MAYARA MORENA DÉL CAMBRE AMARAL WELLER

GENE EXPRESSION IN CATTLE REPRODUCTIVE ORGANS AND MAMMARY GLAND

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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BIOGRAFIA

Mayara Morena Dél Cambre Amaral Weller, filha de Ailton Alves Weller e Remy Gomes do Amaral, nasceu em 4 de abril de 1987 em Cachoeiro de Itapemirim, Espirito Santo, Brasil.

No 2005, iniciou o curso de Zootecnia, pelo Departamento de Zootecnia da Universidade Federal de Viçosa, em Viçosa, MG, onde foi bolsista de iniciação científica, acompanhando e conduzindo pesquisas junto ao Programa de Melhoramento Genético de Suínos do Departamento de Zootecnia da UFV.

Em janeiro de 2010, graduou-se em Zootecnia, pela Universidade Federal de Viçosa. Em março do mesmo ano, iniciou o Mestrado pelo Programa de Pós-Graduação em Genética e Melhoramento Animal da Universidade Federal de Viçosa.

Em 16 de fevereiro de 2012, submeteu-se ao exame final de defesa de dissertação para obtenção do título de *Magister Scientiae* em Genética e Melhoramento, na Universidade Federal de Viçosa.

Em março de 2012, iniciou o curso de Doutorado em Genética e Melhoramento na Universidade Federal de Viçosa.

Em 29 de fevereiro de 2016, submeteu-se ao exame final de defesa de tese para obtenção do título de *Doctor Scientiae* em Genética e Melhoramento, na Universidade Federal.

SUMÁRIO

RESUMO

WELLER, Mayara Morena Dél Cambre Amaral, D. Sc, Universidade Federal de Viçosa, fevereiro de 2016. **Expressão gênica em órgãos reprodutivos e glândula mamária em bovinos.** Orientadora: Simone Eliza Facioni Guimarães. Coorientador: Marcos Inácio Marcondes.

Este estudo foi dividido em 3 experimentos objetivando-se: 1) investigar os efeitos da supernutrição materna sobre o desenvolvimento gonadal e expressão gênica hipofisário-gonadal em fetos bovinos no terço final da gestação. Vinte e sete vacas secas multíparas foram alimentados ad libitum (E) ou em nível de mantença (M) de mesma dieta. Doze vacas de H (n = 6) e M (n = 6) gestantes de fetos do sexo feminino foram submetidos à eutanásia aos 199 e 268 dias de gestação (DG; n = 3 para H ou M em cada DG). Quinze vacas de H (n = 6) e M (n = 9) gestantes de fetos masculinos foram sacrificados aos 139, 199 e 241 DG (n = 2 para H e n = 3 para M em cada DG). 2) Avaliar os efeitos de diferentes planos nutricionais no desenvolvimento do parênquima mamário (PAR), na expressão de marcadores lipogênicos (*CD36*, *ACCA*, *FASN*, *ADIPOR1*), nas concentrações de hormônios no sangue e expressão hepática dos genes GHR, IGF1, *IGFBP2* e *IGFBP3* em novilhas pre-púberes. Dezoito novilhas foram alimentadas 1 de 3 planos nutricionais (n = 6 / tratamento) para sustentar um ganho médio diário (GMD), como segue: alto ganho (AG-1 kg/d), baixo ganho (BG- 0,5 kg/d) ou mantença (MA). 3) Elucidar se os padrões de expressão dos genes *ESR1*, *GDF9*, *FSHR*, *LHR*, *BMPR2*, *TGFB1*, *TGFB2*, *BMP15*, *BMP6*, *BMP7*, *CYP19A1*, *HSD3B1*, *IGFR*, *IGF1* e *IGF2* poderiam estar envolvido na modulação do início da puberdade em novilhas da raça Brahman. No primeiro experimento, os números de folículos primordiais e totais foram menores nos ovários dos fetos oriundos de vacas alimentadas E do que das vacas M. Estes resultados foram opostos para folículos pré-antrais e antrais. As proporções volumétricas e os diâmetros dos cordões seminíferos foram menores nos testículos dos fetos oriundos das vacas alimentadas E em comparação as vacas M. A expressão pituitária do gene *FSH* foi maior nos fetos fêmeas de vacas alimentadas E do que das vacas M, independentemente do DG, ao passo que a expressão do gene *LHB* não diferiu. As expressão ovarianas fetais dos genes *P450* aromatase, *StAR*, *BMPR2*, *TGFB1*, *GDF9*, *FSHR*, *Bax* e *CASP3* foram maiores dos fetos oriundos das vacas alimentadas E do que

das vacas M, independentemente do DG. As expressões testiculares fetais dos genes *StAR*, *HSD17B3*, *IGF1*, *IGF2* e *IGF1R* foram maiores dos fetos oriundos das vacas alimentadas M do que das vacas E. De modo geral, a ingestão materna ad libitum parece afetar o crescimento folicular ovariano fetal e números de folículos, o qual pode influenciar o tamanho da reserva ovariana em sua prole. Nos fetos machos, a ingestão materna ad libitum parece perturbar o desenvolvimento testicular e pode ter implicações na produção de esperma. No segundo experimento, os pesos dos PAR mamário e relação PAR/GM foram menores nas novilhas de AG do que novilhas de MA e BG, visto que a gordura extraparenquimal mamária foi maior nas novilhas de AG do que em outros grupos. Novilhas alimentadas para AG apresentaram maior fração de lipídica e menor fração proteica no PAR. No entanto, o número de adipócitos intraparenquimais foi semelhante entre os grupos. Novilhas alimentadas para AG tiveram maior concentração sérica de IGF1 do que outros, e a concentração sérica de insulina foi menor nas novilhas de AG em comparação as novilhas de MA e BG. Os genes *GHR*, *IGF1* e *IGFBP3* foram up-regulados enquanto o gene *IGFBP2* foi down-regulado no fígado das novilhas de HG relação aos outros grupos. Os genes *CD36*, *ACCA*, *FASN* e *ADIPOR1* foram-se up-regulados pelo nível de ingestão de nutrientes. No geral, estes resultados demonstram que a elevada ingestão de nutrientes favoreceu ao acúmulo de gordura corporal e na glândula mamária. Estes resultados poderiam servir como preditores do comprometimento do desenvolvimento mamário. Finalmente, os dados qRT-PCR obtidos no terceiro experimento revelaram maior expressão dos genes *FSHR*, *BMP7*, *CYP19A1*, *IGF1* e *IGF1R* em novilhas pre-púberes em comparação as novilhas pós-púberes. Além disso, a expressão do gene *IGF1* foi positivamente correlacionada com a expressão do gene *BMP7* ($P = 0.07$; $r = 0.84$) e a expressão do *HSD3B1* foi negativamente correlacionada com a expressão do gene *CY19A1* ($P = 0.3$; $r = -0.90$). Estes resultados sugerem que a expressão diferencial dos genes ovarianos pode estar associado com as alterações na dinâmica folicular e diferentes tipos de populações celulares, os quais surgiram como consequência da puberdade e a fase lútea. A hipótese que emerge é que dois reguladores-chave da atividade do ovário pré-puberdade, BMP7 e IGF1 modulam a expressão de *FSHR*, *LHR*, *IGFR1* e *CYP19A1*. BMP7 parece regular LHR e up-regular FSHR e CYP19A1, que medeiam a dinâmica folicular ovariana dessas

novilhas. Portanto, BMP7 e IGF1 parecem desempenhar papéis reguladores sinérgicos e foram previstos interagir entre si.

ABSTRACT

WELLER, Mayara Morena Dél Cambre Amaral, M. Sc, Universidade Federal de Viçosa, February, 2016. **Gene expression in cattle reproductive organs and mammary gland.** Adviser: Simone Eliza Facioni Guimarães. Co-adviser: Marcos Inácio Marcondes.

This study was divided in three experiments that aimed to: 1) investigate effects of maternal overnutrition on gonadal development and pituitary-gonadal gene expression in cattle fetuses at mid and late gestation. Twenty-seven multiparous dry cows were fed either high (ad libitum, H) or moderate (M) intake of the same diet. Twelve cows from H (n=6) and M (n=6) intake carrying females fetuses were euthanized at 199 and 268 days of gestation (DG; n=3 for H or M on each DG). Fifteen cows from H (n=6) and M intake (n=9) carrying male fetuses were euthanized at 139, 199 and 241 DG (n=2 for H and n=3 for M on each DG). 2) To evaluate effects of different planes of nutrition on mammary parenchyma (PAR) development, expression of lipogenic marks (CD36, ACCA, FASN, ADIPOR1), concentrations of blood hormones and liver GHR, IGF1, IGFPB-2 and -3 mRNA expression in prepubertal heifers. Eighteen heifers were fed 1 of 3 nutrient intake levels (n = 6/treatment) designed to sustain an average daily gain (ADG), as follows: high gain (HG-1 kg/d), low gain (LG- 0.5 kg/d) or maintenance (MA). 3) To elucidated if the expression pattern of *ESR1*, *GDF9*, *FSHR*, *LHR*, *BMPR2*, *TGFB1*, *TGFB2*, *BMP15*, *BMP6*, *BMP7*, *CYP19A1*, *HSD3B1*, *IGF1*, *IGFR1* and *IGF2* genes could be involved in modulate the timing of puberty in Brahman heifers. In the first experiment, primordial and total follicle numbers were lower in fetal ovaries from H than in M intake cows. These results were reverse for preantral and antral follicles. Volumetric proportion and diameter of seminiferous cord were lower in fetal testis of H than M intake cows. The expression level of *FSHB* was greater in pituitary gland of female fetus from H compared to M intake cows, irrespective of DG, whereas *LHB* gene expression did not differ. In males, *FSHB* and *LHB* gene expression levels were similar between maternal intake groups. Fetal ovarian expression of *P450 aromatase*, *StAR*, *BMPR2*, *TGFBR1, GDF9*, *FSHR*, *Bax* and *CASP3* genes were higher in H than in M intake cows, irrespective of DG. Fetal testicular expression of *StAR*, *HSD17B3*, *IGF1*, *IGF2* and *IGF1R* genes were higher in M than in H intake cows. Overall, maternal H intake seems to affect fetal ovarian follicular growth and number of follicles, which may

affect size of ovarian reserve in their offspring. In male fetus, maternal H intake seems to disturb testicular development and may have implications on sperm production. In the second experiment, mammary PAR weight and MA to MG ratio was lower in HG than MA and LG heifers, whereas mammary extraparenchymal fat was greater in HG heifers than other groups. Heifers fed the HG had a greatest fraction of lipids in their PAR, and smallest fraction of protein in their PAR. However, the number of intraparenchymal adipocytes was similar between the groups. Heifers fed the HG had greater serum IGF1 than others, and serum insulin was lower in MA than HG and LG heifers. The liver *GHR, IGF1* and *IGFBP3* mRNA was higher whereas *IGFBP2* mRNA was lower in HG heifers compared to others. The expression of *CD36*, *ACCA*, *FASN* and *ADIPOR1* were up regulated by nutrient intake level. Overall, these results demonstrated that enhancing nutrient intake favored to fat accumulation on body and mammary gland, also resulted adipocyte hypertrophy in the PAR of HG heifers, which could be a predictor of impaired mammary development. Finally, qRT-PCR data from the third experiment revealed the expression of *FSHR*, *BMP7*, *CYP19A1*, *IGF1* and *IGFR1* mRNA was greater in PRE heifers, when contrasted to POST heifers. In addition, the expression of *IGF1* mRNA was positively correlated with *BMP7* mRNA ($P = 0.07$, $r = 0.84$) and the expression of *HSD3B1* mRNA was negatively correlated with expression of *CY19A1* mRNA ($P = 0.03$, $r = -0.90$). Taken together, these results suggest that the differential expression of ovarian genes could be associated with changes in follicular dynamics and different cell populations that have emerged as consequence of puberty and the luteal phase. The emerging hypothesis is that two key regulators of ovarian activity pre-puberty, BMP7 and IGF1 modulate the expression of *FSHR*, *LHR*, *IGFR1* and *CYP19A1*. BMP7 seems to down-regulate LHR and up-regulate *FSHR* and *CYP19A1*, which mediates the follicular dynamics in heifer ovaries. Thus, BMP7 and IGF1 seem to play synergic regulatory roles and were predicted to interact.

GENERAL INTRODUCTION

Maternal nutrition during pregnancy plays a critical role in the postnatal growth often leading to developmental programming. The resultant changes in phenotypes can enhance susceptibility to diseases in adult offspring (Symonds and Budge, 2009; Williams et al., 2014) such as type 2 diabetes (Jones and Ozanne, 2009; Portha et al., 2011), hypertension (Ingelfinger and Nuyt, 2012) and obesity (Parlee and Macdougald, 2014). Recently, the concept has been broadened and encompasses the effect of maternal nutrition on pre- and postnatal on offspring reproductive capacity (Chadio and Kotsampasi, 2014; Chavatte-Palmer et al, 2014; Zambrano et al., 2014)

So far, the majority of data refer to effects of maternal undernutrition imposed during gestation (Lea et al., 2006; Mossa et al., 2009), whereas only few studies have examined the impact of maternal overnutrition on reproductive organs. For example, recent data in rabbits showed that maternal high-fat diet induced follicular apoptosis in their offspring, regardless of post-natal diet (Leveillé et al., 2014). Maternal high-fat nutrition in rats resulted in early pubertal onset (Sloboda et al., 2009; Connor et al., 2012). Considering these previous results, it is reasonable to speculate that the maternal overnutrition during mid and late-gestation impact on fetal pituitary-gonadal development.

Furthermore, nutritional status between birth and puberty have reported impacts on mammary gland development and future milk production (Radcliff et al., 2000; Lohakare et al., 2012). Increased pre-pubertal average daily gaind (ADG) more than 0.7 kg/d can result in decreased mammary parenchymal growth (Sejrsen et al., 1982, 2000; Capuco et al., 1995) milk yield production (Sejrsen and Purup, 1997; Petitclerc et al., 1999; Radcliff et al., 2000) and increased fat deposition in the mammary gland (Swanson, 1960; Radcliff et al., 1997; Meyer et al., 2006). The mechanisms whereby elevated nutrient intake cause this effect remain unclear. Previously some authors claimed that alterations on GH and leptin hormones is involved as mediator of nutritional-impaired prepubertal mammary parenchyma development (Sejrsen et al., 1983; Silva et al., 2002). However, early evidences indicates that mammary-derived factors can be more important (Akers et al., 2000; Purup et al., 2000a, b). Moreover,

because the GH-IGF axis is essential for normal growth and mammary development they likely mediates tissue growth responses to nutrient intake (Breier, 1999; Purup et al., 2000a,b). Some components of this axis was implicated as mediators of inhibited mammary growth in heifers fed elevated levels of nutrients (Weber et al., 1999, 2000; Berry et al., 2003), but not in another (Meyer et al., 2007).

Overall, the objectives of this present study were to evaluated the effects of nutritional environment during pre natal and pre pubertal period in dairy cattle, and give more insights about that the factors that drives the onset of puberty. The first chapter, provided evidences a new insight about the effects of maternal overnutrition on gonadal development and pituitary-gonadal gene expression in cattle fetuses at mid and late gestation. In the second chapter, we aimed to evaluate the effects of increasing nutrient intake level on hormone concentrations, expression of lipogenic marks (CD36, ACCA, FASN, ADIPOR1) in the parenchyma, and the relationship between liver GHR, IGF1, IGFBP2 and IGFBP3 gene expression with prepubertal bovine mammary gland development. In the third chapter, we aimed to evaluate the changes in specific intraovarian factors and their association with onset of puberty in heifers.

CHAPTER I

(Article I)

Interpretative summary

Maternal nutrition changes gene expression, ovarian and testicular development in cattle fetus

Weller

This study has shown that higher maternal nutrition can influence fetal ovarian follicular numbers and alter testicular structure which in turn may affect their reproductive performance after birth. Higher levels of feeding also altered the expression of key genes involved in ovarian folliculogenesis, steroidogenesis, apoptosis and growth factors. The implication of these results is that maternal nutrition may impact on offspring reproductive performance of dairy cattle and should be managed precisely.

EFFECT OF MATERNAL NUTRITION ON GONADAL DEVELOPMENT

Effect of maternal nutrition and days of gestation on pituitary gland and gonadal gene expression in cattle

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ABSTRACT

This study investigated effects of maternal overnutrition on gonadal development and pituitary-gonadal gene expression in cattle fetuses at mid and late gestation. Twenty-seven multiparous dry cows were fed either high (ad libitum, H) or moderate (M) intake of the same diet. Twelve cows from H (n=6) and M (n=6) intake carrying females fetuses were euthanized at 199 and 268 days of gestation (DG; n=3 for H or M on each DG). Fifteen cows from H (n=6) and M intake (n=9) carrying male fetuses were euthanized at 139, 199 and 241 DG (n=2 for H and n=3 for M on each DG). Fetal gonads and pituitary gland were sampled for gene expression and histological analyses. Sex-specific responses to maternal intake were observed. Primordial ($P = 0.045$) and total follicle ($P = 0.026$) numbers were lower in fetal ovaries from H than in M intake cows. These results were reverse for preantral ($P = 0.041$) and antral follicles ($P = 0.038$). Volumetric proportion ($P = 0.035$) and diameter ($P = 0.034$) of seminiferous cord were lower in fetal testis of H than M intake cows. The expression level of FSHB ($P < 0.05$) was greater in pituitary gland of female fetus from H compared to M intake cows, irrespective of DG, whereas LHB gene expression did not differ. In males, FSHB and LHB gene expression levels were similar between maternal intake groups. Fetal ovarian expression of P450 aromatase, StAR, BMPR2, TGFBR1, GDF9, FSHR, Bax and CASP3 genes were higher ($P < 0.05$) in H than in M intake cows, irrespective of DG. Fetal testicular expression of StAR, HSD17B3, IGF1, IGF2 and IGF1R genes were higher ($P < 0.05$) in M than in H intake cows. The differences in gene expression for steroidogenesis, folliculogenesis and apoptosis may explain the distinct pattern of follicular growth between M and H intake cow´s offspring. By contrast, the lower volumetric proportion, diameter and length of seminiferous cord may relate to decreased gene expression in fetal testis from H intake cows. In conclusion, maternal H intake seems to affect fetal ovarian follicular growth and number of follicles which may impact on size of ovarian reserve in their offspring. In male fetus, maternal H intake seems to disturb testicular development and may have implications on sperm production. The underlying mechanism of differential gene expression and the impact on offspring reproductive potential should be the focus of further research, especially considering larger sample size, reducing the chance for type I errors.

Key words: Bovine species, fetal development, overnutrition, real-time PCR, reproduction

INTRODUCTION

Maternal nutrition status has been considered as a major cause of developmental programming. The resultant changes in phenotypes can enhance susceptibility to diseases in adult offspring (Symonds and Budge, 2009; Williams et al., 2014) such as type 2 diabetes (Jones and Ozanne, 2009; Portha et al., 2011), hypertension (Ingelfinger and Nuyt, 2012) obesity (Parlee and Macdougald, 2014) and alterations in offspring reproductive function (Chadio and Kotsampasi, 2014; Chavatte-Palmer et al, 2014; Zambrano et al., 2014)

Many studies reported detrimental effects of maternal undernutrition on offspring reproductive development. In rats, female offspring of undernourished mothers had reduction in ovarian follicular number and mRNA levels of FSHR, GDF9, ER, and CYP17A1 genes (Bernal et al., 2010). In sheep, maternal undernutrition delayed follicular development (Rae et al., 2001), increased the expression of apoptotic genes in fetal ovary (Lea et al., 2006), and increased the expression of StAR in male sheep fetuses (Rae et al., 2002). Moreover, heifers born to nutritionally restricted dams had 60% reduction of mean antral follicular count during follicular waves (Mossa et al., 2009). In contrast, the effects of maternal overnutrition on offspring reproductive organs have been less studied. Early puberty has been reported in rodents and rabbit offspring with prolonged or persistent estrus (Sloboda et al., 2009; Connor et al., 2012; Leveillé et al., 2014) and reduction in the number of primordial and total follicles in sheep fetuses (Da Silva et al., 2003). In males, maternal high-fat diet resulted an increased testicular and sperm oxidative stress leading to decrease of fertility in rats (Rodríguez-Gonzáles et

al., 2015). Moreover, Long et al., 2012 reported that maternal obesity altered hypothalamic-pituitary-adrenal function in rat offspring.

Considering these previous results, we hypothesized that the maternal overnutrition during mid and late-gestation impact on fetal pituitary-gonadal development. The objectives of this present study were to determine (1) whether maternal overnutrition alters gonadal germ cell development; and (2) whether maternal overnutrition affects gonadal and pituitary gland gene expression in cattle fetuses at mid and late gestation. In this context, we examined fetal pituitary gonadotropin gene expression and gonadal expression of candidate genes. In female fetuses, we evaluated the expression pattern of key genes involved in ovarian folliculogenesis (ESR-α, FHSR, BMPR2, GDF9, TGFBR1, and TGFB1), steroidogenesis (StAR and P450arom) and anti- or pro-apoptotic factors (Bax, Bcl2, and CASP3). In male fetuses, we evaluated the expression pattern of key genes involved in testis development and function (AR, LHCGR, IGF1, IGFR1, IGF2, StAR and HSD17B3), as well as apoptotic factors (Bax, Bcl2, and CASP3).

MATERIAL AND METHODS

Management, handling and euthanasia of animals were approved by the Institutional Animal welfare and Ethics/Protection commission (certificate number 47/2012) from the Universidade Federal de Viçosa, Brazil. This research is part of a larger project titled “Nutrient requirements of Holstein-Gyr pregnant dairy cattle”.

Experimental design and Animal management

Unsuckled multiparous crossbreed (Holstein x Gyr) cows (n = 62) had their estrus cycles synchronized for the experiment. Estrus synchronization protocol used a vaginal progesterone insert (CIDR, Zoetis, Madson, NJ) for 7 d and with a PGF2α injection (25 mg, Lutalyse, Zoetis, Madson, NJ) was administered at CIDR removal. Cows were monitored for estrus every 12 h and were artificially inseminated using semen from a single bull 12 h after the onset of estrus. On day 40 of gestation, cows were housed in 30 m2 individual pens, of which 8 m2 were covered with concrete flooring. Pens had individual feed bunks and an automatic water system. On day 55 of gestation, the pregnancy was verified, fetal sexing was performed by using transrectal ultrasound (Aloka 500 with a 5-MHz linear probe Aloka, Wallingford, CT), and 27 pregnant cows were randomly selected from the 62 for this experiment. The nutritional regimes were provided from day 60 of gestation until cows achieved their previously designated days of gestation (DG), when they were euthanized for sampling.

Twenty-seven multiparous dry cows with an average initial body weight (BW) of 480 ± 10.1 kg and an age of 5 ± 0.5 years old were fed either high (H, ad libitum) or moderate (M) intake of same diet, during their gestation. Moderate level was designed to meet 100% of requirements for maintenance which was considered to be 1.15% of BW (in dry matter basis) as reported by our research group before Rotta et al. (2014). Every 28 d, cows were weighed in the morning before feeding and after a 16 h fast to obtain the shrunk BW. The shrunk BW was estimated after collecting all of the feed in the feeders, 16 h before weighing, in order to standardize the BW. Feed intake was adjusted based on values of shrunk BW in order to maintain moderate level throughout the entire

gestation period. High level was designed to meet 190% of requirements for maintenance as reported by our research group Rotta et al. (2014). Cows fed Ad libitum (H) were considered obese in this current study; because H cows had an average daily gain about 3.8 times greater than M intake cows. Also cows fed Ad libitum had an increased rib fat thickness during gestation, whereas M intake cows had a constant amount of rib fat thickness (for details on weight and fat measurements see Rotta et al., 2014). All cows were fed corn silage and concentrate based diet at a ratio of 93:7 on a DM basis as a total mixed ration twice daily, with 60% of the amount offered in the morning and 40% in the afternoon feeding (dry matter basis). All cows had ad libitum access to water. The composition of the experimental diet is presented in supplementary data (TS1).

To evaluate the effects of different days of gestation (DG) on pituitary-gonadal developments of female and male fetuses, pregnant cows were euthanized at selected time points. Six cows from either H or M intake carrying female fetuses were randomly divided for euthanasia at 199 and 268 days of gestation. The four groups of treatments therefore comprised of: H-199DG, M-199DG, H-268DG and M-268DG with 3 cows for each treatment. Six H and nine M intake cows carrying male fetuses were randomly divided for euthanasia at 139, 199 and 241 DG. The six groups of treatments thus comprised of: H-139DG, M-139DG, H-199DG, M-199DG, H-241DG and M-241DG with 2 cows from H and 3 cows from M intake for each DG.

Time points set for euthanasia represent important windows of gonadal development in male and female fetuses. We evaluated the female fetuses at 199 and 268 DG to represent two stages of ovarian fetal development. The initial folliculogenesis and steroidogenesis occur at 130 to 140 DG, the greater wave of apoptosis occurs at 120 to

150 DG and antral follicles are first seen in bovine fetal ovaries aged 230 DG (Erickson et al., 1966; Chavette-Palmer et al., 2014). Thus, at 199 DG initial follicullogenesis and apoptosis would have occurred, but no antral follicles would be present. In male fetuses, we evaluated at 139, 199 and 241 DG because the respective time points represent the mid and late stage of gestation where testicular architecture becomes more differentiated, the proliferation of pre-espermatogonia arrest at day 200 DG (Wrobel 2000) and from 210 to 285 DG the interstitium greatly expands while the number of fetal Leydig cells are reduced (Abd-Elmaksoud, 2005). Therefore, we hypothesized that the maternal nutrition could affect these important events during ovarian and testicular development.

Euthanasia procedures were performed on the same calendar day for each DG. Feed was withheld overnight, but cows had ad libitum access to water. Cows were euthanized at Universidade Federal de Viçosa by stunning with a captive bolt and subsequent exsanguination. The gravid uterus was sectioned at the cervix, and the fetuses were immediately removed and weighed. As soon as possible after euthanasia, the reproductive organs and pituitary gland of fetuses were removed and the reproductive organs weighted.

The left ovaries and testicles from the fetuses were promptly dissected, weighed and immersion fixed in Bouin's solution for 24 h and then stocked in ethanol 70ºC. The right ovaries, testicles and pituitary were immediately immersed in 10 ml of RNAholder (BioAgency, São Paulo, Brazil) to protect and conserve the expression profiles of these tissues. After 16 hours, the collected material were removed from RNAholder, drained and stored in an ultra-freezer (-80ºC) until RNA extractions were carried out.

Gonadal histology

Left ovaries and testis were sliced crosswise in 3 mm thick blocks, which were dehydrated in increasing concentrations of ethanol (70ºC, 95ºC and 100ºC), cleared in xylene and embedded in paraffin wax. Paraffin embedded blocks were sectioned serially at 5 µm thick slices and stained with Haematoxylin–Eosin for examination of germ cell development. An average of 30 gonadal sections was examined per fetus. After qualitative evaluation of the slides, images of stained ovarian sections were retrieved from a video camera linked to light microscope (EVOS® XL Core Cell Imaging System, life technologies) at magnification of 40 X and these images were analyzed with ImageJ® 1.48 software (National Institutes of Health, Bethesda, Maryland, USA). The width of the cortical layer was measured at x 20 magnification.

Twenty randomly selected fields per section were examined in each fetal ovary. Ovarian structures were classified as primordial follicles (germ cells surrounded by flattened follicular cells); primary follicles (enlarged oocyte completely surrounded by one to two layers of cuboidal follicular cells); preantral follicles (enlarged oocyte surrounded by 2 or more layers of granulosa cells with no antrum) and antral follicles were distinguished by the presence of an antrum within the granulosa cell layers enclosing the oocyte as described by Cheng et al., (2002). Only primordial, primary, preantral and antral follicles with spherical nucleus which were visible in the section were counted (Figure 1). The number of each type of follicle was corrected according to

the nuclear diameter and thickness of the cut, as described by Gougeon and Chainy, (1987) and the following formula:

Where: dm = nuclear diameter.

After qualitative evaluation of the slides, images of testicular parenchyma were retrieved from a video camera linked to light microscope (EVOS® XL Core Cell Imaging System) and were analyzed with Image J®1.48 software (National Institutes of Health) at magnification of 40 X (Figure 2). Testicular volume of bovine fetuses was equal to fresh left testis weight (g), assuming testicular density is 1 (Russell et al. 1990). The volumetric proportion of testicular parenchyma components was measured by counting points. Twenty randomly selected fields per fetal testis were used. Briefly, a grid of 450 evenly distributed test points was superimposed over the screen image of every microscopic field studied. In each fetus, the number of test points overlying the component of interest (seminiferous cords or interstitium) was counted. The volumetric proportions, described in percentages, were calculated based on a total of 9000 points per fetus.

The diameter of the seminiferous cords was estimated as average value of measuring two perpendicular diameters (maximum and minimum) from 30 randomly chosen cross-sections of seminiferous cords per testis. Seminiferous cords were assumed to be cylindrical and their lengths were estimated as described by Marshall and Plant, (1996) in the following formula:

V_s = (volumetric proportion of seminiferous cords) x (testicular volume)

Where D_s is the diameter of the seminiferous cords.

Quantitative real-time Transcription PCR analysis

Total RNA was isolated from 50 mg of right ovaries and testicles by using RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. Additional treatment with DNase was performed on the columns using the RNase-free DNase Set (Qiagen), according to the manufacturer's recommendations. RNA concentrations were checked by NanoVue Plus Spectrophotometer (GE Healthcare) with an optimal 260/280 ratio between 1.8 and 2.1. Intact 28S and 18S rRNA subunit integrity was assessed by agarose gel electrophoresis to detect degradation of the RNA.

Reverse transcription was performed using GoScript Reverse Transcription System (Promega) and oligo (dT) primers (Invitrogen, Carlsbad, CA). The cDNA concentrations from the samples were estimated on a NanoVue Plus spectrophotometer (GE Healthcare). Finally, the cDNA samples were stored at –20ºC for analysis.

The gene target and reference sequence was recovered from *Bos taurus* nucleotide sequences obtained from the GenBank database. The following genes were evaluated in fetal ovary: *ESR-α, FHSR*, *BMPR-2, GDF-9, TGFβR1, TGFβ1, Bax, CASP3, Bcl2, StAR* and *P450arom*. In fetal testis, samples were evaluated for the following genes: *AR, LHCGR*, *IGF1, IGF1R, IGF2, Bax, Bcl2, CASP3, StAR* and *HSD17β3*. In fetal pituitary samples, *FSHB* and the *LHB* genes were analyzed. These genes´ sequences were used to construct primers by the PrimerQuest program provided by Integrated DNA Technologies, Inc (IDT, Coralville, IA). *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) and *B2M* (beta-2-microglobulin) genes were used as the reference gene for normalization in pituitary and gonadal tissues, respectively. These genes had the lowest gene-stability measure (M) compared to β-actin and ubiquitin C

using geNorm program (Vandesompele et al., 2002). *GAPDH* and B2m had M = 0.3 versus β-actin and Ubiquitin C with M=0.8. The primer pairs of each target and reference genes are listed in Table 2.

Quantitative real-time PCR (qRT-PCR) reactions were performed in different wells and in duplicates using SYBR Green detection with GoTaq PCR Master Mix (Promega) following the manufacturer's instructions in an ABI Prism 7300 Sequence Detection System thermocycler® (Applied Biosystems). The reaction consisted of an initial step at 95ºC for 10 minutes, a second step of 40 cycles with the same temperature for 15 seconds and a final extension step at 60ºC for 60 seconds. After the amplification cycles, an additional gradient step from 60ºC to 95ºC was used to obtain a melting curve. The measurement in qRT-PCR experiment is expressed in cycles to threshold (C_t) of PCR; a relative value that represents the cycle number at which the amount of amplified cDNA reaches the threshold level. Prior to quantification by qRT-PCR, the amplification efficiency and optimal primer concentration was determined for each gene using serial dilution of cDNA. The PCR efficiencies for all primers pairs were obtained using the formula $E = 10^{(-1/slope)} \times 100$, where E is efficiency and *slope* is the gradient of dilution series and are summarized in Table 2.

Statistical Analysis

The experimental design was a completely randomized with a 2 x 2 (female fetuses) and 3 x 2 (male fetuses) arrangement of treatments. Fetal BW, the weight of their reproductive organs and histological data were analyzed by using the GLM

procedures (SAS Inst. Inc., Cary, NC). Day of gestation, maternal intake and their interaction were included as fixed effects in the model. Means were compared by the test of Tukey to the level of 5%.

Statistical analysis of C_t data for each fetal sex was realized using %QPCR_MIXED macro [https://www.msu.edu/~steibelj/JP files/QPCR.html] developed to generate codes in SAS PROC MIXED suitable to analyze data from qRT-PCR, assuming independent random effects for reference and target genes in each biological replicate (Steibel et al., 2009). This statistical method is more accurate, powerful and flexible than existing alternatives for analysis of relative quantification qRT-PCR data (Steibel *et al*., 2009). The following model was used:

$$y_{gikr} = TG_{gi} + C_{gik} + D_{ik} + e_{gikr}$$

where, y_{gikr} corresponds to the C_t value obtained from the thermocycler software for the *gth* gene (reference or targets) from the *rth* well, which corresponds to the *kth* animal submitted to the *ith* treatment (days of gestation and maternal intake combination). TG_{gi} is the effect of the *ith* treatment on the expression of gene *g*; $C_{gik} \sim$ N(0,) is the gene-specific random effect of the *kth* animal; $D_{ik} \sim$ N(0,) is the sample-specific random effect (common to reference and target genes); and $e_{gikr} \sim$ N(0,) is the residual term.

Relative mRNA abundance (fold change) were estimated using the $2^{-\Delta Ct}$ method of Livak and Shmittgen (2001). For each target gene, the comparison of gene expression across treatments, inside each fetal sex, was performed by the CONTRAST statement of the GLM procedure using Student's t -test to the level of 5%. Relative mRNA expression for genes that exhibited differential expression across treatments were presented in box plots graphs (median, maximum, minimum and interquartile range). The entire statistical

results for each pairwise contrast of interest across treatments are shown in the supplementary data (TS2 and TS3). To validate our gene expression results facing the limited sample size, permutation test–based p-values (TS2 and TS3) were calculated using 1,000 resampling (Manly, 1997; Sun et al., 2014). These permutations were performed by shuffling Ct values from different treatments, maternal intake and days of gestation, separately within each gene. These analyses confirmed the consistence of our qRT-PCR results. Analogously, the mentioned permutation tests were also applied to fetal reproductive organs weight and histological data analysis (TS4) and have also confirmed the significant statistical results. Despite of that, the authors suggest the use of larger sample sizes in future research.

RESULTS

Fetal growth and gonadal development

Fetal and reproductive organs weights in females and males are presented in Table 2 and Table 3, respectively. Irrespective of the sex of fetuses, there was no effect of maternal intake on fetal or reproductive organ weights and no interaction between maternal intake and days of gestation (DG). In contrast, there was DG effect on fetal, reproductive organs, ovarian and testicular weights as expected due to normal fetal growth during gestation.

The estimated numbers of primordial and total follicles were lower (P = 0.045; P = 0.026, respectively) in fetuses from H than M intake cows (Table 2). There was no difference P = (0.497) between DG on number of primordial follicles. Interestingly, there

appeared to be a tendency for the number of primary follicles to increase (P = 0.062) with fetal age corresponding to DG. We did not observe interaction between maternal intake and DG on primordial (P = 0.620), primary (P = 0.951), preantral (P = 0.688) and antral (P = 0.551) follicle numbers (Table 2). Primary (P = 0.044), preantral (P = 0.041) and antral follicles (P = 0.038) numbers were higher in fetal ovaries derived from H intake cows, with no interaction between maternal intake and DG. Moreover, DG affected preantral (P = 0.036) and antral follicles (P = 0.048) numbers as these were higher at 268 DG.

Maternal nutrition affected diameter (P = 0.034), lengths (P = 0.042) and volumetric proportion of seminiferous cords (P = 0.035) but no effect of interaction between maternal intake and DG was detected. Diameter and length of seminiferous cord did not differ (P = 0.446; P = 0.524, respectively) with DG (Table 3). Moreover, volumetric proportion of seminiferous cords was decreased (P = 0.045) with DG whereas interstitium volumetric proportion was higher (P = 0.034) at 199 and 241 compared to 139 DG (Table 3).

Fetal pituitary LHβ and FSHβ gene expression

Maternal nutrition altered the expression levels of FSHB (P = 0.004) in fetal pituitary of females, whereas LHB gene expression did not differ (P = 0.18) with maternal intake. FSHB gene had greater (P < 0.05) values in fetal pituitary of females from H intake cows compared to M intake cows, at 199 and 268 DG (Figure 3). We

observed interaction (P = 0.008) between maternal intake and DG on FSHB gene expression in female fetuses. FSHB expression was higher (P < 0.01) at 268 than at 199 DG in female fetuses from both maternal intake groups (Figure 3).

FSHB gene expression levels were similar between maternal intake in fetal pituitary of males (P = 0.59), with effect of DG (P = 0.012) and no significant interaction between maternal intake and DG (P = 0.42). As observed for FSHB, no difference in LHB gene expression levels (P = 0.25) was detected between maternal intake groups, but there was (P = 0.001) an effect of DG. The expression level of FSHB and LHB were higher (P < 0.01) at 199 and 241 compared to 139 DG in male fetuses from both maternal intake groups (Figure 4).

Fetal ovary gene expression

Maternal nutrition altered ovarian P450arom, StAR, BMPR2, TGFBR1, GDF9, FSHR, Bax and CASP3 expression levels. Ovarian fetuses from H intake cows had higher (P < 0.05) expression of these genes compared to M intake cows, at 199 and 268DG (Figure 5). The gene expression levels of P450arom and StAR were higher (P < 0.01) at 268 than 199 days of gestation in both maternal intake groups. In contrast, CASP3 and Bax genes had greater (P < 0.001) values at 199 compared to 268 DG in both maternal intake groups (Figure 5).

Fetal testis gene expression

Maternal nutrition altered testicular expression of StAR and HSD17B3 genes. Fetuses from M intake cows had higher expression ($P < 0.01$) of these genes compared to H intake cows at 139 and 199 DG (Figure 6). As observed for expression of key steroidogenic enzymes, IGF1, IGF2 and IGF1R genes had greater expression in fetal testis derived from M intake than H intake cows, irrespective of DG. Moreover, testicular expression of IGF1, IGF2 and IGF1R were higher at 139 than at 199 DG and at 241 than at 199 DG, in both maternal intake groups (Figure 6).

DISCUSSION

Maternal nutrition has been considered as a major cause of developmental programming (Chavatte-Palmer et al., 2014; Willian et al., 2014). To our knowledge, the current study is the first to provide evidences regarding the effects of maternal overnutrition on pituitary-gonadal gene expression and gonadal germ cell development in cattle fetuses at mid- and late gestation. It is noteworthy, however, that small sample size was a major limitation of this study. Over nutrition during gestation resulted in up-regulation of genes involved in ovarian folliculogenesis, steroidogenesis and pro-apoptosis. Moreover, we observed a reduction in the estimated numbers of primordial and total follicles and increased primary, preantral and antral follicles in fetuses from H intake cows compared to M intake. In contrast, we observed down-regulation of steroidogenic and growth factors genes in male fetuses from H intake cows compared to M intake. This down-regulation of gene expression was accompanied by a decrease in diameter, length and volumetric proportion of seminiferous cords.

The effects were not accompanied by changes in fetal body weights, reproductive organ and gonad weights, since weights were not affected by maternal nutrition and reinforce the concept that maternal nutrition can affect fetal development independently of changes in fetal weight or in organ mass (Harding and Johnston, 1995; Hawkins et al., 2000). The effects might persist until adulthood according to literature evidence in other species (Bernal et al., 2010; Connor et al., 2012; Guzmán et al., 2014) and should be the focus of future research in cattle. Our data point to distinct effects of maternal overnutrition according to the fetus´ sex.

Although we did not measure fetal or maternal endocrine profiles, it is likely that the availability of nutrients, insulin and IGF-I is high in the overnourished cows' circulation would result in a higher fetal nutrient supply with subsequent changes, not only in fetal hormones levels (e.g insulin, IGF-I) but also in metabolic pathways, which in turn could alter gene expression in fetal gonads. Indeed, animal studies have shown that maternal overnutrition alters fetal endocrine status (Zhang et al., 2011; Seet et al., 2015) and gene expression (Bruce et al., 2009; Tong et al., 2009). In humans, maternal obesity is associated with alteration in the homeostasis of cytokines and adipokines, such as adiponectin (Schmatz et al., 2010). Adiponectin influences placental transfer of nutrients and placental insulin-stimulated amino acid uptake in pregnancy (Aye et al., 2013). Moreover, in animal models, Tenenbaum-Gavish and Hod (2013) reported that adiponectin administration causes down regulation of activity and expression of key placental nutrients transporters; it also binds to the adiponection receptor – 2 on the trophoblast cell and activates p38 MAPK and PPAR-α, which inhibits the insulin/IGF-I signaling pathway. Overall, the effects of overnourished cows on fetal pituitary-gonadal gene expression and development should be further investigated.

Effects of maternal nutrition on fetal pituitary gland

In our study, maternal H intake was associated with enhanced expression of FSHB gene in the pituitary gland of female fetuses. We observed expression FSHB was higher at 199 and 268 DG in female fetuses from H intake cows, which indicates that the FSHB expression may be affected by timing of maternal over nutrition. This supports the study by Rhind et al. (2001), which showed development of the hypothalamic-pituitary axis and the synthesis of gonadotropins can be sensitive to pre-natal nutrition in sheep fetus. By contrast, the expression of FSHB in the pituitary gland of male fetuses in our study was similar between H and M intake groups which suggest that gonadotroph cells of male and female fetuses respond differently to maternal nutrition. There was no effect of maternal intake on LHB gene expression in both fetal sexes, which suggests that this hormone is not involved in the observed nutritionally induced changes in ovarian and testis development during the gestational period evaluated.

Effects of maternal nutrition on fetal gonads

Maternal over nutrition during mid and late gestation disturbs ovarian follicular growth and number of follicles with an increment in the number of primary, preantral and antral follicles and reduction in the estimated number of primordial and total follicles, regardless of DG. These changes may be underpinned by observed alterations in the expression levels of key genes involved in folliculogenesis (BMPR2, TGFBR1, GDF9 and FSHR), steroidogenesis (P450arom and StAR) and apoptosis (Bax, CASP3).

Increased ovarian FSHR and pituitary FSHB expression may also positively impact on preantral and antral follicular numbers. The hormone FSH and its receptor FSHR play an important role in follicle progression from primary to advanced stages of follicular development (Richards, 1994). Our results are consistent with previous studies where FSH and its receptor stimulated follicular growth in bovine fetuses' ovaries (Bao et al., 1997; Tanaka et al., 2001). The emerging hypothesis from our study is that maternal over nutrition may trigger increased pituitary expression of FSHB and increased ovarian expression of FSHR with consequence to folliculogenesis, observed as increased numbers of preantral and antral follicles.

The reduction in the total number of ovarian follicles at both DG in fetuses from H compared to fetuses from M intake cows can be largely attributed to a reduction in the number of primordial follicles. These results may reflect a decrease in proliferation and/or an increase in atresia of germ cells that begins at 60 DG and finishes between 150 to 170 DG, when the number of germ cells in the bovine ovary is fixed (Erickson, 1966). This hypothesis is supported by a previous study that reported maternal high-fat diet induced follicular apoptosis in rabbit's female offspring (Leveillé et al., 2014). Bcl2, its antagonist Bax and CASP3 are key regulators of apoptosis (Hussein, 2005), and they are expressed in mouse fetuses´ ovaries, suggesting their role in atresia (Nandedkar and Dharma, 2001). The CASP3 pathways seem to be one of the most important cell death mechanisms in human fetuses (Poljicanin et al., 2013). We observed increased Bax and CASP3 expression with no alteration of Bcl2 expression in fetal ovaries from H intake cows. The imbalance of anti and pro-apoptotic genes could explain the reduction of primordial and total number of follicles in fetuses from H intake cows. Previous studies reported that in rats (Yoon et al., 2002) and humans (Kugu et al., 1998) increased Bax

expression was correlated with follicular atresia. Moreover, studies in mouse fetal ovary showed that apoptosis of germ cells is correlated with increased levels of Bax in the absence of any change in Bcl2 levels (Felici et al., 1999). It is possible that the maternal overnutrition could lead to oxidative stress resulting in greater loss of germ cells and follicles during gestation, which in turn may reduce ovarian reserve and subsequent fertility in their offspring.

Genes of TGFB superfamily are essential growth factors that regulate the mammalian folliculogenesis (Paradis et al., 2009; Piotrowska et al., 2013). The TGFB1 expression did not differ between the maternal intake groups and DG. However the differential expression of GDF9 is likely to underpin the observed differences in follicular growth. Higher numbers of follicles in advanced stages of follicular growth were observed in H intake cows. In agreement, previous studies have shown that GDF9 is required for primordial to primary transition in bovine and rat follicular growth (Bodensteiner et al., 1999; Vitt et al., 2000). Also, GDF9 enhances the progression of early to late-stage follicular growth in rats (Nilsson and Skinner, 2002; Martins et al., 2008). We detected higher expression of BMPR2 and TGFBR1 genes in fetuses from H intake cows. These results indicate that these receptors could interact with GDF9 to stimulate the observed follicular growth, because GDF9 has its biologic effect by binding to TGFBR1 and BMPR2 on the membrane of target cells (Vitt et al., 2002; Mazerbourg et al., 2004). Collectively, these results corroborate the suggestion that the underlying mechanism of maternal overnutrition stimulus on fetal ovaries is the increased abundance of GDF9, BMPR2 and TGFBR1 that would lead to stimulate the folliculogenesis.

No changes in ERα expression levels were observed. However, the expression of the P450arom and StAR gene was higher in fetuses' ovaries from H than M intake cows. The enzyme StAR is a rate-limiting enzyme in steroidogenesis (Lin et al., 1995). The enzyme P450arom catalyzes the conversion of androgens to estrogens (Meinhardt and Mullis, 2002), and it is found in granulosa cells of primary, preantral and antral follicles in fetal bovine ovaries at 190DG (Burkhart et al., 2010). Up-regulation of the P450arom and StAR genes could be the factor in the increment of primary, preantral, and antral follicle numbers in fetuses from H intake cows. This observation corroborate early studies that reported estrogens regulated early follicular growth (Britt et al., 2000; Drummond et al., 2002) and have a direct effect on the number and size of ovarian follicles (Nayudu and Osborn, 1992; Hulshof et al., 1995).

Maternal over nutrition did not affect testis weight probably because not all types of testicular tissue were affected equally, as demonstrated by quantitative histological analysis. Volumetric proportion as well as length and diameter of seminiferous cords were lower, but interstitium volumetric proportion was higher in the fetal testis from H intake cows. Although we did not measure pre-Sertoli cell numbers, the lower seminiferous cords measurement could be related to lower numbers of pre-Sertoli cells, because in fetal testis the seminiferous cords contain only pre-Sertoli cells and one or two pre-spermatogonias (Orth, 1993). Sertoli cells proliferate before and after birth coordinating testicular development in primates (Plant and Marshal, 2001). Their numbers are highly correlated with adult testicular size and sperm production, and alterations of their development directly impact other testicular cells such as Leydig and fetal germ cells, leading to disorders in human adulthood (Sharpe et al., 2003).

The IGF-1 plays a major role in the proper development and function of the testis (Baker et al., 1996; Froment et al., 2004; Griffeth et al., 2014), and it is produced by Leydig, Sertoli, and germ cells; and IGF1R is found in the germ cells and somatic testicular cells in human and rats (Vannelli et al., 1988; Cailleau et al., 1990). Pitetti et al (2013) have reported that inactivation of IGF1R in mouse pre-Sertoli cells reduced its proliferation during late gestation, which resulted in 79% reduction in daily sperm production in adulthood. Our results suggest that lower expression of IGF1, IGF2 and IGF1R receptor genes in fetal testis from H intake cows could explain detrimental changes in testicular structure. Less seminiferous cord tissue could mean lower proliferation of pre-spermatogonia and pre-Sertoli cells, consistent with earlier studies in rats that reported IGF1 and IGF2 as regulators of germ cell proliferation and survival/maturation of Sertoli and Leydig cells during testis development (Sorder et al., 1992; Pitetti et al., 2013). Moreover, according to Hochereau-de Reviers et al. (1987), the diameter of seminiferous tubules in adults is highly correlated with the number of germ cells, so the lower seminiferous cords diameter observed in our study may impact on the number of germ and Sertoli cells per seminiferous tubule at postnatal age. Because each Sertoli cell has a fixed capacity for the number of germ cells that it can support (Orth et al., 1988), reduction of Sertoli cell numbers is likely to detrimentally impact offspring fertility. Up-regulation of IGFs may explain the increased expression of StAR and HSD17B3 genes. In vitro, studies have demonstrated that IGF1 influenced the expression of key steroidogenic enzymes in the mouse testis during prenatal development (Villalpando et al., 2003).

Effects of days of gestation on fetal growth, pituitary gland and gonadal development

As expected, fetal body weight and reproductive organs measured increased in weight during progress of gestation; greater masses were observed at 268 DG for female fetuses and at 241 DG for male fetuses. The greater number of primary, preantral and antral follicles with DG is consistent with Santos et al. (2013) that characterized folliculogenesis in bovine fetal ovary and revealed as fetal age increased, there was tendency for primary and secondary follicles to increase in number. In male fetuses, the volumetric proportion of seminiferous cords decreased during gestation; the greater value was observed at 139 than 199 and 241 DG, whereas interstitium volumetric proportion increased with DG. Moreover, diameter and length of seminiferous cords were similar during gestational period evaluated, consistent with reported by Abd Elmaksoud (2005) that shown an increase in testicular differentiation and size during mid and late gestation in bovine fetuses.

We observed an increased in FSHB gene expression with DG in both sexes, consistent with a previous study in bovine fetuses demonstrating a gradual increase in pituitary content of FSH for both sexes with DG (Workewych and Cheng, 1979). Furthermore, the expression level of LHB was increased with DG in male fetuses, but it was not differentially expressed in female fetuses. Our results differed from Workewych and Cheng (1979), who reported increase in pituitary content of LH for both sexes with DG.

The lower expression of Bax and CASP3 genes in female fetuses at 268 than at 199 DG, irrespective of maternal intake, are consistent with the tendency for the total number of follicles to decrease at 199 DG. These results are supported by Santos et al. (2013) and Erickson (1966), who reported waves of follicular degeneration occurring at this stage of gestation in bovine fetal ovary. Moreover, the higher expression of StAR

and P450arom genes at 268 than at 199 DG, irrespective of maternal intake could be explained by greater number of follicles at late stage that are secreting estrogen. As the follicles grow and differentiate, the production of estrogen increases (Fortune, 1994).

The greater expression of IGF1, IGF2 and its receptor at 139 than at 199 DG may reflect differences in the age of fetuses associated to stage of testicular development and also a stimulatory effect of these growth factors during testis development by increasing cell proliferation and differentiation which in turn increased testicular size. Similarly, the greater expression of IGF1, IGF2 and IGF1R at 241 than at 199 DG observed in our study could reflect an increase in connective tissue related to fetal growth (Abd Elmaksoud, 2005). Despite IGFs up-regulation, StAR and HSD17B3 expression were decreased at later stages of gestation and may reflect differences in steroidogenic capacity across stages of testicular development. In later stages of gestation, pre-Leydig cells are reduced due to regression or dedifferentiation to mesenchymal cells (Abd Elmaksoud, 2005).

CONCLUSIONS

This is the first study to demonstrate that maternal overnutrition during mid-late gestation might alter gonadal development and gene expression in cattle, for both male and female fetuses. Maternal overnutrition affected ovarian follicular growth and the number of follicles in female fetuses, which in turn may reduce their ovarian reserve and subsequent fertility. In male fetuses, testicular development was disturbed, which could have lasting effects on daily sperm production. The underlying mechanism seems to involve differential expression of multiple genes: P450 aromatase, StAR, BMPR2,

TGFBR1, GDF9, FSHR, Bax, CASP3, HSD17B3, IGF1, IGF2 and IGF1R. The functional meaning of the observed differential gene expression in terms of offspring reproductive potential should be the focus of further investigations, especially considering a larger sample size to reduce the chances of type I errors as the small sample size was a major limitation of this study.

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Table 2 - Effects of maternal intake and days of gestation on bovine female fetuses characteristics

	Maternal intake		Days of gestation		SEM[1]	P-value		
	Moderate	High	199	268		DG[2]	MI[3]	MI x DG[4]
Fetal weight (kg)	21.15	23.15	10.94^{B}	34.00^{A}	1.06	<0.001	0.480	0.495
Reproductive organ weight (g)	24.06	21.28	10.85^{B}	34.50^{A}	2.27	<0.001	0.558	0.217
Ovarian weight (mg)	299.83	326.67	257.33^{B}	369.17^{A}	0.08	0.041	0.712	0.543
Max cortical layer width (µm)	240.23	256.84	258.25	238.82	8.49	0.286	0.357	0.375
Min cortical layer width (µm)	200.86	218.84	218.11	201.59	5.25	0.154	0.124	0.170
Numbers of primordial follicles	332.56^{a}	220.90^{b}	216.92	229.94	27.59	0.497	0.045	0.620
Numbers of primary follicles	79.27^{b}	99.98^{a}	80.81	90.45	11.53	0.062	0.044	0.951
Numbers of preantral follicles	4.0^{b}	8.5^{a}	4.06^{B}	7.93^{A}	0.60	0.036	0.041	0.688
Numbers of antral follicles	1.50^{b}	5.80^{a}	3.5^{B}	6.2^{A}	0.80	0.048	0.038	0.551
Total number of follicles	417.33^{a}	332.88^{b}	305.29	334.52	28.46	0.062	0.026	0.877

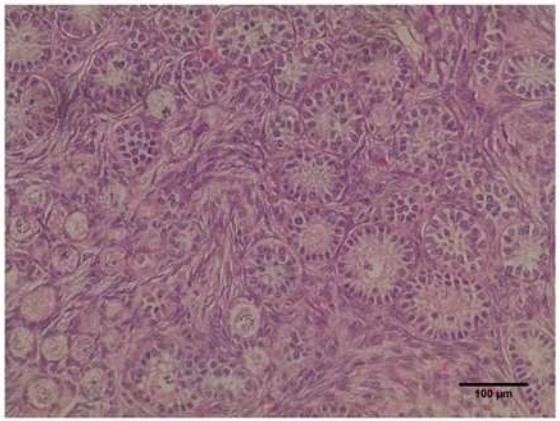
[1]Standard error of the mean. Different lowercase letters represent statistical difference ($P < 0.05$) between maternal intakes. Different uppercase letters represent statistical difference ($P < 0.05$) between days of gestation. [2]Days of gestation main effects. [3]Maternal intake main effects.[4]Interaction between maternal intake and days of gestation.

Table 3 – Effects of maternal intake and days of gestation on bovine male fetuses characteristics

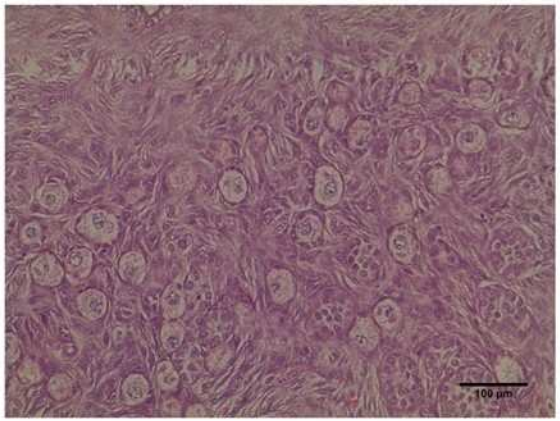
	Maternal intake		Days of gestation			SEM	P-value		
	Moderate	High	139	199	241		DG[1]	MI[2]	MI x DG[3]
Fetal weight (kg)	11.33	10.25	1.77^{C}	9.10^{B}	23.75^{A}	0.79	<0.001	0.397	0.654
Reproductive organ weight (g)	17.30	20.57	4.47^{C}	14.45^{B}	37.91^{A}	1.66	<0.001	0.342	0.607
Testicular weight (mg)	865.33	797.56	316.30^{C}	690.20^{B}	1487.8^{A}	0.08	<0.001	0.460	0.100
seminiferous cords (%)[4]	46.17^{a}	41.43^{b}	45.77^{A}	41.29^{B}	42.80^{B}	0.78	0.045	0.035	0.577
Interstitium (%)[4]	53.83^{b}	58.57^{a}	54.22^{B}	58.61^{A}	57.48^{A}	0.80	0.034	0.042	0.541
Seminiferous cords diameter (μm)	42.60^{a}	38.76^{b}	43.59	41.91	41.50	0.26	0.446	0.034	0.377
Seminiferous cords length (cm/g testis)	0.037^{a}	0.030^{b}	0.031	0.032	0.033	0.004	0.524	0.042	0.124

[1]Days of gestation main effects. [2]Maternal intake main effects. [3]Interaction between maternal intake and days of gestation. [4]%=volumetric proportion. Different lowercase letters represent statistical difference ($P < 0.05$) between maternal intakes. Different uppercase letters represent statistical difference ($P < 0.05$) between days of gestation.

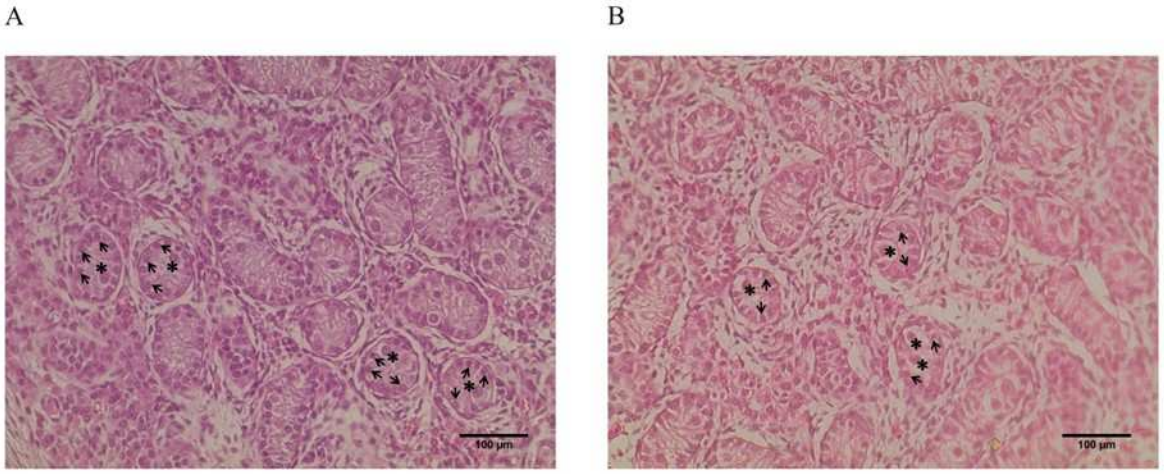
A



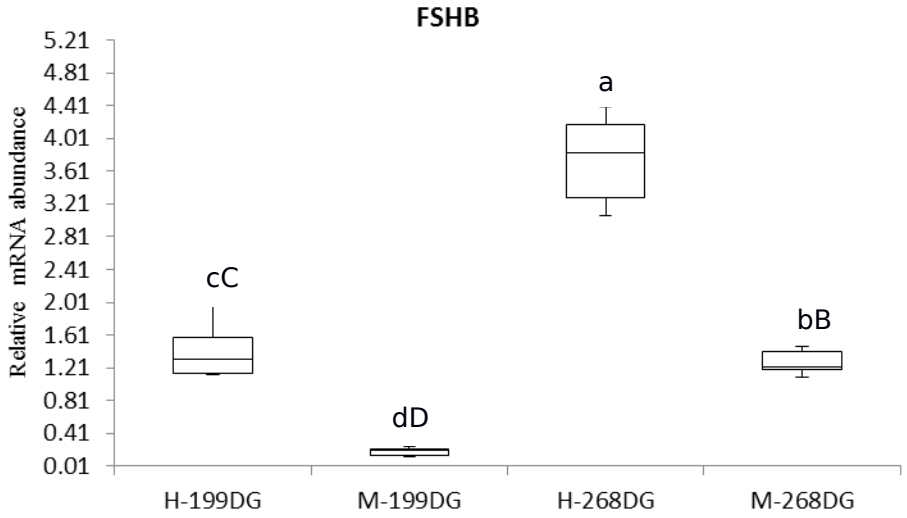
B



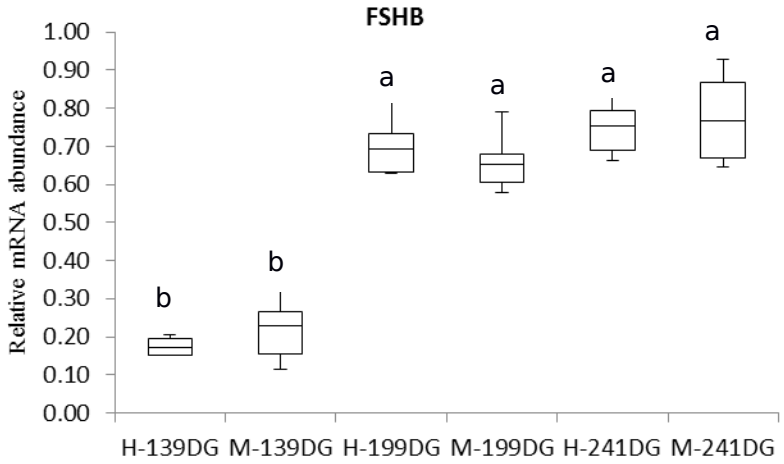
Weller Figure 1

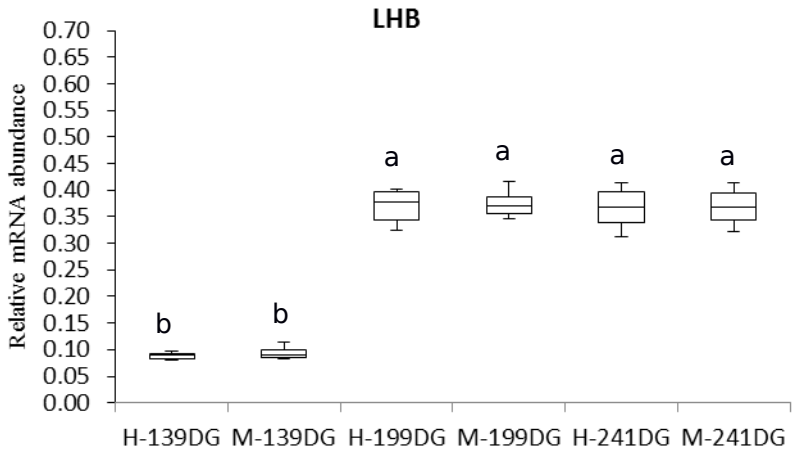


Weller Figure 2



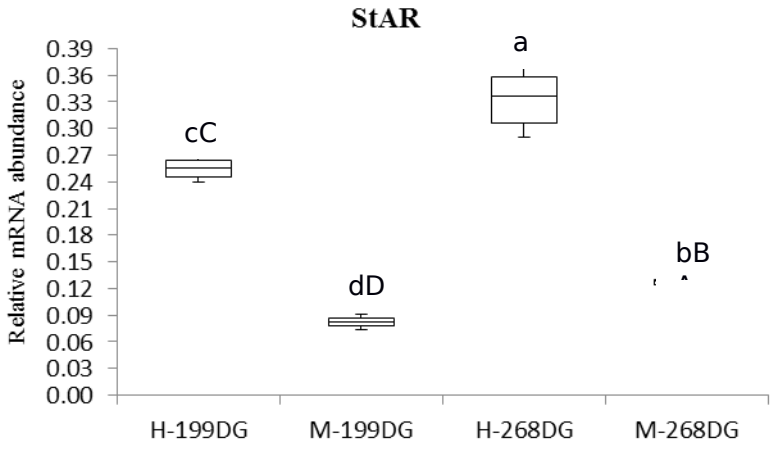
Weller Figure 3


FSHB
Relative mRNA abundance
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
a
a
a
a
b
b
H-139DG
M-139DG
H-199DG
M-199DG
H-241DG
M-241DG


LHB
Relative mRNA abundance
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
a
a
a
a
b
b
H-139DG
M-139DG
H-199DG
M-199DG
H-241DG
M-241DG

Weller Figure 4

A


StAR
Relative mRNA abundance
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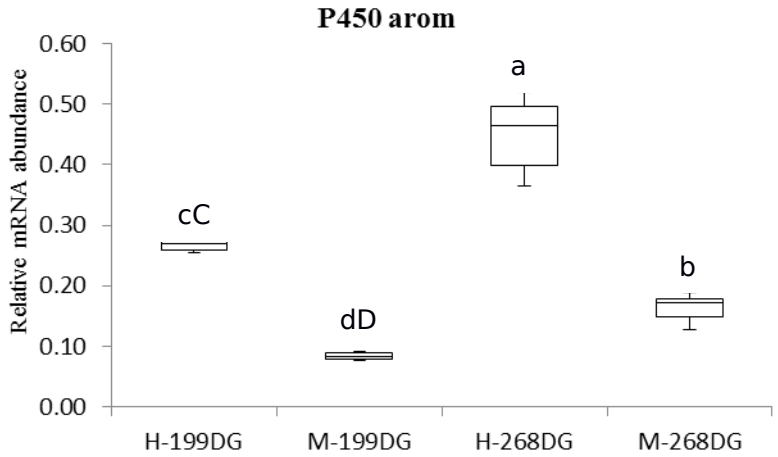
0.36
0.33
0.30
0.27
0.24
0.21
0.18
0.15
0.12
0.09
0.06
0.03
0.00
cC
dD
a
bB
H-199DG
M-199DG
H-268DG
M-268DG

B


P450 arom
Relative mRNA abundance
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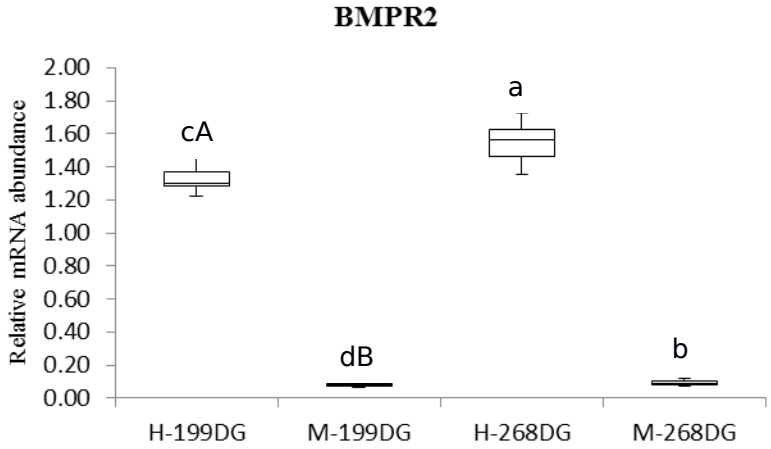
0.50
0.40
0.30
0.20
0.10
0.00
cC
dD
a
b
H-199DG
M-199DG
H-268DG
M-268DG

C


BMPR2
Relative mRNA abundance
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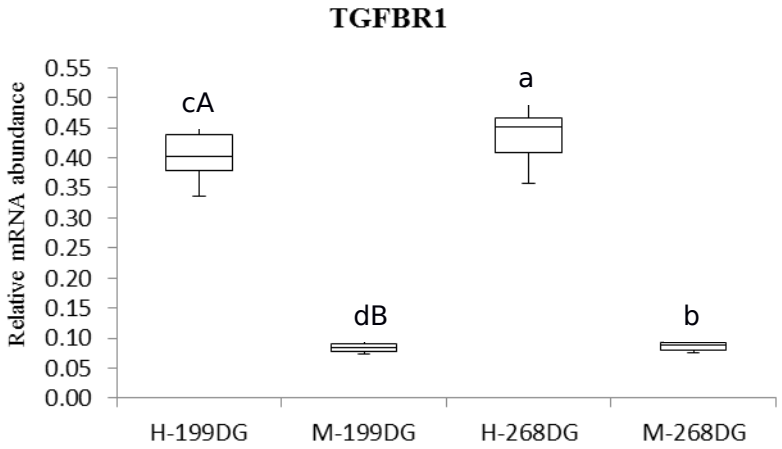
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
cA
dB
a
b
H-199DG
M-199DG
H-268DG
M-268DG

D


TGFBR1
Relative mRNA abundance
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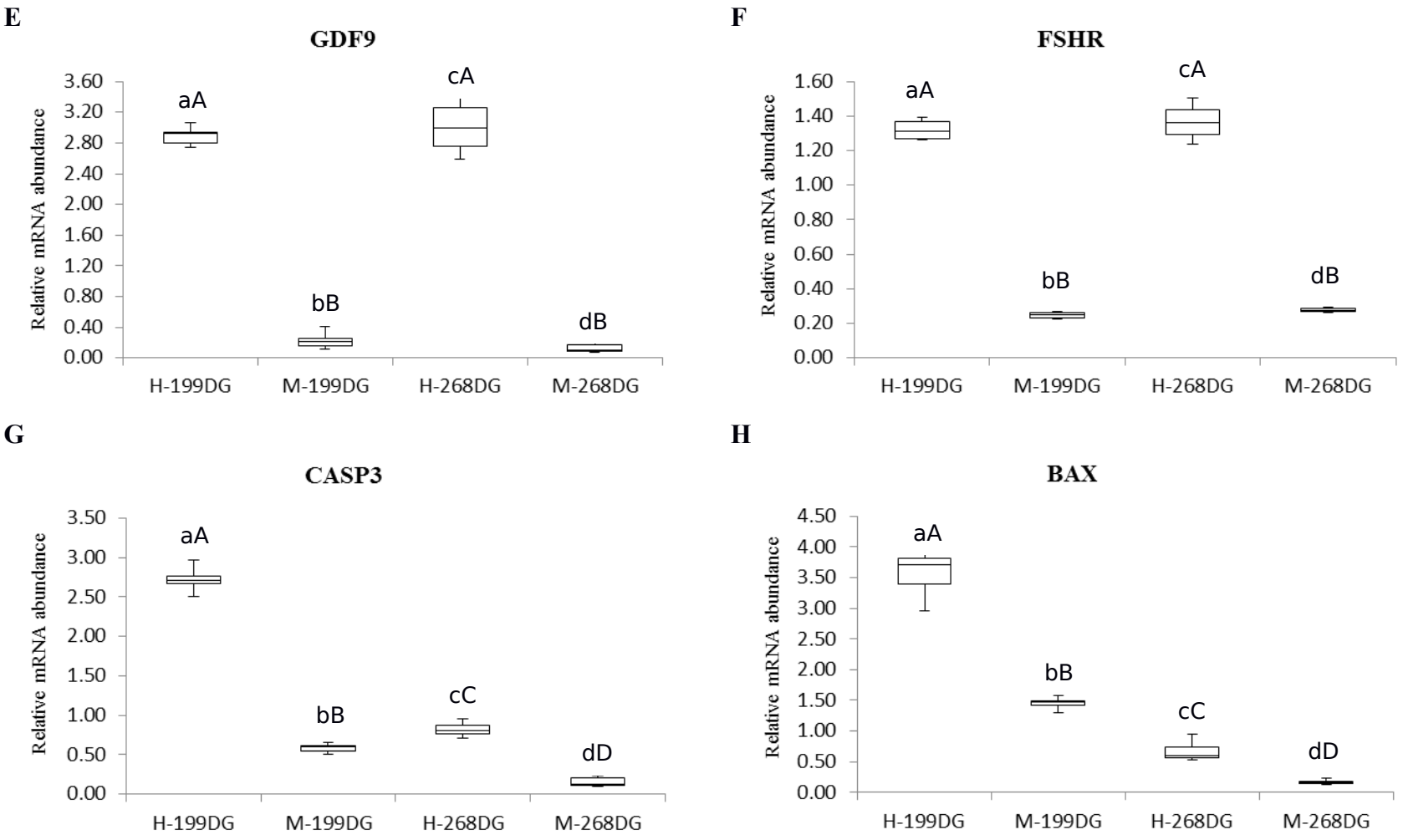
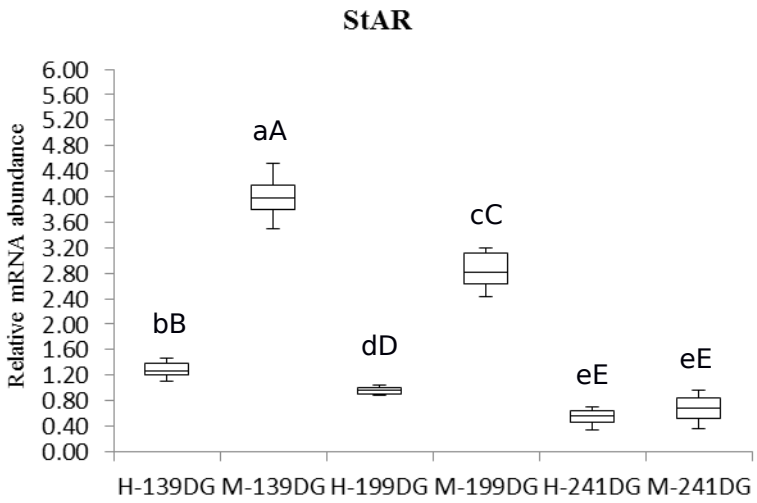
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
cA
dB
a
b
H-199DG
M-199DG
H-268DG
M-268DG



Weller figure 5

A


StAR
Relative mRNA abundance
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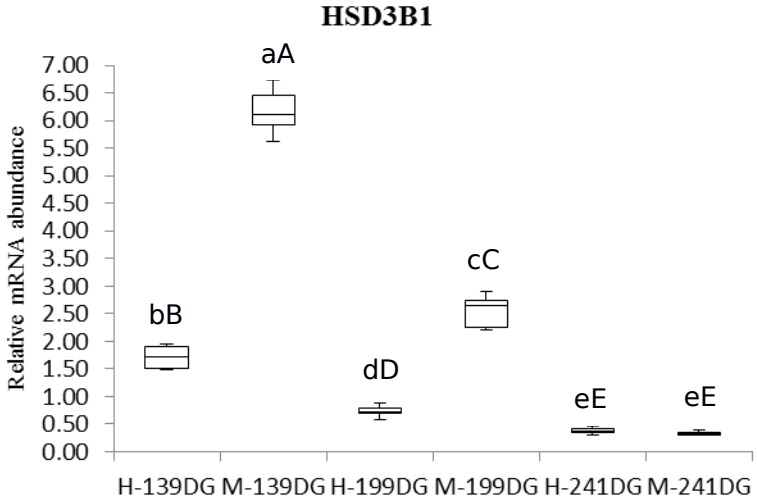
5.60
5.20
4.80
4.40
4.00
3.60
3.20
2.80
2.40
2.00
1.60
1.20
0.80
0.40
0.00
aA
bB
cC
dD
eE
eE
H-139DG M-139DG H-199DG M-199DG H-241DG M-241DG

B


HSD3B1
Relative mRNA abundance
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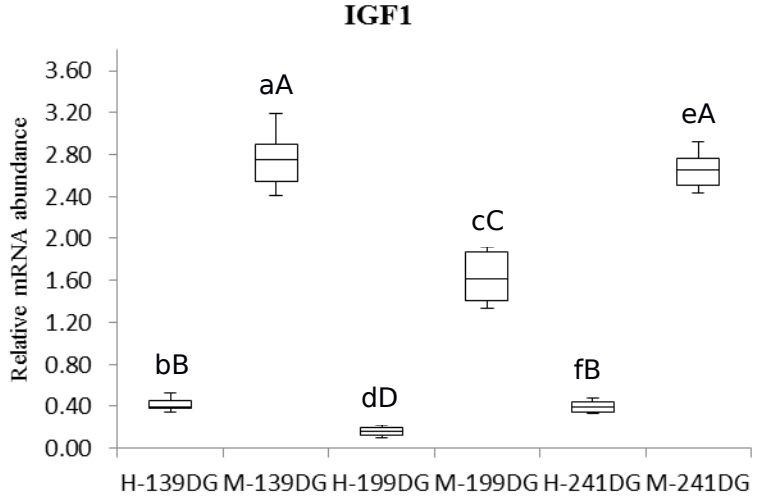
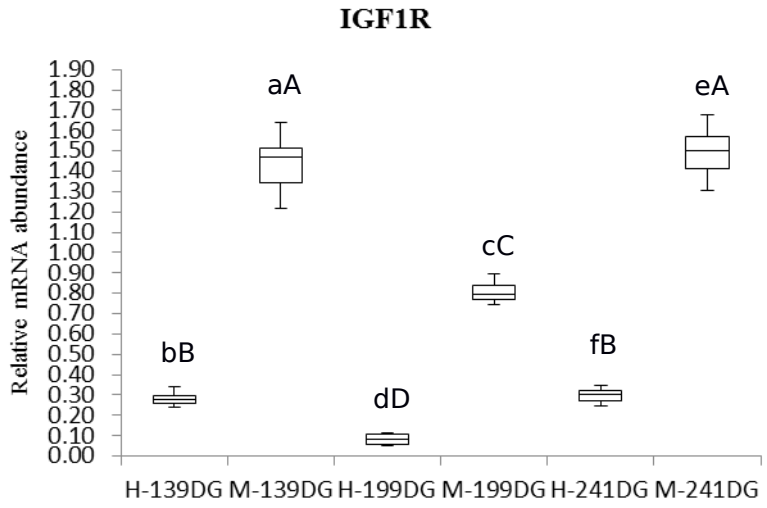
6.50
6.00
5.50
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
aA
bB
cC
dD
eE
eE
H-139DG M-139DG H-199DG M-199DG H-241DG M-241DG

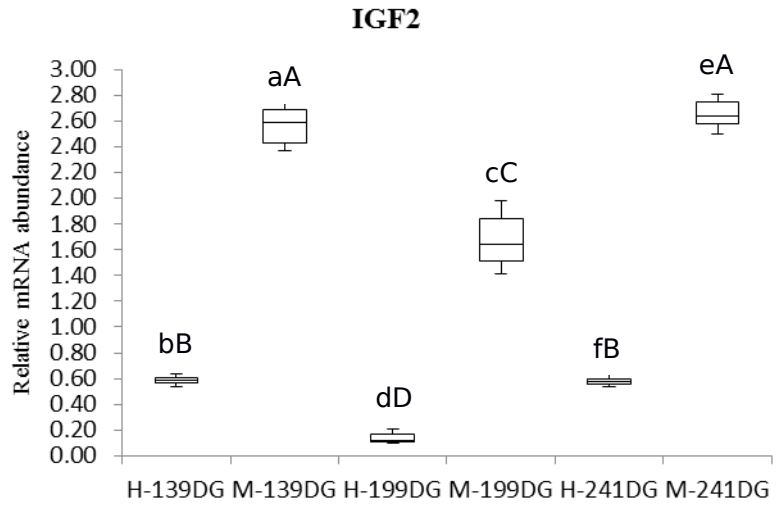
C


IGF1
Relative mRNA abundance
3.60
3.20
2.80
2.40
2.00
1.60
1.20
0.80
0.40
0.00
aA
bB
cC
dD
eA
fB
H-139DG M-139DG H-199DG M-199DG H-241DG M-241DG

D



E



Weller Figure 6

Figure 1. Fetal ovary of a representative bovine fetus from the (A) high and (B) moderate intake cows at 268 days of gestation.

Figure 2. Testicular parenchyma in a bovine fetus from high (A) and (B) moderate intake cows at 199 days of gestation. Each seminiferous cords is composed of pre-espermatogonia (*) centrally located in the middle of the cord and pre-Sertoli cells (arrows) located at the periphery.

Figure 3. Box plot for relative mRNA abundance of *FSHB* in pituitary gland of female fetuses from high (H) and moderate (M) intake cows at 199 and 268 days of gestation (DG). Different lowercase letters represent difference ($P < 0.05$) between maternal intakes within DG. Different uppercase letters represent difference ($P < 0.01$) between DG within maternal intake.

Figure 4. Box plot for relative mRNA abundance of *FSHB* and *LHB* in pituitary gland of male fetuses from high (H) or moderate (M) intake cows at 139, 199 and 241 days of gestation (DG). No difference between maternal intakes within DG, which were represent by lowercase letters. Different uppercase letters represent difference ($P < 0.01$) between DG within maternal intake.

Figure 5. Box plot for relative mRNA abundance of (A) *StAR*, (B) P450arom, (C) *BMPR2*, (D) *TGFBR1* (E) *GDF9,* (F) *FSHR*, (G) *CASP3* (H) *Bax* in fetal ovaries from high (H) and moderate (M) intake cows, at 199 and 268 days of gestation (DG). Different lowercase letters represent difference ($P < 0.05$) between maternal intakes

within DG. Different uppercase letters represent difference ($P < 0.01$) between DG within maternal intake.

Figure 6. Box plot for relative mRNA abundance of (A) *StAR*, (B) *HSD3B1*, (C) *IGF1*, (D) *IGF1R*, (E) IGF2 in fetal testis from high (H) and moderate (M) intake cows at 139, 199 and 241 days of gestation (DG). Different lowercase letters represent difference ($P < 0.05$) between maternal intakes within DG. Different uppercase letters represent difference ($P < 0.01$) between DG within maternal intake.

CHAPTER II

(Article II)

Interpretative summary

Nutrient intake level changes mammary parenchyma growth and gene expression in crossbreed prepubertal heifers

Weller

This study reported that elevated nutrient intake level by prepubertal heifers reduced mammary parenchyma (PAR) growth, changed its composition and increased mammary extraparenchymal fat. Higher expression of PAR lipogenic enzymes in response of augmented nutrient intake, suggesting adipocytes hypertrophy was also observed. These results will provide better understanding of prepubertal mammary gland development and in the future achieve optimal nutritional management for maximizing milk yield.

NUTRIENT INTAKE LEVEL AND MAMMARY GLAND DEVELOPMENT

Effects of nutrient intake level on mammary parenchyma growth and gene expression in prepubertal heifers

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ABSTRACT

This study was designed to evaluate effects of increased nutrient intake of same diet on prepubertal mammary parenchyma (PAR) development in crossbreed dairy heifers. Eighteen heifers between 3-4 mo old were fed 1 of 3 nutrient intake levels (n = 6/treatment) designed to sustain an average daily gain (ADG) as follows: 0.0 kg/d (maintenance; **MA**), 0.5 kg/d (low gain; **LG**), or 1.0 kg/d (high gain; **HG**). Serum blood samples collected on d 42 and 84 after a 12h fast were analyzed for triglycerides (TRI), leptin, insulin and insulin-like growth factor 1 (IGF1). Liver and mammary parenchyma (PAR) were biopsied on d 42 and harvested on d 84 for gene expression analysis. Also, PAR samples were used for biochemical and histological analysis. Mammary PAR weight was lower in HG than MA and LG heifers, whereas mammary extraparenchymal fat was greater in HG heifers than other groups. Heifers fed the HG had a greater fraction of lipids in their PAR than others, and smaller fraction of protein in their PAR than MA heifers. Nutrient intake had no effect on the number of intraparenchymal adipocytes. Only serum insulin and IGF1 were affected by nutrient intake level. Heifers fed the HG had greater serum IGF1 than others, and serum insulin was lower in MA than HG and LG heifers. The liver GHR, IGF1 and IGFBP3 mRNA was higher whereas IGFBP2 mRNA was lower in HG heifers compared to others. The expression of CD36, ACCA, FASN and ADIPOR1 were up regulated by nutrient intake level. Overall, our data demonstrated that enhancing nutrient intake favored to fat accumulation on body and resulted adipocyte hypertrophy in the PAR of HG heifers. The implications of these results to future milk yield remains to be elucidated.

Key words: dairy heifer, mammary gland development, lipogenic gene expression, nutrigenomics

INTRODUCTION

In prepubertal dairy heifers (3 to 10 mo of age), high levels of nutrient intake sustaining average daily gains (ADG) more than 0.7 kg/d can result in decreased mammary parenchymal growth (Sejrsen et al., 1982, 2000; Capuco et al., 1995) milk yield production (Sejrsen and Purup, 1997; Petitclerc et al., 1999; Radcliff et al., 2000) and increased fat deposition in the mammary gland (Swanson, 1960; Radcliff et al., 1997; Meyer et al., 2006). The mechanisms whereby elevated nutrient intake cause this effect remain undefined. Several hypotheses have been proposed to explain this phenomenon. Previous studies focused on hormones such as, growth hormone and leptin that may be involved as mediator of nutritional-impaired prepubertal mammary parenchyma development (Sejrsen et al., 1983; Silva et al., 2002). However, early evidences indicates that mammary-derived factors could be more important (Akers et al., 2000; Purup et al., 2000a, b). Piantoni et al. (2012) reported that doubling nutrient intake from standard feeding rates decreased expression of genes related to molecular transport and lipid metabolism in mammary parenchyma of preweaned heifers. Thus, this finding in preweaned heifers raises the hypothesis that elevated nutrient intake could also alter the expression of genes associated with lipogenesis.

The GH-IGF axis is essential for normal growth and mammary development and likely mediates tissue growth responses to nutrient intake (Breier, 1999; Purup et al., 2000a,b). Some components of this axis have been implicated as mediators of inhibited mammary growth in heifers fed elevated levels of nutrients (Weber et al., 1999, 2000; Berry et al., 2003). In other cases, specific components of the GH/IGF1 axis were not affected by level of nutrient intake (Meyer et al., 2007). These

conflicting results demonstrate the need for further evaluation of the role of GH/IGF1 axis in the development of prepubertal bovine mammary gland.

This study addresses the hypothesis that increased nutrient intake by Holstein x Gyr heifers reduces mammary parenchyma (PAR) weight and increases mammary extraparenchymal fat (MEF). We further hypothesized that this occurs in concert with in alterations in systemic hormone concentrations and local changes, not only on expression of lipogenic enzyme genes in PAR but also on expression of relevant genes in liver. There were 2 experimental objectives: the first was investigate effects of high nutrient intake level on hormone concentrations, expression of lipogenic genes (*CD36*, *ACCA*, *FASN, ADIPOR1*), and mammary composition in prepubertal heifers. The second objective was to examine the relationship between liver *GHR*, *IGF1*, *IGFBP2* and *IGFBP3* gene expression to prepubertal bovine mammary gland development. Therefore, identify molecular mechanisms that are susceptible to different levels of nutrient intake in PAR of prepubertal heifers might help us better understand mammary gland development during this allometric phase and, in the future, achieve practical goals that maximize milk production by suggesting appropriated nutritional management to maximize milk production

MATERIALS AND METHODS

All animal care and handling procedures followed regulations approved by the Institutional Animal welfare and Ethics/Protection commission (certificate number 20/2015) from the Universidade Federal de Viçosa, Brazil.

Experimental design and Animal management

Eighteen crossbred (Holstein × Gyr) heifers with an average initial body weight (BW) of 102.2 ± 3.4 kg and between 3 and 4 month of age were used. Heifers were individually housed and fed for the duration of the 84 d experiment. The experimental unit was heifer. Before the start of the experiment, all heifers were allowed *ad libitum* acess to a corn silage-based diet for 15d to minimize potential compensatory gain effects in the treatment period. Afterwards, heifers were assigned in a completely randomized design to 1 of 3 nutrient intake levels (n=6/treatment) designed to sustain an average daily gain (ADG) of either: 0.0 kg/d (maintenance; **MA**), 0.5 kg/d (low gain; **LG**), or 1.0 kg/d (high gain; **HG**). All heifers were fed twice daily at 08:00 a.m and 04:00 p.m with the same diet throughout the experiment (**Table 1**). The HG diet was formulated based on metabolizable energy (**ME**) requirements of large breed dairy heifers weighing 150 kg and gaining 1.0 kg/d of BW (NRC, 2001). Feed intake for LG was adjusted to support daily gains of 0.5 kg/d; the same was done for MA sot that initial BW was maintained throughout the experiment. All heifers were weighed every 14 days in order to compare the performance achieved within the planned and correcting the amount of supplied diet according to the animal weight.

Blood Sampling and Analysis

Jugular blood samples were collected from each heifer (n = 18) before the morning feeding (after 12 h fast) on days 42 and 84 of the experiment. Blood was collected into evacuated serum tubes. All samples were kept on ice after collection

and were transported to the laboratory within 1 h. Immediately after arrival at the laboratory, the tubes were then centrifuged at 1,340 x g at 4 ºC for 15 min to obtain the blood serum; three 1ml aliquots of serum were individually labeled and frozen at -20 ºC. Subsequently, serum triglycerides (TRI), leptin, insulin, IGF1 and isobutyric acid concentration were measured. Serum TRI was determined using a colorimetric assay kit supplied by Cayman Chemical (Cayman Chemical, Michigan, USA). Intra- and interassay coefficient of variation (CV) for serum TRI were less than 5% and 7%, respectively. Serum leptin was analyzed by enzyme immunoassay method using Elisa kit supplied by DBC (Diagnostics Biochem Inc., Ontario, CA). Intra- and interassay CV for serum leptin were less than 4% and 5%. IGF1 was determined by the enzyme-labeled chemiluminescent immunometric assay (IMMULITE 2000 IGF1 Siemens Healthcare Diagnostics Products Ltd., Gwynedd, UK) immunochemiluminescent method. Intra- and interassay CV for serum IGF1 were less than 4% and 3%, respectively. Serum insulin was determined using an immunochemiluminescent assay kit supplied by Abbott (Abbott Japan Company Ltd., Tokyo, Japan). Intra- and interassay CV for serum insulin were less than 6 % and 7%, respectively. Isobutyric acid was analyzed by high performance liquid chromatography (HPLC) method. Briefly, the samples were centrifuged at 12,000-x g for 10 min to remove cells and the supernatant was treated as described in Siegfried et al. (1984). Samples were analyzed using chromatograph Dionex 3000 Ultimate Dual coupled to a refractive index detector (RI) Shodex RI-101 at 40 °C, and ion exchange column Phenomenex Rezex ROA, 300 x 7.8 mm at 40 °C. The mobile phase used was sulfuric acid (H2SO4) with 5 mM a flow of 0.7 ml /min. The isobutyric acid was measured using standard isobutyric acids with initial concentration of 10mM.

Tissue Sampling

Liver and mammary parenchyma (PAR) biopsies were harvested on day 42 of experiment from three heifers per treatment chosen randomly, and then they were immediately immersed in 5 ml of RNAholder (BioAgency, São Paulo, Brazil). The PAR samples from each heifer were taken from left rear-quarter. After 24 hours, the collected material was removed from RNAholder, drained and stored in an ultra-freezer (-80ºC) until RNA extractions were carried out.

Pre-harvest handling on 84º day was in accordance with good animal welfare practices, and the heifers were harvested by stunning with a captive bolt followed by exsanguination. At harvest, the liver was removed and a sample collected for RNA analysis. Following collection of liver, the whole udder was removed, weighed, skinned and bisected into right and left halves at the median suspensory ligament. The skin and teats from the whole udder were discarded. At this time, the skinned right and left hemi-udder were dissected to collect parenchymal tissue. The weight of PAR tissue was recorded after extraparenchymal fat and lymph nodes were removed. The weight of mammary extraparenchymal fat (MEF) was also recorded. The left skinned hemi-udder was used to obtain samples of PAR for later RNA extraction whereas the right skinned hemi-udders was used to obtain PAR samples that were frozen at -80ºC until later biochemical compositional and histological analyses. The samples of PAR and liver collected for RNA analysis were immediately immersed in 5 ml of RNAholder and the same procedure was performed as mentioned above for the biopsies samples.

The right half of the carcasses were stored in a cold chamber at 4ºC for 18h and after that, they were ground using a ball mill and subsampled for later

determination of body fat mass by ether extract analysis (EE; method 920.85; AOAC, 1990). The final EE was corrected by adding the fat lost during the partial defatting process.

Biochemical Analysis of PAR

Dissected PAR samples were removed from freezer, allowed to partially thaw, and then lyophilized for composition analysis. The lipid content was determined by ether extract (EE; method 920.85; AOAC, 1990). The final EE was corrected by adding the fat lost during the partial defatting process. The nitrogen content was analyzed by a Kjeldahl procedure (AOAC, 1990). The crude protein (CP) content was calculated as N × 6.25.

Histology Analysis of PAR

Formalin-fixed PAR samples were dehydrated in increasing concentrations of ethanol (70ºC, 95ºC and 100ºC), cleared in Histochoice® (Sigma-Aldrich) and embedded in Paraplast (Sigma-Aldrich). Paraplast embedded blocks were sectioned serially at 10 µm thick slices and stained with Haematoxylin–Eosin for examination of number of intraparenchymal adipocytes.

After qualitative evaluation of the slides, 10 randomly selected images of stained PAR sections for each animal were retrieved from a video camera (AxioCam HRc, Zeiss, Göttinger, Alemanha) linked to light microscope (AX-70 TRF, Olympus Optical, Tokyo, Japan) at 10 X magnification (Figure 1) and these images were analyzed by using the ImageJ® 1.48 software (National Institutes of Health,

Bethesda, Maryland, USA). The total number of intraparenchymal adipocytes was counted in each image. Then, the intraparenchymal adipocyte number was averaged by each animal prior to statistical analyses.

Quantitative Real-time Transcription PCR

Total RNA was isolated from 30 mg of liver and PAR tissue by using Trizol (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Additional treatment with DNase was performed on the columns using the RNase-free DNase Set (Qiagen, Valencia, CA), according to the manufacturer's recommendations. RNA concentration was checked by NanoVue Plus Spectrophotometer (GE Healthcare, Munich, Germany) with an optimal 260/280 ratio between 1.8 and 2.1. Purity and integrity were determined with an Agilent RNA 6000 Nano Kit using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Ontario, Canada). The average value of RIN (RNA integrity number) was 7.5.

Reverse transcription was performed using GoScript Reverse Transcription System (Promega, Madison, WI) and oligo (dT) primers (Invitrogen, Carlsbad, CA). The cDNA concentrations from the samples were estimated on a NanoVue Plus spectrophotometer (GE Healthcare, Munich, Germany). Finally, the cDNA samples were aliquoted and stored at –20ºC to prior analysis.

Quantitative real-time PCR (qRT-PCR) was performed in different wells and in triplicates using SYBR Green detection with GoTaq PCR Master Mix (Promega) following the manufacturer's instructions in an ABI Prism 7300 Sequence Detection System thermocycler® (Applied Biosystems). The reaction consisted of an initial step at 95ºC for 10 minutes, a second step of 40 cycles with the same temperature for

15 seconds and a final extension step at 60ºC for 60 seconds. After the amplification cycles, an additional gradient step from 60ºC to 95ºC was used to obtain a melting curve. The measurement in qRT-PCR experiment is expressed in cycles to threshold (Ct) of PCR; a relative value that represents the cycle number at which the amount of amplified cDNA reaches the threshold level. Prior to quantification by qRT-PCR, primer efficiencies for internal control and target genes were determined using 4 dilutions of liver and PAR cDNA according to the equation: % efficiency = $10^{(-1/\text{slope})}$ ×100, where slope is the gradient of dilution series. Primer efficiencies were approximately equal for target and internal control genes and averaged 98%. The coefficient of variation (CV) of Ct values from replicates within each sample was less than 5%, indicating acceptable accuracy and reproducibility (not shown). Expression was declared undetectable in samples with Ct values greater than 36 based on inconsistent amplification (low efficiency) beyond this cycle threshold (Bustin et al., 2009).

Target and internal control genes sequences were recovered from *Bos taurus* nucleotide sequences obtained from the GenBank database. The following genes were measured in PAR: *LEPR*, *CD36, ACCA*, *FASN*, and *ADIPOR1*. In liver samples *GHR*, *IGF1*, *IGF1R*, *IGFBP-3*, and *IGFBP-2* genes were measured. Specific primers were developed with the PrimerQuest software (Integrated DNA Technologies, Inc.; Coralville, IA). The primer pairs of each target and internal control genes are listed in Table 2. In the present study, the genes used for normalization in PAR were mitochondrial GTPase 1 (*MGT1*), ribosomal protein S15a (*RSP15A)* and protein phosphatase-1 inhibition subunit 11 (*PPP1R11*). These same genes were deemed the most suitable control genes for normalization of qRT-PCR data from prepubertal bovine mammary tissue (Piantoni et al., 2008). The genes used for normalization in

liver were glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), *B*-actin (*ACTB*) and 18S ribosomal RNA (18S). The normalization factor was calculated as geometric mean of control genes Ct values for each sample (Vandesompele et al., 2002). The relative mRNA abundance of target genes for each heifer was determined by subtracting the Ct value for geometric mean of the control genes from the target gene Ct ($Ct_{target} - Ct_{geometric\ mean\ control\ genes}$). Relative expression values were estimated as $2^{-\Delta Ct}$ (Livak and Schimttigent, 2001).

Statistical Analysis

Before performing the statistical analysis, we performed the test of normality of variables and they were normally distributed. All data were analyzed using the mixed procedure of SAS (version 9.3, SAS Institute, Cary, NC). Nutrient intake level, day of experiment (42 d and 84 d) and their interaction were included as fixed effects and heifer as random effect. Hormone concentrations, isobutyric acid and qRT-PCR analyses included the days of experiment as repeated measure. The RT-PCR data submitted to SAS consisted of $2^{-\Delta Ct}$ values. Means were separated using the LSMEANS option of SAS and were considered significant when $P \leq 0.05$. The correlation procedure of SAS was used to determine relationships of selected variables.

RESULTS

Body Growth and Mammary Gland Development

Prepubertal body growth and mammary development data are presented in Table 3. Initial BW was not different between the treatments. As intended, the nutrient intake levels effected average daily gain (ADG; $P < 0.001$) and final BW ($P < 0.001$). In addition, body fat mass was increased in heifers fed the high gain and low gain compared to maintenance ($P = 0.014$).

Total mammary gland (MG) weight and mammary extraparenchymal fat (MEF) weight were greater ($P < 0.001$) in HG and LG than MA heifers. However, mammary PAR weight and ratio of PAR to MG were lower ($P = 0.037$; $P = 0.05$, respectively) in HG heifers than MA and LG heifers (Table 3). Nutrient intake level affected the lipid ($P = 0.009$) and protein ($P = 0.008$) content of mammary PAR. However, the number of intraparenchymal adipocytes did not differ ($P = 0.570$) across nutrient intake level.

Metabolites and Hormone Concentrations

There was no effect of nutrient intake level on serum TRI concentration ($P = 0.117$). There was no effect of day experiment ($P = 0.250$) and no interaction between nutrient intake level and day of experiment ($P < 0.001$; Table 4) for serum TRI. Nutrient intake level affected serum insulin concentration ($P = 0.014$) and no interaction ($P = 0.325$) between nutrient intake level and day of experiment was detected. Moreover, the amount of insulin decreased ($P = 0.002$) over the day of experiment. There appeared to be a tendency for serum leptin concentration to increase across nutrient intake levels ($P = 0.061$) with no effect of day of experiment ($P = 0.147$) and no significant nutrient intake level × day of experiment interaction ($P = 0.190$). Serum IGF1 concentration was greater ($P < 0.001$; Table 4) in HG heifers

than LG and MA heifers. No interaction (P = 0.726) between nutrient intake level and day of experiment and no effect of day of experiment (P = 0.172) were detected for serum IGF1. Isobutyric acid was not affected by nutrient intake level. In addition, there was no interaction between nutrient intake and day of experiment and no effect of day of experiment.

Gene expression in liver

No differences in the expression of transcripts for IGFR1 (P = 0.083) due to nutrient intake level and day of experiment was detected. However, nutrient intake level altered the relative mRNA abundance of *GHR* (P < 0.001), *IGF1* (P = 0.008) *IGFBP2* (P = 0.047) and *IGFBP3* (P = 0.036). We observed interaction between nutrient intake and day of experiment on the relative mRNA abundance of GHR (P = 0.007), IGF1 (P = 0.014), IGFBP2 (P = 0.044) and IGFBP3 (P = 0.036). The expression of *GHR*, *IGF1* and *IGFBP3* were higher (P < 0.01) in HG heifers than MA and LG heifers, in both days of experiment (Figure 3). However, the *IGFBP2* mRNA abundance was lower (P < 0.05) in HG heifers than MA and LG heifers, in both days of experiment.

Gene expression in mammary parenchyma

Messenger RNA for *LEPR* was not readily detectable in PAR whereas inclusion of liver mRNA as a positive control for assay verified the ability of the primer to amplify a single product of expected size. Most of PAR samples failed to

reach threshold amplification after 37 cycles of PCR. This indicated that LEPR mRNA is absent in heifers of this age and is not discussed further.

Nutrient intake level affected the relative mRNA abundance of *CD36* ($P < 0.001$), *ACCA* ($P = 0.034$), *FASN* ($P = 0.041$) and *ADIPOR1* ($P = 0.048$). We observed interaction between nutrient intake and day of experiment on the relative mRNA abundance of *CD36* ($P < 0.001$), *ACCA* ($P = 0.036$), *FASN* ($P = 0.014$) and *ADIPOR1* ($P = 0.012$). Mammary PAR from HG heifers had higher ($P < 0.05$) expression of these genes compared to LG and MA heifers at 42 days of experiment (Figure 2).

Correlations

Across all nutrient intake levels, average serum IGF1 concentration (across days) was positively correlated with relative abundance of *IGF1* mRNA (r = 0.80; $P = 0.001$) and *IGFBP3* mRNA (r = 0.76; $P = 0.015$). On the other hand, serum IGF1 concentration was negatively correlated *IGFBP2* mRNA (r = -0.50; $P = 0.025$) in liver, but unrelated with PAR weight. PAR weight was negatively correlated with relative abundance of *IGFBP3* mRNA in liver (r = -0.64; $P = 0.007$). Moreover, the lipid content in PAR was positively correlated with relative abundance of *ACCA* mRNA (r = 0.78; $P < 0.001$) and *CD36* mRNA (r = 0.62; $P = 0.012$) in PAR.

DISCUSSION

Earlier studies have focused largely on establishing a direct link between high-gain diets (ADG > 0.7 kg/d) and impaired development of mammary

parenchyma, mammary growth or both in prepubertal heifers (Sejrsen et al., 1982; Capuco et al., 1995; Sejrsen and Purup, 1997). However, the effects of high nutrient intake level on mRNA expression of lipogenic enzyme genes in PAR, on liver GHR-IGF1-IGFBP axis gene expression and their relationship with mammary growth have yet to be investigated extensively. The present data herein provide evidences that elevated nutrient intake level can induced changes on expression of lipogenic marks in mammary PAR, which leading to increase of lipogenesis in intraparenchymal adipocytes in prepubertal bovine mammary gland. Furthermore, we revealed that increasing feeding level in prepubertal heifers altered concentration of hormone involved with nutritive status and liver GHR-IGF1-IGFBP axis mRNA expression. Thus, we assume that these alterations might be mirrored by changes on heifer´s mammary gland development.

As expected by our study design, the ADG was increased by increasing nutrient intake level. Body fat mass, total mammary gland and extraparenchymal fat weight increased whereas PAR weight and the PAR to MG ratio reduced by enhanced nutrient intake level. These results are consistent with previous studies in which rearing prepubertal dairy at elevated nutrient intake increased fat deposition in the body and extraparenchymal portion of the mammary gland (Capuco et al., 1995; Radcliff et al, 1997; Meyer et al., 2006) and impaired PAR growth (Sejrsen et al., 1982, 2000; Capuco et al., 1995). In addition, increased body fat mass in prepubertal heifers has been associated with decreased mammogenesis (Sejrsen et al., 2000; Silva et al., 2002). The presence of adipose tissue in mammary organoid cultures inhibits proliferation of bovine mammary epithelial cells (McFadden and Cockrell, 1993). These observations and our results, reinforce the hypothesis that accumulation of fat is detrimental to optimal prepubertal mammary parenchyma growth.

Blood metabolites are accepted indicators of nutritive status and metabolism. Thus, we sought to measure circulating TRI, insulin, leptin and IGF1 as indices of lipid and energy metabolism in heifers fed different nutrient intake level to associate this nutritional relationship with changes on heifer´s mammary gland development. As expected, serum TRI were not affect by nutrient intake level. The lack of nutrient intake effect might reflect our diet that was not high in fat and the pancreatic lipase activity and efficiency of TRI clearance that also affect TRI concentrations (Lowe, 2002). This result suggest that pancreatic lipase activity was sufficient to facilitate absorption of a substantial amount of additional fat intake and this addition did not overwhelm TRI clearance.

Previous studies reported that elevated nutrient intake increased leptin concentrations (Block et al., 2003; David Rincker, 2005; Thorn et al., 2006). In addition, Delavaud et al (2000) observed that increased body fat, in turn, increases leptin production. In our study, the heifers fed the high gain rate were fatter and their serum leptin concentration tended to be higher compared to MA and LG heifers. These results corroborate other reports, where circulating leptin concentration is strongly related to body fatness and positively related to feeding level in ruminants (Delavaud et al., 2002; Thorn et al., 2006).

It is well know that IGF1 mediates the mammogenic action of growth hormone via endocrine, paracrine or autocrine mechanisms (Forsyth, 1996; Weber et al., 1999; Purup et al., 2000a). The majority of serum IGF1 is produced by the liver through IGF1 gene expression, which is controlled by GH, as well as by insulin (Phillip et al., 1991; Eleswarapu et al., 2008). Furthermore, Purup et al. (2000b) reported strongly correlation between IGF1 of serum and mammary extracts (r =0.84), suggesting that IGF1 concentrations in mammary tissue are dependent on the

concentration of IGF1 on blood. Similar to previous studies (Capuco et al., 1995; Radcliff et al., 1997, 2004), we observed that serum insulin and IGF1 concentration increase in response to augmented (enhanced) nutrient intake level. Thus, because insulin is involved in mechanisms of partitioning nutrient toward the body tissues and considering insulin role in IGF1 regulation, we can infer that increased insulin as consequence of more uptake of energy from HG heifers could stimulate the IGF1 synthesis. Moreover, we observed that the greater serum IGF1 concentration in heifers fed the high gain rate was strongly associated with greater liver IGF1 mRNA in these heifers. The positive correlation between liver IGF1 mRNA and serum IGF1 concentrations illustrates the dependence of systemic IGF1 on liver IGF1 synthesis (Sharma et al., 1994; Yakar et al., 1999).

Previous studies reported that feeding heifers a high growth diet increased liver growth hormone receptor (GHR) (Capuco 1995; Smith et al., 2002). Although we were unable to detected serum GH concentration in our study, heifers submitted to high feeding level had greater liver *GHR* mRNA as well. Binding of GH to isolated hepatic membranes is highly correlated with GHR mRNA expression (Radcliff et al., 2003). An increase in relative mRNA abundance of *GHR* increase the capacity for liver GH binding and increase GH-dependent responses like IGF1 synthesis and release. Greater GH binding could also increase clearance of GH from blood (Lapierre et al., 2000) which could explain the lower serum GH concentration in heifers fed the high gain rate observed in the previous studies and likely could happen in our HG heifers. Moreover, it is reasonable to assume that increasing liver GHR mRNA in heifers fed the high gain rate could be mediate by the increase in serum insulin concentrations in these heifers and a subsequent increase in nuclear factors controlling liver-specific genes, like GHR. This observation is consistent with

previous studies showing that insulin is required for the normal function of the hepatic somatotropic axis (Dunger and Cheetham, 1996). For instance, loss of insulin or reduction in human hepatic insulin sensitivity caused a reduction on GHR mRNA amount (Schwartzbauer and Menon, 1998). Moreover, Butler et al. (2003) reported that insulin infusion increased GHR mRNA and IGF1 mRNA in liver of post-partum dairy cows.

At first sight, it seems contradictory that HG heifers had lower PAR weight even though had greater serum IGF1 concentration. This contradictory effect can be explained by changes in IGF binding proteins. Bioavailability of IGF1 is modulate by a group of IGF-binding proteins, IGFBP1-6 that regulated the circulating IGF1 concentration and action (Firth and Baxter, 2002). Nutrient intake is a major regulator of biological action of IGF1 through changes in IGFBPs (Smith et al., 1995; Raush et al., 2002; Wu et al., 2008, 2010). We evaluated the abundance of *IGFBP2* and *IGBPB3* mRNA because they are the most important in blood circulation (Kenneth, 2001). IGFBP3 is the major IGFBP in serum and in most situations suppresses the mitogenic action of IGF1 by forming a ternary complex involving IGF1, IGFBP3 and an acid labile subunit. This complex is responsible for hinder IGF1 from crossing the capillary walls to the extravascular tissue, and hence retain IGF1 in the blood (McCusker, 1998; Firth and Baxter, 2002). IGFBP2 is involved in the delivery of IGF1 to tissues, because when IGFBP2 is bound to IGF1 in smaller complex that can cross the capillary endothelium (Bars et al., 1990). Radcliff et al (2004) observed that changes in serum IGFBP-2 were closely related to the changes in expression of *IGFBP2* mRNA in the liver. In this study, high feeding level resulted in lower expression of *IGFBP2*, higher expression of *IGFBP3* and no changes on *IGFR1* expression. These results are in agreement with Raush et al.

(2002) that reported greater serum IGF1 and lower IGFBP2 and greater IGFBP3 in heifers fed high feeding level. Interestingly, our study revealed a significant negative correlation between serum IGF1 and liver *IGFBP2* expression and positive correlation between serum IGF1 and liver *IGFBP3* expression. Thus, these results suggest that feeding high level, although increase serum IGF1 concentration, might impair the mammary PAR growth by elevating IGFBP3 that reduced the bioavailability of IGF1 for stimulate mammogenesis.

Interestingly, our study revealed that heifers fed the high gain rate had lower PAR weight and smaller PAR to MG ratio, with more lipid content in their composition. Such response we attributed to the fact that enhancing intake nutrients to 2.5 times of heifers fed the maintenance up regulated genes for lipogenic enzymes, such as *FASN*, *ACCA* and the *CD36* (thrombospondin receptor) gene that is involved with fatty acids uptake into cells. Supporting this idea, earlier studies reported that promoters of the genes for lipogenic enzymes, including those for *FASN* and *ACCA*, are regulated by dietary state and hormones (Girard et al., 1994; Hillgarther et al., 1995). Insulin, for instance, increase transcription of these genes (Goodridge, 1987; Girard et al., 1994; Hillgarther et al., 1995). These previous evidences corroborate to our data that enhancing nutrient intake lead to altered insulin profile over the long-term and can alter the extent of lipogenesis in mammary PAR. Moreover, transcriptomic study in mammary PAR of preweaned heifers reported that enhancing nutrient intake to twice of conventional calves rearing program decrease the most genes associated with lipid production whereas up regulated *CD36* gene (Piantoni et al., 2012).

Another gene up regulated by enhancing nutrient intake was ADIPOR1, the receptor for adiponectin (ADIPOQ). The ADIPOQ, pro-adipogenic factor, is

expressed in mammary fat pad (MFP) and it is involved to hypertrophy of adipocytes (Lefterova and Lazar, 2009). Thus, it likely that enhanced nutrient intake might increase the adiponectin secretion from MFP and contribute to the increased in size of the intraparenchymal adipocytes by accumulation of lipid.

CONCLUSIONS

In conclusion, we revealed that enhancing nutrient intake altered the prepubertal mammary development by changes not only in concentrations of insulin and IGF1 but also changes on liver and PAR gene expression. Our data demonstrated a decrease in parenchyma weight whereas increase on extraparenchymal fat and lipid content. Also, the increased on lipogenic enzymes in PAR by the nutrient intake suggest adipocyte hypertrophy in mammary PAR of HG heifers compared to others groups. The implications of these results to future milk yield remains to be elucidated.

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Table 1. Composition and content of nutrients in the experimental diet

Ingredient	% of DM
Corn silage	59
Corn	22.3
Soybean meal	9.4
By pass protein soybean meal	7.7
Urea	0.3
Mineral mix[1]	1.2
Nutrient	
CP	18.3
NDF	34
NFC	39.8
TDN	73.6
EE%	2.2

[1]Supplement contained per kg: limestone - 40 g, flower of sulphur -15 g, dicalcium phosphate - 15 g, potassium iodate - 4 mg, zinc sulfate - 0.2 mg, copper sulfate – 60 mg, cobalt sulfate - 1.5 mg, manganese sulfate – 5 mg, sodium selenite – 1 mg.

Table 2. Gene names, primer pair sequences, annealing temperatures and amplification efficiencies of each target

Gene symbol[1]	Accession number[2]	Primer sequence (5'- 3')	Amplicon (pb)
LEPR	NM_001012285.2	F- TCAGCTACGTCTCTGCTAGT R-GATTCCTAGGCCATCCAATCTC	90
CD36	NM_001278621.1	F- TGAGGCAGACACAACAAGAG R- CCAGTTGGAAGTCCGATGAA	112
ACCA	NM_174224.2	F-TGAAGAAGCAATGGATGAACC R-TTCAGACACGGAGCCAATAA	108
FASN	XM_005220997.2	R- ATCAACTCTGAGGGGCTGAA F- CAACAAAACTGGTGCTCACG	122
ADIPOR1	NM_001034055.1	R-AGGACAACGACTACCTACTG F-GTGTGGATGCGGAAGATG	103
GHR	NM_176608.1	F-CCTCAACTGGACTCTACTGAAC R-CCAGGATTATCCATCCCATCTT	112
IGF1	NM_001077828.1	F- AGCAGTCTTCCAACCCAATTA R-ACAGGGCCAGATAGAAGAGA	103
IGF1R	NM_001244612.1	F-GTATGGAGGAGCCAAGCTAAA R-GTCTTGGCCTGAACGTAGAA	123
IGFBP2	NM_174555.1	F-CAACCTCAAACAGTGCAAGATG R-CTGCTCGTTGTAGAAGAGATGAC	112
IGFBP3	NM_174556.1	F-CTCCACTTCATGCCTTAGCA R-GACAGGGCGTTCTTCTTCTT	120
MTG1	NM_001034035.2	R- GTGGGATATGCCCTTGACTATAA F- GGACTCTCATCTTAGGCTTTGT	104
RSP15A	NM_001037443.2	F- GGAGTGATCAGCCCTAGATTTG R- AGCTGAGGTTGTCAGTACAATG	108
PPP1R11	XM_005223603.1	F-GGAAGTGGGTTTGGGAGAAT R-CCCAAGCAGTATCACCTACATC	117
GAPDH	NM_001034034.2	F- GATGCTGGTGCTGAGTATGT R- GCAGAAGGTGCAGAGATGAT	113
18S	NR_036642.1	F- GCCGCTAGAGGTGAAATTCT R- TCGGAACTACGACGGTATCT	129
ACBT	NM_173979.3	F- ACTCCTGCTTGCTGATCCACATCT R-AAGATCAAGATCATCGCGCCTCCA	109

[1]Genes: leptin receptor (*LEPR*), trombospondin receptor (*CD36*), acetyl-CoA carboxylase alpha (*ACCA)*, fatty acid synthase (*FASN*), adiponectin receptor 1 (*ADIPOR1*), growth hormone receptor (*GHR*), insulin-Like growth factor 1 (*IGF1*), insulin-like growth factor 1 receptor (*IGF1R*), insulin-like growth factor binding protein 2 (*IGFBP2*), insulin-like growth factor binding protein 3 (*IGFBP3*), mitochondrial GTPase1 (*MTG1*), ribosomal protein S15a (*RSP15A*), protein phosphatase-1 inhibition subunit (*PPP1R11),* glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)*,* 18S ribosomal RNA (*18S*) and B-actin (*ACBT*). [2]Accession number at Genbank (htpp://www.ncbi.nlm.nih.gov).

Table 3. Effects of prepubertal nutrient intake on final body weight (BW), average daily gain (ADG) and mammary parenchyma (PAR) development in heifers (means ± SEM)

	Prepubertal nutrient intake[1]			
Item	MA	LG	HG	*P-value*
Initial BW, kg	98.16 ± 6.08	104.33 ± 6.08	104.17 ± 6.08	0.721
Dry matter, kg	1.48	2.56	3.78	<0.001
ADG, kg/d	0.105^{c} ± 0.02	0.500^{b} ± 0.02	0.907^{a} ± 0.02	<0.001
Final BW, kg	107^{c} ± 7.30	146.33^{b}± 7.30	181.20^{a} ± 7.99	<0.001
Body fat mass, g/kg of BW	331.2^{b} ± 13.58	390^{a} ± 14.87	395.7^{a} ± 16.63	0.014
MG weight, kg[2]	0.621^{b} ± 111.2	1.210^{a} ± 111.2	1.405^{a} ± 111.2	<0.001
MEF weight, kg[3]	0.313^{c} ± 0.68	0.603^{b} ± 0.75	1.027^{a} ± 0.75	<0.001
PAR weight, kg	0.178^{b} ± 0.40	0.356^{a} ± 0.44	0.237^{b} ± 0.44	0.037
Protein, g/kg of PAR	128.7^{a} ± 6.95	109.7^{b} ± 6.95	91.90^{b} ± 6.95	0.009
Ether extract, g/kg of PAR	861.1^{b} ± 7.35	878.7^{b} ± 7.35	900.7^{a} ± 7.35	0.008
N° intraparenchymal adipocytes	451.95 ± 67.46	357.62 ± 55.08	397.42 ± 55.08	0.570
PAR:MG	0.211^{a} ± 0.03	0.195^{a} ± 0.02	0.097^{b} ± 0.02	0.050

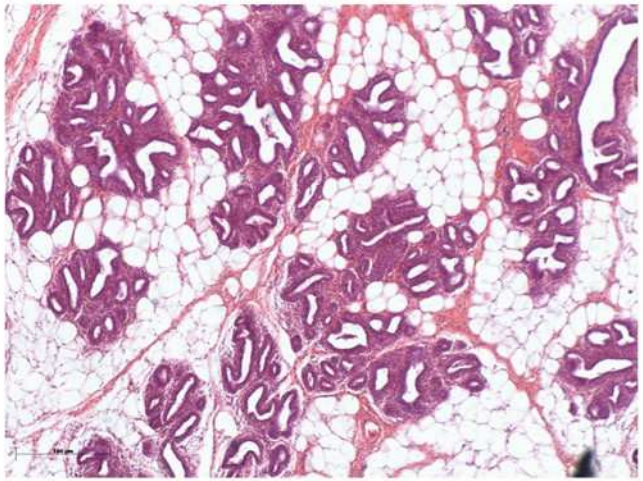
[1]Prepubertal nutrient intake: MA= maintenance, HG= high gain (1kg/d), LG= low gain (0.5kg/d). [2]MG= mammary gland. [3]MEF= mammary extraparenchymal fat. [a-c]Means within a row with different superscripts differ (P <0.05).

Table 4. Effects of prepubertal nutrient intake on blood metabolite concentrations in heifers during experiment (means ± SEM)

Item	Prepubertal nutrient intake[1]			Day of the experiment		*F-test, P-value*		
	MA	LG	HG	42	84	PNI[3]	Day[4]	PNI x day[5]
Triglycerides, mg/dL	20.20 ± 0.48	21.46 ± 0.45	20.75 ± 0.43	21.11 ± 0.39	20.50 ± 0.35	0.117	0.250	0.137
Insulin, µU/mL	0.58^{b} ± 0.08	0.96^{a} ± 0.07	1.20^{a} ± 0.13	1.22^{a} ± 0.08	0.68^{b} ± 0.07	0.014	0.002	0.325
Leptin, ng/mL	1.24 ± 0.10	1.22 ± 0.08	1.40 ± 0.08	1.17 ± 0.07	1.33 ± 0.07	0.061	0.147	0.190
IGF1, ng/mL	102.17^{c} ± 25.48	159.97^{b} ± 23.77	281.83^{a} ± 22.66	165.22 ± 16.18	200.72 ± 17.48	<0.001	0.172	0.726
Isobutyric acid, mmol/L	0.207 ± 0.02	0.240 ± 0.02	0.178 ± 0.02	0.221 ± 0.02	0.196 ± 0.02	0.257	0.160	0.427

[1]Prepubertal nutrient intake: MA= maintenance, HG= high gain (1kg/d), LG= low gain (0.5kg/d). [2]standard error of the mean.[3]PNI= prepubertal nutrient intake main effect.[4]day= day of the experiment main effects.[5]interaction of main effects. Different lowercase letters represent statistical difference between prepubertal nutrient intake levels.

A



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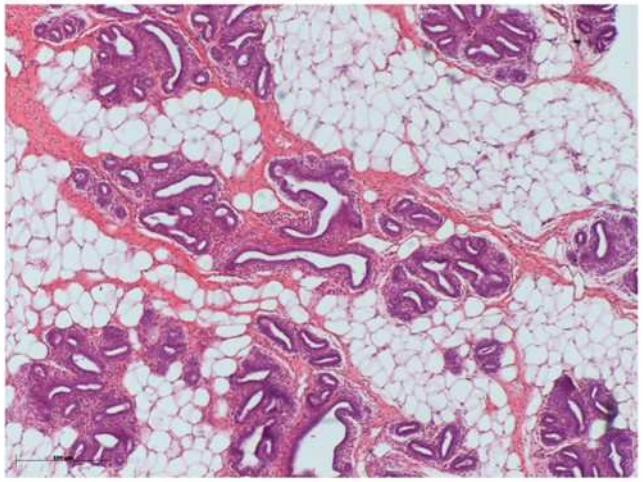
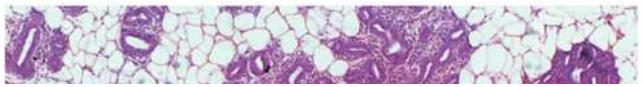
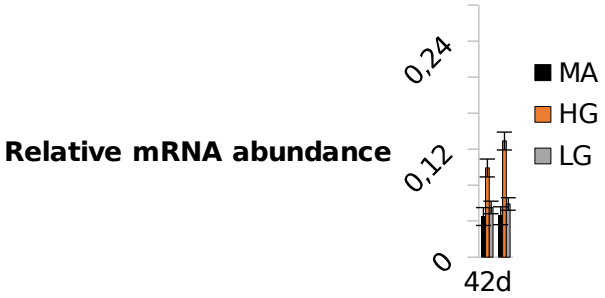


C

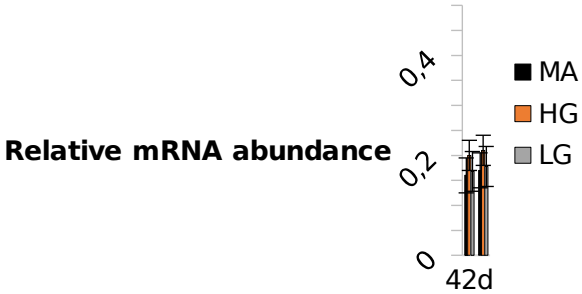


Weller Figure 1

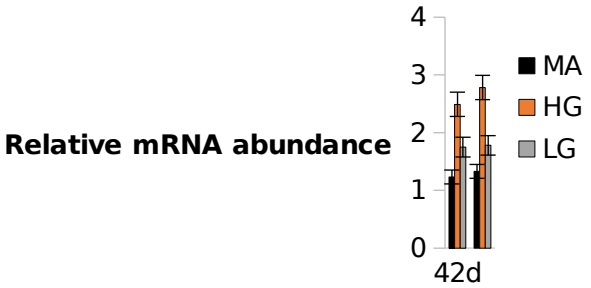
GHR


Relative mRNA abundance
0,24
0,12
0
42d
MA
HG
LG

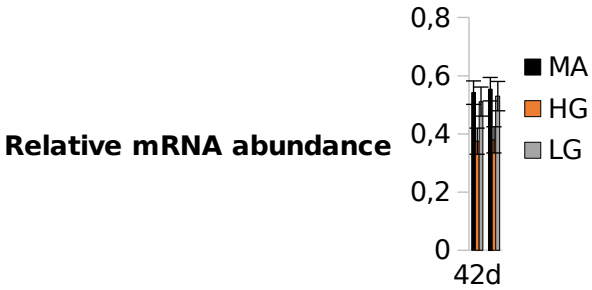
IGFR1


Relative mRNA abundance
0,4
0,2
0
42d
MA
HG
LG

IGF1


Relative mRNA abundance
4
3
2
1
0
42d
MA
HG
LG

IGFBP2


Relative mRNA abundance
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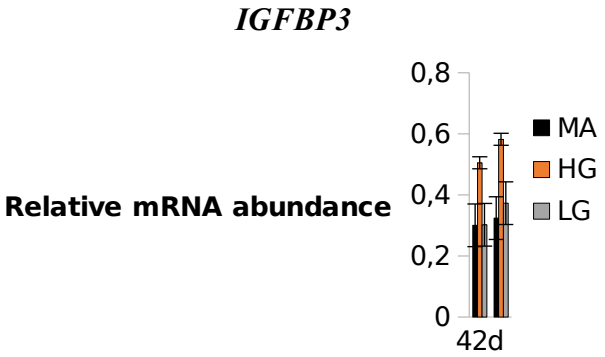
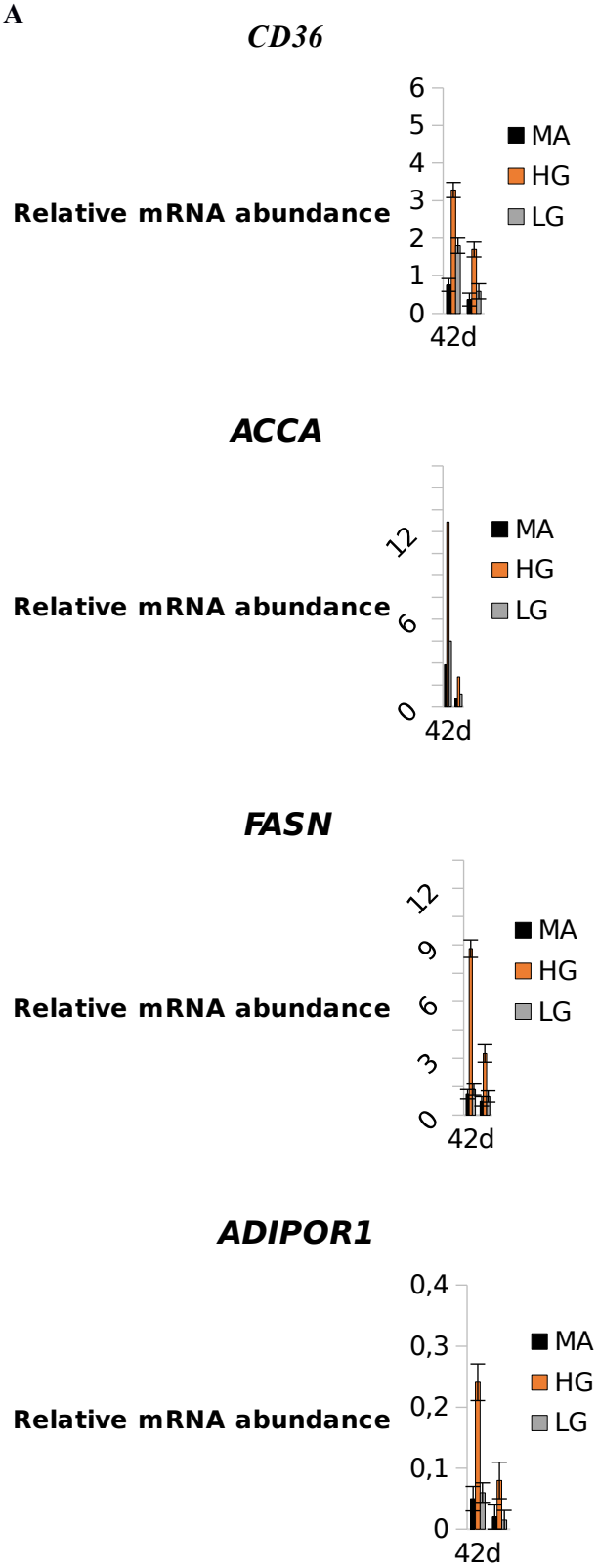
0,6
0,4
0,2
0
42d
MA
HG
LG



Weller Figure 2

A



Weller Figure 3

Figure 1. Representative mammary parenchymal from the (A) maintenance, (B) high gain and (C) low gain heifers at the slaughter (84ªday).

Figure 2. Comparative relative mRNA abundance of (a) *CD36*, (b) *ACCA*, (c) *FASN*, and (d) *ADIPOR1* in mammary parenchyma from prepubertal heifers fed 1 to 3 nutrient intake treatments within day. **P* < 0.05 compared with maintenance (MA) and low gain (LG) heifers at 42 days of experiment.

Figure 3. Comparative relative mRNA abundance of (A) *GHR*, (B) *IGFR1*, (C) *IGF1*, (D) *IGFBP2* and (E) *IGFBP3* in liver from prepubertal heifers fed 1 to 3 nutrient intakes throughout the experiment. Different lowercase letters represent difference (P < 0.05) between nutrient intakes within day of experiment.

CHAPTER III

(Article III)

BMP7 and IGF1 predicted to modulate ovarian activity in pre versus post-pubertal Bos indicus heifers

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ABSTRACT

Background

Members of the transforming growth factor-beta superfamily such as bone morphogenetic proteins 6, 7, 15 and two isoforms of transforming growth factor-beta (*BMP6*, *BMP7*, *BMP15*, *TGFB1* and *TGFB2*) and insulin-like growth factor system act as local regulators of ovarian follicular development. To elucidate if these factors could modulate or influence the onset of puberty, the expression pattern of these genes as well as receptors of estrogen receptor 1 (*ESR1),* growth differentiation factor 9 (*GDF9*)*,* follicle stimulating hormone receptor *(FSHR),* luteinizing hormone receptor (*LHR*), bone morphogenetic protein receptor, type 2 (*BMPR2*), type 1 insulin-like growth factor receptor (*IGFR1*), and key steroidogenic enzymes cytochrome P450 aromatase and 3-β-hydroxysteroid dehydrogenase (*CYP19A1* and *HSD3B1)* was evaluated in pre (PRE) and post-pubertal heifers (POST).

Results

The expression of *FSHR*, *BMP7*, *CYP19A1*, *IGF1* and *IGFR1* mRNA was greater in PRE heifers, when contrasted to POST heifers. The expression of *LHR* and *HSD3B1* was lower in PRE heifers than POST. Pair-wise correlations predicted 4 gene-gene interactions from co-expression data: *IGF1* and *BMP7* ($P = 0.07$, r = 0.84); *IGF1* and *IGF1R* ($P = 0.10$, r = 0.79); *LHR* and *HSD3B1* ($P < 0.01$, r = -0.97); and *HSD3B1* and *CY19A1* ($P = 0.03$, r = -0.90).

Conclusion

Differential expression of ovarian genes could be associated with changes in follicular dynamics and different cell populations that have emerged as consequence of puberty and the luteal phase. The emerging hypothesis is that two key regulators of ovarian activity pre-puberty, *BMP7* and *IGF1* modulate the expression of *FSHR*, *LHR*, *IGFR1* and *CYP19A1*. *BMP7* seems to down-regulate *LHR* and up-regulate *FSHR* and *CYP19A1*, which mediates the follicular dynamics in heifer ovaries. Thus, *BMP7* and *IGF1* seem to play synergic regulatory roles and were predicted to interact.

Keywords: Bovine species, Intraovarian factors, Quantitative real time PCR, Pubertal development

BACKGROUND

Ovarian activity and hormones are paramount for pubertal development and normal reproductive performance [1]. Improving the reproductive performance of *Bos indicus* cattle is an industry priority in tropical and subtropical regions of the world, because of its impact on farm productivity [2]. *Bos indicus* breeds have a later onset of puberty (16 to 40 months of age), which has a negative impact on their overall reproductive performance [3-6]. Although, the heritability of age at puberty measured by first detected *corpus luteum* (CL) has been reported to be moderate (0.52 to 0.57) [7], the phenotypic identification of animals that undergo puberty at an early age is expensive. Enhancing our comprehension of ovarian genes and their interactions involved in bovine puberty could have practical implications in the animal breeding context.

The regulation of ovarian activity is an integrated process that involves FSH and LH hormones, their receptors, ovarian steroids and intra-ovarian factors [8]. One of the most important intra-ovarian factors includes members of the transforming growth factor-beta (*TGFB*) superfamily, such as bone morphogenetic proteins 6, 7 and 15 (*BMP6*, *BMP7* and *BMP15*) and two isoforms of transforming growth factor-beta (*TGFB1* and *TGFB2*), which are expressed by ovarian somatic cells and oocytes in a stage-specific manner throughout folliculogenesis [9,10]. These genes function as local regulators of ovarian follicular development and subsequently affect fertility [11,12,13]. Knockout experiments indicate that the different isoforms of *TGFB* are responsible for diverse physiological functions [14,15]. An *in vivo* study showed that *BMP7* promotes the "recruitment" of primordial follicles into the growing follicle pool, while inhibiting progesterone production and ovulation [16]. Similar to *BMP7*, *BMP15* and *BMP6* are part of a group of luteinization inhibitors [17]. *In vitro*, *BMP7*

induced expression of follicle stimulating hormone receptor (*FSHR*) mRNA in human granulosa cells [18]. In contrast, *BMP15* suppressed *FSHR*, and luteinizing hormone receptor (*LHR*) expression [19]. Given the discussed roles affecting ovulation, these intraovarian factors could modulate or influence the onset of puberty that leads to the first ovulation event.

The insulin-like growth factors (IGFs) system plays a key role in follicular development and female fertility [20,21,22]. Insulin-like growth factor 1 (*IGF1*) and insulin-like growth factor 2 (*IGF2*) have been reported to act in synergy with gonadotropins *LH* and *FSH* to stimulate growth and differentiation of ovarian follicles and subsequent synthesis and secretion of estradiol and progesterone production [23-26]. Moreover, experiments in cattle [27,28] showed that *IGF2* was the main intrafollicular IGF ligand regulating follicular growth, and highly expressed in theca cells. All of these previous reports suggest that the IGFs and *TGFB* superfamily genes may have different roles in ovarian activity, before and after puberty, related to endocrine regulation of puberty.

So far, studies have focused on the endocrine regulation of the hypothalamic-pituitary-ovarian system for onset of puberty [29, 30, 31]. Information about specific changes in ovarian gene expression associated with puberty is sparse. Thus, the aim of the present study was to elucidate the expression pattern of candidate genes, such as, *ESR1, GDF9, FSHR, LHR, BMPR2, TGFB1, TGFB2, BMP15, BMP6, BMP7, IGF1, IGFR1, IGF2*, and key steroidogenic enzymes such as, cytochrome P450 aromatase (*CYP19A1*) and 3-β-hydroxysteroid dehydrogenase (*HSD3B1)* in pre (PRE) and post-pubertal heifers (POST). Evidence of differential expression will help to support or disprove the hypothesis that these genes modulate or are influenced by the onset of puberty.

RESULTS

Serum progesterone concentrations, body weight and condition

Average serum concentration of progesterone for the PRE and POST *Bos indicus* Brahman heifers were 0.4 ± 0.2 and 2.0 ± 0.7 ng / mL, respectively (n = 6 per group). The difference in progesterone concentration corresponds to the observation of CL in POST heifers (Figure 1). Associated data collected prior to euthanasia of PRE and POST heifers indicated no significant difference in body weight or condition score between PRE and POST heifers. Body weight averages were 338 ± 54.17 and 362.6 ± 38.62 kg ($P = 0.38$), and condition score averages were 3.5 ± 0.44 and 3.75 ± 0.41 ($P = 0.18$), respectively.

Ovarian gene expression

Ovarian gene expression was measured with real-time quantitative PCR in 3 contrasts: 1) average gene expression from both ovaries PRE versus POST heifers; 2) average expression of PRE ovaries versus only ovaries with a CL in POST; and 3) ovaries with CL versus ovaries without using only POST heifers.

In the contrast between PRE versus POST heifers (both ovaries), *FSHR* ($P < 0.05$), *BMP7* ($P < 0.05$), *IGF1* ($P < 0.05$) and *IGFR1* ($P < 0.01$) expression were greater in PRE heifers, whereas *LHR* ($P < 0.05$) expression was lower in PRE heifers (Table 1). The abundance of key steroidogenic enzyme genes, such as, *CYP19A1* ($P < 0.05$) was greater in PRE heifers, whereas *HSD3B1* mRNA expression was greater ($P < 0.01$) in POST heifers.

In the contrast between both ovaries from PRE versus only ovaries with CL from POST heifers, *FSHR* ($P < 0.05$), *CYP19A1 ($P < 0.05$)* and *TGFB1* ($P < 0.01$) expression was greater in PRE heifers, whereas expression of *LHR* ($P < 0.05$), and *HSD3B1* ($P < 0.01$) were greater in POST heifers' ovaries with CL (Table 1).

In the third and last contrast, we did not observe any differential gene expression between ovaries with a CL versus the contralateral ovary (without CL) from POST heifers.

Pair-wise correlations predicted 4 gene-gene interactions from co-expression data. The significant correlations were between: *IGF1* and *BMP7* ($P = 0.07$, $r = 0.84$); *IGF1* and *IGF1R* ($P = 0.10$, $r = 0.79$); *LHR* and *HSD3B1* ($P < 0.01$, $r = -0.97$); and *HSD3B1* and *CY19A1* ($P = 0.03$, $r = -0.90$). Other correlations were not significant ($P > 0.1$).

DISCUSSION

This study has demonstrate that puberty onset is associated with changes in ovarian gene expression of gonadotropin receptors (*FSHR*, *LHR*), key steroidogenic enzymes (*HSD3B1*,*CYP19A1*) and important intra-ovarian factors and their receptor, such as *BMP7*, *TGFB1* and *IGFR1*. The regulation of ovarian activity is determined by co-ordinated action between FSH and LH with their receptors, ovarian steroids, and intra-ovarian factors [8]. Genes of the *TGFB* superfamily, such as *GDF9*, *BMP6, BMP7, BMP15, TGFB1 and TGFB2* and their cell receptors are known paracrine and autocrine modulators of ovarian function and fertility [9-13]. The IGF system plays a key role in follicular development and is additional important intra-ovarian factor involved in pubertal development [23-26]. Our results fit with current theory and point to specific intra-ovarian factors relevant to puberty in *Bos indicus* heifers.

We did not detect differential gene expression between ovaries from POST heifers with a CL versus the contralateral ovary (without a CL), which confirms the systemic effect of circulating progesterone on ovarian dynamics. Therefore, we focussed on the differences between PRE and POST heifers. In the present study, the significant differences in ovarian gene expression between two physiological states, PRE and POST, are likely related to onset of puberty and influenced by progesterone signalling. It is important to note that the comparison is between PRE heifers which had never experienced a luteal phase and the related progesterone signalling, while the POST heifers were in their second diestrus with the corresponding levels of progesterone being produced by CL.

We identified greater expression of *FSHR* in the ovaries of PRE than POST heifers which could be related to follicular waves characterized by synchronous development of groups of growing follicles, one of which became dominant and achieved the greatest diameter, supressing the growth of the other smaller subordinate follicles. Transrectal ultrasonography has detected the sequential growth and regression of large follicles in heifers, near the time of first ovulation [35]. FSH and its receptor FSHR play an important role in follicle progression from the primary to the advanced stage of follicular development [36]. Studies reported the expression of *FSHR* on granulosa cells in primary and secondary follicles, which is evidence for the role of FSH in follicular development at early stages [37,38]. Our findings are further supported by previous studies that demonstrated an increase in FSH binding sites in the rat ovary during the pre-pubertal period [39,40]. The lower expressions of *FSHR* in POST heifers is in accord with the high progesterone concentrations during luteal phase because progesterone exerts a negative feedback inhibition on GnRH and suppress further follicular growth and maturation [41].

The POST heifers exhibited greater *LHR* mRNA expression than PRE heifers. These animals are in luteal phase of their estrus cycle and it is well know that LH has essential role for maintenance and normal function of the CL [42]. Therefore, LHR signalling is likely to play has an essential role in suppressing ovarian activity in the remainder of the diestrus ovary.

The bone morphogenetic proteins (BMPs) are important for the regulation of follicular development, ovulation and CL morphogenesis [12,16,19]. Interestingly, in the present study, no significant differences in mRNA abundance of *GDF9, BMP15, BMP6 TGFB2* and their receptor *BMPR2* were observed between the PRE versus POST contrasts. Although these genes are known as modulators of mammalian folliculogenesis, and were therefore selected for investigation, it seems they are not involved with the changes in follicular dynamics observed in this contrast of PRE vs. POST. The specific BMP associated to pubertal development seems to be *BMP7*.

In the PRE vs. POST contrast, *BMP7* was differentially expressed. Our findings demonstrated higher abundance of ovarian *BMP7* in PRE than in POST heifers, which may be associated with the different physiological phases represented in this contrast. The ovaries of PRE heifers are likely to be constantly initiating in follicular recruitment. The recruitment stage is characterised by the presence of a cohort of growing follicles at the beginning of the follicular wave. Our finding is consistent with previous studies showing that *BMP7* promoted the recruitment of primordial follicles into the growing follicles and enhanced oestradiol secretion when secretion of progesterone was concomitantly suppressed [43, 44,45]. Because progesterone is important in the process of ovulation [46] inhibition of progesterone production by *BMP7* suggests a functional role as luteinization inhibitor delaying ovulation [47,48]. Furthermore, in an *in vitro* study *BMP7* increased *FSHR* mRNA

expression in rat and human granulosa cells [49,18] while it decreased *LHR* expression in human granulosa cells [18]. In this context, the results of the present study suggest an important mechanism of *FSHR* and *LHR* regulation by *BMP7* in pre-pubertal heifers. Greater ovarian *BMP7* contributes to increased FSH sensitivity of granulosa cells via up-regulation of *FSHR* and down-regulation of *LHR*, thus promoting folliculogenesis (recruitment, selection and atresia) and suppressing ovulation [18].

We observed differential expression of key steroidogenic genes *CYP19A1*, *HSD3B1* between PRE and POST heifers. *CYP19A1* codes for the key regulator enzyme in the steroid biosynthesis pathways: P450aromatase, which catalyses the conversion of androgens to estrogens [50]. The up-regulation of *BMP7* may explain the increased expression of *CYP19A1* in PRE heifers. An *in vivo* study demonstrated that rat ovaries treated with BMP-7 had enhanced expression of *CYP19A1,* which in turn increased estradiol production, as well as increased the number of preantral and antral follicles [43,51].

Greater expression of *HSD3B1* mRNA in POST heifers could be the result of the presence of an active CL and consequent progesterone signalling. Theca-and granulosa-derived luteal cells express the enzyme *HSD3B1* that converts pregnenolone to progesterone [52]. In short, the greater expression of *HSD3B1* is necessary for production of progesterone, which is generally not occurring in PRE heifers.

TGFB isoforms (TGFB1, TGFB2) are multifunctional regulatory molecules because it can either stimulate or inhibit proliferation, differentiation and other critical cell functions according to species, stage of differentiation of ovarian cells and presence of others growth factors [44,53,54]. In rodents, *TGFB1* increased

proliferation of FSH-stimulated granulosa cells [55]. However, in cattle (*TGFB2*) and sheep (*TGFB1* and *TGFB2*) these growth factors have inhibitory effects on granulosa cell proliferation [56,57]. The effect of *TGFB* isoforms are species specific and might be different in *Bos indicus* and *Bos taurus* animals. *TGFB1* mRNA and protein expression in bovine granulosa cells decrease during progress of folliculogenesis [58]. Furthermore, in cattle *TGFB1* was present in granulosa cells of earliest stage of development (early preantral, early antral follicles) but absent in larger more advanced follicles [59]. We observed greater expression of *TGFB1* mRNA in PRE heifers, which highlights the important role of *TGFB1* in modulating both granulosa cell growth and differentiation. We can speculate that the proliferation inhibition induced by *TGFB1* may promote differentiation of granulosa cell in order to acquire *FSHR* and express steroidogenic enzymes which allow the cell to be more responsive to *FSH* and enables secretion of ovarian steroids. An improved understanding of the mechanisms of *TGFB1* in follicular growth and pubertal development in bovine ovary requires further investigation. Especially, it will be relevant to understand the interactions between *BMP7* and *TGFB1* in regulation of bovine folliculogenesis.

Intrafollicular *IGF1* and *IGF2* have been reported to act in synergy with the gonadotropins *LH* and *FSH* to stimulate growth and differentiation of ovarian follicles and subsequent synthesis and secretion of estradiol and progesterone production [23-26]. The type 1 IGF receptor (*IGFR1*) mediates most of the actions of both *IGF1* and *IGF2* [60] and its expression increases during follicular development [61]. In this current study, we observed greater expression of *IGF1* and *IGFR1* in PRE compared to POST heifers. Our findings suggest that *IGF1*, as well as, *BMP7* could be underpinning to increased expression of *FSHR* and *CYP19A1* mRNA found in PRE heifers ovaries. This is in agreement with a previous *in vivo* study that

showed *IGF1* can regulate *FSHR* gene expression and indirectly aromatase expression [62]. *FSHR* expression has been shown to be severely reduced in pre-antral follicles of *IGF1* knockout mice, as well as, aromatase expression and was restored to wild type expression levels after 2 weeks of exogenous IGF1 supplementation [63]. Reinforcing this hypothesis an *in vivo* study showed that FSH enhanced *IGFR1* expression [62]. Thus, local IGF1 creates a positive feedback loop where IGF1 enhances FSH action and FSH enhances IGF1 action through mutual receptor up-regulation.

CONCLUSION

Taken together, our findings suggest that *BMP7* and *IGF1* interact and regulate intrafollicular steroidogenesis and follicular response to gonadotropins during pubertal development. The emerging hypothesis is that two key regulators of ovarian activity pre-puberty, *BMP7* and *IGF1* play synergic roles modulating the expression of *FSHR*, *LHR* and *IGFR1* and steroidogenesis (*CYP19A1*). *BMP7* seems to down-regulate *LHR* and up-regulate *FSHR* and *CYP19A1*, which mediates the follicular dynamics in PRE heifer ovaries. Up-regulation of *IGF1* expression pre-puberty correlates with increased levels *FSHR* and *CYP19A1*. Furthermore, *TGFB1* likely plays important role in follicular dynamics in pre-puberty. The role of these genes and predicted interactions in puberty merits further research, to elucidate the molecular pathways in cattle ovarian tissue.

In summary, our results showed that differential expression of ovarian genes could be related to changes in follicular dynamics and differences in cell populations that have emerged as consequence of puberty and the luteal phase.

METHODS

Animal management and puberty observation

Management, handling and euthanasia of animals were approved by the Animal Ethics Committee of The University of Queensland, Production and Companion Animal group (certificate number QAAFI/279/12). Twenty Brahman heifers, which were not pedigree animals, but had a characteristic Bos indicus phenotype and were typical beef industry animals were sourced as young weaners born at the same season (< 250 kg) and kept at grazing conditions, from two commercial herds in Queensland, Australia. After being sourced, heifers were kept at the Gatton Campus facilities of the University of Queensland; they were all under the same conditions and pasture based diet until project end. Precise day of birth information was not available for these heifers as they were sourced from industry; the effect of small age differences (about 2 month) in pubertal development is possible but could not be tested in this experiment.

Heifers were examined every 2 weeks for observation of pubertal development, from October 2012 to May 2013. Ovarian activity was observed using ultrasonography (HS-2000(VET), Honda Electronics Inc.). Pubertal status was defined by presence of a corpus luteum (CL) observed with the ultrasound [32]. Euthanasia plans were based on date of first CL observation. Six heifers were chosen to be pre-pubertal (PRE) and six heifer post-pubertal (POST). The POST heifer when identified was then paired with PRE heifer which was randomly drawn from the remaining animals, and processed on the same day. The animals were weighted and the body condition (BCS) was measured. POST heifers were euthanized on the luteal

phase of their second estrus cycle, confirmed by CL presence on ovarian tissue post euthanasia (Figure 1). Euthanasia was carried out by stunning with a captive bolt followed by exsanguination. Concentrations of progesterone were measured with a radioimmunoassay from blood samples collected at exsanguination, to confirm that POST heifers had a functional CL, which was observed at euthanasia. Radioimmunoassay was performed by the Laboratory for Animal Endocrinology of the University of Queensland (Dr. Stephen Anderson).

Post-euthanasia, left and right ovaries were harvest and preserved by snap freezing in liquid nitrogen, then kept at -80ºC until RNA extractions were carried out. In total, 24 ovaries (2x 6 PRE and 2 x 6 POST heifers) were processed separately for RNA extraction and quantitative real-time PCR (qRT-PCR) measurements.

RNA extraction and quantitative real-time Transcription PCR analysis

Prior to RNA extraction, ovarian tissue was homogenized to form a uniform sample representative of the whole organ, using liquid nitrogen; left and right ovaries were kept separately. Total RNA was isolated separately from 25mg of the homogenized sample of left and right ovaries from PRE and POST heifers, using Trizol method (Life Technologies, Inc.). The total RNA was re-suspended in RNase-free ultrapure water and stored at -80ºC until further use. RNA concentrations were measured by Nanodrop ND-1000 spectrophotomer (Thermo Fisher Scientific, Wilmington, DE) with an optimal 260/280 ratio between 1.8 and 2.1. Intact 28S and 18S rRNA subunit integrity was assessed by Bioanalyser (RIN 6.9 or above for all samples).

Reverse transcription was performed using GoScript Reverse Transcription System (Promega) and oligo (dT) primers (Invitrogen). The reactions were performed with 6 μg of total RNA and 2 μl of 50 μM oligo (dT)23VN primer, following the manufacturer's recommended protocol. The cDNA concentrations from the samples were estimated on a Nanodrop ND-1000 spectrophotomer (Thermo Fisher Scientific). Finally, the single-stranded cDNA samples were stored at –20ºC until analysis by quantitative real-time PCR.

Quantitative real-time PCR reactions were performed in triplicate using SYBR® Select Master Mix (Applied Biosystem) following the manufacturer's instructions in a ViiA™ 7 Real-Time PCR System (Applied Biosystem). The coefficient of variation (CV) of Ct values from replicates within each sample was low < 5% indicating acceptable accuracy and reproducibility (not shown). The oligonucleotide primers used for the reactions were designed using PrimerQuest software provided by Integrated DNA Technologies, Inc. from Bos taurus sequences available in GeneBank database. In the present study, GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control gene because it was stably expressed in our study. The list of primer sequences used for each target gene is listed in Table 2.

Prior to performing qRT-PCR, the amplification efficiency and optimal primer concentration were determined for each gene using serial dilution of cDNA. For this purpose, four concentrations of cDNA (0.625, 2.5, 10, and 40 ng/reaction) and 2 primer dilutions (100, 200 nM) were tested. The PCR efficiencies for all primers pairs were obtained using the formula $E = 10(-1/slope) \times 100$, where E is efficiency and slope is the gradient of dilution series in the linear phase. These results are summarized in Table 1. Samples were amplified separately in triplicate using a

ViiA™ 7 Real-Time PCR System (Applied Biosystem, Foster City, CA) with the following amplification program: an initial step at 95ºC for 10 min, a second step of 40 cycles of 95°C for 20 s, and a final extension step at 60°C for 30 s. After the 40 amplification cycles, all samples were subjected to a melting curves analysis, in which they were heated in increments of 1°C per 30 s from 60ºC to 94°C to ensure the absence of non-specific products.

Statistical Analysis

Progesterone, body weight and condition score data were analysed in a completed randomized design using SAS (version 9.1.3), and means were compared by the t-test to the level of 5%. Statistical analysis of Ct data was performed using %QPCR_MIXED macro developed in SAS (version 9.1.3) [https://www.msu.edu/~steibelj/JP_files/QPCR.html] developed to generate codes in SAS PROC MIXED suitable to analyse data from qRT-PCR, assuming independent random effects for reference, and target genes in each biological replicate [33]. The following model was used:

$$y_{gikr} = TG_{gi} + C_{gik} + D_{ik} + e_{gikr}$$

where, y_{gikr} corresponds to the Ct value obtained from the thermocycler software for the g*th* gene (reference or targets) from the r*th* well, which corresponds to the k*th* animal in ith physiological state (PRE or POST); TG_{gi} is the effect of the i*th* physiological state on the expression of gene g; $C_{gik} \sim N(0,)$ is the gene-specific random effect of the k*th* animal; $D_{ik} \sim N(0,)$ is the sample-specific random effect (common to reference and target genes); and $e_{gikr} \sim N(0,)$ is the residual term.

The expression for each gene was calculated using ΔCt method (target gene Ct – GAPDH gene Ct) as previously reported [34], where Ct is the PCR cycle number at which the fluorescence generated within a reaction crosses an arbitrary threshold. For each target gene, the comparison of gene expression between physiological state (PRE or POST) was performed by CONTRAST statement of the GLM procedure (SAS software) using Student's t -test to the level of 5%. The "estimates" generated by CONTRAST analyses were used to calculate fold-change (relative expression) for pair-wise contrast of interest and were obtained by using 2-(Estimate). The contrast between PRE versus POST heifers (both ovaries) was analysed considering the average of Ct values from right and left ovary of each heifer for each gene. The other two contrasts were: 1) both ovaries from PRE versus only ovaries with CL from POST heifers; and 2) ovaries from POST heifers without CL versus ovaries from POST heifers with CL. Once the efficiency (E) of the qRT-PCR reaction was close to 100%, one PCR cycle of difference between two samples means twice as much expression in the first sample in comparison with the second.

Pair-wise correlations between gene expression values were calculated and its significance was tested using R software for analyses. Significant correlations were interpreted as predicted gene interactions.

AUTHOR´S CONTRIBUTIONS

MMDCAW, MRSF, LRPN, SAL, and SSM participated in the design of the study. MRSF, LRPN, LK, JPAR, SE, GBBH, EP carried the field trial and euthanasia

of heifers. BV contributed handling of tissue samples and RNA extraction. MMDCAW performed qRT-PCR experiment. MMDCAW and MK performed statistical analyses. MMDCAW, MRSF, SEFG, GBBH, SAL, SSM drafted and revised the manuscript. All authors read and approved the final manuscript.

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Table 1. Fold change values for each candidate gene in pre-pubertal compared to post-pubertal heifers.

	ESR1	*FSHR*	*LHR*	*GDF9*	*CYP19A1*	*HSD3B1*	*BMP6*	*BMP7*	*BMP15*	*IGF1*	*IGFR1*	*TGFB1*	*TGFB2*
PRE vs POST[a]	1.13	2.30*	-2.03*	1.38	2.03*	-7.11**	1.33	1.47*	1.63	2.41*	1.92**	1.08	1.31
PRE vs POST-CL[b]	1.05	2.07*	-2.53*	1.36	2.48*	-6.51*	1.40	1.29	1.59	1.09	1.83	2.79**	1.26

[a]Contrast: both ovaries from pre-pubertal (PRE) versus post-pubertal (POST); [b]Contrast: both ovaries from pre-pubertal (PRE) versus ovaries with CL (corpus luteum) from post-pubertal (POST); Negative values mean lower expression in ovaries from pre-pubertal compared to post-pubertal heifers. *P < 0.05; **P < 0.01.

Table 2. Gene names, primer pair sequences, annealing temperatures and amplification efficiencies of each target.

Gene [a]	GenBank Accession no.	Primer sequence (5'-3')	Amplicon (bp)	Efficiency (%)
ESR1	NM_001001443.1	F- CTCTCTGCCTTTGCTACCTTAC R- CCCGGGTCACAAATAGCTAAA	114	98.87
FSHR	NM_174061.1	F- CATGCTCATCTTCACCGACTT R- GACCAGGAGGATCTTTGACTTG	112	99.64
LHR	NM_174381.1	F- GAGTGGCTGGGATTATGACTATG R- CAGCCAAATCAGGACTCTAAGG	127	99.87
BMPR2	NM_001304285.1	F- GTACCGGCATGACCACTATATC R- AGTCTTCTTCGGTCAGTTGTAAG	120	99.74
TGFB1	NM_001166068.1	F- TGCTTCAGCTCCACAGAAA R- GTATCCAGGCTCCAGATGTAAG	149	99.74
TGFB2	NM_ 001113252.1	F- CACGAATGGCTCCACCATAA R- AGCGTGCTTCTAGTTCTTCAC	127	98.67
GDF9	NM_174681.2	F- GCATTCCCTCCACCCTAAA R- GGTGACGGGACAATCTTACA	113	99.89
BMP15	NM_ 001031752.1	F- GTAGTGAGGTTCGTGAGTTCTG R- TAGGGAGAGGTTTGGTCTTCT	111	98.85
BMP6	XM_005223892.2	F- TTCTCAACGACGCCGATATG R- ACCCTCAGGAATCTGGGATAG	122	98.56
BMP7	NM_ 001206015.1	F- ATGACAGCTCCAACGTCATC R- AGAGACCCAGGATCCAGAAA	123	98.99
CYP19A1	NM_174305.1	F- GTGTCCGAAGTTGTGCCTAT R- GACCTGGTATTGAGGATGTGTC	101	98.95
HSD3B1	NM_174343.3	F-ACACCAGCACCATAGAAGTG R-CTTGCTGTATGGGTATGGAGAG	113	98.88
IGF1	NM_001077828.1	F- TCCCATCTCCCTGGATTTCT	105	98.86

		R- GGGTTGGAAGACTGCTGATT		
IGFR1	NM_001244612.1	F-GTATGGAGGAGCCAAGCTAAA	123	98.98
		R-GTCTTGGCCTGAACGTAGAA		
IGF2	NM_174087.3	F-CATTGTGGGAAGGTGTGTC	113	99.02
		R- TTACATTCTGTGGGCTGTGG		
B2M	NM_173893.3	F- ACCTGAACTGCTATGTGTATGG	134	99.95
		R- GTGGGACAGCAGGTAGAAAG		
GAPDH	NM_001034034.2	F- GATGCTGGTGCTGAGTATGT	113	99.96
		R- GCAGAAGGTGCAGAGATGAT		

[a]Gene Symbol: estrogen receptor α (*ESR1*), follicle stimulating hormone receptor (*FSHR*), luteinizing hormone receptor (*LHR*), bone morphogenetic protein receptor, type 2 (*BMPR2*), transforming growth factor beta 1 (*TGFβ1*), transforming growth factor beta 2 (*TGFβ2*), growth differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*), bone morphogenetic protein 6 (*BMP6*), bone morphogenetic protein 7 (*BMP7*), cytochrome P450 aromatase (*CYP19A1*), 3-β-hydroxysteroid dehydrogenase (*HSD3B1*), insulin-like growth factor 1 (*IGF1*), type 1 insulin-like growth factor receptor (*IGFR1*), insulin-like growth factor 2 (*IGF2*), beta-2- macroglobulin (*B2M*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

GENERAL CONCLUSION

In summary, this research was the first to provide evidences regarding the effects of maternal overnutrition on pituitary-gonadal gene expression and gonadal germ cell development in cattle fetuses at mid- and late gestation. Over nutrition during gestation resulted in up-regulation of genes involved in ovarian folliculogenesis, steroidogenesis and pro-apoptosis. Moreover, maternal overnutrition affected ovarian follicular growth and the number of follicles in female fetuses, which in turn may reduce their ovarian reserve and subsequent fertility. In male fetuses, testicular development was disturbed, which could have lasting effects on daily sperm production. The biological significance of the observed differential gene expression in terms of offspring reproductive potential should be the focus of further investigations.

We have also provided further insight into the effects of different levels of nutrient intake in mammary PAR development of prepubertal heifers. We reported that elevated nutrient intake level can induced changes on expression of lipogenic marks (*CD36*, *ACCA, FASN, ADIPOR1*) in mammary PAR, which leading to increase of lipogenesis in intraparenchymal adipocytes in prepubertal bovine mammary gland. Furthermore, we revealed that increasing feeding level in prepubertal heifers increased body fatness and altered concentration of hormone involved with nutritive status and liver GHR-IGF1-IGFBP axis mRNA expression. Thus, we inferred that increased fat accumulation on body and mammary gland could be predictor of impaired mammary gland development.

We have also provided evidences regarding the putative role of intraovarian factors in modulating the timing of puberty in Brahman heifers. Our findings suggest that *BMP7* and *IGF1* interact and regulate intrafollicular steroidogenesis and follicular response to gonadotropins during pubertal development. The emerging hypothesis is that two key regulators of ovarian activity pre-puberty, *BMP7* and *IGF1* play synergic roles modulating the expression of *FSHR*, *LHR* and *IGFR1* and steroidogenesis (*CYP19A1*). *BMP7* seems to down-regulate *LHR* and up-regulate *FSHR* and *CYP19A1*, which

mediates the follicular dynamics in prepubertal heifer ovaries. Furthermore, *TGFB1* likely plays important role in follicular dynamics in pre-puberty.

From the perspective of dairy cattle industry, the current studies provide additional knowledge about the relationship between the maternal nutritional status and fetal gonadal development and across the feeding regimes and prepubertal mammary gland development. Future studies based on the results from this thesis can be useful for testing hypothesis and to create novel strategies to enhance the reproductive efficiency, and accelerate growth rates for prepubertal heifers without compromising future milk production.