.

In pig production, both litter size and embryonic viability are important economic traits. Nutritional pressures on the lactating sow have impaired the quality of the subsequent litters. This study was divided in three experiments that aimed to: 1) investigate the follicular dynamics and gene expression pattern in corpus luteum (CL), granulosa cells (GC) and denuded oocytes during the estrous cycle in pig breeds with high (commercial-line: n=24) and low (local Brazilian Piau: n=21) ovulation rates and prolificacy; 2) investigate the expression of genes involved in angiogenesis, apoptosis and encoding intrafollicular growth factors in CL, GC and denuded oocytes recovered from commercial sows characterized as high (n=5) and low (n=5) birth weight litter phenotype at Day 5 of gestation; 3) use a refined feed restriction model to investigate global changes in expression in Day 9.5 embryos recovered from commercial sows breed at the "first heat" (FH, n=3) or at second post-weaning estrus (skip). Skip sows were further divided in subgroups according their previous catabolic state in lactation: highly (CH, n=4), moderately (CL, n=4) and non-catabolic (NC, n=4). In the first experiment, the low ovulating Piau gilts were associated with a different pattern of follicle development, with lower numbers of small follicles at day 18, fewer large follicles at days 0 and 18 ($P \leq 0.05$) and a higher proportion of atretic follicles at days 0 and 18 ($P \leq 0.05$). Compared to commercial-line gilts, less prolific Piau gilts showed higher expression of apoptotic genes during luteolysis (*CASP3* and *FASL,* $P \leq 0.05$), decreased expression of *TGFBR2* and *BAX* mRNA in CL ($P \leq 0.05$), higher expression of apoptotic genes (*FAS*, *BCL2* and *CASP8,* $P \leq 0.05$) in GC, and a greater abundance ($P \leq 0.05$) of genes controlling oocyte secreted factors (*GDF9*, *BMP15* and *BMP6)*, suggesting underlying mechanisms controlling differences in follicular development, ovulation rate and inherent prolificacy in this pig breed. In the second experiment, quantitative real time PCR (QPCR) analysis revealed that apoptotic genes were differentially expressed between commercial high and low groups in CL and GC. Most of the

angiogenesis-related genes investigated were higher expressed in CL tissue in the low group. Among intrafollicular growth factors, only *IGFR1* and *BMPR2* were differently expressed in GC and denuded oocytes, respectively. Findings from the present study suggest that differences in CL vascularity and function, as well as in follicle development, may be in part, driving differences between-litter variation in birth weight in contemporary sows. Finally, microarray data from the third experiment revealed different pattern of distribution of biological functions across treatments for the commercial sows. Validation by QPCR showed a differential expression for *CYR61 and MYOF*, specifically for the female sex, in the pair-wise contrast FH*vs*NC and CL*vs*NC, respectively ($P \leq 0.05$). Additional contrasts in female sex were marginally significant ($P \leq 0.10$) for *MYOF*, *BCSL1*, *CYR61*, *RAD21* and *SOD1*, but no difference was found for *ETFA, ACDSB, TFPI2* and *TNFRSF21*. Furthermore, skip sows showed higher total corpus luteum weight, total corpus luteum average and litter growth average compared to "first heat" sows. These results suggest that the differential expression observed in female embryos may be an adaptive response to the intrauterine conditions, which may mediate epigenetic programming in the offspring. This study reinforces and provides additional insights into the role of nutrition and maternal metabolic state in determining the dynamics of early embryonic development and embryo quality in pigs.

RESUMO

SILVA, Priscila Vendramini; D.S., Universidade Federal de Viçosa, Setembro, 2012. **Expressão gênica em porcas de alta de baixa taxa de ovulação, alto e baixo peso médio de leitegada ao nascimento e efeito do estado catabólico na expressão gênica de embriões**. Orientadora: Simone Eliza Facioni Guimarães. Co-orientadores: Paulo Sávio Lopes e Fabyano Fonseca e Silva.

Na produção de suínos, tanto o tamanho da leitegada quanto a viabilidade embrionária são características de importância econômica. As pressões nutricionais sobre a porca em lactação tem prejudicado a qualidade das leitegadas subsequentes. Este estudo foi dividido em três experimentos objetivando-se: 1) investigar a dinâmica folicular e padrão de expressão gênica no corpo lúteo (CL), células da granulosa (CG) e oócitos desnudados durante o ciclo estral em porcas com alta (linhagem comercial: n=24) e baixas (local Piau brasileira: n=21) taxas de ovulação e prolificidade, 2) investigar a expressão de genes envolvidos na apoptose, angiogênese e genes codificantes de fatores de crescimento intrafoliculares no CL, na CG e em oócitos desnudados recuperados de porcas comerciais de alto e de baixo peso médio de leitegada ao nascimento (n=5 por grupo) no dia 5 de gestação; 3) utilizar o modelo de restrição alimentar para investigar a expressão gênica em embriões coletados no Dia 9,5 de gestação de porcas comerciais acasaladas no primeiro (FH, n=3) e no segundo estro após o desmame (*Skip*, n=12). As porcas *skip* foram divididas em subgrupos de acordo com seu estado catabólico em: altamente (CH), moderadamente (CL) e não-catabólicas (NC). No primeiro experimento, a raça Piau apresentou distinto padrão de desenvolvimento folicular, com menor número de pequenos folículos no dia 18, poucos folículos grandes nos dias 0 e 18 ($P \leq 0,05$) e maior proporção de folículos atrésicos nos dias 0 e 18 ($P \leq 0,05$). Comparadas com as de linhagem comercial, porcas da raça Piau apresentaram maior expressão de genes apoptóticos durante luteólise (*CASP3* e *FASL*, $P \leq 0,05$), menor expressão dos transcritos *TGFbR2* e *BAX* no CL ($P \leq 0,05$), maior expressão de genes apoptóticos (*FAS*, *BCL2* e *CASP8*, $P \leq 0,05$) na CG, e maior abundância de fatores de crescimento secretados por oócitos (*GDF9*, *BMP15* e *BMP6*, $P \leq 0,05$), sugerindo que mecanismos intrínsecos à raça Piau controlam diferenças quanto ao desenvolvimento folicular, taxa de ovulação e prolificidade. No segundo experimento, a análise quantitativa de PCR em tempo real (QPCR) revelou que os genes apoptóticos foram

diferencialmente expressos no CL e na CG entre as porcas comercias de alto e de baixo peso médio de leitegada ao nascimento. A maioria dos genes relacionados com angiogênese apresentou maior expressão no CL em porcas de baixo peso médio de leitegada ao nascimento. Dentre os fatores de crescimento intrafoliculares, apenas *IGFR1* e *BMPR2* apresentaram expressão diferencial nas CG e nos oócitos desnudados, respectivamente. Resultados do presente estudo sugerem que as diferenças na vascularização e na função do CL, bem como no desenvolvimento folicular, podem ser em parte, atribuídas a variação no peso de leitegada ao nascimento observada em porcas comerciais. Finalmente, os dados de microarranjo obtidos no terceiro experimento revelaram distinto padrão de distribuição das funções biológicas entre os tratamentos. Validação dos resultados por meio do QPCR demonstrou diferença de expressão para os genes *CYR61* e *MYOF*, especificamente para o sexo feminino, nos contrastes FH*vs*NC e CL*vs*NC, respectivamente ($P \leq 0,05$). Contrastes adicionais para o sexo feminino foram marginalmente significativos ($P \leq 0,10$) para os genes *MYOF*, *BCSL1*, *CYR61*, *RAD21* e *SOD1*, mas nenhuma diferença foi observada para os genes *ETFA*, *ACDSB*, *TFPI2* e *TNFRSF21*. Além disso, porcas acasaladas no segundo estro após o desmame apresentaram maior peso de corpo lúteo total, maior peso médio de corpo lúteo e maior taxa de crescimento de leitegada, comparadas com as porcas acasaladas no primeiro estro após o desmame. Estes resultados sugerem que a expressão gênica diferencial verificada no sexo feminino pode ser uma resposta adaptativa frente às condições intrauterinas, que podem desencadear programação epigenética nos descendentes. Este estudo reforça e auxilia o entendimento do papel da nutrição e do estado metabólico materno na dinâmica do desenvolvimento e na qualidade embrionária em suínos.

General Introduction

The pig is one of the most economically important domesticated animals and the major meat consumed worldwide according to the United States Department of Agriculture (http://www.fas.usda.gov/dlp/circular/2012/livestock_0412.pdf). The worldwide demand for a protein source of high quality has promoted an increase in pig production in developing countries (McManus et al. 2010). For instance, Brazilian swine production is experiencing a change of position in international scenario in recent years, and emerging as one of majors exporters of pork according to Abipecs (*Associação Brasileira da Indústria Produtora e Exportadora de Carne Suína*, 2010).

Traditionally, pig breeding programs have focused on the genetic improvement of production traits, such as growth rate, lean meat percentage and feed efficiency. However, as these traits have reached desirable values, more attention has been given to the genetic improvement of reproductive traits (Lopes et al. 2001). Among the reproductive traits, the litter size and pre-weaning mortality of piglets are important components in reducing costs, resulting in an increase in the number of piglets weaned per sow per year and therefore, greater economic return for producers (Aherne, 1994). More recently, birth litter weight and weaning weight has been associated with improved postweaning performance and therefore included in swine genetic programs (Bergstrom et al. 2009, Schinckel et al. 2010).

Genetic regulation of litter size is complex and affected by multiple components such as ovulation rate, fertilization rate and embryonic/fetal survival (Distl, 2007). In pigs, fertilization rate is the less significant component, generally superior to 95% (Soede et al. 1992). Therefore, litter size is mainly determined by the balance between ovulation rate and embryonic and/or fetal losses. According Foxcroft et al. (1997, 2006) the pattern of prenatal losses in commercial sows maybe changing due to ongoing selection for ovulation rate, associated with increased rate of embryonic survival in the pre-implantation period. As a consequence, a large proportion of prenatal loss has been attributed to the post-implantation period, which is primarily limited by uterine capacity, defined as the number of conceptuses that can be maintained by the uterus until term (Christenson et al. 1987). Further losses may occur during later gestation due to the effects of intrauterine crowding that impair placental development (Almeida et al. 2000, Vonnahme et al. 2002; Town et al. 2004). In addition, the nutritional status of the lactating sow may also increase embryonic loss (Foxcroft et al. 1997).

The pre-implantation period comprises some critical events, such as embryonic disk

formation, changes in morphology and expansion (elongation) of the extra-embryonic and primordial placental tissue (Blomberg et al. 2010). These events are finely regulated by thousands of genes related with important biological process, such as compaction/cavitation, metabolism, transcription/translation, DNA methylation and histone modification, oxidative stress, response to or production of growth factors, cytokine signalling, cell cycle regulation and apoptosis (Ruddock-D'Cruz et al. 2007). Equally important for conceptus implantation and survival is a synchrony between the embryo and the uterine environment (Geisert and Schmitt, 2001), or maternal ability to respond appropriately to embryo derived signals (Hansen, 2002).

Classical methods of selection have allowed only slow genetic progress for litter size due to its low heritability, 0.10 to 0.15 (Avalos and Smith, 1987, See et al. 1993, Spotter and Distl, 2006). Major advances in genetic improvement of reproductive characteristics were obtained through the association of molecular genetics with quantitative genetics. The identification of candidate genes underlying, or associated with, phenotypic trait through quantitative trait loci (QTLs) has allowed identification of candidate genes. Several candidate genes with an important role in reproductive physiology can be cited, such as the estrogen receptor (*ESR*) (Rothschild et al. 1996), binding protein 4 retinol (*RBP4*) (Ollivier et al. 1997; Rothschild et al., 2000), prolactin receptor (*PRLR*) (Vincent et al., 1998), epidermal growth factor (EGF) and follicle stimulating hormone β (fSHb) (Linville et al., 2001). However, the use of marker assisted selection is limited by the numbers of isolated and/or known genes of interest available for inclusion in genetic programmes, considering the whole genome. More recently, efforts to sequence the pig genome have resulted in the release of the first draft of the swine genome sequence with an overall depth of 4X coverage (Sscrofa 9) available in September 2009 (Archibald et al. 2010). The second draft (Sscrofa10.2) has been released in the current year (http://www.ncbi.nlm.nih.gov.ilsprod.lib.neu.edu/Traces/wgs/?&val=AEMK&size=50&page=2). Along with the information derived from genomic studies the development of new tools on microarray techniques comprising DNA variants or expressed tags of many genes or even the whole genome have been useful for in depth understanding about gene regulation and its implications for biological and physiological processes of the reproductive traits. In this context, understanding the molecular and biochemical mechanisms required for normal follicular development and oocyte growth, as well as the those leading to differences in embryo survival resulting from divergence in follicle and oocyte quality, is therefore of great practical and economic importance (Webb et al., 2007, Paradis et al., 2009).

Overall, the present study aimed to clarify the biological question regarding the genetic control of intrafollicular mechanisms responsible for normal preovulatory follicle development in the pig. The first chapter provided a new insight into genetic diversity of the local Brazilian Piau breed and a commercial line, specifically in regard to recruitment, selection and the establishment of the preovulatory follicle population. The Piau breed was introduced by Portuguese settlers in the 16th century and is composed for Iberian breeds with influences of Dutch and African breeds (Vianna, 1985). These breeds have acquired adaptive traits in specific local environments over time, and are considered as (fat-type), yielding larger amount of fat and lower amount of carcass in comparison to commercial line (Benevenuto Júnior, 2001). Since 1998, a nucleus of conservation of this breed has been allocated at Pig Breeding Farm at Universidade Federal de Viçosa. Several studies have been conducted by our group since then, such as QTL mapping using divergent crosses (Guimarães and Lopes, 2000; Pires et al., 2008; Paixão et al., 2008; Silva et al., 2011), association studies between expression of candidate genes and intramuscular content (Serão et al., 2011), phylogeny and genetic diversity (Schierholt et al. 2008, Sollero et al. 2008, Souza et al. 2009) and transcriptional profiling during skeletal muscle development (Sollero, 2010). Divergences between Piau and commercial line is also extended for reproductive traits, such as ovulation rate, total born, total born alive and birth weight litter (data presented in the first chapter). Therefore, comparative studies using Piau and commercial line may contribute to the identification of genes related to ovulation rate, oocyte quality, and consequently embryonic development and survival, in the pig. This may be more evident considering that local breeds may have allelic variants no longer found in European lineages; continuously selected for commercial purposes (Vianna et al., 1956). Moreover, local breeds may represent an important genetic and ecological component of biodiversity (Hall and Bradley, 1995).

In pig production, continued selection for increased litter size for important quality traits, defined as number of pigs born live, survivability and between-litter variability in litter average birth weight have become a major research focus in genetic selection programs, particularly in higher parity sows (Foxcroft, 2012). Foxcroft and Town (2004) reported that a considerable amount of variation in growth performance after birth may be determined during fetal development in the uterus, in a process known as pre-programming. The period of intrafollicular development of the oocyte is also an important factor determining the growth potential of weaned pigs, as well as conceptuses-endometrial interactions within the oviduct and uterus (Pope, 1994). From the perspective of pork production systems, the low birth weight of the individual pigs within a litter, and within-litter variation in birth weight are

already of considerable economic interest, since they have been associated with reduced growth potential and poor carcass quality (Foxcroft et al. 2007; 2009). In the second chapter the biological origins of low birth weight litters in commercial line of mature sows were used to investigate the role of these intrafollicular factors in creating the differences in corpus luteum vascularity and function, as well as follicle and oocyte quality. It is also becoming increasingly apparent that nutritional or other environmental stimuli, acting both in utero and during critical periods of relative plasticity beyond birth, may exert detrimental effects on cellular structure and function and, consequently, on the postnatal growth and risk of developing chronic disease in adulthood (Jones et al. 2009). Lactational and nutritional management strategies are likely to influence the ovarian function through gonadotrophin-mediated effects and/or through direct effects of nutrients and metabolic hormones (Prunier and Quesnel, 2000, Soede et al. 2009). In the third chapter a refined feed restriction model known to negatively affect litter weaning weight and embryonic development of the next litter was used to investigate how the previous catabolic state of lactating primiparous sows affects embryonic gene expression using microarray analysis.

Microarray technologies have been widely applied in the past decade and broaden the number of applications from gene screening and target identification to emerging approaches such as developmental biology, disease classification, pathways studies, drug discovery and toxicology (Russo et al. 2003). The purpose of a microarray is to detect the presence and abundance of labelled nucleic acids in a biological sample, which will hybridise to the DNA on the array via Watson–Crick duplex formation, and which can be detected via the labelling. In the majority of microarray experiments, the labelled nucleic acids are derived from the mRNA of a sample or tissue, and so the microarray measures gene expression. The power of a microarray is that there may be many thousands of different DNA molecules bonded to an array, and so it is possible to measure the expression of many thousands of genes simultaneously (Stekel, 2003). However, a cautionary interpretation of differentially expressed data is needed to have a comprehensive picture of the events that occurs during early embryonic development, implantation and maternal-embryo communication (Almiñana and Fazeli, 2012). This knowledge may contribute to an overall understanding of molecular interactions as key drivers for understanding complex cellular processes and the respective biological functions (Dreher et al. 2012).

Finally, in the general conclusion the overall impact of this research on our understanding of the complex mechanisms required for follicle development and the extension of the nutritional effect on gene embryonic expression will be mentioned.

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CHAPTER 1

(Article 1)

Follicular dynamics and gene expression in granulosa cells, corpus luteum and oocyte in gilts from breeds with low and high ovulation rates

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Abstract

Follicular dynamics and the expression of candidate genes using real-time PCR were compared during the estrous cycle of pig breeds with high (commercial-line: n=24) and low (local Brazilian Piau: n=21) ovulation rates and prolificacy. Gilts were slaughtered on days 0, 4, 10, and 18 of the estrous cycle and visible ovarian follicles were classified by follicular diameter. Recovered cumulus-oocyte complexes were classified as normal or atretic and frozen in liquid nitrogen until RNA extraction. Low ovulation rates/prolificacy in Piau gilts was associated with a different pattern of follicle development, with lower numbers of small follicles at day 18, fewer large follicles at days 0 and 18 ($P \leq 0.05$) and a higher proportion of atretic follicles at days 0 and 18 ($P \leq 0.05$). Compared to commercial-line gilts, less prolific Piau gilts showed higher expression of apoptotic genes during luteolysis (*CASP3* and *FASL,* $P \leq 0.05$), decreased expression of *TGFBR2 and BAX* mRNA in CL ($P \leq 0.05$), higher expression of apoptotic genes (*FAS*, *BCL2* and *CASP8,* $P \leq 0.05$) in GC, and a greater abundance ($P \leq 0.05$) of genes controlling oocyte secreted factors (*GDF9*, *BMP15* and *BMP6)*, suggesting underlying mechanisms controlling differences in follicular development, ovulation rate and inherent prolificacy in this pig breed.

Key words: gene expression, growth factors, ovulation, litter size

Resumo

A dinâmica folicular e o perfil de expressão de genes candidatos obtido por meio do PCR em tempo real foram comparados durante o ciclo estral em linhagens de alta (linhagem comercial: n=24) e de baixa (raça local Brasileira Piau: n=21) taxa de ovulação e prolificidade. As porcas foram abatidas nos dias 0, 4, 10 e 18 do ciclo estral e folículos ovarianos visíveis foram classificados de acordo com o diâmetro folicular. Células do cumulus foram coletadas e classificadas como normal ou atrésicas, e em seguida foram congeladas em nitrogênio líquido, até o momento de extração de RNA. A baixa taxa de ovulação/prolificidade da raça Piau foi associada com diferente padrão de desenvolvimento folicular, caracterizado como pequeno número de folículos no dia 18, poucos folículos grandes no dia 0 e 18 ($P \leq 0,05$) e maior proporção de folículos atrésicos no dia 0 e 18 ($P \leq 0,05$). Comparadas com as da linhagem comercial, as porcas menos prolíficas da raça Piau apresentaram maior expressão de genes apoptópticos durante a luteólise (*CASP3* e *FASL,* $P \leq 0,05$), decréscimo da expressão do *TGFBR2* e *BAX* mRNA no corpo lúteo ($P \leq 0,05$), maior expressão de genes apoptóticos (*FAS*, *BCL2* e *CASP8,* $P \leq 0,05$) nas células da granulosa, e maior abundância ($P \leq 0,05$) de genes que controlam a secreção de fatores de crescimento pelos oócitos (*GDF9*, *BMP15* e *BMP6*), sugerindo que mecanismos intrínsecos controlam diferenças no desenvolvimento folicular, taxa de ovulação e prolificidade inerentes a raça Piau.

Palavras-chave: expressão gênica, fatores de crescimento, ovulação, tamanho de leitegada.

Introduction

As in sheep, differences in ovulation rate in different breeds of pigs are presumably linked to differences in intra-ovarian regulatory mechanisms and overall prolificacy. For example, according to Manabe *et al.* (2004), the higher ovulation rate in the Meishan sow is related to differences in both follicular recruitment and atresia. The same group reported that a lower ovulation rate and a smaller number of pigs born in local Hungarian Mangalica sows, in comparison with other European breeds, was also related to differences in follicle recruitment and atresia (Rátky *et al.* 2005). It was also postulated that follicles of Meishan sows provide a "better" environment for oocyte maturation than follicles of Large-White hybrid sows, which may also contribute to the prolificacy of Meishan females (Bazer *et al.* 1988a; Haley and Lee, 1993, Hunter and Picton, 1995). Similarly, different patterns of follicle and oocyte development may exist between prolific commercial breeds and the less prolific Brazilian Piau breed, and comparative studies using these breeds may contribute to the identification of genes related to ovulation rate, oocyte quality, and consequently embryonic development and survival, in the pig. Preliminary studies comparing different traits in contemporary commercial sows with sows from the less prolific Brazilian Piau breed (Peixoto *et al.* 2006) also revealed differences in ovulation rate (OR) (15.5 ± 1.9 *vs* 11.1 ± 2.4, $P \leq 0.05$), number of total pigs born (TB) (14.3 ± 3.7 *vs* 9.3 ± 2.7, $P \leq 0.05$) and pigs born alive (BA) (12.7 ± 3.1, 7.9 ± 2.6, $P \leq 0.05$) (unpublished data). As the Piau breed has never been subjected to intensive genetic selection, it may carry allelic variants that are no longer found in highly selected and more prolific commercial lines.

The growth and development of ovarian follicles requires a series of coordinated events that lead to follicular somatic cell differentiation, oocyte maturation and ovulation (Bonnet *et al.* 2008). In farm species, the number of ovulatory follicles is mainly regulated by the pituitary gonadotrophins FSH and LH (Hunter *et al.* 2004). However, it is well established that the ovulatory process is also influenced by endocrine and paracrine pathways that involve local growth factors secreted from granulosa cells (GC) and the growing oocytes (Manabe *et al.* 2004). Most recent studies have focused on the role of bone morphogenetic proteins (BMPs) belonging to the transforming growth factor β family in the control of follicle development in mammals (Glister *et al.* 2004; Feary *et al.* 2007; Paradis *et al.* 2009). The BMPs modulate a wide range of cellular functions, such as proliferation and differentiation, steroidogenesis, metabolism and apoptosis (Shimasaki *et al.* 2004; Juengel and McNatty, 2005; Gilchrist *et al.* 2008, Gilchrist and Ritter, 2011) and can also affect the quality of the

oocyte and consequently embryonic development and survival (Hunter *et al.* 2004; Hunter and Paradis, 2009). The bi-directional communication between oocyte and somatic cells is also considered to be essential for follicular and theca cell development, and for oocyte maturation (Singh *et al.* 1993; Gilchrist *et al.* 2004). However, once having initiated development, the majority of follicles become atretic before ovulating due to apoptosis of GC, irrespective of the stage of follicular development (Tilly, 1996; Guthrie, 2005). The fate of follicles during follicular development is, therefore, determined by the balance between pro-apoptotic and survival molecules. The molecules and the processes in which they are involved may be summarized as follows: (i) atresia: B/linfoma-2 cell family members (Bcl2), tumor necrosis factor (TNF) and caspases, (ii) follicle selection: Bcl2, Bax, FSH, inhibin, Fas ligand (*FASL*), caspases, and (iii) luteolysis: Fas/FasL, caspase-3 (*CASP3*), Bax, BMP ligands and receptors (Hussein, 2005). However, the precise role of these proteins and genes in regulating follicle selection and apoptosis in the pig has not been completely elucidated (Inoue *et al.* 2011). Since ovulation rate and oocyte quality are important determinants of reproductive efficiency, understanding the regulation of follicular growth leading to ovulation is crucial (Webb *et al.* 2007).

As there are no gene expression data available to explain the lower prolificacy of the Brazilian Piau breed, the aims of the current study were: (1) to investigate and compare the estrous cycle length and the dynamics of follicle growth between the less prolific Piau and commercial-line sows; (2) to elucidate the expression pattern of candidate genes during the estrous cycle using quantitative real-time PCR in both genetic lines, as a means of better understanding the mechanisms controlling ovarian follicular development in the pig.

Materials and Methods

Animals

This experiment was conducted with approval from the Universidade Federal de Viçosa Animal Care Committee (Protocol # 34/2010). The 24 commercial-line (Landrace x Large-White x Pietrain) and 21 Brazilian Piau breed gilts used were obtained from the pig farm at the Universidade Federal de Viçosa (Viçosa, MG, Brazil). Estrous behavior was evaluated during the first to seventh consecutive estrous periods. The onset of estrus was checked once a day using a mature boar and designated as day 0. Groups of gilts were then slaughtered by electrical stunning on days 0, 4, 10 and 18 of the estrous cycle, with n=6 gilts per day of the estrous for commercial and n=5 gilts per day of the estrous cycle for Piau

breed, except day 18, with n=6 Piau gilts. Selected time frame covers the initial phase of follicle development (day 0 to 4), which is characterized as being gonadotrophin-independent and during which initial recruitment and growth of primordial follicles and preantral follicle relies mostly on local ovarian factors (Foxcroft *et al.* 1994). Day 10 to 18 of the cycle corresponding to the final selection phase when the preovulatory follicle population has been established (Grant *et al.* 1989, Hunter and Wiesak, 1990). The animals were fed to appetite with a recommended diet twice daily and had free access to water.

Tissue collection

Ovaries were collected immediately after slaughter and transported on ice to the laboratory. Before processing the ovaries, the number of corpora lutea were recorded in order to estimate the ovulation rate. All visible ovarian follicles were classified by follicular diameter as small (≤3 mm), medium (3-6 mm) and large (≥ 6 mm) and individual follicles were aspirated using a 21 gauge needle attached to a 1 mL disposable syringe. Recovered cumulus-oocyte complexes (COCs) were classified according to morphological criteria under a stereoscopic microscope as: (I) surrounded with intact layers of cumulus cells, (II) having a partial cumulus cell layer, (III) naked oocytes, (IV) having expanded cumulus cells, and (V) degenerated oocytes. Due to the limited amount of material available, oocytes with the same size and in morphological classifications I, II and III, were pooled within sow and classified as normal/healthy; classifications IV and V were pooled within sow and classified as degenerated/atretic oocytes. The oocytes classified as normal showed a homogeneous cytoplasmic pigmentation and unexpanded zona pellucida, whether or not they were surrounded by cumulus cells, while atretic oocytes showed an abnormal shape, a lack of cytoplasm pigmentation and the zona pellucida was not intact. After oocyte isolation, follicular fluid was centrifuged for 5 min at 6000 x *g* at 4°C to collect GC that received the same classification (normal or atretic) as the COCs from which they were harvested. The aspirated oocytes were denuded from attached cumulus cells by repeated pipetting in 1X PBS with 0.1% (w/s) polyvinyl alcohol (PVA) and immediately frozen in liquid nitrogen until RNA extraction (Costa et al., 1997). The corpora lutea from each sow were dissected and treated with RNAlater (Ambion, Austin, TX) and stored at – 20 ºC for 48h. After freezing, the corpora lutea of each sow were thawed and ground in liquid nitrogen and the ground tissue kept at -70 ºC until RNA extraction.

RNA isolation and cDNA synthesis

Immediately after classification of COCs, GC from follicles with the same classification from each animal were washed twice in 1X PBS by centrifugation at 5000 x *g* for 6 min at 4°C, stored in RLT Buffer provided by RNeasy Mini Kit (Qiagen, Valencia, CA) and frozen at -20°C until RNA extraction. The GC samples were thawed at 36°C for 5 min and extracted according manufacturer's instructions. RNA isolation from luteal tissue was performed with 30 mg of grounded tissue using the same kit cited above. Oocyte samples were thawed on ice and total RNA isolation was performed using RNeasy Micro Kit (Qiagen, Valencia, CA) according manufacturer's instructions. All samples were DNase treated using the on-columm DNase digestion with the RNase free DNase Set (Qiagen, Valencia, CA) and the RNA was eluted in 30 µL for GC and corpora lutea, and 10 µL for oocytes. The samples were quantified using the NanoVue Plus spectrophotometer (GE Healthcare Bio-Scinces, Psicataway, NJ, USA) and RNA integrity was verified using Agilent 2100 Bioanalyser Nano Kit (Agilent Technologies, USA). Oocyte, GC and CL total RNA was reverse transcribed with ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions using 5 µM of oligo dT. cDNA synthesis was performed using 1 µg of GC and CL total RNA and with 6 µL of the oocyte total RNA. The cDNA was then stored at – 20 ºC until analysis by quantitative real-time PCR.

Quantitative real-time PCR

Quantitative real-time PCR was performed using SyBr Green GoTaq qPCR Master Mix (Promega, Madison, WI, USA) according to the manufacturer's instructions. The primer oligonucleotides used for the reactions were designed using "PrimerQuest" software (Integrated DNA Technologies, Inc., Coralville, IA) from swine sequences available in GenBank (htpp://www.ncbi.nlm.nih.gov). In the present study, GAPDH was used as a reference gene against which all gene expression was normalized. The list of primer sequences and expected PCR product lengths are shown in Table 1. Reactions were performed in duplicate in 96-well optical reaction plates sealed with optical adhesive film using 12.5 µl of 2X SyBr Green GoTaq qPCR Master Mix. Prior to quantification by quantitative real-time PCR, the amplification efficiency and optimal primer concentration was determined for each gene using serial dilution of cDNA from each cell type. The PCR efficiencies for all primers pairs were obtained using the formula $E = 10^{-1/slope}$, where E is efficiency and *slope* is the gradient of the dilution series in the linear phase. The samples were

amplified separately using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) by the amplification program: 40 cycles of 30 s of melting at 95ºC followed by 30 s of annealing and extension at 60 ºC. After the 40 amplification cycles, all samples were subjected to a melt curve analysis in which they were heated at 1 ºC/30 s increments from 60 ºC to 94 ºC to validate the absence of non-specific products.

Statistical analysis

The Chi-square test of independence was used to evaluate the distribution of follicles within different follicle size classes and the sample size deviation was corrected using the Fisher exact test. Statistical tests were performed using the PROC FREQ from SAS statistical package (SAS Institute, Cary, NC, USA), with a significance level of 5% and confidence intervals for the independent proportions were then constructed by the PROP.TEST from the statistical R software system (R Development Core Team, 2008). The number of CL and data on the inter-estrous interval were analyzed using SAS PROC Univariate to verify differences in ovulation rate and estrous cycle length between genetic groups.

The expression for each gene was calculated using the ΔC_t method (target gene C_t – GAPDH gene C_t) for all individual samples, where C_t reflects the PCR cycle number at which the fluorescence generated within a reaction crosses an arbitrary threshold.The gene expression differences were estimated using the method of $2^{-\Delta Ct}$ (Livak and Schimittgen, 2001). For statistical analysis six follicle classifications were used: i) large normal ii) large atretic iii) normal medium; iv) medium atretic v) small normal vi) small atretic and compared between genetic group x day of estrous cycle. However, as follicle classifications varied by genetic line and day of cycle, not all comparisons were possible. The comparisons were made using the SAS GLM PROCEDURE and defined as (commercial - Piau) for GC and oocytes. For corpus luteum (CL), the contrast comparisons were made using the same model with balanced samples, regardless of follicle classification.

Results

Dynamics of follicle development

Compared to commercial-line gilts, the less prolific Piau gilts tended to have a shorter estrous cycle length (19.4 ± 1.71 *vs* 20.0 ± 1.66 d, respectively; *P=0.068*) and a different pattern of follicle development. As well as having a lower OR (15.5 ± 1.9 *vs* 11.1 ± 2.4 in commercial-line vs Piau, respectively; $P \leq 0.05$).

The preovulatory population of follicles for both Piau and commercial gilts reached 6 to 10 mm in diameter. The distribution of both small ($P \leq 0.05$) and large ($P \leq 0.01$) ovarian follicles during the estrous cycle differed between commercial and Piau gilts throughout the estrous cycle, as illustrated in supplementary Table 1 (Appendix 1). The confidence interval for the difference between proportions allowed the identification of days of the estrous cycle on which the follicular number from Piau and commercial-line were significantly different (Figure 1). The pattern of follicle development in Piau breed was characterized as a lower number of small follicles at day 18, and large follicles at day 0, reflecting their expected lower-ovulation rate compared to the commercial-line.

The proportion of normal and atretic follicles during the estrous cycle for all size follicles in commercial and Piau breed is illustrated in Figure 2. The pattern of atresia seems to be different between breeds, with a higher atresia rate in day 10 for commercial and day 18 for Piau breed. In addition to the decline in the number of small and medium sized follicles observed between days 10 and 18 of the estrous cycle for both groups, the incidence of atresia in the total follicle population increased from 17.3% before selection (Day 10) to 50% before estrous (Day 18) in the Piau breed compared to an decrease in atresia from 59.3% to 25.7% in the commercial-line gilts.

Corpus luteum mRNA expression profiles

Few differences between the genetic lines were observed for mRNA expression of apoptosis related genes in the CL during the estrous cycle (Figure 3). *BCL2* mRNA abundance, a major anti-apoptotic molecule, was similar in commercial and Piau breed at day 0, whereas mRNA abundance of the pro-apoptotic factor *BAX* was higher in commercial-line gilts on day 4 and 18 ($P \leq 0.05$ and $P \leq 0.01$, respectively). One of the components of the Fas

system, *FASL* was higher expressed in Piau breed at day 18 (P≤0.01). However, no differences in expression were observed for *FAS* and the cascade initiator caspase-8 (CASP8). *CASP3* mRNA abundance, which is one of the downstream components of *CASP8* was higher in Piau breed on day 0 and 4 (P≤0.05 and P≤0.01, respectively). Similar results were observed for *TGFBR2*, with higher expression in commercial-line gilts on day 0 and day 4 (P≤0.0001 and P≤0.05, respectively) than in the Piau breed.

Granulosa cell mRNA expression profiles

Figure 4 illustrates mRNA expression between the commercial and Piau breed for components of the death receptor apoptotic pathway, with the exception of *FASL*, which could not be detected in the GC with the current primer set, and *TGFBR2*. In large normal follicles, *TGFBR2* mRNA abundance was higher in the commercial-line on day 0; however, no significant difference was observed in large atretic follicles. *FAS* mRNA expression in large atretic follicles was higher in Piau than commercial gilts (*P*≤0.05) on day 0, while no significant difference was observed in large normal follicles. At day 0, medium normal follicles showed higher *BCL2* mRNA abundance in GC in the Piau breed (*P*≤0.01) than in commercial-line. *CASP8* mRNA expression was higher in Piau breed for medium normal follicle category at day 18 of the estrous cycle. *CASP3* mRNA expression was higher in commercial-line for medium atretic follicles at day 4. In small normal follicles from day 4 *CASP3* mRNA expression was higher in commercial-line while *CASP8* mRNA expression was higher in the Piau breed. Additional comparisons between follicle size/health status were limited by the number of experimental units at specific days of estrous cycle.

Oocyte mRNA expression profiles

Figure 5 illustrates the contrast of oocyte gene expression for growth differentiation factor 9 (*GDF9)*, bone morphogenetic protein 15 (*BMP15)* and bone morphogenetic protein 6 (*BMP6)* between breeds according to follicle size on a particular day of the estrous cycle. Interestingly, for all genes analyzed negative values were obtained, meaning that the Piau breed had higher expression than the commercial-line. The relative amount of *GDF9* in large normal and atretic follicles at day 18 was higher (*P*≤0.05) in the Piau group. In medium normal follicles, *GDF9* mRNA abundance was higher in the Piau group at day 10, while no differences were observed for medium atretic follicles. For small follicles no differences in

GDF9 mRNA abundance were observed in either normal or atretic follicles on the analyzed days. *BMP15* mRNA expression was higher at day 18 in the Piau breed in medium normal and small atretic follicles ($P \leq 0.05$) than in the commercial-line. Only one contrast was significant for *BMP6* mRNA abundance, which showed higher expression in the Piau breed for small normal follicles at day 10 ($P \leq 0.05$).

Discussion

The key ovarian phenotypic traits of the less prolific Piau breed were a lower OR, tendencies for a shorter estrous cycle than in the more prolific commercial-line gilts. A shorter estrous cycle has also been observed in the hyperprolific Meishan breed when compared to nonprolific breeds (Bazer *et al.* 1988b). It has been postulated that the longer behavioral estrus in Meishan breed would result in recovery of follicles relatively earlier to ovulation; as a result, Meishan sows maintained a large number of follicles during the follicular phase, which keeps constant with no reduction in the proliferating pool, contributing for their high prolificacy (Bazer et al. 1988b). Despite showing a tendency for shorter estrous cycle as observed in Meishan, the Piau breed represents the other extreme of prolificacy. Besides the lack of information about the precise time course of events during the estrous cycle in Piau breed, findings from the present study indicate decrease in the number of small follicles (≤ 3mm diameter) in early follicular phase (Day 18) as illustrated in Figure 1, panel A. The reduction in the number of small follicles along with higher rate of atresia at Day 18 (see Figure 2) suggests a block of follicle replacement in the proliferating pool, an opposite situation that occurs in Meishan, which may be the primary determinants of low prolificacy in this breed.

The observation of fewer small follicles, and the higher rate of atresia in large follicles, limits the number of follicles available for selection and subsequent ovulation in the less prolific Piau compared to commercial-line gilts at day 18 of estrous cycle and is consistent with reports that sows with a higher OR have a larger number of follicles in the proliferating pool (Clark *et al.* 1973) and maintain a higher number of follicles during follicular phase than do non-prolific breeds (Miller *et al.* 1998). However, Piau gilts also had lower incidence of atresia in medium and small sized follicles at days 4 and 10. This pool of follicles begins to grow after ovulation and will constitute the pool of follicles in the recruitment phase, which occurs at 14 and 16 of the estrous cycle in pigs (Foxcroft and Hunter, 1985). Collectively, these observations corroborate the suggestion that OR is a complex trait related to differences in both follicular recruitment and atresia (Manabe *et al.*

2004).

It is possible that the different pattern of follicle growth observed in the present study is caused by different timing of the LH surge between breeds, linked to differences in the estrous cycle length between Piau and commercial gilts, as comparative studies reported that behavioral estrous in the hyper-prolific Meishan pig occurred earlier, relative to the LH surge and ovulation, than in Large-White sows (Hunter *et al.* 1993).

The follicular dynamics between breeds were further corroborated by gene expression analysis using quantitative real-time PCR. Genes of TGF-β superfamily, such as BMPs, GDF9 and their receptors are known modulators of mammalian folliculogenesis, and were selected for study. The expression of *BMP6*, *BMP15* and *GDF9* transcripts have recently been reported in porcine oocytes from preovulatory follicles, as well as their cell receptors (Paradis *et al.* 2009; Sun *et al.* 2010). However, less information is available about the mechanisms underlying their physiological role in follicular development between species, or differences in their expression in divergent breeds of pigs. Therefore, a comparative study using the less prolific Piau breed and more prolific contemporary commercial-line gilts provided the opportunity to identify genes related to ovulation rate and oocyte quality, and consequently embryonic development and survival in these two breeds.

Interestingly, in this study the significant contrasts between two breeds identified higher expression of oocyte secreted factors in Piau breed than the commercial-line. As they act as key regulators of oocyte maturation and folliculogenesis (McNatty *et al.* 2004; Juengel and McNatty, 2005, Gilchrist *et al.* 2008) they may be related to the differences in ovulation rate between breeds. The local Piau breed showed higher *GDF9* mRNA abundance in large normal and large atretic follicles at day 18 and in medium normal follicles at day 10. *BMP15* mRNA expression was detected in the oocyte, consistent with previous studies in the pig (Quinn *et al.* 2004; Zhu *et al.* 2008; Paradis *et al.* 2009), with higher expression in medium-sized healthy follicles and small-sized atretic follicles in the Piau breed at day 18. Working with *in vitro* oocytes, Liu *et al.* (2008) reported that these two genes and their encoded proteins were differentially expressed during the maturation process, especially during cumulus cell expansion. Therefore, these data suggest that the lower prolificacy of the Piau breed may be related to differences in expression of both oocyte secreted factors, resulting in changes in the timing of cumulus cell expansion, and potentially also affecting granulosa cell steroidogenesis. Furthermore, it is possible that the differential expression for *GDF9* and *BMP15* verified in the present study is due to relative differences in the stage of follicular

maturation, as previous studies reported that the Meishan preovulatory follicle is in a more advanced state of maturation than that of the Large-White (Hunter *et al.*, 1993, Faillace and Hunter, 1994, Xu *et al.*, 1998).

BMP6 mRNA expression was also detected in porcine oocytes in this study, corroborating previous findings (Zhu *et al.* 2008, Paradis *et al.* 2009). The differential expression of *BMP6* mRNA from Piau oocytes may play an important role in FSH-dependent follicle development and in the regulation of luteinization, again affecting the difference in ovulation rate between breeds. It has been suggested in rats that *BMP6* mRNA derived from GC is lost during selection of the dominant follicle and that *BMP6* mRNA is strongly expressed in GC during atresia (Erickson and Shimasaki, 2003). Moreover, investigation into the mechanism of action found that *BMP6*, unlike *BMP15* and *GDF9*, does not have proliferative properties on rat GC and is able to suppress FSH-induced progesterone production (Otsuka *et al.* 2001). Therefore, the results from the present study suggest breed differences in the *BMP6*, *BMP15* and *GDF9* mRNA expression profile, and indicate that a larger number of follicles can escape from atresia during early folliculogenesis in the Piau breed, resulting in the lower incidence of atresia at days 4 and 10 compared to commercial-line gilts. These results initially seem contradictory, since the Piau breed is the less prolific. However, oocyte secreted factors may be involved in the recruitment process, leading to differences in oocyte quality (Gilchrist *et al.* 2008) and the number of tertiary and atretic follicles between lines, as suggested by Manabe *et al.* (2004). Also associated with their lower prolificacy, differences in oocyte-expressed factors may be contributing to the ability of Piau to use smaller follicles for selection and ovulation, as described for the Meishan breed (Miller *et al.* 1998).

Once the pool of follicles initiates its growth, atresia and ovulation are the only possible fates (Knox *et al.* 2005). Atresia limits the number of oocytes capable of fertilization and embryonic development, and is physiologically important for the elimination of degenerated oocytes (Guthrie *et al.* 1995; Guthrie, 2005). It is not clear whether this process differs between pig breeds. In the current study, mRNA expression of candidate genes involved in apoptotic pathways in granulosa and luteal cells differed between breeds during the estrous cycle. The Fas-FasL system has been reported in many species as the major mechanisms regulating GC (Matsuda-Minehata *et al.* 2008) and luteal cell apoptosis (Juengel *et al.* 1993; Rueda *et al.* 1997; Sakamaki *et al.* 1997). In the current study, *FAS* mRNA was more abundant in GC from healthy than atretic follicles during estrous cycle, consistent with previous findings in several species (Dharma *et al.* 2003, Inoue *et al.* 2006, Porter *et al.*

2001). However, in contrast, Inoue *et al.* (2006) reported that *FAS* mRNA expression increased in GC from atretic compared to healthy follicles**.**

We also detected *FAS* and *FASL* mRNA expression in pig luteal tissue as reported in murine and human studies (Kondo *et al.* 1996; Sakamaki *et al.* 1997). Interestingly, in the current study no differences were observed for the *FAS* transcript in CL between breed groups, while *FAS* mRNA expression in GC was significantly higher in large atretic follicles from Piau gilts than from commercial females at day 0. According to Manabe *et al.* (2004), differences in the initiation of GC apoptosis between species indicate local mechanisms of regulation, mainly the apoptotic stimuli induction mechanism. In this context, the current study indicates that apoptosis signaling may be differently activated in atretic follicles between distinct breeds. Therefore, it can be suggested that the follicle in the Piau breed provides a different environment for follicle apoptosis than in commercial-line follicles, which may contribute to the lower prolificacy of Piau breed. Many reports have shown that apoptosis occurs during luteolysis, and it is also established that it plays a role in the CL regression at the end of the estrous cycle (Rueda *et al.*, 1997). In the present study the amount of transcript that encodes the membrane protein Fas were similar in CL between breeds, while its ligand *FASL* mRNA was higher expressed in Piau CL, which may result in a more effective apoptosis signaling towards luteolysis in this breed.

Several genes belonging to the *bcl2* family, including both anti-apoptotic and pro-apoptotic family members, are known to be expressed in luteal cells of various species (Rodger *et al.* 1995; Rueda *et al.* 1997; Goodman *et al.* 1998). The *Bcl2:Bax* expression ratio within a cell is related to its potential to become apoptotic, and it has been proposed that Bax homodimers promote cell death, while Bcl2 homodimers function as repressors of cell death (Oltvai *et al.* 1993). Findings in the present study indicate that *BCL2* mRNA abundance in CL was not different between breeds during the estrous cycle. However, mRNA abundance of the pro-apoptotic factor *BAX* was higher in commercial-line gilts at day 4 and 18, resulting in a higher ratio of *BAX: BCL2* mRNA compared to Piau breed, a change consistent with bax-mediated apoptosis (Rueda *et al.* 1997). This indicates a possible occurrence of increased ratio of apoptosis in CL of commercial than in Piau breeds. Similar higher expression of *BAX* mRNA in the regressing CL was reported during luteolysis in cattle and human (Rueda *et al.* 1997; Sugino *et al.* 2000; Pretheeban *et al.* 2010). Although in the current study the expression of *BCL2* mRNA was similar between breeds at day 10, previous studies in human demonstrated higher expression of *BCL2* mRNA in the mid-luteal phase CL (Sugino *et al.* 2000). Taken together, these findings suggest that *BAX* gene may be important in the

regulation of the CL lifespan by controlling the rate of apoptosis and potentially underlies differences in estrous cycle length and cyclicity between the breeds. However, it is important to emphasize that these events may also be regulated at the post-translational level and modulated by interactions with other molecules such as p53 tumor suppressor protein (Miyashita *et al.* 1994). In addition, the abundance of transcripts is not always associated with differences in protein secretion (Griffin *et al.* 2002) and studies at proteomic level are also required.

Many findings indicate that Bcl2 family proteins modulate apoptosis of GC in mammals, where *BCL2* over-expression is related with reduced follicular atresia and increased litter size (Hsu *et al.* 1996; Choi et a., 2004). In the current study *BAX* mRNA abundance in GC was similar between breeds during estrous cycle, while *BCL2* mRNA abundance of medium-sized healthy follicles was higher in the Piau breed compared to commercial-line gilts at day 0, again suggesting decreased apoptosis in Piau GC during early follicular development. *CASP3* has been reported as an essential molecule for the apoptosis of GC. Many studies in GC have described changes in caspase-3 protein expression and activity associated with the progression of atresia in ovarian follicles (Boone and Tsang 1998; Berardinelli e*t al.* 2004). In the current study *CASP3* mRNA abundance was significant higher in GC from medium-sized atretic and small-sized healthy follicles at day 4 in commercial-line gilts than in the Piau breed. However, little other information is available about *CASP3* mRNA expression pattern in different breeds of pigs during the estrous cycle. During the early luteal phase in the pig, a new group of medium-sized follicles starts to growth between days 3 and 8 (Guthrie *et al.* 1995). Although the functional life-span of these follicles is unknown, it is likely that within a day or two after ovulation the medium-sized growing follicles will be eventually deleted by atresia, as described in cattle (Sirois and Fortune, 1988). Therefore, the data on *CASP3* mRNA expression in medium- and small-sized follicles in commercial-line gilts suggest that this breed may be more susceptible to atresia than the Piau breed at day 4, driving the different pattern of follicular development observed between breeds. These findings also agreed well with the higher incidence of atresia (23.1% *vs* 14.5%) at day 4 in commercial-line gilts compared to the Piau gilts in the total follicle population, as shown in Figure 2.

It is also known that apoptosis occurs during luteolysis in the ovary and it is also recognized that controlled cell death is important to physical removal of the CL from the ovary at the end of estrous cycle (Rueda *et al.* 1997; Tilly, 1998). Previous studies reported *CASP3* expression in CL during luteolysis induced by prostaglandin in the rat (Boone and

Tsang, 1998) and in sheep (Rueda el al. 1999) CL. Moreover, abundant expression of *CASP3* in the human CL was reported and considered to be important for luteal regression (Krajewska *et al.* 1997). However, little is known regarding the expression and role of caspases in the pig CL. In the current study *CASP3* mRNA was expressed in luteal cells throughout estrous cycle and its abundance was higher in the Piau breed at day 0 and day 4. Apoptotic cell death during luteolysis is important to maintain estrous cyclicity (Manabe *et al.* 2004). However, premature disruption of normal CL function could result in reduction of reproductive efficiency due to irregular estrous cycles and loss of pregnancy (Rueda *et al.* 1997). In this context, the present results indicate that apoptosis mediated by *CASP3* may be differently regulated between the breeds studied and may reflect differences in the estrous cycle length and the time of luteal regression. Recent findings have addressed roles for TGFβ in the follicle maturation and luteinization processes in the pig (Paradis *et al.* 2009, Sriperumbudur *et al.* 2010). In the current study, *TGFBR2* mRNA expression was decreased in Piau CL at day 0 and day 4 compared to the commercial-line, indicating that the regression process may differ between breeds. In addition, the *TGFBR2* mRNA expression in this study was higher in GC from large follicles of the commercial-line gilts at day 0 and may mediate the differences in follicular dynamics leading to differences in ovulation rate and oocyte quality in gilts from the different breeds. This finding is further supported by Sriperumbudur *et al.* (2010), who suggested that *TGFBR2* may have roles in mediating the luteinization process in post-ovulatory porcine follicles.

Conclusions

In summary, the findings from this experiment indicate differences in the estrous cyclicity and follicular development in the less prolific Piau gilt than in commercial-line gilts. The pattern of follicular growth in Piau gilts is characterized by a small population of pre-ovulatory follicles, combined with a higher incidence of atresia in the pre-ovulatory stage of the cycle compared to commercial-line gilt. The gene expression profiles observed were consistent with the different follicular dynamics between the breeds, with the relative expression of *BCL2* and *BAX* genes influencing important decisions between proliferation and atresia in CL and GC of gilts of the different breeds. It is important to remember that transcriptional profiling has many levels of regulation, such as RNA processing, translation and degradation; therefore, the mRNA abundance observed in this study is only an initial indicator of overall activities of the genome during follicular development. Moreover, RNA abundance may not correspond to protein levels and does not provide information about

protein modification, activity or location (Zeng et al. 2004). Additional techniques like RNA interference (RNAi) could be used in future experiments to down-regulate the expression of specific genes in vitro culture systems. For example, RNAi has been used investigate the potential role of growth factors in mediating oocyte regulation of cumulus cells expansion (Gui and Joyce, 2005) and to confirm the pro-activity of apoptotic genes in granulosa cells during atresia (Sai et al. 2012). Apoptosis in the CL, mediated by differences in *FASL* mRNA, *CASP3* mRNA and by decreased expression of *TGFBR2* mRNA verified in Piau gilts compared to commercial-line at day 0 and 4 of estrous cycle, may also reflect the tendency for a shorter estrous cycle length and faster luteal regression in the Piau breed. Finally, the higher expression of oocyte secreted factors (*GDF9*, *BMP15* and *BMP6*) in Piau oocytes may play a role in inhibition of the luteinization process and also affect follicle development and induce the lower ovulation rate that is a key component of the reduced prolificacy of this breed.

Taken together, our results support the hypothesis that differential expression of genes and/or gene pathways controlling follicle growth mediate the different pattern of follicle development observed between the breed studied. This may affect not only ovulation rate but also oocyte and embryo quality.

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Table 1. List of primer sequences and quantitative real-time PCR quality control data for pig candidate gene expression analysis

Gene Symbol[1]	Accession number[2]	Cell type[3]	Primer	Oligonucleotide sequence (5'→3)'	Product size (bp)	$R^{2(4)}$	Slope of calibration curve	Efficiency (%)
GAPDH	AF017079	GC	Forward	GCAAAGTGGACATTGTCGCCATCA	124	0.99	-3.33	2.00
		CL	Reverse	AGCTTCCCATTCTCAGCCTTGACT		0.99	-3.32	1.99
		Oocyte				0.99	-3.21	2.05
GDF9	NM001001909	Oocyte	Forward	TGGTGCAGAACATCATCCACGAGA	100	0.99	-3.32	2.00
			Reverse	GGCTCAATGGCCAACACACTCAAA				
BMP15	NM001005155	Oocyte	Forward	AAGCTTGGACGGAGATGGATGTCA	162	0.99	-3.53	1.92
			Reverse	GAAGGCAGTGTCCAGGGATGAAA				
BMP6	EU693015	Oocyte	Forward	GGCGGTGACGGCTGCAGAAT	150	0.98	-3.42	1.96
			Reverse	CACACGACGCGGGTGTCCAA				
FASL	NM213806	CL	Forward	AGGCCTGTGTCTCCTTGTGATGTT	125	0.99	-3.30	2.00
			Reverse	TTTGGCTGGCAGACTCTCTGAGTT				
FAS	NM213839	GC	Forward	AGGTGATGATGCCCAAGTGACTGA	149	0.99	-3.53	1.92
		CL	Reverse	AGTCAGCATGTTTCCGTTTGCCAG		0.99	-3.55	1.91
BCL2	NM214285	GC	Forward	TACGGAAACAATGCAGCAGCTGAG	123	0.99	-3.41	1.96
		CL	Reverse	TGGTCATTTCCGACTGAAGAGCGA		0.99	-3.55	1.91
BAX	NM138761	GC	Forward	TTTCTGACGGCAACTTCAACTGGG	122	0.99	-3.73	1.85
		CL	Reverse	TGTCCAGCCCATGATGGTTCTGAT		0.99	-3.28	2.01
TGFBR2	EF396957	GC	Forward	TGAGTCCTTCAAGCAGACGGATGT	134	0.99	-3.33	2.00
		CL	Reverse	TGGAACCAAAGGGTGGCTCATAGT		0.99	-3.76	1.85
CASP8	NM001031779	GC	Forward	TGCCTCCGGTTACAACTACATCCT	112	0.99	-3.31	2.01
		CL	Reverse	AACTTGAGGGAAGCCAGGTCATCA		0.99	-3.76	1.85
CASP3	NM214131	GC	Forward	ATGCTGCAAATCTCAGGGAGACCT	159	0.99	-3.59	1.90
		CL	Reverse	CACCATGGCTTAGAAGCACGCAAA		0.99	-3.26	2.02

1) Gene symbol: glyceraldehyde-3-phosphate dehydrogenase *(GAPDH),* growth differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*), bone morphogenetic protein 6 (*BMP6*), Fas ligand (*FASL*), Fas protein (*FAS*), B/linfoma-2 cell (*BCL2*), BAX protein (*BAX*), transforming growth factor β receptor 2 (*TGFBR2*), caspase-8 (*CASP8*), caspase-3 (*CASP3*). 2) Accession number at Genbank ((htpp://www.ncbi.nlm.nih.gov). 3) Cell type which amplicons are generated: granulosa cell (GC), corpus luteum (CL). 4) Coefficient of determination (R^2).

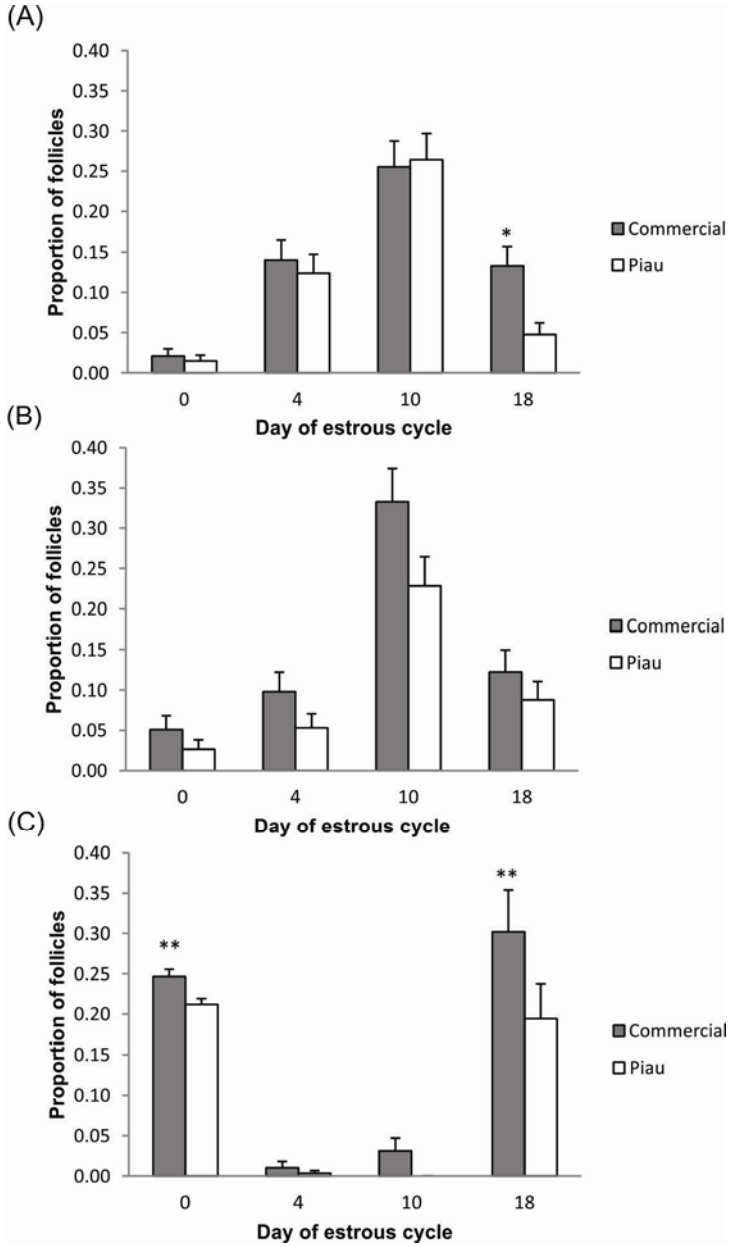

Figure 1. Confidence intervals for the proportion of (A) small, (B) medium and (C) large follicles during the estrous cycle in commercial and Piau gilts. Asterisks indicate significant differences (*P≤0.05; **P≤0.01) between genetic groups.

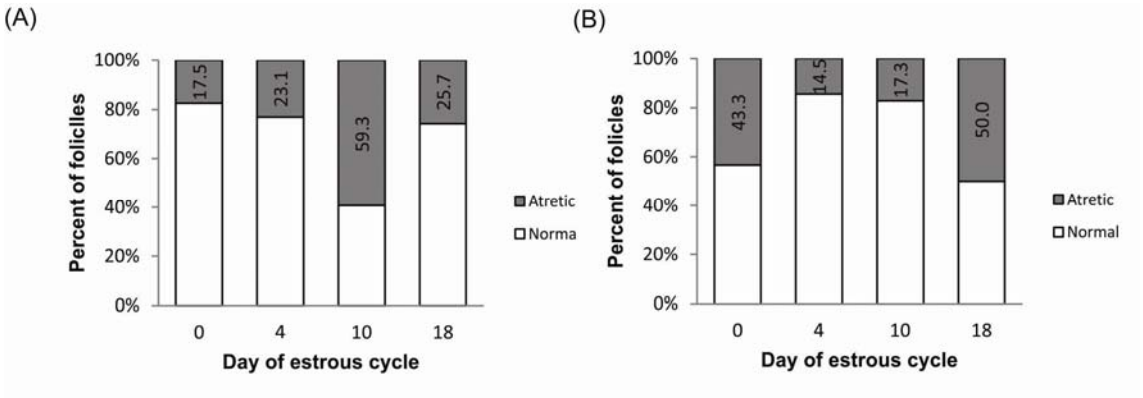

Figure 2. Percentage of normal and atretic follicles during the estrous cycle for all follicle sizes in (A) commercial and (B) Piau sows.

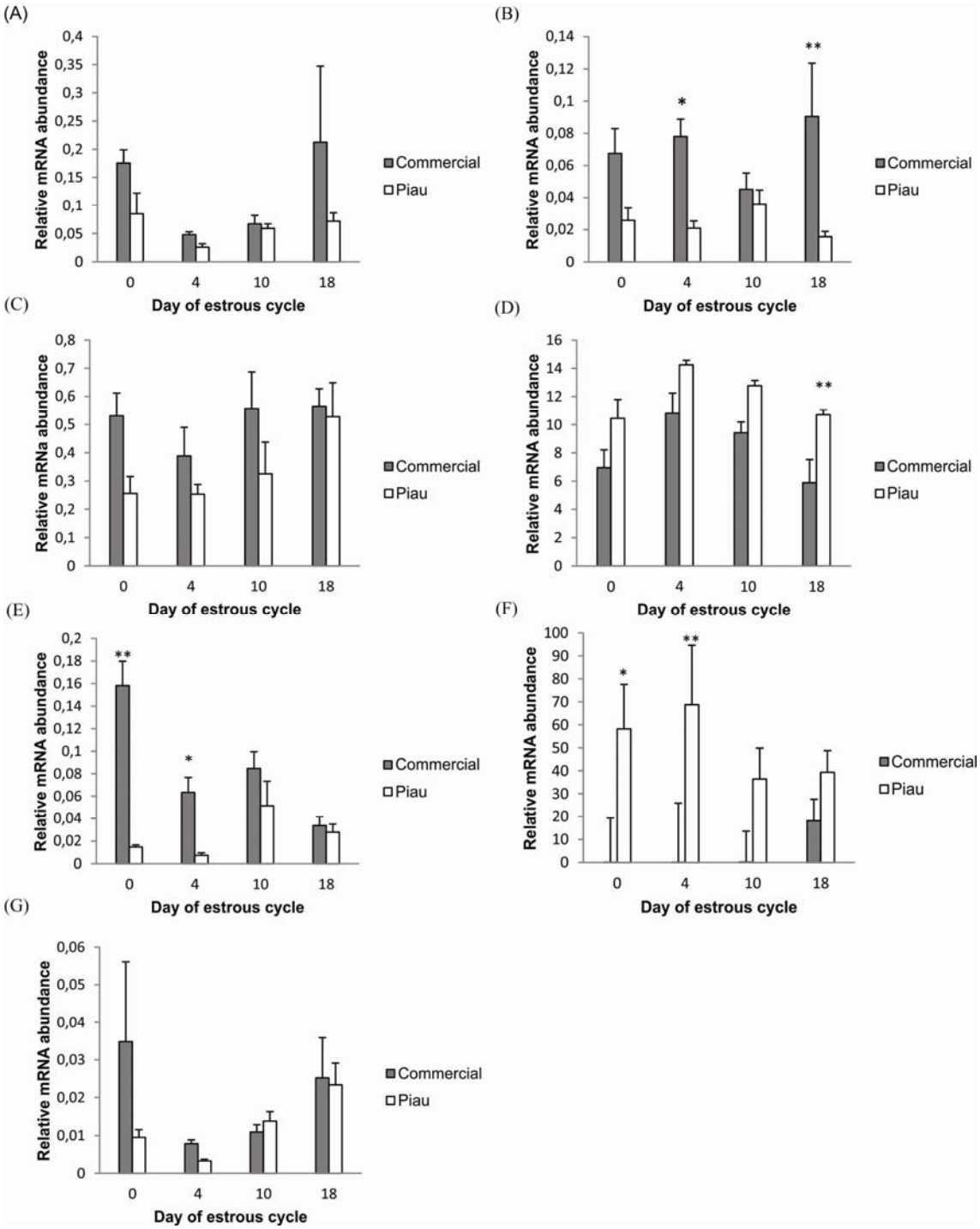

Figure 3. Comparison of mRNA abundance in corpus luteum from commercial and Piau genetic group (A) *BCL2*, (B) *BAX,* (C) *FAS*, (D) *FASL*, (E) *TGFBR2*, (F) *CASP3,* (G) *CASP8* throughout estrous cycle. Relative mRNA abundance is expressed as lsmeans of $2^{-\Delta Ct}$ ± S.E.M. Asterisks indicate significant differences (*P≤0.05; ** P≤0.01).

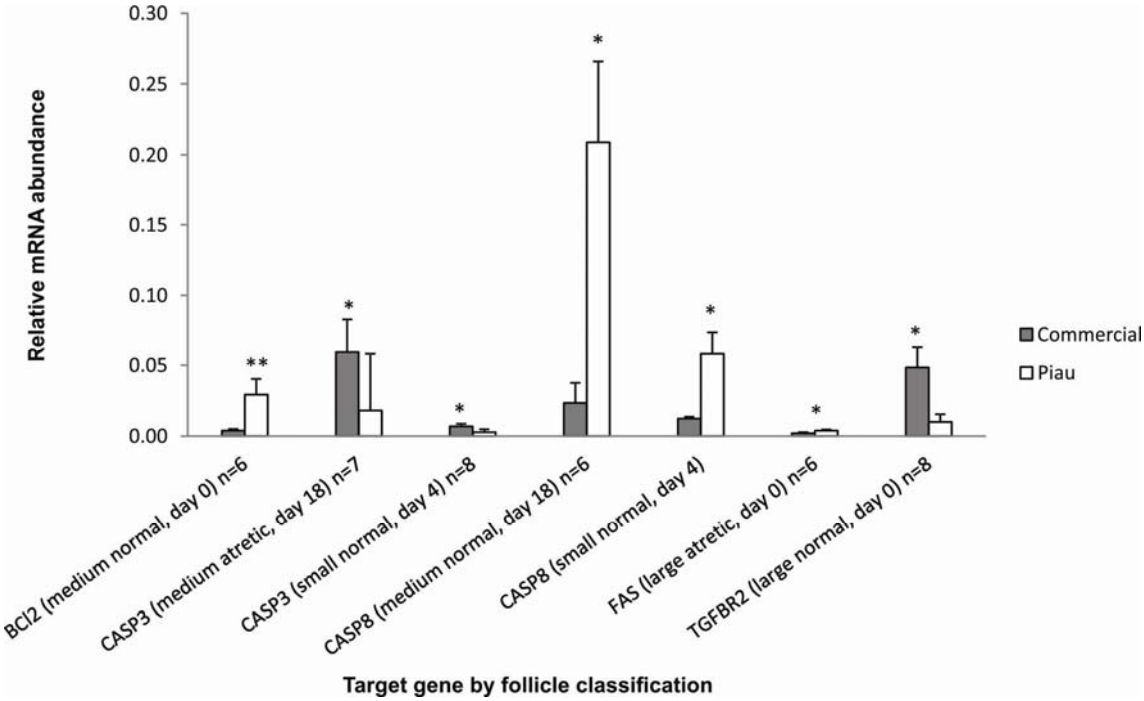

Figure 4. Comparison of mRNA abundance in granulosa cells from commercial and Piau genetic group for target genes by follicle classification on particular days of the estrous cycle and number of animals. Relative mRNA abundance is expressed as lsmeans of $2^{-\Delta Ct}$ ± S.E.M. Asterisks indicate significant differences (*P≤0.05; ** P≤0.01).

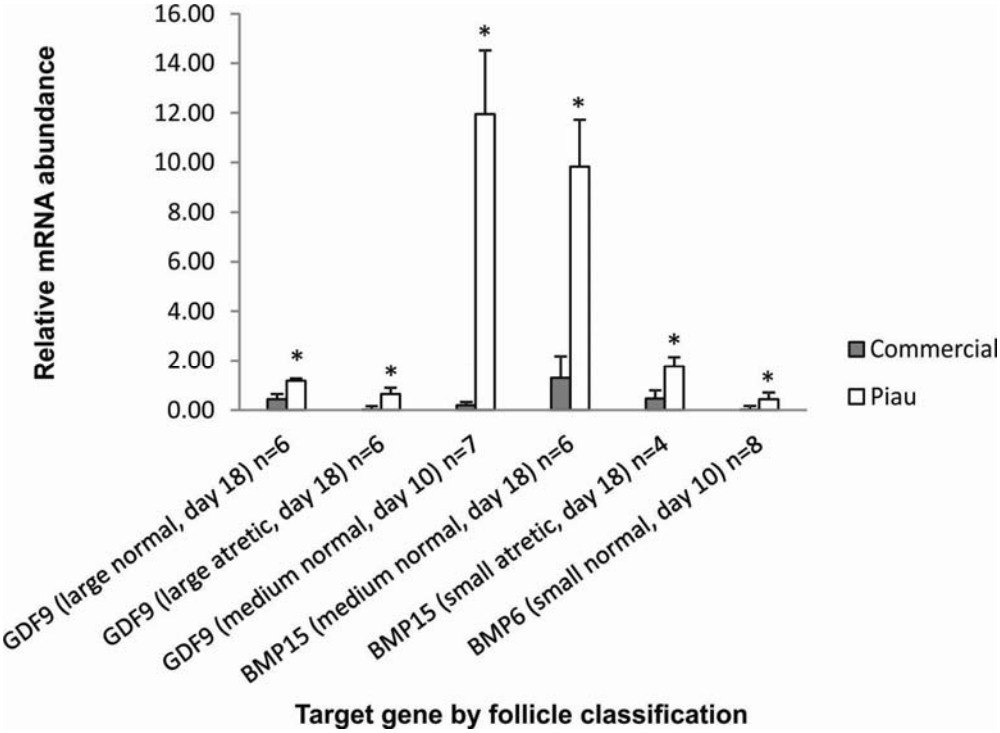

Figure 5. Comparison of mRNA abundance in denuded oocytes recovered from commercial and Piau genetic group for target genes by follicle classification on particular days of the estrous cycle and number of animals. Relative mRNA abundance is expressed as lsmeans of $2^{-\Delta Ct}$ ± S.E.M. Asterisks indicate significant differences (*P≤0.05; ** P≤0.01).

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CHAPTER 2

(ARTICLE 2)

Gene expression in commercial sows with low and high birth weight litter phenotype

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Abstract

In the present study expression of candidate genes controlling angiogenesis, apoptosis and encoding intrafollicular growth factors were analyzed in corpus luteum (CL), granulosa cells (GC) and denuded oocytes recovered from commercial sows characterized as high and low birth weight litter phenotype at Day 5 of gestation. Quantitative real time PCR analysis revealed that the analyzed apoptotic genes were differentially expressed between high and low groups in CL and GC. Most of the angiogenesis-related genes investigated were higher expressed in CL tissue in the low group. Finally among intrafollicular growth factors, only *IGFR1* and *BMPR2* were differently expressed in GC and denuded oocytes, respectively. Findings from the present study suggest that differences in CL vascularity and function, as well as in follicle development, may be in part, driving differences between-litter variation in birth weight in contemporary sows.

Key words: intrauterine growth retardation, postnatal growth potential, swine

Resumo

O presente estudo teve como objetivo a investigação de genes candidatos relacionados com apoptose, angiogênese e codificadores de fatores de crescimento intrafolicular no corpo lúteo (CL), células da granulosa (CG) e oócitos desnudados coletados no dia 5 de gestação em porcas caracterizadas como de alto e de baixo peso médio de leitegada ao nascimento. A análise quantitativa de PCR em tempo real revelou que os genes apoptóticos analisados foram diferencialmente expressos entre os grupos de alto e baixo peso médio de leitegada no CL e na CG. A maior parte dos fatores angiogênicos foi significativamente mais expressa no CL nas porcas de baixo peso médio de leitegada ao nascimento em relação às porcas de alto peso médio de leitegada. Finalmente, dentre os fatores de crescimento intrafoliculares, apenas *IGFR1* e *BMPR2* foram diferentemente expressos nas CG e nos oócitos desnudados, respectivamente. Os resultados do presente estudo sugerem que diferenças na vascularização do CL e em sua função, assim como no desenvolvimento folicular, podem ser em parte, atribuídas a variações de peso de leitegada ao nascimento observada nas linhagens comercias.

Palavras-chave: retardo do crescimento intrauterino, potencial de crescimento pós-natal, suíno.

Introduction

In pig production, both litter size and birth weight are important economic traits. As a complex phenotypic trait, litter size can be partitioned into some main components (ovulation rate, uterine capacity and embryonic survival). Ongoing selection for litter size has increased ovulation rate but is associated with crowding of embryos in utero, limited placental development, fetal growth restriction and ultimately low litter average birth weight (Foxcroft et al. 2006). Birth weight is an important factor for piglet survival and post natal growth potential. Low birth weight litters takes to reduced growth and poor carcass quality in comparison with high birth weight (Rehfeldt and Kuhn, 2006). It seems that low birth weight is a characteristic of a subpopulation of sows that occurs due to an imbalance between ovulation rate and uterine capacity, which create uterine crowding in early gestation. This crowding negatively impacts placental and fetal development, irrespective of the final litter size born (Foxcroft et al., 2006).

The biological origins of low birth weight litters in mature sows have become a major research focus. In contrast to effects of uterine crowding, the growth performance after birth appears to be determined and essentially pre-programmed during fetal development in the uterus (Foxcroft and Town, 2004). Recent studies have suggested that gene imprinting, interactions between metabolism, nutrition, and methylation state during oocyte maturation and early embryonic development may be components of the low birth weight phenotype involved in contemporary sow populations (Kelly and Trasler, 2004; Reik, 2007; Burdge et al., 2007 Park et al., 2009; Foxcroft et al., 2009).

To provide further insight into the mechanisms regulating the low birth weight phenotype in contemporary sow populations, gene expression patterns in granulosa cells, denuded oocytes and corpus luteum from sows with a high and low birth weight litter at Day 5 of gestation were evaluated in the present study using quantitative real time PCR.

Materials and methods

Animals

A total of 10 sows Landrace x Large White sows (CPig; Saskatoon, Canada) were characterized as having a high or low birth weight phenotype (BTWA) based on records of average litter birth weight over at least 4 farrowing events. Sows (n = 5 per group) were pair-matched at weaning by their average litter weight and fed with a standard gestation diet. Selected sows were synchronized using porcine luteinizing hormone (pLH) injection at the onset of standing heat, bred accordingly to normal herd protocols and euthanized at day 5 of gestation.

Tissue Collection

The ovaries were removed immediately after slaughter to 50 ml Falcon tube containing 1X PBS. Before processing the ovaries, the number (of corpora lutea) and size of all visible follicles were recorded for the left and right ovaries of each animal. Follicles from the right and left ovaries were aspirated using a 21 gauge needle attached to a 1 mL disposable syringe. Aspirated oocytes from the left and right ovaries of each sow were isolated and denuded from attached cumulus cells by vortexing at low speed for 5 minutes in 150 µl PBS in a 1.5 ml microcentrifuge tube. The denuded oocytes were observed under a dissecting microscope to ensure that they were free of cumulus cells and then transferred to a fresh tube in a minimal volume of PBS and snap frozen on dry ice. After oocyte isolation, the follicular fluid from the left and right ovaries were recovered and centrifuged for 5 min at 13000 x g at 4°C to collect granulosa cells (GC). The pellets were washed twice with 1X PBS followed by centrifugation at 200 g for 5 min. The supernatant were discarded and the GC from each ovary side per sow were disrupted and homogenized in the RLT Buffer provided by RNeasy Mini Kit (Quiagen, Valencia, CA), and stored at -20°C until RNA extraction. The corpora lutea from the left and right ovaries of each sow were dissected, weighted and snap frozen in liquid nitrogen and stored at – 70 ºC until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA from the left and right ovaries of each animal was extracted from granulosa cells and corpora lutea using RNeasy Mini Kit (Qiagen). The GC samples were thawed at 36°C for 5 min and extracted according manufacturer's instructions. RNA extraction of corpora lutea recovered from the left and right ovaries of each sow was performed with 30 mg of grounded tissue using the same kit cited above. Total RNA from denuded oocytes recovered from the left and right ovaries of each animal was isolated using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA, USA). The RNA quality of the samples was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). The RNA was quantified using NanoDrop-100 equipament (NanoDrop Technologies, Wilmington, USA). All samples were DNase treated using DNA-free (Ambion) as suggested in the protocol and the RNA were eluted in RNase free water (Ambion). All samples were stored at -80°C until cDNA synthesis. Denuded oocytes, granulosa cells and corpora lutea total RNA were reverse transcribed with MultiScribe (ABI) according the manufacturer's instructions, using 5 µM of oligo dT. RNaseOUT (Invitrogen) were also added to the reaction at a concentration of 2 U/µl. cDNA synthesis were performed using 2 µg of Total RNA. After reverse transcription, GC and CL cDNA were diluted to 20 ng/µl and the oocyte cDNA to an equivalent amount of 0.25 oocytes/2 µl with nuclease-free water.

Quantitative real-time PCR

Quantitative real time RT-PCR was performed using KAPA SYBR® FAST qPCR Master Mix (Kapabiosytems, Boston, MA, USA) according to manufacturer's instructions. The primer oligonucleotides were designed using "PrimerQuest", software available from Integrated DNA Technologies, Inc. (Coralville, IA) from swine sequences available in GenBank (htpp://www.ncbi.nlm.nih.gov). The list of primer sequences and expected PCR product lengths are shown in Table 2. Reactions were performed in duplicate in 96-well optical reaction plates sealed with optical adhesive film using 10 µl of 2X KAPA SyBr qPCR Master Mix in a total volume of 20 µl. Prior to quantification by quantitative real-time PCR, the amplification efficiency and optimal primer concentration was determined for each gene using serial dilution of cDNA from each cell type. The PCR efficiencies for all primers pairs were obtained using the formula $E = 10^{-1/slope}$, where E is efficiency and *slope* is the gradient of the dilution series in the linear phase. The samples were amplified separately using the ABI 7900HT

thermocycler (Applied Biosystems, Foster City, CA) by the amplification program: 45 cycles of 30 s of melting at 95ºC followed by 30 s of annealing and extension at 60 ºC. After the 45 amplification cycles, all samples were subjected to a melt curve analysis in which they were heated at 1 ºC/30 s increments from 60 ºC to 94 ºC to validate the absence of non-specific products. The housekeeping genes *18S* rRNA, *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase), *PPIA* (cyclophilin A) and *HPRT* (hypoxanthine-guanine phosphoribosyltransferase) were tested for utilization as endogenous control. These genes were firstly selected based on their amplification and dissociation curves, and further evaluated using the geNorm software (Vandesompele et al., 2002). The endogenous controls considered more stable for utilization in CL (*GAPDH* and *PPIA*), GC (*GAPDH, 18S, PPIA* and *HPRT*) and oocyte (*GAPDH*, *18S* and *PPIA*) analysis. Data relating to gene assessment as a reference gene are available from the authors upon request.

The following angiogenic-related genes were analyzed in CL: vascular endothelial growth factor A (*VEGFA*), vascular endothelial growth factor receptor 2 (*VEGF2R*), fibroblast growth factor 2 (*FGF2*), angiopoietin 1 (*ANGPT1*), angiopoietin 2 (*ANGPT2*), TEK tyrosine kinase (*TIE2*), metalloproteases 2 (*MMP2*) and metalloproteases 9 (*MMP9*). Genes related with apoptosis were also investigated in CL and GC such as B/linfoma-2 cell (*BCL2*), BAX protein (*BAX*). Additional genes encoding intrafollicular growth factors were investigated in GC and/or denuded oocytes: epidermal growth factor (*EGF*), epidermal growth factor receptor (*EGFR*), insulin-like growth factor 1 (*IGF1*), insulin-like growth factor 2 (*IGF2*), insulin-like growth factor receptor 1 (*IGFR1*), insulin-like growth factor receptor 2 (*IGF2R*), insulin-like growth factor binding protein 2 (IGFBP2), insulin-like growth factor binding protein 4 (*IGFBP4*), bone morphogenetic protein 15 (*BMP15*), bone morphogenetic protein receptor 1 A (*BMPR1A*), bone morphogenetic protein receptor 1 B (*BMPR1B*), bone morphogenetic protein receptor 2 (*BMPR2*), transforming growth factor β receptor 1 (*TGFBR1*) and growth differentiation factor 9 (*GDF9*).

Statistical Analysis

After real time PCR run data obtained as CT values (threshold cycle) were normalized against the selected reference genes previously evaluated for each cell type. The gene expression values (arbitrary units) were estimated using the method of $2^{-\Delta Ct}$ (Livak and

Schimittgen, 2001) and analyzed using repeated measures ANOVA in a model including the fixed effects of Group, ovary side and the covariate Parity. Gene expression was measured in the right and left ovaries of each animal, and the side of measurement was modeled using the sow as the subject of repeated measures fitting a compound symmetry covariance structure. Outliers were removed from the data in order to achieve normality of the studentized residuals using Shapiro-Wilk's test at alpha=0.05. All statistical procedures were performed using the MIXED and UNIVARIATE procedures from SAS 9.2 (Statistical Analysis System Institute, Inc., Cary, NC, USA).

Reproductive data were analyzed in a model including the fixed effects of Group and the covariate Parity. Data were tested for normality of the studentized residuals using Shapiro-Wilk's test at alpha=0.05. All statistical analyses were performed using the MIXED and UNIVARIATE procedures from SAS 9.2. Pearson correlation coefficients were performed to determine relationships between birth weight litter average (BWTA) and total number of piglets born (TB); and BWTA and corpus luteum number (CL) in high and low groups.

Results and Discussion

In the present study, sows classified as high showed higher BWTA (1552.9±98.0 *vs* 1052.6±88.9, respectively; *P*=0.0003), but lower ovulation rate compared to low phenotype sows (21±3.1 *vs* 26±2.8, respectively; *P*=0.0656), while the TB did not differ statistically between the groups (13±1.0 *vs* 13.5±1.1, respectively; *P*=0.4509) as illustrated in Table 1. Despite the overall negative relationship between BWTA and CL (r=-0.59; P=0.0718), no relationships were significantly established between TB and BWTA for high and low group. The covariate parity does not show effect on BWTA, TB and CL variables. These data support the hypothesis of Foxcroft et al. (2004) that low-weight piglets occur as a result of high ovulation rate, linked with crowding of embryos in the uterus during early pregnancy resulting in inefficient or impaired placental development.

In order to identify functional differences in ovarian function between high and low BWTA sows, genes involved in angiogenesis, apoptosis and encoding intrafollicular growth factors were investigated at Day 5 of gestation. This time is physiologically similar to early luteal phase of the estrous cycle, where successive

populations of primordial (non-developing) follicles initiate growth and provide the pool of intermediate sized follicles (3-6 mm) that will eventually be recruited into the preovulatory population after luteolysis (Wiesak et al. 1992).

Recent findings have demonstrated differences in expression of genes related to the angiogenesis in preovulatory follicles of sows (Paradis et al. 2009), suggesting that differences may be present with respect to CL function and vascularity between subgroups of contemporary sows. Relative expression data for CL are illustrated in Figure 1. For the CL samples, *BAX* mRNA abundance was higher expressed in high compared to low group ($P \leq 0.05$); while *BCL2* mRNA abundance was more expressed in low than high group ($P \leq 0.05$). Both *BCL2* and *BAX* are components of bcl2 family, which act as regulators of programmed cell death or apoptosis (Rueda et al. 1997). The balance between these molecules is related to the cell potential to become apoptotic. It has been established that free *BAX* promotes apoptosis, while *BCL2* as homodimers act as repressors of cell death (Oltvai et al. 1993; Oltvai and Korsmeyer. 1994; Simonian et al. 1996). Thus, the proportion *BAX/BCL2* mRNA is increased in the high group compared to the low group; which according to the mechanism of apoptosis mediated by *BAX* described by Rueda et al. (1997) suggests that apoptosis in CL of the group of high BWTA may be occurring more prominently. In granulosa cells there was no significant difference for *BAX* mRNA abundance between groups; however, the anti-apoptotic transcript *BCL2* was higher expressed in high compared to low group, suggesting that apoptosis in granulosa cells may be less pronounced in the high group, following an opposite pattern observed in CL (Figure 2).

After ovulation the ruptured follicle is transformed into the corpus luteum, which is responsible for the production and secretion of progesterone (Senger et al. 2003). This process involves expression of angiogenesis-related genes, which may be finely regulated to allow rapid tissue growth and luteal regression enabling the return to estrus cycle and repeated chances of fertilization (Ribeiro et al. 2006). Several factors operate in this process to promote mitosis and differentiation of endothelial cells, such as vascular endothelial growth factors and proteases required for extracellular matrix remodelling and formation of new vascular tubes (Ferrara and Davis-Smyth, 1997; Pitzel et al. 2000).

In the present study there were no differences in mRNA transcript abundance for (*ANGPT1, ANGPT2*, *TIE2*, *EGF*, *EGFR* and *MMP2*) in CL between groups. It is well

accepted that a surge of angiogenesis occurs within the CL during its initial development, which is characterized by increased proliferation of endothelial cells and secretion of vascular endothelial growth factors (VEGF), and up-regulation of angiopoietins (ANGPT) (Christenson et al. 1996; Wulff et al. 2001). However, it is not clear whether this involves a second wave of angiogenesis in the CL of pregnancy (Fraser and Wulff, 2003). ANGPT1 is necessary to maintain and stabilize blood vessels, while the ANGPT2 acts as a natural antagonist for ANGPT1. These angiopoietins compete for the same receptor, Tie2, so the proportion of ANGPT2/ANGPT1 is important for vascular stability. A high ratio ANGPT2/ANGPT1 induces destabilization of blood vessels, which is a prerequisite for vascular formation (Schams and Berisha, 2004). Although the groups of commercial sows used in this study presented differences in birth weight litter average, both groups exhibited a similar ratio ANGPT2/ANGPT1 and its receptor *TIE2* in CL in early pregnancy, indicating no difference in vasculature instability rate, which is required for vasculature formation. Further studies should be done to verify the biological activity of angiopoietins, once the transcriptional profiling is only an initial indicator of overall genome activities. This may provide valuable insight of the role of those factors in capillaries proliferation needed for the supply of nutrients and precursors required for adequate growth of pre-ovulatory follicles (Berisha et al., 2000).

In the present study, VEGFA mRNA expression was also similar between high and low group. Since VEGF is the main angiogenic factor related in proliferation, migration and survival of endothelial cells, the pattern of VEGF mRNA abundance in early pregnancy may be associated with CL renewal in pigs (Ribeiro et al. 2006). The VEGF/VEGFR2 pathway has been associated with the functionality of luteal blood vessels during pregnancy and might also be involved in regulating vascular permeability and progesterone release into the bloodstream (Kashida et al. 2001).

During angiogenesis, additional molecules produced by micro vascular endothelial cells known as metalloproteases (*MMP2* and *MMP9*) are required to degrade the perivascular basement membrane and to allow endothelial cells migration through the extracellular space (Goldberg et al. 1996). In the present study *MMP9* mRNA tended to be higher expressed in high compared to low group ($P \leq 0.10$), corroborating previous studies that reported differential expression and enzyme activity of *MMP9* in CL at different stages of the luteal phase and at basal levels during early

pregnancy in the pig (Pitzel et al. 2000, Ribeiro et al. 2006). Evidence of *MMP9* involvement in extensive tissue remodelling during CL formation have been reported, suggesting that *MMP9* is the main metalloprotease required to create an appropriate environment for the subsequent rapid proliferation of luteal cells (Zhao et al 1996; Goldberg et al. 1996). Results from the present study agree with findings of Ribeiro et al. (2006), which reported major alterations in the MMP9 activity at beginning of the estrus cycle (days 1 to 5 after ovulation) are related with the switch of vascular quiescence to angiogenesis during early CL formation . Therefore, findings from the present study indicate that higher *MMP9* mRNA abundance verified in high BWTA group may indicate differences in angiogenesis initiation between groups.

In vitro studies have shown that FGFs influence several cellular functions, including cell proliferation, differentiation, matrix formation and cellular movement (Gospodarowicz, 1989). *FGF2* mRNA was expressed in high compared to low group (*P≤0.01*), corroborating previous findings of expression of acidic and basic FGF (aFGF, bFGF) in porcine uterus in both pregnant and cycling gilts (Katsahambas et al. 1996). Despite several studies in cattle linking *FGF2* to embryonic survival and fertilization through trophectoderm modulation by interferon-τ (IFNT) synthesis (Roberts 1991, Inskeep and Dailey 2005), little is known about *FGF2* role in determining early stages of porcine conceptus development and/or maternal interactions during pre-implantation period. Evidences that FGFs interact synergistically with transforming growth factors (TGFβ) (Larson et al. 1992) and may influence steroid production (Sardoillet et al. 1992) and extracellular matrix (ECM) deposition (Baird et al. 1990) have been reported. Result from the present study indicates that higher abundance of *FGF2* transcripts in high group may be related with intensive angiogenesis through up-regulation of VEGFR2 in endothelial cells, suggesting a more efficient capillary network formation required for the endocrine function of pregnant CL in high birth weight sows (Pepper et al., 1998).

It is generally accepted that IGF1 plays an important role in folliculogenesis and proliferation of granulosa cells (Mao et al, 2004). IGF1 gene is constitutively expressed and did not differ significantly between the stages of development, follicle size or day of the estrous cycle (Zhou et al., 1996; Liu et al., 2000; Paradis, 2009; Silva et al. 2011). However, IGF1 bioavailability can be finely regulated by the presence of follicular

tissue IGFPBs (Guthrie, 2005). Most of the cellular actions of IGF1 are mediated by the binding to the type I receptor that is expressed in the granulosa cells and interstitial theca cells (LeRoith et al. 1995). However, the GC response to the IGF1 stimulation may be variable according to the specie and stage of follicular development (Mazerbourg et al. 2003). In this study, all the components of IGF system (*IGF1*, *IGF2*, *IGF2R*, *IGF1R*, *IGFPB2* and *IGFBP4*) did not differ between the high and low groups.

There were no differences in expression between treatments for any of the analyzed oocyte secreted factors investigated. Relative expression for denuded oocytes is illustrated in Figure 3. Oocyte secreted factors, such as BMPs, GDF9 and their receptors (BMPR1A, BMPR1B and BMPR2) are involved in the regulation of mammalian folliculogenesis (Gilchrist et al, 2004, 2011; McNatty et al, 2004). The expression of transcripts *BMP6*, *BMP15* and *GDF9* were recently reported in oocytes and pre-ovulatory follicles in the pig, as well as their cellular receptors (Paradis et al. 2009; Sun et al, 2010). BMPs modulate a wide range of cellular functions such as proliferation and differentiation, steroidogenesis, metabolism and apoptosis (Shimasaki et al., 2004, Juengel and McNatty, 2005, Gilchrist et al, 2008), and can also affect the oocyte quality and subsequent embryo development (Hunter et al 2004; Paradis and Hunter, 2009). A lack of differences in gene expression between BWTA groups in the present study is consistent with previous findings by Paradis et al. (2009) that oocyte secreted receptors, though abundantly expressed, show little or none variation in mRNA abundance through preovulatory follicular development. Despite of recognized role of BMP6, BMP15 and GDF9 in mediating most of the effects that the oocyte has on porcine cumulus cell metabolism and oocyte developmental competence, the pattern expression observed in the present study does not preclude the possibility that post-translational modification or processing could regulate the biological activity of those regulating ovarian follicle growth and oocyte competence.

Conclusions

In summary, findings from the present study indicate differences in genes related with CL function and follicular development between subsets of commercial-line sows characterized as high and low birth weight litter average phenotype. In the present study, analysis of angiogenic-related genes in CL and GC showed that apoptosis

mediated by *BAX* and *BCL2* may be differentially regulated between groups of high and low BWTA, suggesting that apoptosis rate may be more pronounced in CL, but less prominent in GC in high compared to low group. Angiogenesis-related genes including *FGF2* and *MMP9* were highly expressed in high BWTA group, suggesting a more intensive activity of vascularization required for adequate blood supply and CL maintenance during gestation, which may be improved in high BWTA sows. Finally, genes related in cell proliferation, differentiation and steroidogenesis such as *IGF system* and oocyte secreted factors did not differ in GC and denuded oocytes between BWTA sows. Taken together, our results support the hypothesis of Foxcroft et al. (2004) that low average birth weight litters are a consequence of ongoing selection for high ovulation rates, linked to crowding of embryos *in utero* in early gestation and detrimental effects on placental development.

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Table 1. Reproductive data of high and low birth weight sow phenotypes

Parameter	High	Low	*P-value*
CL	21±3.1	26±2.8	0.0656
BWTA	1552.9±98.0	1049.5±88.9	0.0003
TB	13.0±1.0	13.5±1.1	0.4509

USASK/UofA/CPIG, 2010. Data show the mean ± s.e.m. CL: corpus luteum, TB: total born, BWTA: birth weight litter average.

Table 2. List of primer sequences and quantitative real-time PCR quality control data for pig candidate gene expression analysis

Gene Symbol	Accession number	Primer	Sequence 5' – 3'	Amplicom (pb)	Efficiency (%)
18S	NR_002170	F	TCCTTGGATGTGGTAGCCGTTTCT	108	1.93
		R	TCAACTTTCGATGGTAGTCGCCGT		
ANGPT1	NM_213959	F	CATGTGCAAATGTGCCCTCATGCT	104	1.98
		R	TTCCATGGTTCTGTCCCGCTGTAT		
ANGPT2	NM_213808	F	TCTTCAGAGACTGCGCAGAAGCAT	80	1.99
		R	AGTGGAGTTAGGAAAGGTCAGCGT		
BAX	XM_003127290	F	AACACAGCATGGAACAGAACAGGC	104	1.96-1.98
		R	ACCGCTTTCATATCCCATCACCCT		
BCL2	EF681866	F	TTGTGGCCTTCTTTGAGTTCGGTG	108	1.97-2.0
		R	GGTTCAGGTACTCAGTCATCCACA		
BMP15	NM_001005155	F	TTCATCCCTGGACACTGCCTTCTT	88	1.92
		R	TTCCAGGCCTCTGGGAAGAAGTTT		
BMPR1A	EU693016	F	GGCTTTGCTCGTTTCTATGGCTGT	103	1.95
		R	ACGACGTCTGCTTGAGATGCTCTT		
BMPR1B	AY065994	F	AAACGAGGTCGACATACCACCCAA	86	1.94
		R	TCCTGTTCAAGCTCTCATCCAGCA		
BMPR2	EU693017	F	ACAACAGGCGTGTGCCAAAGATTG	87	1.91
		R	TGCTGTCAGTGTGATGGATCGAGT		
EGF	NM_214020	F	TTGTATTGGTGCGATGCCAAGCAG	114	1.99
		R	AAACACAGCTACCGCAAATGGGTG		
EGFR	NM_214007	F	ATCTGTAACCCGCTGTGCTCATCA	91	1.97
		R	ATTCCTTGCCGCGGCTAAAGTTTC		
GAPDH	AF017079	F	GCAAAGTGGACATTGTCGCCATCA	124	1.99-2.05
		R	AGCTTCCCATTCTCAGCCTTGACT		
GDF9	AY649763	F	TGGTGCAGAACATCATCCACGAGA	100	1.93
		R	GGCTCAATGGCCAACACACTCAAA		
HPRT	DQ845175	F	GGACTTGAATCATGTTTGTG	91	1.91
		R	CAGATGTTTCCAAACTCAAC		
IGF1	NM_214256	F	ATCTGTAACCCGCTGTGCTCATCA	91	1.96
		R	ATTCCTTGCCGCGGCTAAAGTTTC		
IGF1R	NM_214172	F	TGTGTTCTTCTACGTCCAGGCCAA	87	1.97
		R	ACTATCAACAGAACGGCCACTGGT		
IGF2	NM_213883	F	GCCATCGGAAGTGAGCCAAATTGT	115	1.97
		R	AAGGGTACAGAGATCTCAGAAGGG		
IGF2R	XM_003121108	F	TCGTTTGCAATGGCGACCTTTACC	108	1.97
		R	CCAGCGCGGTTTCAAAGTCAAAGA		
IGFBP2	NM_214003	F	AAGATGTCTCTGAATGGGCAGCGT	127	1.99
		R	CCTGCTGCTCGTTGTAGAAGAGATGA		
IGFBP4	NM_00123129	F	CACCCATGAGGACCTCTACATCAT	139	1.99
		R	AAGCTTCACTCCCGTCTTCCGGT		
MMP2	NM_214192	F	AGGACAAGTGGTGCGTGTGAAGTA	92	1.98
		R	TGCAGCTGTTATACTCCTTGCCGT		
MMP9	DQ132879	F	ACGTGAAGACGCAGAAGGTGGATT	88	1.98
		R	AAGATGTCGTGTGTGTTCAAGGGC		
PPIA	AY266299	F	ATCTTGTCCATGGCAAATGCTGGC	94	1.97-1.99
		R	CCACATGTTTGCCATCCAACCACT		
TGFBR1	DQ519378	R	ATGTTGGTATGCCAATGGAGCAGC	86	1.97
		F	TGATGCCTTCCTGCTGACTGAGTT		
TIE2	XM_001926034	F	TCAGCTAAGCACTCCCTTGTTGCT	118	1.98
		R	TGGAGTTAGGAAAGGTCAGCGTGT		
VEGFA	NM_214084	F	TGTGCCCACTGAGGAGTTCAACAT	80	1.97
		R	TCATCTCTCCTATGTGCTGGCCTT		
VEGFR2	AJ245446	F	AGTGGAGGTGACAGATTGCAGTGA	93	1.96
		R	AAGCACTTGTAGGCTCCAGTGTCA		

Gene symbol: ribosomal 18S rRNA (*18S*), angiopoietin (*ANGPT1*), angiopoietin 2 (*ANGPT2*), BAX protein (*BAX*), B/linfoma-2 cell (*BCL2*), bone morphogenetic protein 15 (*BMP15*), bone morphogenetic protein receptor 1 A (*BMPR1A*), bone morphogenetic protein receptor 1 B

(*BMPR1B*), bone morphogenetic protein receptor 2 (*BMPR2*), epidermal growth factor (*EGF*), epidermal growth factor receptor (*EGFR*), glyceraldehyde-3-phosphate dehydrogenase *(GAPDH),* growth differentiation factor 9 (*GDF9*), hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), insulin-like growth factor 1 (*IGF1*), insulin-like growth factor receptor 1 (*IGFR1*), insulin-like growth factor 2 (*IGF2*), insulin-like growth factor receptor 2 (*IGF2R*), insulin-like growth factor binding protein 2 (IGFBP2), insulin-like growth factor binding protein 4 (*IGFBP4*), metalloproteinase 2 (*MMP2*), metalloproteinase 9 (*MMP9*), cyclophilin (*PPIA*), transforming growth factor β receptor 1 (*TGFBR1*), TEK tyrosine kinase (*TIE2*), vascular endothelial growth factor A (*VEGFA*), vascular endothelial growth factor receptor 2 (*VEGFR2*). Accession number at Genbank (htpp://www.ncbi.nlm.nih.gov).

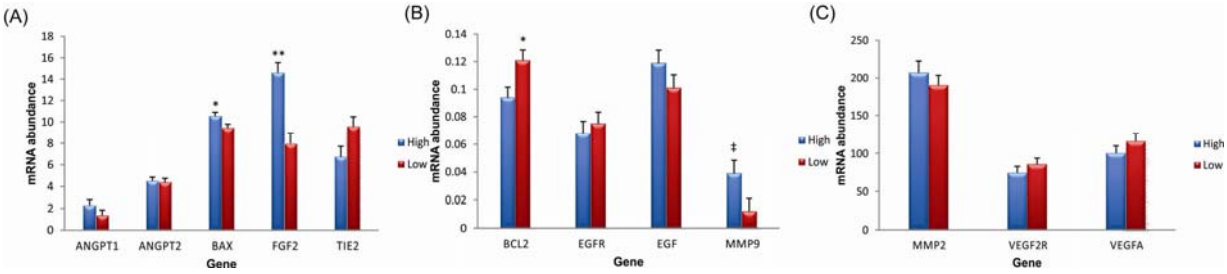

Figure 1. Comparison of mRNA abundance in corpus luteum from high and low birth weight litter phenotype sows (A) *ANGPT1*, *ANGPT2*, *BAX*, *FGF2*, *TIE2;* (B) *BCL2*, *EGFR*, *EGF*, *MMP9* and (C) *MMP2*, *VEGFR2*, *VEGFA*. Relative mRNA abundance is expressed as lsmeans of $2^{-\Delta Ct}$ (x 1000) ± S.E.M. Asterisks indicate significant differences (*P≤0.05; **P≤0.01) or exhibited a tendency for differential expression (‡P≤0.10).

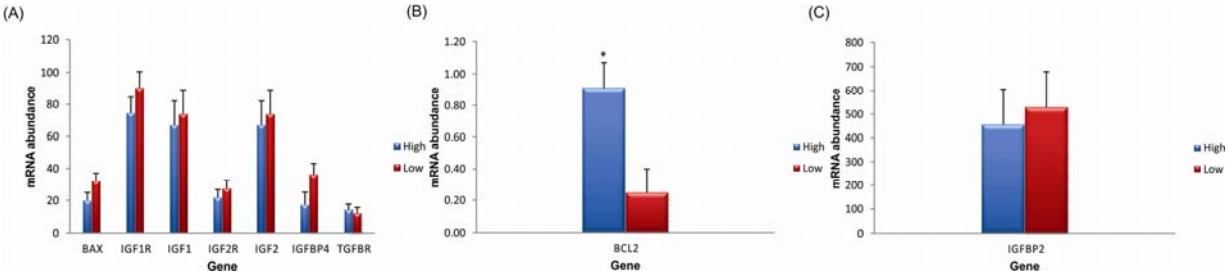

Figure 2. Comparison of mRNA abundance in granulosa cells from high and low birth weight litter phenotype sows (A) *BAX*, *IGF1R, IGF1, IGF2R, IGF2, IGFBP4, TGFBR;* (B) *BCL2* and (C) *IGFBP2*. Relative mRNA abundance is expressed as lsmeans of $2^{-\Delta Ct}$ (x 1000) ± S.E.M. Asterisks indicate significant differences (*P≤0.05; ** P≤0.01).

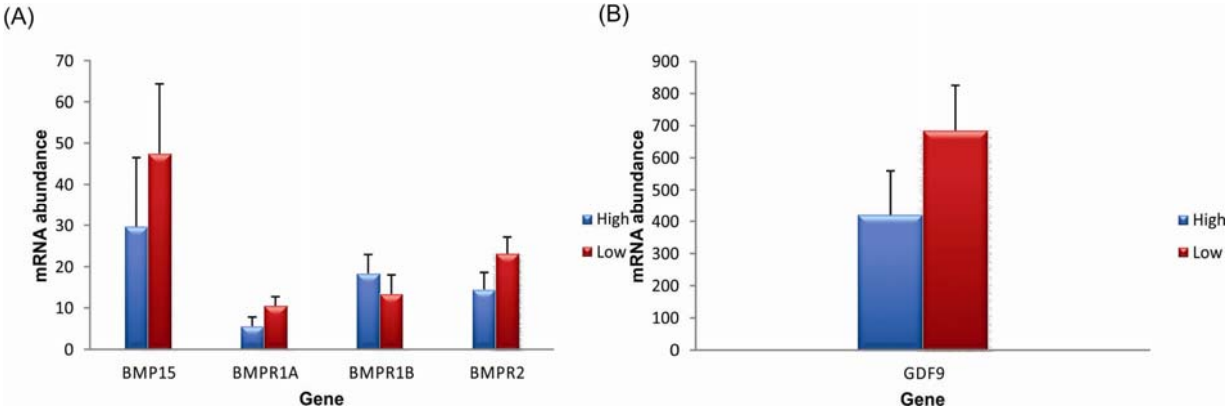

Figure 3. Comparison of mRNA abundance in denuded oocytes from high and low birth weight litter phenotype sows (A) *BMP15, BMPR1A, BMPR1B, BMPR2* (B) *GDF9*. Relative mRNA abundance is expressed as lsmeans of $2^{-\Delta Ct}$ (x 1000) ± S.E.M. Asterisks indicate significant differences (*P≤0.05; ** P≤0.01).

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CHAPTER 3

(Article 3)

Effects of sow metabolic status on subsequent embryonic gene expression

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Abstract

The current study investigated how the previous catabolic state of lactating primiparous sows affects early embryonic gene expression. All sows were feed restricted during the last week of lactation and breed at the "first heat" (FH) or at second post-weaning estrus (*skip*). Skip sows were further divided in subgroups according their previous catabolic state in lactation: highly (CH, n=4), moderately (CL, n=4) and non-catabolic (NC, n=4). Microarray data analysis in Day 9.5 embryos revealed different pattern of distribution of biological functions across treatments. Validation by QPCR showed a differential expression for *CYR61 and MYOF*, specifically for the female sex, in the pair-wise contrast FH*vs*NC and CL*vs*NC, respectively (P≤0.05). Additional contrasts in female sex were marginally significant (P≤0.10) for *MYOF*, *BCSL1*, *CYR61*, *RAD21* and *SOD1*, but no difference was found for *ETFA, ACDSB, TFPI2* and *TNFRSF21*. Furthermore, skip sows showed higher corpus luteum weight, total corpus luteum average and average litter growth, compared to first heat sows. Results suggest that the differential expression observed in female embryos may be an adaptive response to the intrauterine conditions, which may mediate epigenetic programming in the offspring. This study reinforces and provides additional insights into the role of nutrition and maternal metabolic state in determining the dynamics of early embryonic development and embryo quality in pigs.

Key words: catabolism, embryo quality, epigenetics, microarray analysis

Resumo

No presente estudo investigou-se o efeito do estado catabólico de porcas primíparas sobre a expressão gênica em embriões na fase inicial de desenvolvimento. Todas as porcas foram submetidas à restrição alimentar na última semana de lactação, e acasaladas no primeiro (*first heat*, FH) ou segundo estro após o desmame (*skip*). Porcas do grupo *skip* foram posteriormente divididas em subgrupos, de acordo com seu estado catabólico na lactação: altamente catabólicas (CH), moderadamente catabólicas (CL) e não catabólicas (NC). Análise de microarranjo, realizada a partir de embriões de 9.5 dias, revelou distintos padrões de distribuição de funções biológicas entre os tratamentos. Validação dos resultados por meio do QPCR demonstrou diferença de expressão para os genes *CYR61* e *MYOF*, especificamente para o sexo feminino, nos contrastes FH*vs*NC e CL*vs*NC, respectivamente ($P \leq 0.05$).Contrastes adicionais para o sexo feminino foram marginalmente significativos ($P \leq 0.10$) para os genes *MYOF*, *BCSL1*, *CYR61*, *RAD21* e *SOD1*, mas nenhuma diferença foi observada para os genes *ETFA, ACDSB, TFPI2* e *TNFRSF21.* Além disso, porcas *skip* apresentaram maior peso de corpo lúteo total, peso médio de corpo lúteo e taxa de crescimento de leitegada, comparado com as porcas do grupo controle. Resultados sugerem que a expressão gênica diferencial verificada no sexo feminino pode ser uma resposta adaptativa frente às condições intrauterinas, que podem desencadear programação epigenética nos descendentes. Este estudo reforça e auxilia no entendimento do papel da nutrição e do estado metabólico materno na dinâmica do desenvolvimento e na qualidade embrionária em suínos.

Palavras-chave: análise de microarranjo, catabolismo, qualidade embrionária, epigenética

Introduction

The possibility that maternal nutritional status during gestation could affect growth and tissue development of offspring and program their later phenotype is an emerging challenge in pig production (Sarr et al. 2010). It is known that nutritional status influences the reproductive axis via alteration of gonadotrophin secretion (Miller et al. 1998) and involves both central and local ovarian effects (Foxcroft et al. 1995). The nutritional restriction has negative effects on subsequent reproductive performance (Foxcroft, 1997). Furthermore, feed restriction in the sow during late lactation affect oocyte quality and reduce litter size and embryonic survival, which are likely related to alterations in follicular growth and maturation (Zak et al. 1997a, 1997b, Quesnel et al. 2009). Previous studies using a lactational feed restriction model in primiparous sows showed that increased catabolic state limits the growth of all surviving embryos and cause a selective decrease in female embryo survival (Vinsky et al. 2006), suggesting that metabolically induced changes in embryonic survival may be sex specific in the pig. A further refined model was used by Patterson et al (2011) to confirm a detrimental effect of restriction in late lactation on embryonic weight at Day 30 of the subsequent pregnancy, but reported the absence of any effect on embryonic survival. Based on these results, Oliver et al. (2011) reported changes in expression of embryonic and placental genes involved in early embryonic development.

Experiments linking maternal nutritional state to prenatal programming are of particular relevance to understand role of nutrition and metabolic state in determining oocyte/embryonic quality in the pig and the dynamic of early embryonic survival (Foxcroft et al. 2009). According Vinsky et al. (2006), gene imprinting may explain how feed restriction during lactation limits conceptus growth and fetal development, and causes the selective loss of female embryos. However, molecular mechanism mediating nutritional effects on embryonic survival in pigs are not fully understood.

The present study were based on a refined model used by Patterson et al. (2011), in respect to variation in sow weight and individual voluntary feed intake in early lactation. In order to investigate factors affecting the recovery of sows from the effect of feed restriction in late lactation, Day 9.5 embryos were recovered from subsets of sows that had either been breed at the first or at second post-weaning estrus. Therefore, the aims of the present study were: 1) to elucidate how the previous catabolic state of lactating primiparous sows affects embryonic gene

expression; 2) to further test the hypothesis that the effect of previous catabolic state on early embryonic development may be gender specific in the pig.

Materials and methods

Animals, feeding and treatments

A total of 169 Landrace x Large White sows (Genex Hybrid; Hypor, Regina, Canada) were used in the main experiment, using a pattern of feed restriction in the last week of lactation and estimates of relative catabolic state at weaning as described in details by Patterson *et al.* (2011). Sows were fed using a standardized step-up feeding *regimen* until Day 14 of lactation. In the last 7 days of lactation before weaning all sows were restricted, allowed to feed 60% of their predicted feed intake. Sows were pair matched according to their body weight and randomly assigned to first heat or skip treatment. After weaning, "first heat" sows (FH, n=3) were bred at the first and "skip-a-heat" sows (skip, n=12) at the second post-weaning estrus. All sows were artificially inseminated using fresh mixed Duroc boar semen (3.0×10^9 morphologically normal spermatozoa per 50 mL dose). Based on previous analysis by Patterson *et al.* (2011), the skip sows were divided in subgroups according to their previous catabolic state in highly (CH, n=4), moderately (CL, n=4) and non-catabolic (NC, n=4).

Embryo collection

Pregnant sows were euthanized on Day 9.5, with the day of last insemination being defined as Day 1 of gestation, at the Swine Research and Technology Centre. Reproductive tracts were recovered from sows immediately after being killed. Sow measurements during lactation and litter characteristics at Day 9.5 were recorded for all animals and can be visualized at Table 1. Details and additional information about sow and litter performance are described by Patterson et al. (2011). Embryos were recovered from each uterine horn with a single flush of 30 mL of warm mDPBS/BSA injected into the uterine lumen by using a sterile syringe with a blunt needle inserted through the uterine wall at the base of each uterine horn. The flushing medium was gently massaged toward the utero-tubal junction; where flushing was collected into a sterile tube 50 mL (Corning, NY, USA) through a funnel-tipped glass tube inserted through a small incision in the uterine wall. The flushing were transferred to a Petri dish and kept warm during the collection procedure. Embryos were visualized using magnifying lens, individually separated and

washed three times in 1 X PBS before transferred to a tube with minimum amount of buffer. Embryos were then snap-frozen in liquid nitrogen and stored at -80°C.

DNA extraction and sex-typing PCR

The DNA was extracted from 25 mg of tissue using Purelink 96 (Invitrogen, Carlsbad, CA, USA), according to manufacturer's protocol. The embryos were then sex-typed by PCR using an adapted protocol, which amplifies part of the SRY region. Details of primers sequence and sex-typing PCR are fully described by Oliver *et al*. (2011).

RNA extraction, mRNA amplification and labeling

Total RNA was extracted from each embryo using TRIzol (Invitrogen) adapted protocol and further treated with DNase I (Qiagen, Valencia, CA) treatment to remove genomic DNA, as described by Oliver *et al*. (2011).The RNA integrity was verified using Agilent 2100 Bioanalyser Pico Kit (Agilent Technologies, Mississauga, ON, Canada). The amplified anti-sense RNA (aRNA) was produced using the RiboAmp HS RNA amplification kit (Applied Biosystems, Foster City, CA, USA) in two successive rounds of in vitro T7 RNA transcription. Following the amplification reaction, the aRNA output was quantified using the Nanodrop (NanoDrop 2000, Thermo Scientific, Walton, MA, USA). Two micrograms of aRNA from each sample were used for labeling. Probes were labeled using the ULS Fluorescent Labeling Kit for Agilent arrays with Cy3 or Cy5 dyes (Kreatech Diagnostics, Amsterdam, Netherlands) according to the manufacturer's instructions. The labeled product was then purified using the Picopure RNA extraction kit (Applied Biosystems) to remove uncoupled dyes. Concentration and labeling efficiencies were measured using the Nanodrop (NanoDrop 2000, Thermo Scientific).

Microarray hybridization and image processing

The microarray slides used in the present experiment were developed by the EmbryoGENE Porcine Version 1 (EMPV1) microarray platform and constructed by Agilent Technologies. It features a total of 43,795 probes specific for embryo related biological process (Tsoi et al. 2012). Samples were pooled within sow (sows, n=15; pools, n=120) and balanced for sex for each subgroup, except the FH group (2 pools of female and 1 pool of male) and hybridized on microarray using a reference design for a total of fifteen arrays. The reference sample consisted

of equivalent amounts of each experimental unit. The hybridization was done according to Agilent standard protocols. Briefly, the amount of 825 ng of each labeled sample were incubated in a solution containing 2X Blocking Agent and 1X Fragmentation Buffer in a volume of 55 ml at 65°C for 15 min, and were then put on ice. Further 55 µL of 2X GEx Hybridization Buffer was added for a final volume of 110 µl. The hybridization mix was added onto the array and hybridization was performed at 65°C for 17 h using a rotating oven. After the hybridization step, slides were washed for 3 min at room temperature with Gene Expression Wash Buffer 1 (0.005% Triton X-102 added) followed by Gene Expression Wash Buffer 2 (0.005% Triton X-102 added) for 3 min at 42°C. A final wash was done with Acetonitrile and Drying and Stabilization Solution for 30 sec at room temperature before air-drying of the slides. Slides were scanned using the GenePix scanner (Molecular Devices) and features were extracted using ArrayPro 6.4 (Media Cybernetics, Bethesda, MD).

Microarray data analysis

Microarray data analysis was performed using the Flex Array 1.2 (Genome Quebec, genomequebec.mcgill.ca/FlexArray). Data were normalized by a simple background subtraction, and a locally weighted regression (loess) within-array normalization (Yang et al. 2002), prior to statistical analysis. The Cy3 and Cy5 log normalized intensities were derived from the loess-normalized log Cy5/Cy3 intensities and analyzed using linear models from the LIMMA R package in bioconductor (Smyth, 2005). Genes were considered differentially expressed at a fold-change >2.0 and a P-value <0.05; P-values were adjusted using the Benjamini and Hochberg false discovery rate (FDR) method by Storey and Tibshirani (2003).

Ingenuity functional and network analysis

Data were analyzed the Ingenuity Pathways Analysis software (Ingenuity® Systems, www.ingenuity.com). The functional analysis identified the biological functions that were most significant to the gene variants in the data set. Potential canonical pathways were further identified from the IPA knowledge base of canonical pathways that were most significant based on the input data set. Finally, IPA software was used to identify the network gene interactions that are particularly enriched for the input genes, which are called focus genes by the IPA software. The network construction is based on the selective interconnectivity with the focus

genes with each other and additional molecules stored in the IPA knowledge base. The statistical association between each biological function, the canonical pathways and gene networks assigned to that data set was determined by Fisher's exact test, which calculates the p-value or probability that each biological function and canonical pathways to that network is due to chance alone.

cDNA synthesis

Total RNA were reverse transcribed using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to manufacturer's instructions with random primers and RNAseOUT enzyme included. The cDNA synthesis were performed with 1 µg of total RNA from individual embryos and further diluted to 20ng/µl with nuclease-free water (Ambion).

Quantitative real-time PCR

Quantitative real-time PCR was performed using KAPA SYBR® FAST qPCR Master Mix (Kapabiosytems, Boston, MA, USA) for validation of microarray results. Nine differentially expressed genes were selected for validation: *mitochondrial chaperone (BCS1L), Acil-Coenzima A desidrogenase (ACDSB), double-strand-break repair protein rad21 homolog (RAD21), cysteine-rich, angiogenic inducer, 61 (CYR61), Myoferlin (MYOF), tissue factor pathway inhibitor 2 (TFPI2), tumor necrosis factor receptor superfamily, member 21 (TNFRSF21), electron-transfer-flavoprotein, alpha polypeptide (ETFA) and superoxide dismutase 1, soluble (SOD1).* The primer oligonucleotides used for the reactions were designed using "PrimerQuest" software (Integrated DNA Technologies, Inc., Coralville, IA) from swine sequences available in GenBank (htpp://www.ncbi.nlm.nih.gov). In the present study, *cyclophilin (PPIA)* was considered a suitable reference gene (data not shown).The list of primer sequences and expected PCR product lengths are shown in Table 2. Reactions were performed in duplicate in 96-well

optical reaction plates sealed with optical adhesive film using 10 µl of 2X KAPA SyBr qPCR Master Mix in a total volume of 20 µl. Prior to quantification by quantitative real-time PCR, the amplification efficiency and optimal primer concentration was determined for each gene using serial dilution of cDNA from each cell type. The PCR efficiencies for all primers pairs were obtained using the formula $E = 10^{-1/slope}$, where *E* is efficiency and *slope* is the gradient of the dilution series in the linear phase. The samples were amplified separately using the ABI 7900HT thermocycler (Applied Biosystems, Foster City, CA) by the amplification program: 45 cycles of 30 s of melting at 95ºC followed by 30 s of annealing and extension at 60 ºC. After the 45 amplification cycles, all samples were subjected to a melt curve analysis in which they were heated at 1 ºC/30 s increments from 60 ºC to 94 ºC to validate the absence of non-specific products.

Quantitative real-time PCR statistics

In the current experiment, subgroups of sows from different catabolic status represented the four levels of the first factor (FH, CH, CL and NC) and the embryonic sex the two levels (male and female) of the second factor. Thirty and two samples representing both factors were analyzed for nine target genes and for the *PPIA* reference gene. Statistical analysis were performed based on a linear mixed model analyses using the %QPCR_mixed macro developed in SAS (v.9.1.3) applied by Steibel at al. (2009). This method allows accommodation of more complex experimental designs and testing general hypotheses assuming independent random effect for the control and target genes in each biological replicate. The threshold to cycle (CT) data of each sample in each gene of interest was used in a completely randomized design.

$$\mathrm{Y}_{\mathrm{gikr}} = TG_{\mathrm{g}i} + B_{gik} + D_{ik} + \varepsilon_{ikr}$$

Where Y_{gikr} is the CT obtained from the thermocycler for the *gth* gene (target or *PPIA*) from the *rth* well corresponding to the *kth* animal subjected to the *ith* treatment; TG_{gi} is the effect of treatment *i* in the expression of gene *g*, $B_{gik} \sim N(0, \sigma_{Bg}{}^2)$ is the gene-specific random effect of the *kth* animal, $D_{ik} \sim N(0, \sigma_D{}^2)$ is the random sample-specific effect (common to both genes) and

$e_{gikr} \sim N(0, \sigma_e^2)$ is the residual term. The treatments (*i*) consisted of the combination of two factors: sow subgroup and sex effect.

Results

Sow and litter characteristics

Sow and litter characteristics from restricted sows at first (FH) and second post-weaning estrus (Skip) are summarized in Table 1. Sow body weight did not differ among treatment groups either at Day 1 or between Day 14 to 21 of lactation. All sows showed the same weaning to estrus interval (WEI) and number of embryo recovered (P>0.05). There were differences between treatments in total corpus luteum (CL) weight and total CL average (P≤0.01). Regarding energy mobilization, the subpopulation of first heat sows showed lower average litter rate (P≤0.05) compared to skip sows, despite mobilizing similar amount of body tissue (P>0.05) to support litter weight gain. Although ovulation rate and follicle size were not different, there was a difference in embryo recovery rate between treatments (P≤0.05).

Microarray data analysis

The microarray analysis identified 72 genes differentially expressed in a CH*vs*FH pairwise comparison, 340 genes in a CH*vs*CL comparison and 151 genes in a CH*vs*NC comparison. There were 27 genes present in all of the pairwise comparisons, named as commonly expressed (CE) genes. The number of differentially expressed genes up and down-regulated between pair-wise comparisons generated by Flex Array software were annotated by the Ingenuity Pathway Analysis (IPA) software as mapped or novel genes (Table 3). The IPA analysis was performed to indicate functional processes, putative networks and well-defined canonical pathways based on the uploaded list of genes compared to the Ingenuity knowledge base. IPA analysis resulted in the generation of a total of 42 gene networks, 303 molecular and cellular functions and 474 canonical pathways significantly associated with the data set (data not shown).

Based on the IPA curated ontology we determined which molecular and cellular functions were most significantly associated with the gene set in each comparison. The top 10 molecular and cellular functions are illustrated in Table 4. These findings, addressed early embryonic genes

to multiple biological functions including macromolecule metabolism (i.e. lipid, nucleic acid, and carbohydrate), cell morphology (such as shape change and spreading of cells, structural integrity of plasma and mitochondrial membrane and cytosol viscosity), cellular assembly and organization (i.e. biogenesis and structure of plasma membrane, remodelling of actin cytoskeleton and biogenesis of mitochondria) and small molecule biochemistry (such as cleavage and metabolism of acyl-coenzyme A, redistribution of phosphatidic acid and fatty acid metabolism). Interestingly, biological functions showed a different pattern of distribution based on their scoring between pair-wise comparisons. For example, the CH*vs*CL showed as top-scoring functions: cell death, cellular function and maintenance (cell morphogenesis, binding and organization), DNA process (replication, recombination, and repair), and nervous system development and function (i.e. migration, development, myelination, proliferation and neurological process). The CH*vs*NC comparison showed as most significant functions: tissue morphology (i.e. proliferation and transformation of tumor cells), post-translational modification (i.e. acetylation and heterodimerization of protein, activation of protein kinase) and cellular movement (i.e. rearrangement and cell migration). Finally, CE genes showed distinct top functions such as metabolic and neurological diseases (i.e. metabolic disorder, neurodegeneration) and developmental disorder (i.e. inborn error of metabolism).

The canonical pathway analysis defined a different number of pathways for each group with $p \leq 0.01$; two of the top 5 pathways are common for all comparisons and are related with carbohydrate and aminoacid metabolism. The top 5 canonical pathways are listed and ranked for significance of genes enrichment, as illustrated in Table 5.

The IPA analysis also defined a set of 20 genes for each group with $p \leq 0.05$; that were fold change up or down-regulated. The genes *MYOF*, *ETFA* and *SOD1* were found in all comparisons, with expression values above 2. Table 6 shows the top molecules fold change up or down-regulated in each pair-wise comparison.

Several networks were constructed using IPA network algorithm, in which molecular interactions and regulatory processes are represented. Figure 2 shows the top network for each comparison, that were generated based on their composite score, which represents the negative log of the p-value for the likelihood these molecules would be found together by random. A higher score indicates greater statistical significance that molecules depicted in the network are interconnected. Legends used in the network diagrams are illustrated in Figure 3.

The most significant top network was identified by IPA analysis for the CH*vs*NC comparison, with 26 of the 35 focus genes; only one of them down-regulated. The related processes for this comparison were free radical scavenging, cellular development, cellular growth and proliferation. Within the up-regulated genes, are included several enzymes such as: cytochrome c, somatic (*CYCS*), superoxide dismutase 1, soluble (*SOD1*), methylmalonyl CoA mutase (*MUT*). Also; growth factor (neuregulin 1 - *NRG1*); transcriptional factors (SERTA domain containing protein 2 - *SERTAD2,* AF4/FMR2 family, member 4 - *AFF4,* nuclear factor (erythroid-derived 2)-like 2 - *NFE2L2*) and cytokine (nicotinamide phosphoribosyltransferase – *NAMPT*) has also been identified in this network.

The second top scoring network was identified in the CH*vs*FH comparison, which includes cell morphology, cardiovascular system development and function, organismal development. This network comprised 19 of the 35 focus genes differentially expressed between treatments including histones (*HIST1H2AB/HIST1H2AE* and *HIST1H2AG*), enzymes (*GNAS, SOD1,* ephrin-A5 - *EFNA5*) among other (Figure 2). Also, this network contains molecules interactions with important complexes such as *NFkB* (nuclear factor kappa-light-chain-enhancer of activated B cells) that controls DNA transcription; and *PI3K* (Phosphatidylinositol 3-kinases) complex that modulates apoptosis, survival and proliferation; however they were not statistically significant between groups. The *laminin complex* was up-regulated in the CH group and is related with cell-cell adhesion and nervous system development.

The CH*vs*CL comparison showed 25 of the 35 focus genes found in the IPA knowledgebase; 21 were up-regulated in the CH treatment and were 7 down-regulated. These factors were associated with the following processes cellular assembly and organization, cellular development, and reproductive system development and function. The RNA polymerase II complex (*RNA POL II*) was down-regulated in CH treatment and show direct interaction with polymerase (RNA) II (*POLR2J*), binding to *pinin* (*PNN*) and to the splicing regulator (RPAP3), as illustrated in Figure 2. Within this network, the transcriptional factors AF4/FMR2 family, member 4 (*AFF4*) and Nipped-B homolog (*NIPBL*) were up-regulated, and WD repeat domain 77 (WDR77) was down-regulated. Also, genes involved in protein folding were identified, such as heat-responsive protein 12 (*HRSP12*) and the *chaperonin heat shock 60kDa* (*HSPD1*) both up-regulated in CH group.

Finally, the top network for the CE genes comprised 15 of 33 genes and was basically

related to cell death, lipid metabolism and small molecule biochemistry. Among the differentially expressed gene two complexes *GSN-PI3K-PIP2-Src* and *NFkB-TBP* were identified. Other molecule type as enzymes such as argininosuccinate synthase 1 (*ASS1*), superoxide dismutase 1(*SOD1*) and tyrosine phosphatase (*PTPN13*), and a transcriptional regulator TATA box binding protein (*TBP*) were included in this network.

Quantitative real-time PCR

In order to validate the results of microarray, 9 known genes have been selected for confirmation of their differential expression using real-time PCR based on their relevant function in cellular and metabolic process such as macromolecule metabolism and energy production *(ACADSB, ETFA, BCSL1),* signal transduction and cell cycle pathways *(CYR61, TNFRSF21, SOD1)*, as well as in the cellular functions and maintenance *(RAD21, MYOF, TFPI2)*. Some of those genes (*CYR61, SOD1* and *TFPI2*) were included in three of four top scoring networks illustrated in Figure 2.

The liner mixed model analysis used Ct from both biological and technical replicates as input data and generated a gene-specific ANOVA table for each target gene (data not shown). For all analyzed genes there was no evidence of interaction between catabolic state of the sow and gene expression, and no effect of sex in the normalized expression of each gene to the expression of *PPIA* (control gene). Interaction between sow catabolic state and sex on gene expression was also not significant, however, interaction of gene, sow catabolic state and sex were evident for all target genes ($P \leq 0.01$). The %QPCR_mixed macro output provided log-fold change estimates for pair-wise contrast of interest, which includes the estimated differences in Ct between the target gene and control. The log-fold change of each target gene for the interaction: gene, sow catabolism and embryo sex were further converted to fold change, as illustrated in Figure 4. A significant difference was observed only for *CYR61 and MYOF*, specifically for the female sex, in the pair-wise contrast FH*vs*NC and CL*vs*NC, respectively ($P \leq 0.05$). Additional contrasts in female sex were marginally significant ($P \leq 0.10$) for *MYOF*, *BCSL1*, *CYR61*, *RAD21* and *SOD1*, but no difference was significant for *ETFA, ACDSB, TFPI2* and *TNFRSF21*. Interestingly, CL*vs*NC contrast showed higher number of genes differently expressed (*MYOF*, $P \leq 0.05$; *BCSL1* and *RAD21*, $P \leq 0.10$).

The comparison of fold changes between microarray and QPCR is illustrated in Table 7. Most of the results agreed in terms of values and direction. Although the QPCR fold changes found for the effect of sow catabolism of gene expression did not reach the significance level ($P \leq 0.05$), there was a tendency for differential expression ($P \leq 0.10$) for *BCSL1* in the CH*vs*CL contrast.

Discussion

The current study investigated how the previous catabolic state of lactating primiparous sows affects early embryonic gene expression. Sow and litter characteristics were measured in a subpopulation of skip and first heat sows, including their previous catabolic state in lactation. Overall, skip sows showed higher CL weight and total CL average compared to first heat sows. These findings corroborates the results obtained in the omega-3 fatty acids (O3FA) supplementation experiment, which reported differences in CL weight between control and O3FA-treated sows (Smit, 2007). In the context of the results of Smit (2007), Paradis et al. (2009) reported differences in angiogenesis-related genes in pre-ovulatory follicles produced from skip-a-heat models, suggesting that differences may be present with respect to CL function and vascularity between control and O3FA-treated sows, even prior to ovulation. Alteration in CL function may involve hormones and intermediary metabolites modification, which in turn alters follicle mileau and the oviductal uterine tract, where oocyte and embryo will develop (Ashworth et al. 2009). Thus, functional differences in CL may be seen as an additional component controlling pre-natal development and survival (Foxcroft et al. 2000; 2006).

No difference in (WEI) was observed between “first heat” and skip group in the present study, although increased WEI have been reported in feed restricted compared to fed to appetite sows in earlier studies (Zak et al. 1997a). The result of the present study confirm the observation of Patterson et al. (2011) that selective pressure to improve reproductive performance have changed the biology of commercial sows over time, as a consequence; sows are becoming more resistant to the negative effects of lactational catabolism. In this sense, sow response in terms of nutrient partitioning is likely to be affected, though the mechanism by which occurs it is not fully understood (Oliver et al. 2011).

Some divergent results have been reported using the lactational feed restriction model in primiparous sows, in relation to embryonic development of the next litter (Zak et al. 1997a;

Vinsky et al. 2006, Patterson et al. 2011). According to Foxcroft et al. (2009), the most consistent response to a previous catabolic state is a reduction on embryonic weight, independent of embryo sex. In the current experiment, previous sow catabolism led to a reduction of average litter growth at Day 9.5 of gestation in first heat sows compared to skip sows (Table 2). This is in agreement with the notion that skip sows had a greater period of recovery from anabolic state, which in theory, would contribute to their improved fertility and embryo quality compared with sows bred at first estrus (Clowes et al. 1994).

A global transcriptome profiling of Day 9.5 embryos recovered from sows with different catabolic state was performed using the EMPV1 enriched platform (Tsoi et al. 2012) by Agilent technology. Based on the IPA curated ontology the major classical molecular and cellular functions significantly associated with the CE gene list for all pair-wise comparisons included: cell morphology, protein synthesis and cellular assembly and organization, which overlap with function identified in pig embryos during elongation process, day 11 to 12 (Blomberg et al. 2008). Additional functions as RNA post-transcriptional modification, molecular transport were also reported in ruminants (Hue et al. 2007).

The high incidence of embryonic losses in early gestation is related with the initial elongation process of the blastocyst, which indicates an important phase of developmental period (Bennett & Leymaster, 1989). In pigs, transition from spherical to tubular and filamentous form occurs around day 10 to 12, and precedes the initial attachment of the blastocyst to the endometrium (Geisert et al. 1982, Foxcroft et al. 2000). Based on recent studies, Hue et al (2007) reported that specific genes categories may be temporally expressed during the elongation process. Our findings identified pathways related to general metabolism (lipid metabolism, nucleic acid metabolism, carbohydrate metabolism); cell multiplication (DNA replication) and cell shape remodelling (tissue development and cell morphology). These findings are in agreement with initial elongation process, which is marked by intense cellular growth and multiplication, cell shape remodelling and hyperplasia (Geisert et al. 1982, Pusateri et al. 1990, Bazer et al. 1993, Blomberg et al. 2006). Pathways related with end of elongation process were also identified in the present study, such as cell-to-cell signalling and interaction, small molecule biochemistry and cellular assembly and organization (Table 4) (Blomberg et al. 2006, Hue et al. 2007). Protein synthesis pathway observed in this study is also in agreement with the onset on estrogen verified along elongation process (Godkin et al. 1982, Gadsby et al. 1990).

Interestingly, several transcripts related to the nervous system development and

neuregulin signalling pathway were identified in CH*vs*CL comparison (Tables 4 and 5), which coincides with embryonic disc formation that initiates around day 9 of gestation in pigs (Oestrup et al. 2009). Pathways containing genes related with actin cytoskeleton signalling were also identified for all pair-wise comparisons, considered critical processes in the nutritional programming of embryonic development (Swali et al. 2011).

The current study revealed different pattern of distribution of biologically related genes/gene pathways across treatments, with a more discrepant pattern of top scoring functions for the CH*vs*CL pair-wise comparison (Table 4). This data indicates variability not only among sows in terms of catabolic state, as previously reported at Day 30 (Patterson et al. 2011), but also in relation to embryonic gene expression among litters. The heterogeneity of embryonic gene expression in response to the nutritional challenge is likely caused by inherent sensitivity of litter to epigenetic mechanisms that impair embryonic development in early gestation of all surviving embryos, as suggested by Vinsky et al. (2006). The litter sensitivity to feed restricted sows also seems to be related to the developmental stage by the time feed restriction occurred (Foxcroft et al. 2009). In the present study, all sows were feed restricted during 3rd week of lactation, which is coincident with the final stage of oocyte maturation and the establishment of oocyte imprinting (Lucifero et al. 2002). Therefore, imprinting of oocytes may be one mechanism by which catabolism in the sow affects embryonic survival and development (Foxcroft et al. 2009).

Following microarray analysis, nine genes were selected for QPCR validation; four of them encode products directly involved in energy production and free scavenging radicals, which are products of mitochondrial fatty acid β- oxidation (FAO). Mitochondria are energy supplying organelles, whose functional integrity is essential for cellular survival and development. In addition, mitochondria can provide adenosine triphosphate (ATP) for fertilization and pre-implantation embryo development (Torner et al., 2004). The Short chain acyl-CoA dehydrogenase (SCAD, encoded by *ACADSB*) catalyzes the first step of (FAO), while *ETFA* act as an electron acceptor for this reaction (Matsuhara et al. 1989, Indo et al. 1991). In this study, results provided by microarray analysis showed that *ACADSB* and *ETFA* mRNA were higher expressed in CH than CL or NC group as illustrated in Figure 4; however, these results were not validated by QPCR. It is important to say that QPCR validation was performed using individual embryos in order to estimate the sex effect, while microarray analysis was done using pool of embryos balanced by sex, thus counting only for sow catabolic status. Therefore, the inclusion of distinct effects may be driving differences in fold-changes between these two methodologies in

terms of sensitivity and statistical power. The expression of *ACADSB* and *ETFA* mRNA has been previously reported in fetal and embryonic tissue in humans and rat (Nagao et al. 1993, Oey et al. 2006). Since directly involved in energy production, these genes may be important to meet embryo energy required during compaction and cavitation process (Sturmey et al. 2009, Hamatani et al. 2006).

Evidence that intrauterine nutrient supply affects development and postnatal traits in pigs has been reported by several studies showing that growth-retarded individuals at birth display impaired oxidative phosphorylation and mitochondrial dysfunction (Selak et al. 2003; Simmons, 2006; Wang et al. 2008). It has been also postulated that mitochondrial changes generated from excessive reactive oxygen species is closely associated with the oxidative energy production followed by the release of cytochrome-c and the activation of caspase cascade (Thouas et al., 2004; Kinnally and Antonsson, 2007). Further two genes related with this process were selected in the present study. The first is the product of mitochondrial chaperone (*BCSL1*) gene, involved in the complex III of the mitochondrial respiratory system, which is tightly coupled to ATP generation and reactive oxygen species production (Wallace et al. 2005). In the present study, the fact that *BCSL1* mRNA tended to be 3.9 fold higher expressed in CH sows compared to CL (P=0.0719), and 4.3 fold higher expressed in NC sows compared to CL sows, specifically in female embryos (P=0.0586) (Figure 4), is consistent with recent findings using feed restriction model, where several enzymes involved in pathways related to glycolysis, lipid metabolism and transport were up-regulated in piglets prenatally exposed to low protein diet (Sarr et al. 2010). This led us to postulate a possible link between the *BCSL1* mRNA abundance in embryos recovered from less catabolic sows, and the reduced litter weight gain in those pigs later in life. Furthermore, the detrimental effect of intrauterine nutrient restriction on postnatal lean growth rate and carcass quality have been specially noted in individuals of female sex (Zambrano et al., 2006, Gondret et al., 2006; Rehfeldt et al., 2008) corroborating the results from the present study, which showed significant contrasts in female but not in male embryos. Therefore, the finding that at least one mitochondrial fatty acid oxidation enzyme was affected by previous sow catabolic state suggests the existence of an active fatty acid oxidation in Day 9.5 embryos, as reported in the human fetus (Oey et al., 2005) and in the pig (Sarr et al., 2010). These results are also consistent with evidence that late stage oocyte and pre-implantation embryos can use endogenous lipid as substrate for energy production (Oey et al. 2005, Oey et al., 2006, Sturmey et al. 2009).

The second gene related with free scavenging radicals is the *SOD1*, which binds copper

and zinc ions and is responsible for destroying free superoxide radicals (Fridovich, 1975). The production of reactive oxygen species is the main reason for oxidative damages of DNA (Wang et al. 2009). Several mitochondrial dysfunctions such as the structural, spatial and genetic abnormalities in the oocyte, may influence normal embryo development and the viability of post-implantation embryos or result in abnormal foetuses (Wang et al. 2009). In the current study, the 8.04 fold higher expression of *SOD1* mRNA in embryos recovered to CL compared to NC sows, in the female sex (P=0.078) illustrated in Figure 4, corroborates (Thompson and Al-Hasan, 2012) suggestion, that intrauterine conditions may be one of the key down-stream mediators that initiates epigenetic programming of the offspring. In addition, reactive oxygen substances may act as signalling molecules that induce transcription of several genes related to oxygen sensing, cell differentiation, and proliferation (Schafer and Buettner, 2001; Burton, 2009). Furthermore, oxidative stress can be generated by other conditions than maternal nutritional status (Thompson and Al-Hasan, 2012), and may be further complicated by post-transcriptional regulation mediated by microRNAs (Milani et al. 2011). Therefore, understanding the cell-specific responses to intrauterine stress in early development may be an important component of developing strategies to mitigate detrimental long-term consequences during adulthood.

In pigs, muscle fibre formation occurs during prenatal development (Buckingham et al. 2003), and involves myoblasts proliferation and fusion (Wigmore and Evans, 2002). Maternal nutrition during gestation has been found to affect muscle fibre number in pigs (Dwyer and Stickland 1992; Dwyer et al. 1994) and ewes (Costello et al. 2008). *MYOF* is a required component of normal muscle development, highly expressed during muscle development and regeneration (Davis et al., 2000; Doherty et al., 2005). A lack of differences in *MYOF* expression between treatments in the present study is consistent with recent results of Oliver et al (2011), which reported no effect of feed restriction in myogenesis-related genes at Day 30 of gestation. This observation is further supported by the fact that primary muscle fibre development is resistant to nutritional manipulation (Dwyer et al. 1994). However, in the present study, *MYOF* was 4.8 fold higher expressed in NC compared to CL ($P \leq 0.05$), and showed a tendency for 4.5 higher expression in FH than CL sows (P=0.056), specifically in female sex (Figure 5). Recent findings demonstrate that myoferlin is a critical mediator of postnatal muscle growth mediated by IGF1 (Demonbreun et al. 2010). Thus, is likely that reduced concentrations of IGF1 in feed restricted sows impair ovarian responsiveness to the gonadotrophic stimulation at weaning and alter subsequent follicular development and ovulation rate. In turn, follicular and oocyte quality

influences embryonic development and survival (Vallet et al. 2009). Therefore, these results may indicate a different expression in male and female embryos as an adaptive response to the nutritional status of the sow through IGF1 signalling pathways. This study has practical implications for meat production in pigs, once the number of muscle fibres in postnatal life relies on the hypertrophy of the existing fibres (Rehfeldt and Kuhn 2006). In this context, a focus on the expression of embryonic genes involved in early myogenesis may be an important component of genetic breeding programs.

Results from the present study also revealed molecules involved in cell shape remodelling, cell assembly and organization such the metalloproteases (*CYR61*) and their inhibitors (*TFPI2*). *CYR61* is an extracellular matrix-associated angiogenic inducer, member of the CCN family of secreted matrix cellular proteins, which act as a ligand of integrin receptors to promote cell adhesion, migration, and proliferation (Babic et al. 1998, Grzeszkiewicz et al. 2001). Previous experiments have shown that Cyr61-null mice suffer embryonic death due to a failure in chorioallantoic fusion, placental vascular insufficiency and compromised vessel integrity (Mo et al. 2002). In the current study, *CYR61* mRNA was 4.9 fold up-regulated in CH sows compared to Con group ($P \leq 0.05$), which also tended to be 6.3 fold up-regulated in NC than Con sows, specifically in female embryos ($P=0.0728$) (Figure 5). These findings indicate a role of *CYR61* either directly or indirectly in the activation and/or expression of genes involved in angiogenesis and matrix remodelling through multiple signalling pathways (Zhou et al 2005, Chen et al. 2006), which may be driving sex differences in embryonic survival, as previously suggested by Vinsky et al. (2006). This is further supported by the concept that *CYR61* acts as a determinant factor in the genetic reprogramming that occurs in response to cells submitted to mechanical stress (Zhou et al. 2005).

The protein encoded by TFPI2 gene is a serine proteinase inhibitor that limits the ability of the metalloproteases to degrade extracellular matrix components (ECM) (Petersen et al. 1996). TFPI2 is widely expressed in various adult human tissues and highly abundant in the full-term placenta (Butzow et al. 1988). Activation of plasmin pathway through TFPI2 might be required for tissue remodelling during early embryonic implantation and placentation (Foxcroft 2000). In the present study, TFIP2 microarray data was not confirmed by QPCR as illustrated in Figure 5, suggesting that nutritional environments did not affect the mechanism by which TFPI2 reduce matrix degradation determining embryo implantation in pigs.

An additional gene involved with pre-implantation development was evaluated in the

present study. Results obtained for tumor necrosis factor receptor superfamily, member 21 (*TNFRSF21*) was similar expressed between all pair-wise comparisons by QPCR (Figure 5). The protein encoded by (*TNFRSF21*) is a member of the TNF-receptor superfamily. This receptor has been show to activate NF-kappa-B complex and mitogen-activated protein kinase (MAPK), both related with cell apoptosis (Nguyen et al. 2007). Indeed, the MAPK pathway is one of the active pathways during pre-implantation development (Kues et al. 2008); while *TNFRSF21* has been associated with neuronal cells differentiation in mice through NF-kappa-B signalling pathway (Gurok et al. 2004). This result indicates that *TNFRSF21* may exert a role in apoptotic signaling pathways in embryonic development, which may be reflecting an early formation of embryonic disc during elongation, with consequent degradation (apoptosis) of trophoblasts cells (Blomberg et al. 2008). In addition, studies with knockout mice suggested that TNF-receptor gene members may be involved in inflammation and immune regulation (ENTREZ, RefSeq, Jul 2008), suggesting its importance in maternal recognition and regulation of placental growth (Grunig and Antczak, 1995; Schäfer-Somi, 2003).

RAD21 is a component of cohesion complex, involved in several processes such as chromosome segregation and morphology, DNA-damage repair and apoptosis (Nasmyth and Haering, 2009; Peters et al. 2008). Recent study has been show that *RAD21* depletion causes overall changes in gene expression similar to those observed in Nanog depletion, demonstrating *RAD21* functional role in the pluripotency transcriptional network (Nitzsche et al. 2011). The tendency of 3.4 fold higher abundance of *RAD21* mRNA observed in female embryos recovered from CL compared to NC sows (P=0.092) (Figure 5), could then indicate alterations in chromosome organization in response to the intrauterine environment, supporting embryonic stem cell expression program, that occur along major morphological transitions such as compaction and blastocyst formation (Nitzsche et al. 2011). Mitotic divisions occur prenatally and ensure that the female is born with a complete supply of germ cells, which remain arrested in the first meiotic prophase before their recruitment into a growing follicle. The purpose of the nuclear arrest is to inactivate the DNA in female gamete so that it may not be vulnerable to possible insults during female lifetime (Senger et al. 2003). Thus, our results is also consistent with the hypothesis of Zeng et al. (2004) that genes related to DNA repair machinery and involved in cellular response to DNA damage, may become overrepresented as an attempt to ensure genomic integrity of the female germ line.

Conclusions

Overall, the model of feed restriction used in this study was mainly associated with changes in gene expression in female embryos. These results support the suggestions of Vinsky et al. (2006) that the effects of previous catabolism in the weaned sow on embryonic expression in early gestation may be sex specific in the pig. Findings from the present study indicate a distinct pattern of distribution of biological functions across treatments that may explain the sensitivity of embryos to changes in intrauterine environment. Differential expressed genes related to energy metabolism, angiogenesis, tissue remodelling and chromosome organization were further validated, representing potential process for promoting early embryonic survival. Taken together, these results suggest that intrauterine conditions may be one of the key down-stream mediators that trigger epigenetic programming of the offspring. Biological pathways leading to embryonic development are quite complex and involve gene imprinting, interactions between metabolism and methylation state; therefore, this study provides additional knowledge to clarify the relationships between early nutritional environment and postnatal development in the pig.

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Table 1. Sow and litter characteristics from restricted sows bred at first (FH) or second (Skip) post-weaning estrus.

Parameter	FH (n=23)	Skip (n=34)	*P*-value
Sow characteristics during lactation			
Sow weight, Day 1 of lactation (kg)	177.21±12.25	180±11.30	NS
Sow weight loss, Day 14–Day 21 of lactation (kg)	12.80±4.68	9.74±28.5	NS
Weaning to estrous interval (days)	6.17±3.01	5.36±1.10	NS
Ovulation rate (OR)	19.4±0.8	19.0±0.6	NS
Follicle size (mm)	0.77± 0.070	0.72±0.047	NS
Number of embryos recovered	16.5±4.6	18.1±4.7	NS
Embryo recovery rate (% of OR)	87.9	98.1	0.04
Total CL weight (g)	8.22±1.40	9.38±1.64	0.005
Total CL average (g)	0.422±0.055	0.478±0.0632	0.001
Litter characteristics			
Days of gestation when euthanised	9.13±0.09	9.36±0.35	NS
Average litter growth (g day^{-1})[A]	2323.60±354.90	2500±310.64	0.027
Average piglet growth rate (g day^{-1})[B]	234.15±34.37	236.34±37.23	NS
Input body (MJ ME day^{-1})	-35.044±21.44	-41.38±21.40	NS

[A] Average daily gain litter from day 14 of lactation to weaning.
[B] Average daily gain piglet from day 14 of lactation to weaning.
Data shown are means±s.e.m.

Table 2. List of genes selected for QPCR analysis

Gene Symbol[1]	Accession number[2]	Primer	Oligonucleotide sequence (5'→3')	Product size (bp)	$R^{2(3)}$	Slope	Efficiency
PPIA	AY266299	Forward	ACTGCCAAGACTGAGTGGTTGGAT	119	0.99	-3.03	2.1
		Reverse	TTCTTGCTGGTCTTGCCATTCCTG				
ACADSB	AK233370	Forward	AACGGGTCAAAGATGTGGATCACC	91.00	0.99	-3.29	2.01
		Reverse	TTCCTTTATACCCAAGGGCAGGGT				
SOD1	AY610279	Forward	GGCAATGTGACTGCTGGCAAAGAT	112.00	0.99	-3.35	1.99
		Reverse	TTTCATGGACCACCATTGTGCGG				
MYOF	XM_001926023	Forward	TTGGACATGATCCCGGACCTCAAA	143	0.99	-3.05	2.13
		Reverse	GGCAAGGAGAAGTCTTGGCATCTT				
TFPI2	AK234322	Forward	TACTGGCTGTGGAGGGAATGACAA	109	0.99	-3.57	1.91
		Reverse	GGCAAGGAGAAGTCTTGGCATCTT				
TNFRSF21	XM_003128430	Forward	CCACTTTCGTTCCCAAAGGCATGA	115	0.99	-3.19	2.06
		Reverse	ACTGAGCTTGTGTTGTCAGGGACT				
CYR61	XM_001927740	Forward	TGGTGAAGGAAACCCGCATCT	88	0.99	-3.6	1.9
		Reverse	GTCTTGCTGCATCTCTTGCCCTTT				
BCS1L	NM_001243676	Forward	TGCGGAGAACCCAGTAAAGTACCA	125	0.98	-3.3	2.01
		Reverse	ACATACTCCTTCAGGTCTACTCGC				
ETFA	NM_001244403	Forward	TGGAACCAAATGTGACAAGGTGGC		0.99	-3.21	2.05
		Reverse	ACCAATGGTGTCAGTTCCTCTGGA	120			
RAD21	XM_003481348	Forward	ATGCTTCATGGTCTTCAGCGAGC	97	0.99	-3.28	2.02
		Reverse	CTGCAGCTTGCTTTCTGTTCGTGT				

1) Gene symbol: 2) Accession number at Genbank (htpp://www.ncbi.nlm.nih.gov). 3) Coefficient of determination (R^2).

Table 3. Number of differentially expressed genes up and down-regulated between pair-wise comparisons annotated by IPA as mapped or unmapped transcripts.

Pair-wise Comparisons	Expression Status	Mapped on IPA*	Novel genes (unmapped)
CHvsCon	Up-regulated	47	9
	Down-regulated	14	2
	Total	**61**	**11**
CHvsCL	Up-regulated	85	27
	Down-regulated	194	34
	Total	**279**	**61**
CHvsNC	Up-regulated	75	12
	Down-regulated	48	16
	Total	**123**	**28**

Differentially expressed genes between pair-wise comparisons: (1) CH*vs*FH, catabolic high *versus* first heat sows; (2) CH*vs*CL, catabolic high *versus* moderately; (3) CH*vs*NC, catabolic high *versus* non-catabolic. * corresponds to unique transcripts.

Table 4. Ingenuity ontology for differentially expressed genes in each pair-wise comparison and commonly expressed genes between treatments.

	CH*vs*FH (72)[1]		CH*vs*CL (340)[2]		CH*vs*NC (123)[3]		Commonly expressed (27)[4]	
Mollecular and cellular functions (Top 10)	**Gene count**	**P-value (range)**	**Gene count**	**P-value (range)**	**Gene count**	**P-value (range)**	**Gene count**	**P-value (range)**
Cell Morphology	14	2.72E-05-4.55E-02	45	2.18E-07-1.45E-02			7	2.66E-04-4.61E-02
Lipid Metabolism	10	6.46E-05-4.55E-02			17	6.33E-05-3.25E-02	7	4.07E-05-4.28E-02
Nucleic Acid Metabolism	6	6.46E-05-4.04E-02						
Small Molecule Biochemistry	16	6.46E-05-4.55E-02			28	6.33E-05-3.25E-02	12	4.07E-05-4.28E-02
Carbohydrate Metabolism	6	1.07E-04-2.62E-02			8	2.95E-04-3.25E-02	8	4.07E-05-4.28E-02
Cellular Assembly and Organization	18	1.07E-04-4.55E-02	68	9.84E-07-1.45E-02	23	2.95E-04-3.25E-02	11	4.07E-05-4.28E-02
Molecular Transport	11	1.07E-04-4.55E-02			12	2.95E-04-3.25E-02	12	4.07E-05-4.28E-02
Cell-To-Cell Signaling and Interaction	11	2.15E-04-4.55E-02	34	1.03E-05-1.45E-02				
Tissue Development	19	2.15E-04-4.86E-02	38	1.03E-05-1.45E-02				
Gene Expression	5	2.24E-04-4.55E-02						
Cell Death			76	4.02E-07-1.54E-02				
Cellular Function and Maintenance			57	9.84E-07-1.45E-02				
Protein Synthesis			48	3.87E-06-1.46E-02	10	4.20E-04-2.18E-02	5	5.6E-04-8.18E-03
DNA Replication, Recombination, and Repair			16	1.49E-05-1.06E-02				

(Continued)

Mollecular and cellular functions (Top 10)	CH*vs*FH (72)[1]		CH*vs*CL (340)[2]		CH*vs*NC (123)[3]		Commonly expressed (27)[4]	
	Gene count	P-value (range)	Gene count	P-value (range)	Gene count	P-value (range)	Gene count	P-value (range)
Nervous System Development and Function			31	4.09E-05-1.45E-02				
Cancer			98	1.03E-05-1.46E-02	34	3.37E-04-3.95E-04		
Tissue Morphology					9	4.03E-04-3.25E-02		
Post-Translational Modification					13	4.02E-04-3.53E-02		
Cellular Movement					16	4.25E-04-3.25E-02		
Metabolic Disease							16	4.68E-04-6.71E-03
Neurological Disease							9	6.83E-04-4.34E-02
Developmental Disorder							7	8.38E-04-3.61E-02

Differentially expressed genes between pair-wise comparisons: (1) CH*vs*FH, catabolic high *versus* first heat sows; (2) CH*vs*CL, catabolic high *versus* moderately; (3) CH*vs*NC, catabolic high *versus* non-catabolic.

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Table 5. Top canonical pathways for differentially expressed genes in each pair-wise comparison and for commonly expressed genes between treatments.

	CH*vs*FH (72)[1]		CH*vs*CL (340)[2]		CH*vs*NC (123)[3]		Commonly expressed (27)[4]	
Canonical Pathways (Top 5)	**P-value**	**Genes**	**P-value**	**Genes**	**P-value**	**Genes**	**P-value**	**Genes**
Propanoate Metabolism	8.90E-04	MUT, ACADM,LDHB	2.55E-04	ACSL3, AUH, ACADSB, MUT, ACADM, LDHB	2.69E-04	ACSL3, ACADSB, MUT, ACADM	2.14E-04	ACADSB, MUT, ACADM
Valine, Leucine and Isoleucine Degradation	1.42E-03	MUT, ACADM,ACAA2	8.19E-05	HSD17B10, AUH, ACADSB, OXCT1, MUT, ACADM, ACAA2	5.00E-04	ACADSB, OXCT1, MUT, ACADM	3.45E-04	ACADSB, MUT, ACADM
Role of Tissue Factor in Cancer	5.66E-03	CFL2, CYR61, ITGB5	1.61E-03	ITGB1, RPS6KB1, PTPN11, ITGAV, PIK3R4, CYR61, ITGB5				
Ephrin Receptor Signaling	1.97E-02	GNAS, CFL2, EFNA5						
Actin Cytoskeleton Signaling	3.37E-02	TIAM1, CFL2, GSN						
Pyruvate Metabolism			6.17E-04	GRHPR, ACSL3, DLAT, MDH1, PDHB, LDHB	3.29E-05	GRHPR, ACSL3, ME1, MDH1, PDHB		

(Continued)

	CH*vs*FH (72)[1]		CH*vs*CL (340)[2]		CH*vs*NC (123)[3]		Commonly expressed (27)[4]	
Canonical Pathways (Top 5)	**P-value**	**Genes**	**P-value**	**Genes**	**P-value**	**Genes**	**P-value**	**Genes**
Glyoxylate and Dicarboxylate Metabolism					5.85E-03	GRHPR, MDH1		
Neuregulin Signaling			2.55E-03	ITGB1, RPS6KB1, HSP90B1, PTPN11, ERRFI1, PRKCH				
Germ Cell-Sertoli Cell Junction Signaling					1.12E-02	PLS1, TUBA1A, TUBA1C, GSN		
β-alanine Metabolism							4.34E-03	ACADSB, ACADM
Assembly of RNA Polymerase I Complex							1.83E-02	TBP
Assembly of RNA Polymerase III Complex							2.63E-02	TBP

Differentially expressed genes between pair-wise comparisons: (1) CH*vs*FH, catabolic high *versus* first heat; (2) CH*vs*CL, catabolic high *versus* moderately; (3) CH*vs*NC, catabolic high *versus* non-catabolic.

Table 6. Fold change up- and down-regulated genes for pair-wise comparisons.

		Expression Value		
Gene symbol	**Entrez Gene name**	**CH*vs*FH[1]**	**CH*vs*CL[2]**	**CH*vs*NC[3]**
HIST1H2AB/HIST1H2AE	histone cluster 1, H2ae	-1.705		-1.339
HIST3H2A	histone cluster 3, H2a	-1.601		
HIST1H2AG	histone cluster 1, H2ag	-1.522		
SLC28A3	solute carrier family 28 (sodium-coupled nucleoside transporter)	-1.509		
HIST1H2AA	histone cluster 1, H2aa	-1.220		
NHP2L1	NHP2 non-histone chromosome protein 2-like 1	-1.174		
TMEM111	transmembrane protein 111	-1.151		
HIST1H4A	histone cluster 1, H4a	-1.127		
UBB	ubiquitin B	-1.100		
HIST1H2AC	histone cluster 1, H2ac	-1.081		
MYOF	Myoferlin	+1.615	+2.407	+1.661
STC2	stanniocalcin 2	+1.652		
LAMB1	laminin B1	+1.747		
GNAS	adenylate cyclase-stimulating G alpha protein	+1.933		
ETFA	Electron-transfer-flavoprotein, alpha polypeptide	+1.954	+2.191	+1.848
NID2	nidogen 2 (osteonidogen)	+2.041		+1.776
SOD1	superoxide dismutase 1, soluble	+2.052	+2.384	+1.878
CHCHD2	Coiled-coil-helix-coiled-coil-helix domain containing 2	+2.073		
LPGAT1	lysophosphatidylglycerol acyltransferase 1	+2.210		
CHAC1	ChaC, cation transport regulator homolog 1	+3.430	+2.270	
AKAP12	A kinase (PRKA) anchor protein 12		+2.241	
FN1	fibronectin 1		+2.217	
GOLGA4	golgi autoantigen, golgin subfamily a, 4		+2.183	
MYH10	myosin, heavy chain 10, non-muscle		+2.105	
RAD21	Double-strand-break repair protein rad21 homolog		+2.007	+1.532
CHMP4B	charged multivesicular body protein 4B		+2.004	

(Continued)

		Expression Value		
Gene symbol	**Entrez Gene name**	**CH*vs*FH[1]**	**CH*vs*CL[2]**	**CH*vs*NC[3]**
BCS1L	mitochondrial chaperone BCS1		-1.534	-1.332
RPL18A	ribosomal protein L18a		-1.679	
ADAMDEC1	ADAM+like, decysin 1		-1.744	-1.657
CDA	cytidine deaminase		-1.757	
ILVBL	acetolactate synthase-like protein		-1.785	
NPR2	natriuretic peptide receptor B/guanylate cyclase B		-1.788	-1.574
PRMT7	protein arginine methyltransferase 7		-1.864	
RPS3	ribosomal protein S3		-1.974	
GPR124	G protein-coupled receptor 124			-1.417
CACNG8	calcium channel, voltage-dependent, gamma subunit 8			-1.338
POLR1D	polymerase (RNA) I polypeptide D			-1.32
NDUFA7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7			-1.31
SSR2	signal sequence receptor, beta (translocon-associated protein beta)			-1.31
HSF4	heat shock transcription factor 4			-1.28
ANXA1	annexin A1			+1.51
ACADM	Acyl-CoA dehydrogenase, C-4 to C-12 straight chain			+1.58
ANK3	ankyrin 3, node of Ranvier (ankyrin G)			+1.67
LPGAT1	lysophosphatidylglycerol acyltransferase 1			+2.25
CHAC1	ChaC, cation transport regulator homolog 1			+2.54

Differentially expressed genes between pair-wise comparisons: (1) CH*vs*FH, catabolic high *versus* first heat sows; (2) CH*vs*CL, catabolic high *versus* moderately; (3) CH*vs*NC, catabolic high *versus* non-catabolic. Expression value (+) up-regulated, (-) down-regulated.

Table 7. Comparison of Fold Changes between distinct pair-wise comparisons

	Microarray Fold Change (P≤0.05)			QPCR Fold Change (P≤0.10)		
Gene Symbol	CHvsCon	CHvsCL	CHvsNC	CHvsCon	CHvsCL	CHvsNC
BCS1L	0.54(NS)	-1.53	-1.33	2.34(NS)	3.37	1.00(NS)
ACADSB	2.13	1.3	1.35	2.10(NS)	2.70(NS)	1.31(NS)
RAD21	1.63(NS)	2.01	1.53	1.82(NS)	2.37(NS)	0.88(NS)
CYR61	1.4	1.24	1.04	3.30(NS)	2.99(NS)	0.93(NS)
MYOF	1.62	2.41	1.66	1.22(NS)	2.65(NS)	0.84(NS)
TFPI2	1.27	1.53	1.41	2.27(NS)	1.95(NS)	0.86(NS)
TNFRSF21	1.27	1.23	1.04	1.67(NS)	2.63(NS)	0.76(NS)
ETFA	1.95	2.19	1.85	2.27(NS)	1.95(NS)	0.86(NS)
SOD1	2.05	2.38	1.88	2.02(NS)	3.39(NS)	0.71(NS)

Pair-wise comparisons: CH*vs*FH, catabolic high *versus* first heat sows; CH*vs*CL, catabolic high *versus* moderately; CH*vs*NC, catabolic high *versus* non-catabolic. NS: not significant

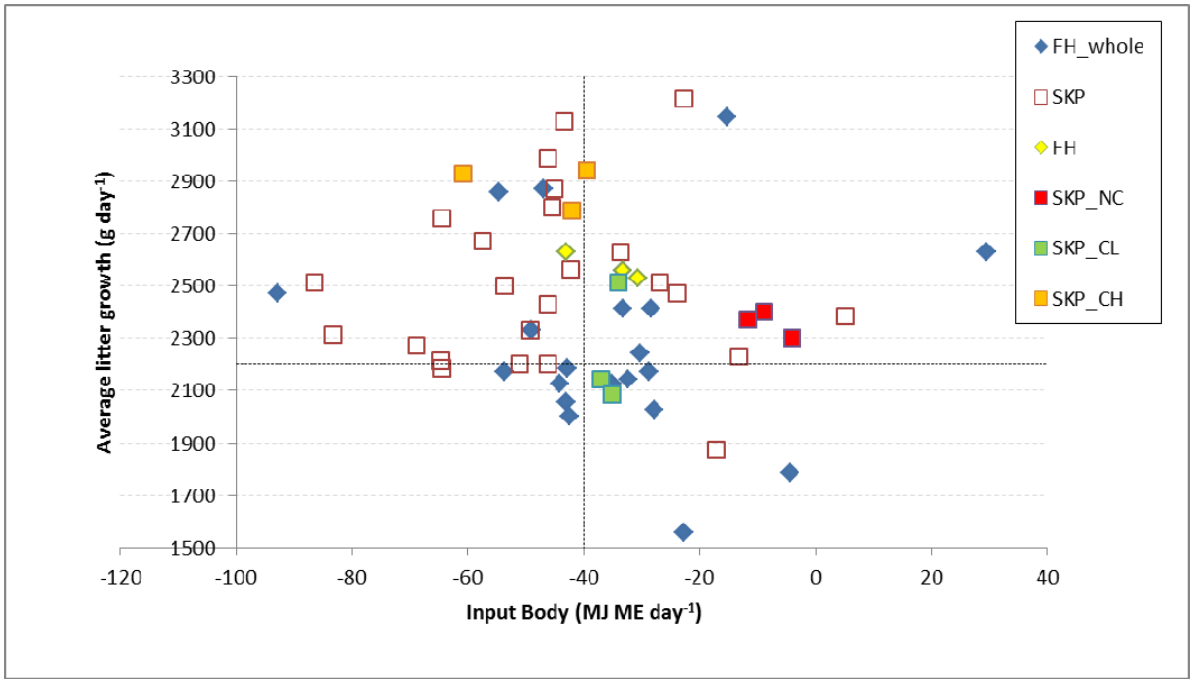
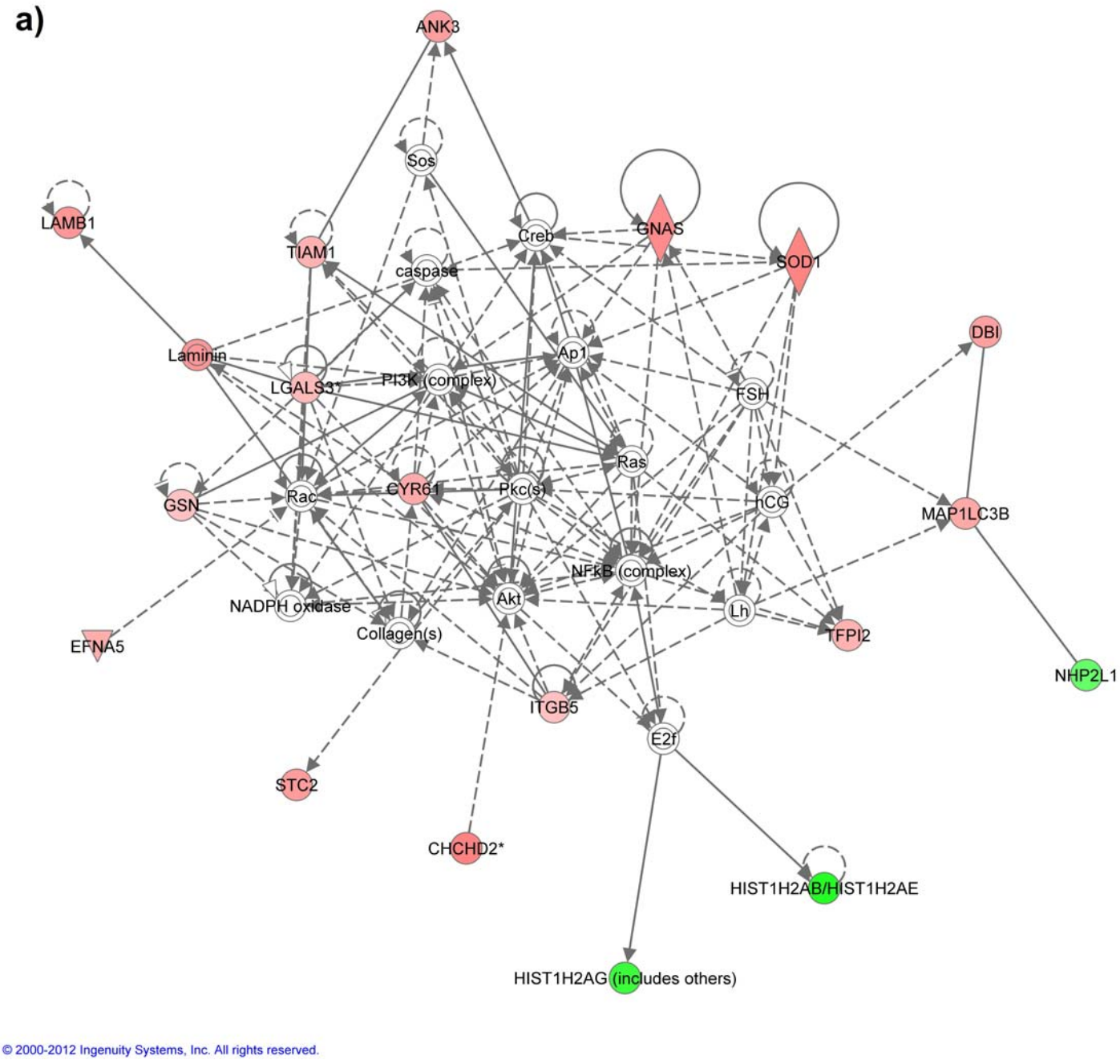
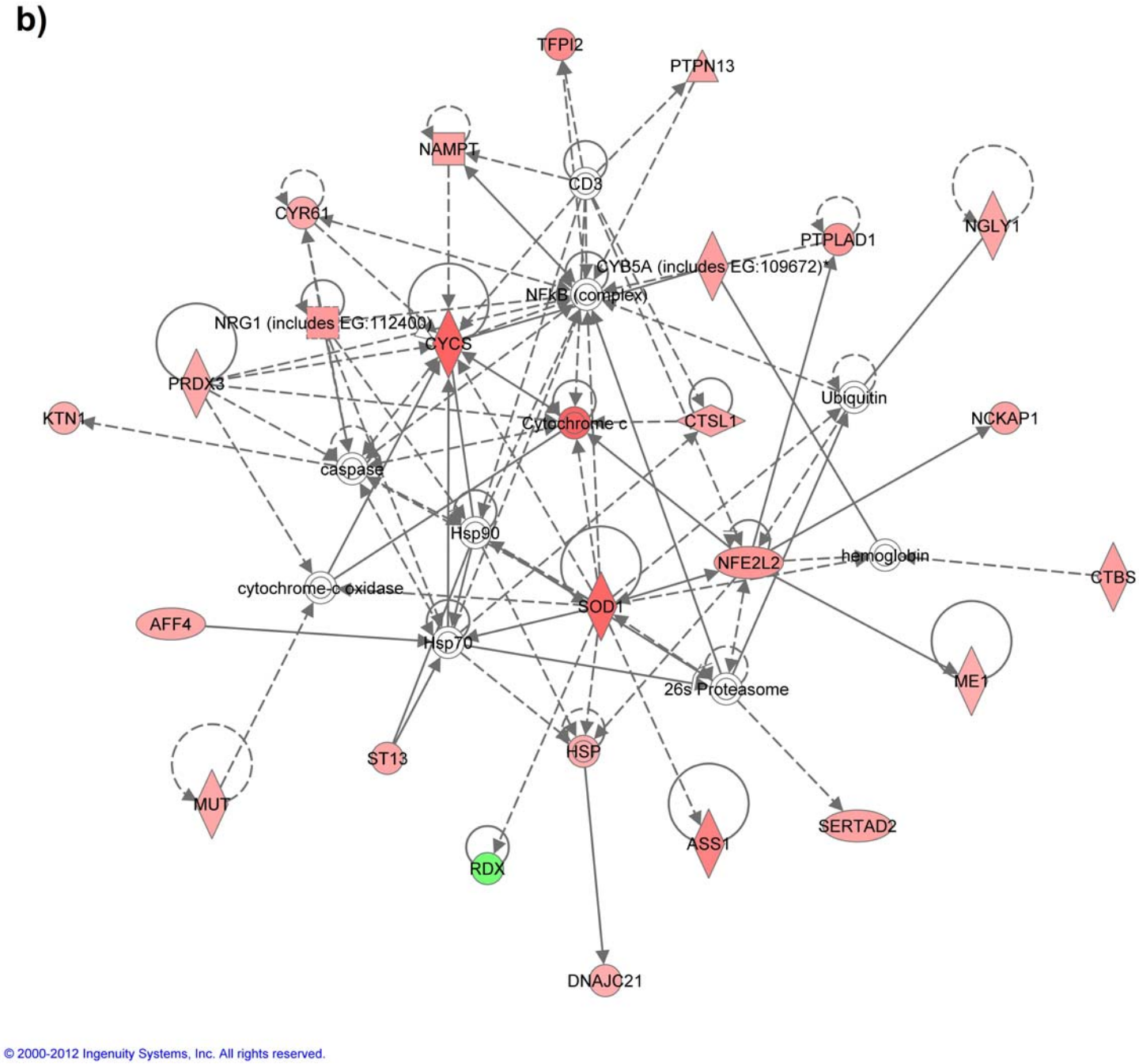


Figure 1. Relationship betweeen the sow subset used for microarray analysis in the present study and the whole dataset. (◆) Whole dataset of first heat sows; (◇) Subset of first heat sows; (□) whole dataset Skip sows; (■) Subset of Skip NC; (■) Subset of Skip CL and Subset of Skip CH (■). (University of Alberta, preliminary data).

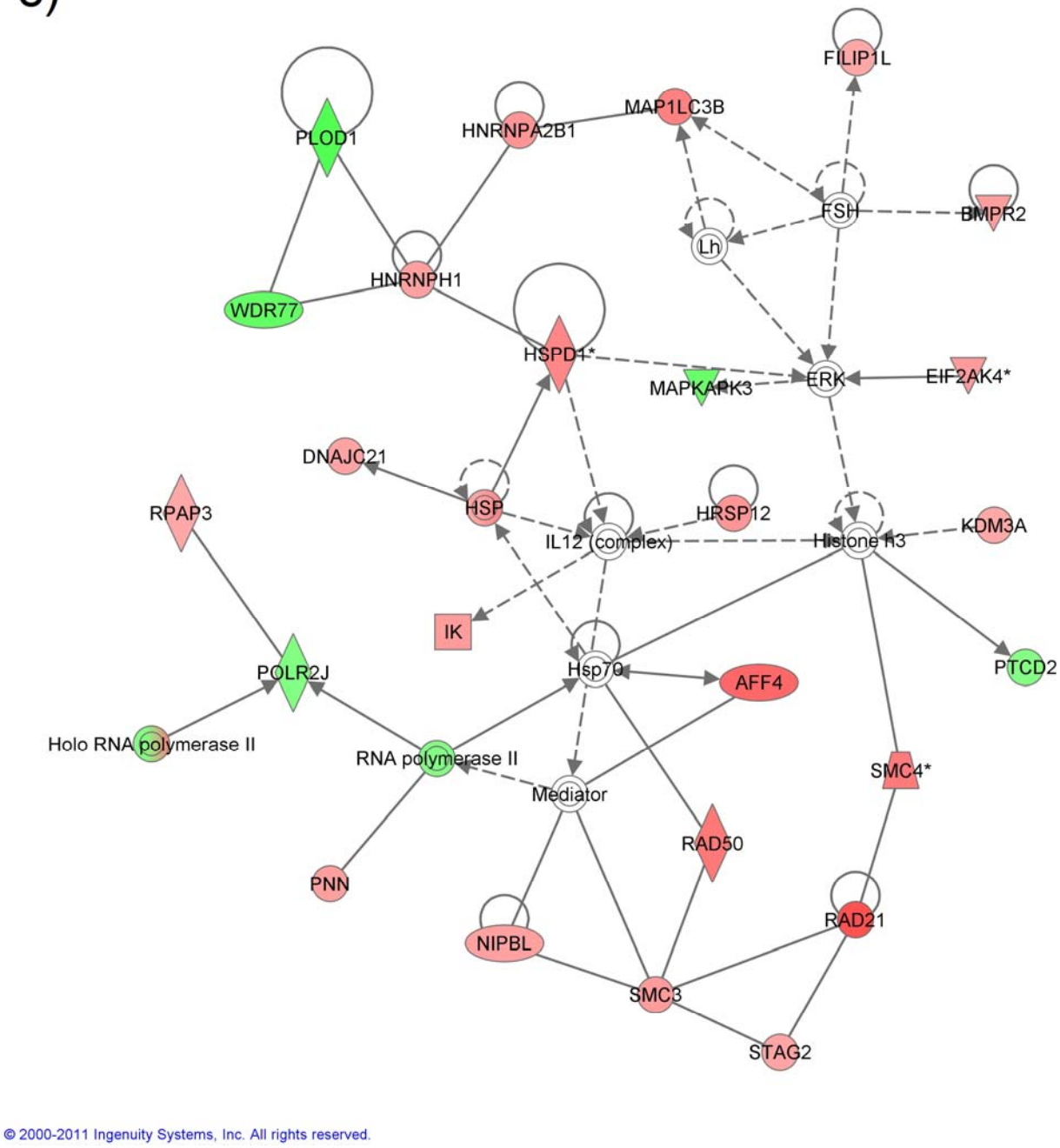


CHvsFH Top Scoring Network
Cell Morphology, Cardiovascular System Development and Function, Organismal Development

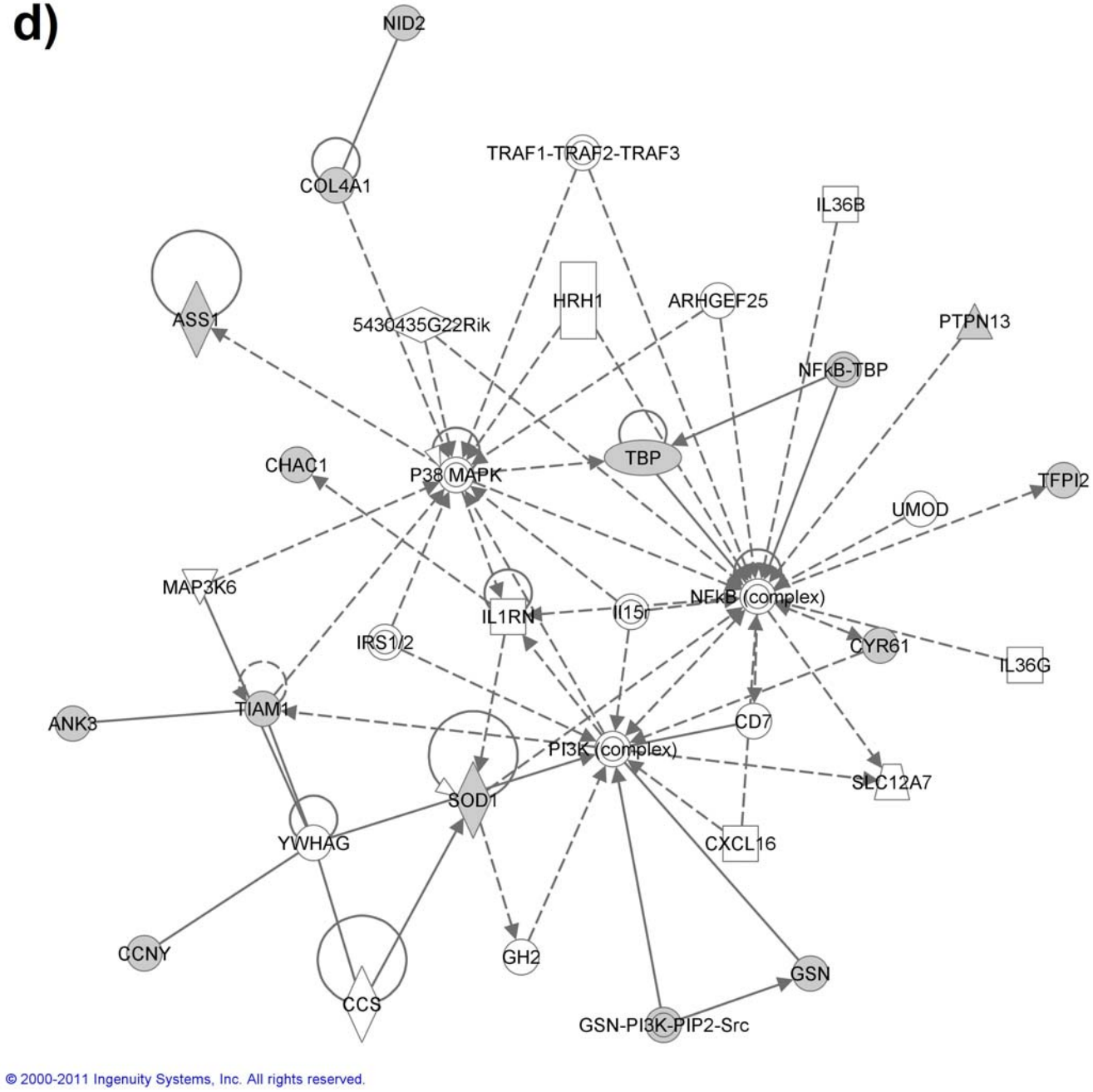


CHvsNC Top Scoring Network
Free Radical Scavenging, Cellular Development, Cellular Growth and Proliferation

c)



CHvsCL Top Scoring Network
Cellular Assembly and Organization, Cellular Development, Reproductive System Development and Function



Commonly expressed genes Top Scoring Network
Cell Death, Lipid Metabolism, Small Molecule Biochemistry

Figura 2. Top ranking gene/protein networks determined by IPA analysis in **a)** CHvsFH (Network score 41); **b)** CHvsNC (Network score 50); **c)** CHvsCL (Network score 44); **d)** for the commonly expressed genes (Network score 30). Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line).Solid lines show direct interaction (binding/physical contact); dashed line, indirect interaction supported by the literature but possibly involving one or more intermediate molecules that have not been investigated definitively. Molecular interactions involving only binding are connected with a solid line (no arrowhead) since directionality cannot be inferred. The

intensity of the node color indicates the degree of up- (red) or down- (green) regulation.

*indicates that there was more than one probe for this gene tested and the most significant

was placed into the analysis.

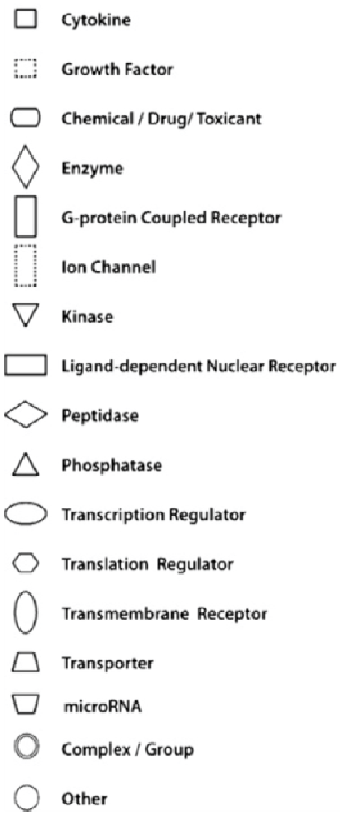


Figure 3. Network shapes representing the functional class of the gene product. Figure legend for the IPA network (https://analysis.ingenuity.com/pa/info/help/help.htm#legend.htm.)

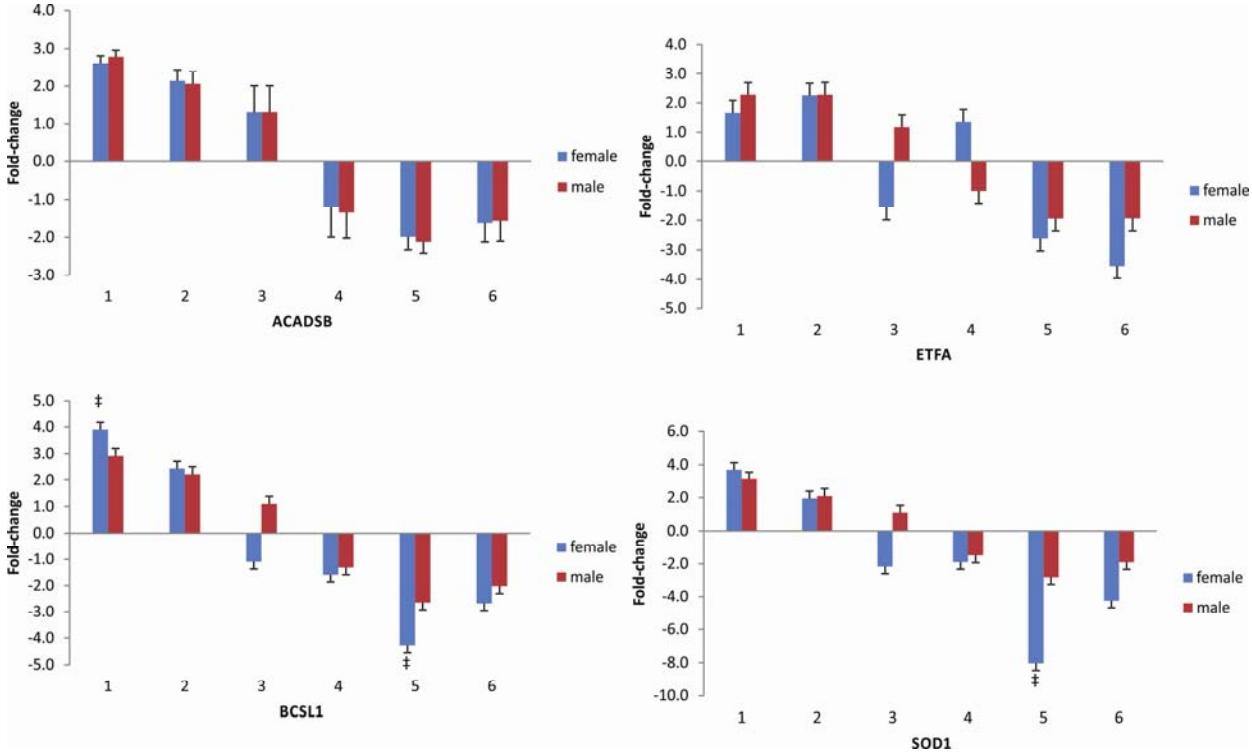


Figure 4. QPCR validation of *ACADSB*, *ETFA*, *BCSL1* and *SOD1* genes selected based on their differential expression by microarray analysis. The relative quantity was normalized to *PPIA*. In order to estimate the effect of sow catabolism on embryo sex, two distinct bars were generated to illustrate the contrasts between pair-wise comparisons. The Fold-change shown by the bars are the mean±standart error; red bars (female), blue bars (male). Numbers indicate the following contrasts: 1. catab:CH-CL|sex; 2. catab:CH-FH|sex, 3. catab:CH-NC|sex, 4. catab:CL-FH|sex, 5. catab:CL-NC|sex, 6. catab:FH-NC|sex. Genes that were significantly differentially expressed (*P ≤0.05) or marginally significant (‡P≤0.10) between treatments are indicated.

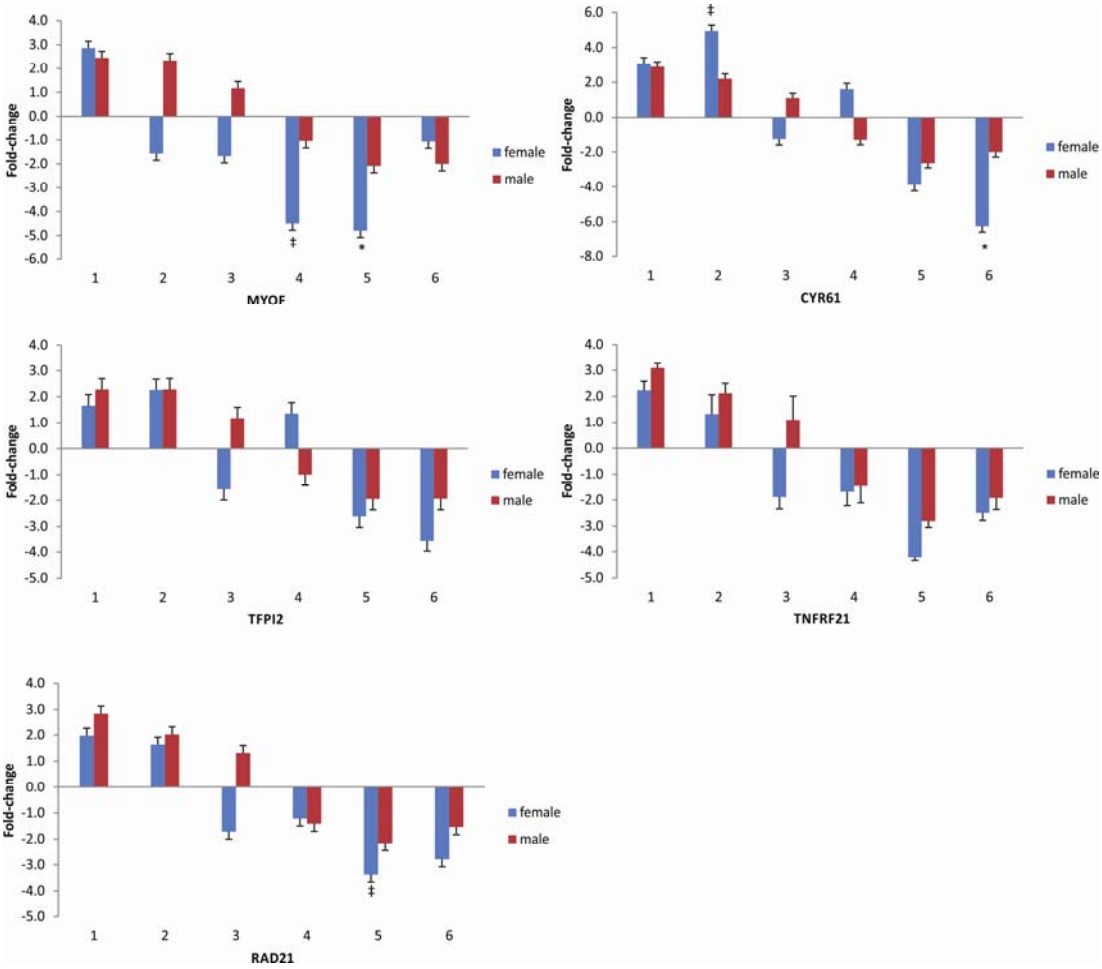


Figure 5. QPCR validation of *MYOF*, *CYR61, TFPI2, TNFRF21* and *RAD21* genes selected based on their differential expression by microarray analysis. The relative quantity was normalized to *PPIA*. In order to estimate the effect of sow catabolism on embryo sex, two distinct bars were generated to illustrate the contrasts between pair-wise comparisons. The Fold-change shown by the bars are the mean±standart error; red bars (female), blue bars (male). Numbers indicate the following contrasts: 1. catab:CH-CL|sex; 2. catab:CH-FH|sex, 3. catab:CH-NC|sex, 4. catab:CL-FH|sex, 5. catab:CL-NC|sex, 6. catab:FH-NC|sex. Genes that were significantly differentially expressed (*P ≤0.05) or marginally significant (‡P≤0.10) between treatments are indicated.

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General Conclusions

Overall, this research provides new data regarding genetic control of follicle development between high and low ovulating breeds. The pattern of follicular growth in Piau gilts was firstly described and related with differential expression of genes involved in proliferation and atresia in GC and CL during estrous cycle. In addition, the higher expression of soluble growth factors (*GDF9*, *BMP6* and *BMP15*) secreted by Piau oocytes may be inducing the lower ovulation rate that is a key component of the reduced prolificacy of this breed.

We have also provided further insight into the role of intra-ovarian factors in the preovulatory follicle development and CL function as key mediators of the increased variability in birth weight and growth performance reported in greater parity sows. We reported that genes related with apoptosis, angiogenesis may be driving differences in both vascular formation and blood supply which can be impaired in low BWTA sows. Also, differential expression of genes related with cell proliferation, differentiation and steroidogenesis indicates that differences in follicular recruitment and selection process may be present between BWTA sows, even prior to ovulation.

We have also used a feed restriction model to investigate the mechanisms by which nutrition and metabolic state affect embryonic development in early gestation. We confirmed previous findings that sows that become highly catabolic during lactational feed restriction have reduced average litter weights compare to sows that do not show such high levels of catabolism (Patterson et al. 2011). We reported changing patterns of gene expression in female embryos, corroborating the hypothesis of Vinsky et al. (2006) that the effects of previous catabolism in the weaned sow on embryonic expression in early gestation may be sex specific in the pig. Moreover, a distinct pattern of distribution of biological functions was observed across treatments as a reflection of changings in the intrauterine environment. Genes related to energy metabolism, angiogenesis, tissue remodeling and chromosome organization were further validated, representing major events of pre-implantation stages, such as transition from maternal to embryonic genome activation, cell-to-cell adhesion or compaction, and differentiation of cells to inner cell mass and

trophectoderm at blastocyst stage. In addition, economically carcass traits such as back fat depth, lean meat percentage and animal health and longevity may also be affected by sow catabolism, either direct in utero, or as developing follicles/oocytes prior to ovulation (Oliver et al. 2012).

From the perspective of swine industry, the current study provides additional knowledge to clarify the relationships between the embryo and uterine environment, in terms of the size and uniformity of the litter born. Future studies based on the results from this thesis may be useful for testing hypothesis and to create novel strategies to enhance the reproductive efficiency, without compromising prenatal programming on postnatal growth later in life.

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Appendix 1

Supplementary Table 1. **Proportion of follicles within genetic group during the estrous cycle.**

	Day of estrous cycle					
Follicle size classification (mm)	Genetic Group	0	4	10	18	(χ^2) P value
Small (≤3 mm)	Piau	0.015	0.124	0.264	0.048	
	Comercial	0.021	0.14	0.255	0.133	0.039*
Medium (3-6 mm)	Piau	0.027	0.053	0.229	0.088	
	commercial	0.051	0.098	0.333	0.122	0.70
Large (≥ 6 mm)	Piau	0.212	0.003	0.000	0.194	
	Comercial	0.247	0.010	0.031	0.302	0.0001**

Asterisks indicate significant differences (*P≤0.05; **P≤0.01) between genetic groups within follicle