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# Poor Maternal Nutrition during Gestation Alters Placental IGF-I, IGF-II, and IGFBP-3 mRNA Expression in Sheep

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**Abstract**

Insulin-like growth factors (IGF) modulate placental and fetal growth and development through nutrient sensing and endocrine signaling. We hypothesized that poor maternal nutrition during gestation would alter IGF-I, IGF binding protein (IGFBP)-2, and IGFBP-3 mRNA expression in the ovine placenta, but would not affect IGF-II mRNA expression. Pregnant ewes (n=57) were individually fed: 60% (restricted fed; RES), 100% (control fed; CON), or 140% (over fed; OVER) of National Research Council requirements for TDN starting at day 30±0.2 of gestation. Ewes were euthanized and cotyledon and caruncle samples were collected at days 45, 90, and 135 of gestation. Relative mRNA expression of IGF-I, IGF-II, IGFBP-2, and IGFBP-3 was quantified using real-time PCR. Data were analyzed using the MIXED procedure in SAS. Relative IGF-I mRNA expression was less at day 45 than at days 90 and 135 in the caruncle ($P < 0.001$; d45: 0.96 ± 0.06, d90: 1.28 ± 0.06, d135: 1.38 ± 0.05). In the caruncle, IGFBP-2 expression was less at day 45 than at days 90 and 135 ($P < 0.001$; d45: 0.67 ± 0.20, d90: 1.90 ± 0.20, d135: 1.65 ± 0.18). There was no detectable impact of diet or time on IGF-II or IGFBP-3 expression in the caruncle ($P > 0.12$). In the cotyledon, IGF-I expression tended to be greater in RES than OVER, which was similar to CON ($P = 0.08$; RES: 1.10 ± 0.06, OVER: 0.89 ± 0.07, CON: 0.96 ± 0.07). Relative IGF-II mRNA expression was greater in RES than OVER cotyledons ($P = 0.01$; RES: 1.96 ± 0.31, OVER: 0.54 ± 0.32, CON: 1.30 ± 0.35). Relative IGFBP-2 mRNA expression decreased over time in the cotyledon ($P < 0.001$; d45: 1.26 ± 0.12, d90: 0.93 ± 0.12, d135: 0.59 ± 0.11). Relative IGFBP-3 mRNA expression was less in RES than in OVER or CON cotyledons ($P = 0.009$; RES: 0.03 ± 0.57, OVER: 3.62 ± 0.66, CON: 1.84 ± 0.64). In response to poor maternal diet, changes in IGF expression in the cotyledon were greater

than in the caruncle, suggesting a potential mechanism by which maternal-fetal exchange may be modified to restrict placental and fetal growth.

## Acknowledgements

I would first like to thank my honors thesis advisor Dr. Sarah Reed for providing me with the tools and skills necessary for success in this project and for her continuous guidance and mentorship throughout the scientific process. Thank you to my undergraduate and honors advisor Dr. Steven Zinn for supporting me throughout this project and the entirety of my undergraduate career. Thank you to all of the faculty and staff within the Animal Science department for providing me an incomparable undergraduate education. This project was inspired by the wealth of knowledge I learned in the classrooms of the George White and Young buildings as well as in the animal units around Horsebarn Hill. Finally, I would like to thank my friends and family for supporting me throughout my academic career.

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# Review of Literature

## *Introduction*

Fetal development is a tightly regulated period of rapid growth. Therefore, modulations to the intrauterine environment throughout gestation can negatively impact normal patterns of fetal growth and development in offspring with persistent effects in the early postnatal period through to maturity. Maternal nutrition, defined as the diet the mother consumes before breeding, throughout gestation, and during lactation (American Journal of Public Health, 1973) has the potential to influence the intrauterine environment by modulating the availability of nutrients, growth factors, and hormones to the developing fetus (McMullen et al., 2005). Poor maternal nutrition during gestation due to either overnutrition or undernutrition can have detrimental anatomical and physiological effects on the offspring including decreased placental vascularization, decreased birth weight, decreased muscle development, and increased fat deposition (Redmer et al., 2004; Hoffman et al., 2014; Reed et al., 2014). These effects alter postnatal growth of the offspring, indicating the importance of maternal nutrition during gestation on offspring development.

## *Normal Placental Development*

Ruminants have a cotyledonary placenta, which consists of a maternal contribution, the caruncle, and a fetal contribution, the cotyledon. The caruncle and the cotyledon interdigitate at discrete sites in the placenta called placentomes. Materno-fetal exchange of nutrients, gases, and waste products occurs at the placentomes. As described by Reynolds et al. (1997), the fetal membranes attach to the endometrial epithelium of the uterus on days 15 to 18 of gestation with the interdigitation of the caruncle and the cotyledon beginning on day 23 of gestation. By day 30

of gestation, the placentomes are well established and interdigitation is complete. The individual placentomes and the placenta itself continue to grow in size until day 90 of gestation, when the placenta achieves its maximum weight in sheep (Redmer et al., 2004). In sheep, the majority of placental growth occurs in the first two-thirds of pregnancy, while approximately 90% of fetal growth occurs in the last third of pregnancy (day 90 to day 145; Redmer et al., 2004).

Insulin-like growth factors (IGF) are nutritionally sensitive endocrine factors that regulate fetal and placental growth and development. The IGFs regulate processes involved in development by promoting cellular proliferation, differentiation, migration and aggregation, and inhibiting apoptosis (Jones & Clemmons, 1995; Carter et al., 2005). IGF-I and IGF-II have complimentary yet overlapping roles in optimizing nutrient partitioning to the fetus to optimize growth and development. IGF-I regulates fetal growth by partitioning nutrients to the fetus in response to nutrient availability (Sferruzzi-Perri et al., 2010). IGF-II acts directly on the placenta to modulate the thickness and surface area of the materno-fetal interface (Sibley et al., 2004; Sferruzzi-Perri et al., 2010). IGF-II is more abundant in maternal plasma than IGF-I irrespective of reproductive state in many species, including sheep (Sferruzzi-Perri et al., 2010; Kadakia & Josefson, 2016).

The bioavailability and biological actions of IGFs are regulated by IGF binding proteins (IGFBP). All six of these binding proteins specifically bind to IGF-I and IGF-II; however, they do so with differing affinities. While IGFBP-1, -3, and -4 bind IGF-I and IGF-II with similar affinities, IGFBP-2, -5, and -6 preferentially bind to IGF-II (Rajaram et al., 1997; Reynolds et al., 1997). It is well documented that IGFBPs function to limit the bioavailability of IGFs in circulation; however, this family of six high-affinity binding proteins either inhibits or potentiates the actions of IGFs on metabolism and mitogenesis based on the tissue and the post-

translational modifications of individual IGFBPs (Rajaram et al., 1997; Reynolds et al., 1997; Carter et al., 2005; Denley et al., 2005). The specific actions of the IGFBPs in placental tissue are not yet fully understood. The IGFBPs are not only capable of modulating IGF-induced cell proliferation but most of the IGFBPs have also been shown to act in IGF-independent pathways (Rajaram et al., 1997; Denley et al., 2005; McMullen et al., 2005). Due to the complexity of the IGFBP functions and the lack of understanding of the pathways with which they exert their IGF-independent effects, it is clear that the significance of changes in concentrations of the six IGFBP in placental tissue requires further study.

*Potential Mechanisms for Altered Placental Development*

Poor maternal nutrition during gestation has been associated with a decrease in birth weight of offspring and a decrease in placental weight (Gadd et al., 2000; Osgerby et al. 2004; McMullen et al., 2005). Both high and low planes of maternal nutrition throughout gestation have similar negative effects on the growth of the fetus (Gadd et al., 2000; Hoffman et al., 2014). IGF-I concentration in plasma increases over the course of gestation (Carter et al., 2005). Plasma IGF-I concentrations correlate with nutritional status, as IGF-I decreases in under-fed ewes compared with control-fed ewes and increases in over-fed compared with control-fed ewes (Gallaher et al., 1998; Gadd et al., 2000; McMullen et al., 2005). Relative mRNA expression of IGF-I in placental tissue is not well described as IGF-I mRNA concentrations fall below the limit of detection for *in-situ hybridization* assays in maternal nutrient restriction experiments shortly after the invasion of the fetal villi (Reynolds et al., 1997; McMullen et al., 2005). While IGF-I expression is both nutritionally and time-point sensitive in maternal plasma and serum, IGF-II mRNA expression remains constant throughout gestation and is nutritionally independent in

maternal plasma, maternal serum, and placental samples (Gadd et al., 2000; Osgerby et al., 2004; McMullen et al., 2005).

Previous studies have presented conflicting results regarding the placental expression of IGFBP-2 mRNA in ewes exposed to poor maternal nutrition. McMullen et al. (2005) and Gadd et al. (2000) report that the concentrations of IGFBP-2 mRNA are reduced in nutrient restricted ewes during early gestation and increased in ewes exposed to high plane rations in mid-gestation, respectively. However, Osgerby et al. (2004) reported that mRNA expression of IGFBP-2 throughout gestation was nutritionally and time-point independent. While the effects of IGFBP-2 in the placenta are not well understood, IGFBP-2 has been shown to be mitogenic in IGF-independent pathways in human uterine endometrial epithelial cells and osteosarcoma cells (Firth & Baxter, 2002).

IGFBP-3, the most abundant binding protein in placental tissue in women, exhibits both nutritionally and time-point sensitive mRNA expression (Forbes & Westwood, 2008). Concentrations of IGFBP-3 mRNA were reduced in the caruncles at day 90 in maternal undernutrition experiments (Osgerby et al., 2004; McMullen et al., 2005). Relative mRNA expression of IGFBP-3 was increased in fetal placental tissue of over-fed ewes compared with control-fed ewes (Gadd et al., 2000). The direct effects of IGFBP-3 on placental tissue is not well understood; however, IGFBP-3 has been reported to have IGF-independent growth inhibitory effects in IGF Receptor 1-knockout mice (Firth & Baxter, 2002). In opossum kidney cells, intact IGFBP-3 inhibited cell growth due to direct nuclear actions (Rechler & Clemmons, 1998). Several studies have found evidence that intact IGFBP-3 has a mediating role in a wide range of growth effectors, such as TGFβ, retinoic acid, antiestrogens, vitamin D analogs, and TNFα (Firth & Baxter, 2002). Fragments and proteolyzed forms of IGFBP-3, which have little to

no affinity for IGFs, have also been shown to inhibit mitogenesis in the fibroblasts of chick embryos and mice via IGF-independent pathways (Firth & Baxter, 2002).

*Objectives and Hypothesis*

The placenta is an important determinant of fetal growth rate. Therefore, relative expression of IGFs and IGFBPs in the placenta can be used as an indicator of the effect of environmental stressors on the growth and development of the fetus throughout gestation. The objective of this study is to determine alterations in transcription of IGF-I, IGF-II, IGFBP-2, and IGFBP-3 by poor maternal nutrition throughout gestation. This study is the first comprehensive study to describe the effects of both undernutrition and overnutrition on the relative mRNA expression of IGFs and IGFBPs over the course of gestation. Given the great implications of maternal stressors, including poor maternal nutrition during gestation, on postnatal growth and overall health of the animal, it is important to understand the effects of maternal stressors on fetal development. Examining the effects of both underfeeding and overfeeding ewes throughout gestation provides further insight into optimal nutrient requirements for lamb production as well as the duration a fetus can be exposed to poor maternal nutrition before the onset of the detrimental effects associated with poor maternal nutrition. Based on results of related studies, we hypothesized that poor maternal nutrition during gestation would cause a change in the mRNA expression of IGF-I, IGFBP-2, and IGFBP-3 in the ovine placenta, but would not affect the mRNA expression of IGF-II.

## Materials and Methods

*Animals and Sample Collection*

All animal procedures were reviewed and approved by the University of Connecticut's Institutional Animal Care and Use Committee (protocol number: A13-059).

Animal procedures were previously reported in Pillai et al. (2017). For this project, multiparous Western White-Faced ewes (n= 57) were randomly assigned one of three diets: restricted (RES; 60%), control (CON, 100%), or over-fed (OVER; 140%) based on National Research Council requirements for total digestible nutrients starting at d30 $\pm$ 0.2 of gestation (Figure 1). Ewes were euthanized via intravenous injection of Beuthanasia-D Special (0.22 mL/kg; Merck Animal Health; Summit, NJ) at days 45, 90, and 135 of gestation. Placental samples from the cotyledon and caruncle (n= 4 to 7 ewes per treatment per time point) were collected during necropsy, immediately snap frozen in liquid nitrogen, and stored at -80°C. While fetal tissues were also collected at parturition, placental tissue was not recovered.

*RNA Extraction*

Tissue was homogenized using a Qiagen TissueLyser Bead Homogenizer (Qiagen, Valencia, CA). Total RNA was extracted using a standard phenol-chloroform extraction. The quantity of RNA was determined using a Nanodrop spectrophotometer (Thermoscientific, Lafayette, CO).

*Real-Time Quantitative Polymerase Chain Reaction*

Isolated RNA was reverse transcribed to complimentary DNA using a SuperScript III kit (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Briefly, 1 μL OligodT primer

(Invitrogen), 1,000 ng total RNA, 1 μL dNTP mix (Invitrogen), and sterile water were mixed to a total reaction volume of 10 μL. The mixture was heated to 65°C for 5 minutes and incubated on ice for 1 minute. A cDNA master mixture composed of 4 μL of 5x First-Strand Buffer (Invitrogen), 1 μL DDT (Invitrogen), 1 μL RNase Out (Invitrogen), 1 μL SuperScript III (Invitrogen), and 5 μL RNase Free water (Invitrogen), was then added to the reaction. Reverse transcription was performed with a standard protocol starting at 25°C for 5 minutes, 50°C for 60 minutes, and 70°C for 15 minutes. Complimentary RNA was removed by adding 2 units of *Escherichia coli* RNase H to the reaction and incubating the mixture at 37°C for 60 minutes. Primers were designed for genes involved in the somatotropic axis (IGF-I, IGF-II, IGFBP-2, and IGFBP-3; Table 1) using NCBI BLAST and synthesized by Integrated DNA Technologies (Coralsville, IA). All primers with the exception of IGF-II were validated by Hoffman et al. (2014). The IGF-II primer was validated by Xing et al. (2014). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using SYBR Green Master Mix (Invitrogen) and the ABI 7900 HT Fast Real-time PCR machine (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The total volume of the reaction mixture was 25 μL containing 12.5 μL SYBR Green Master Mix (Invitrogen), 0.225 μL each of 10 nM forward and reverse primer (Integrated DNA Technologies), 7.05 μL of nuclease free water, and 5 μL generated cDNA. Real-time qPCR was performed using standard cycling conditions (stage 1: 50°C for 2 minutes, stage 2: 95°C for 10 minutes, stage 3: 95°C for 15 seconds and 60°C for 1 minute for 40 cycles). Ct values were utilized to calculate the $^{\Delta\Delta}$Ct values to determine changes in gene expression. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as its expression did not vary between treatments or time points. Gene expression was expressed as fold change relative to controls at day 45.

*Statistical Analysis*

Data were analyzed using the PROC MIXED procedure (SAS Institute, Inc., Cary, NC) with main effects of diet, time point, and the interaction of diet and timepoint. The PDIFF statement was utilized to determine differences between means. Data are reported as mean ± SEM, with $P < 0.05$ considered significant and $0.05 < P < 0.10$ considered a tendency.

## Results

*Day of gestation altered IGF-I and IGFBP-2 mRNA expression in the caruncle.*

In the caruncle, relative IGF-I mRNA expression was less at day 45 than at days 90 and 135 of gestation ($P < 0.001$; d45: 0.96 ± 0.06, d90: 1.28 ± 0.06, d135: 1.38 ± 0.05; Figure 2). Relative mRNA expression of IGFBP-2 was less at day 45 than at days 90 and 135 of gestation ($P < 0.001$; d45: 0.67 ± 0.20, d90: 1.90 ± 0.20, d135: 1.65 ± 0.18; Figure 2). There was no detectable impact of time on relative mRNA expression of IGF-II or IGFBP-3 in the caruncle ($P > 0.05$; Figure 2). Dietary treatment did not significantly alter relative mRNA expression of IGF-I, IGF-II, IGFBP-2, or IGFBP-3 in the caruncle ($P > 0.05$).

*Maternal diet altered IGF-II and IGFBP-3 mRNA expression in the cotyledon.*

In the cotyledon, expression of IGF-I tended to be greater in RES than OVER with CON intermediate (main effect of diet: $P = 0.08$; RES: 1.10 ± 0.06, OVER: 0.89 ± 0.07, CON: 0.96 ± 0.07; Figure 3). Relative IGF-II mRNA expression was greater in RES than OVER cotyledons with CON intermediate (main effect of diet: $P = 0.01$; RES: 1.96 ± 0.31, OVER: 0.54 ± 0.32, CON: 1.30 ± 0.35; Figure 3). Relative IGFBP-3 mRNA expression was less in RES than in

OVER or CON cotyledons (main effect of diet: $P = 0.009$; RES: 0.03 ± 0.57, OVER: 3.62 ± 0.66, CON: 1.84 ± 0.64; Figure 3). Maternal diet had no detectable effect on relative mRNA expression of IGFBP-2 in the cotyledon ($P > 0.05$; Figure 3).

*Day of gestation altered IGFBP-2 mRNA expression in the cotyledon.*

Relative IGFBP-2 mRNA expression decreased over time in the cotyledon ($P < 0.001$; d45: 1.26 ± 0.12, d90: 0.93 ± 0.12, d135: 0.59 ± 0.11; Figure 4). There were no significant detectable effects of time on relative mRNA expression of IGF-I, IGF-II, or IGFBP-3 in the cotyledon ($P > 0.05$; Figure 4).

**Table 1. Primer sequences for real-time qPCR.**

| Gene | Primer Sequences | References |
|---|---|---|
| IGF-I | | Hoffman et al., 2014 |
| Forward | 5'-CCAGACAGGAATCGTGGATG-3' | |
| Reverse | 5'- ACTTGGCGGGCTTGAGAG-3' | |
| IGF-II | | Xing et al., 2014 |
| Forward | 5'-TTCTTCCAATCTGACACCTG-3' | |
| Reverse | 5'-AGGCAGGGCGATCAGCGGACGGTGA-3' | |
| IGFBP-2 | | Hoffman et al., 2014 |
| Forward | 5'- CCCTACACATCCCCAACTGT-3' | |
| Reverse | 5'- CAGTGTTGGGGTTCACACAC-3' | |
| IGFBP-3 | | Hoffman et al., 2014 |
| Forward | 5'- CAGAGCACAGACACCCAGAA-3' | |
| Reverse | 5'- CACAGTTGGGAATGTGGATG-3' | |
| GAPDH | | Hoffman et al., 2014 |
| Forward | 5'-GGCGTGAACCACGAGAAGTATAA-3' | |
| Reverse | 5'- CCTCCACGATGCCAAAGTG-3' | |

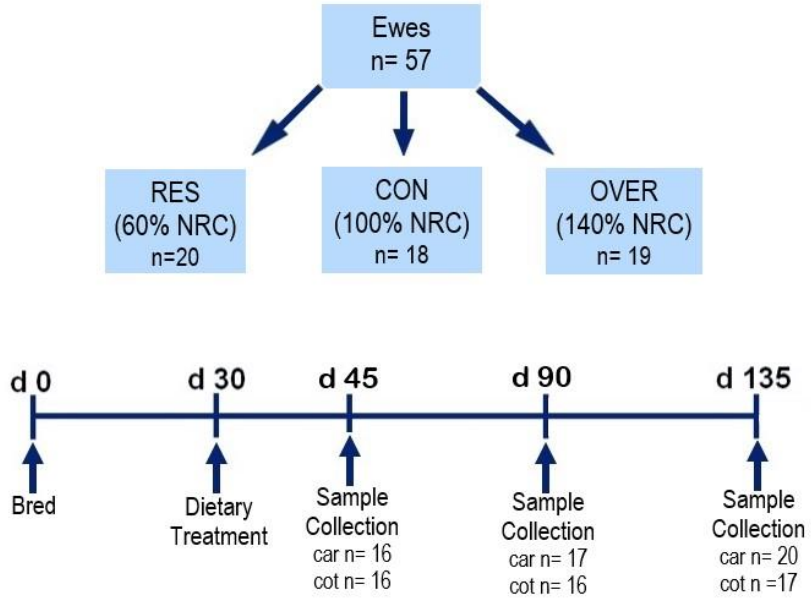


**Figure 1. Experimental Design.** Experimental design, adapted from Pillai et al. (2017). Pregnant ewes (n = 57) were randomly assigned to one of three dietary treatments beginning at d 30 ± 0.2 of gestation (CON = 100% NRC; RES = 60% NRC; OVER = 140% NRC). At day 45, 90, or 135 of gestation, ewes were euthanized and placental samples were collected during necropsy. The placental samples were dissected into two distinct tissues: the caruncle (car) and cotyledon (cot). The samples were placed into individual tubes, snap frozen in liquid nitrogen, and stored at -80°C (n = 4 to 7 ewes per treatment combination per tissue).

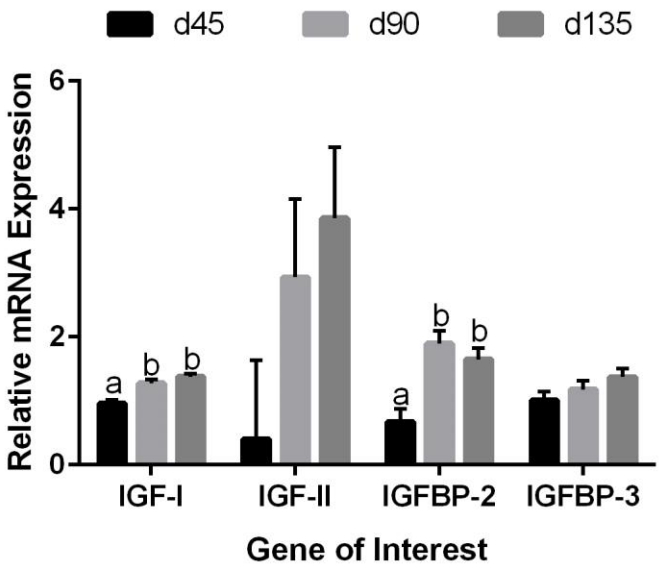


**Figure 2. Day of gestation altered IGF-I and IGFBP-2 mRNA expression in the caruncle.** Gene expression of insulin-like growth factor (IGF)-I, IGF-II, IGF binding protein (BP)-2, and IGFBP-3 was analyzed in the caruncle. Relative mRNA expression of IGF-I and IGFBP-2 were less at day 45 than at days 90 and 135 ($P < 0.001$ for both genes). Data are expressed as mean ± SEM. Within each gene, means with different letters are different at $P < 0.05$.

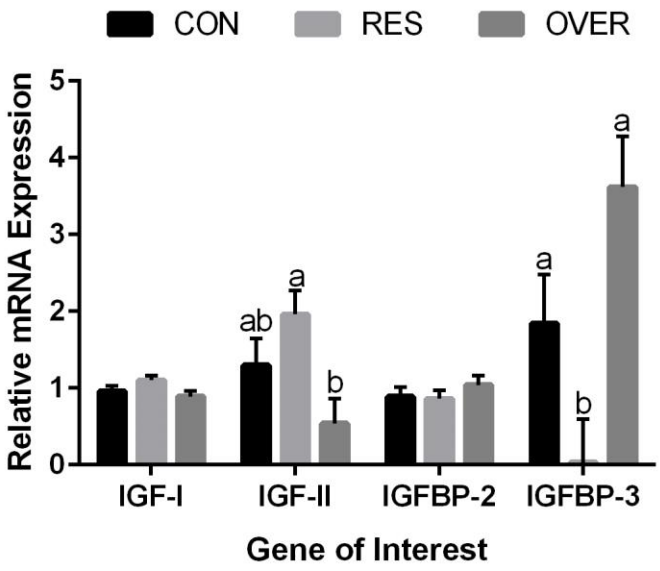


**Figure 3. Maternal diet altered IGF-II and IGFBP-3 mRNA expression in the cotyledon.** Gene expression of insulin-like growth factor (IGF)-I, IGF-II, IGF binding protein (BP)-2, and IGFBP-3 was analyzed in the cotyledon. Relative IGF-II mRNA expression was greater in RES than OVER cotyledons (main effect of diet: $P = 0.01$). Relative IGFBP-3 mRNA expression was less in RES than in OVER or CON cotyledons (main effect of diet: $P = 0.009$). Data are expressed as mean ± SEM. Within each gene, means with different letters are different at $P < 0.05$.

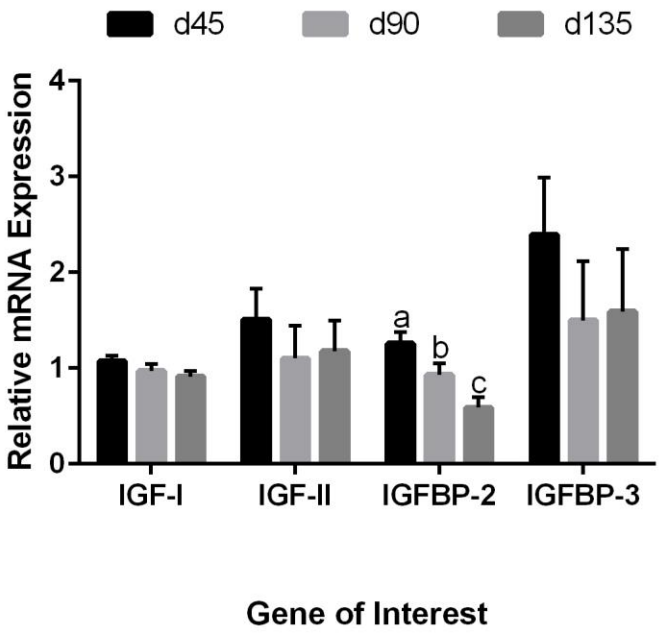


**Figure 4. Day of gestation alters IGFBP-2 mRNA expression in the cotyledon.** Gene expression of insulin-like growth factor (IGF)-I, IGF-II, IGF binding protein (BP)-2, and IGFBP-3 was analyzed in the cotyledon. Relative IGFBP-2 mRNA expression decreased over time ($P < 0.001$). Data are expressed as mean ± SEM. Within each gene, means with different letters are different at $P < 0.05$.

## Discussion

Previous studies investigating the effects of poor maternal nutrition during gestation on placental expression of IGFs and IGFBPs have focused on either restricted- (Gallaher et al., 1998; Osgerby et al., 2004; McMullen et al., 2005) or over-feeding (Gadd et al., 2000). Additionally, the models utilized in previous experiments studied only the acute effects of poor maternal nutrition (Gallaher et al., 1998; Osgerby et al., 2004; McMullen et al., 2005). The aim of this study was to determine the effects of both over- and under-feeding throughout gestation on placental mRNA expression of IGF-I, IGF-II, IGFBP-2, and IGFBP-3 in both the caruncle and the cotyledon. The caruncle and cotyledon were analyzed separately to identify potential mechanisms through which changes in expression of the IGFs and IGFBPs induced by poor maternal nutrition in either of these tissues may contribute to the restricted placental and fetal growth observed in previous studies (Gadd et al., 2000; Osgerby et al., 2004; Redmer et al., 2004; Carter et al., 2005; Pillai et al., 2017). We found that maternal diet altered the expression of IGF-II and IGFBP-3 in the cotyledon, but did not alter expression of any of the IGFs or IGFBPs studied in the caruncle.

### *Maternal diet altered IGF-II and IGFBP-3 mRNA expression in the cotyledon.*

In this study, relative IGFBP-3 mRNA expression was less in RES than in OVER or CON cotyledons. This result is consistent with the observations of Gallaher et al. (1998) in which IGFBP-3 concentrations were decreased in fetal plasma in under-fed ewes. This result is also consistent with an observed reduction of plasma IGFBP-3 in underfed guinea pigs (Carter et al., 2005). IGFBP-3 has an equal binding affinity for IGF-I and IGF-II and binds 95% of circulating IGF-I and IGF-II (Reynolds et al., 1997). The decrease in placental expression of

IGFBP-3 in restricted-fed ewes concurrent with the increase in IGF-II would allow for an increase in the bioavailability of IGF-II. The increase in bioavailable IGF-II would then allow for an increase in placental surface area and decrease in placental thickness, similar to those effects observed in guinea pigs (Roberts et al., 2008; Forbes & Westwood, 2008; Forbes & Westwood, 2010; Sferruzzi-Perri et al., 2010). Roberts et al. (2008) reported that plasma IGF-II concentrations correlate positively with placental surface area and negatively with placental thickness in guinea pigs. When interpreted in this context, the increase in IGF-II expression in RES cotyledons compared with OVER cotyledons is intuitive physiologically. A restricted-fed ewe has less nutrients available for the fetus and the ewe will partition nutrients to the fetus at the expense of her own requirements. The increase in placental expression of IGF-II would cause a concurrent increase in placental surface area and decrease in the thickness of the placenta, allowing for the passage of nutrients from maternal to fetal circulation to occur at a more rapid rate. Similarly, the decrease in IGF-II expression in the OVER cotyledons would decrease placental surface area while increasing barrier thickness. The decrease in the rate of passage of nutrients from maternal to fetal circulation due to these physiological changes to the placenta would protect the fetus from over-exposure to nutrients, allowing for a more optimal intrauterine environment for growth. IGFBP-2 expression was not impacted by maternal diet in this study, potentially allowing an increase in bioavailability of IGF-II which may result in the above described physiological changes in the placenta. However, the nutritional dependence of IGF-II expression in the cotyledons is contradictory to *in-situ hybridization* studies that indicate that placental expression of IGF-II is nutritionally independent (Gadd et al., 2000; Osgerby et al., 2004; & McMullen et al., 2005). This discrepancy in results could be due to the different methods utilized in the experiments. The *in-situ hybridization* studies utilized an image analysis

system and quantified the results using optical density units. In this study, however, we utilized real-time qPCR which is more sensitive and accurate in quantifying the concentrations of compounds such as IGFs and IGFBPs (Biedermann et al., 2004; Thermo Fisher Scientific Inc., 2020).

IGFBP-3 has an equal binding affinity for IGF-I and IGF-II and binds 95% of circulating IGF-I and IGF-II (Reynolds et al., 1997). In this study, relative IGFBP-3 mRNA expression was less in RES than in OVER or CON cotyledons. This result is consistent with the observations of Gallaher et al. (1998) in which IGFBP-3 concentrations were decreased in fetal plasma in under-fed ewes and with an observed reduction of plasma IGFBP-3 in underfed guinea pigs (Carter et al., 2005). The decrease in placental expression of IGFBP-3 in restricted-fed ewes concurrent with the increase in IGF-II would allow for an increase in the bioavailability of IGF-II. The increase in bioavailable IGF-II would then allow for an increase in placental surface area and decrease in placental thickness, similar to those effects observed in guinea pigs, which would allow for the limited nutrients of the RES ewes to be more easily passed via the feto-maternal interface of the placentome to the fetus (Roberts et al., 2008; Sferruzzi-Perri et al., 2010). The nutrients would be partitioned to the fetus to allow for the fetus to grow at its maximum potential at the expense of the nutrient-restricted ewe.

*Day of gestation altered IGF-I in the caruncle and IGFBP-2 mRNA expression in both the caruncle and cotyledon.*

Over the course of gestation, fetal demands for nutrients increase in preparation for the rapid period of fetal growth during late gestation (Redmer et al., 2004). Maternal IGF-I serum concentrations increase to accommodate the changes in nutrient demand, partitioning more

nutrients to the fetus to allow for optimal growth of the fetus at expense of the ewe. Previous studies indicate that maternal serum IGF-I concentrations increase over gestation (Carter et al., 2005). Relative mRNA expression of IGF-I was less at day 45 than at days 90 and 135 in the caruncle. This change in mRNA expression was not observed in the cotyledon. The observed change in IGF-I expression in the maternal but not the fetal contribution to the placentome is consistent with the idea that IGF-I is essential in controlling nutrient partitioning (Sferruzzi-Perri et al., 2010). The relative expression of IGFBP-2 in the caruncle mirrored the changes observed in IGF-I expression. While IGFBP-2 preferentially binds to IGF-II, it still has the capacity to bind to IGF-I and mitigate its bioavailability (Rajaram et al., 1997; Reynolds et al., 1997). The expression of IGF-II has been characterized as being time-point independent during gestation in sheep (Gadd et al., 2000; Osgerby et al., 2004; & McMullen et al., 2005). These results suggest that IGFBP-2 may be expressed in a similar pattern as IGF-I in the caruncle to maintain IGF-II concentrations throughout gestation while having a lesser effect on the bioavailability of IGF-I. The increase in IGFBP-2 decreases the bioavailability of IGF-II while having a more minimal impact on the bioavailability of IGF-I. This allows IGF-I concentrations to increase to meet the nutritional demands of the fetus throughout gestation while ensuring its concentrations remain within an appropriate physiological range.

The majority of fetal growth occurs in the last third of pregnancy (day 90 to 145; Redmer et al., 2004). IGF-II regulates fetal growth by acting directly on the placenta to influence nutrient delivery to fetal cells (Sferruzzi-Perri et al., 2010). IGFBP-2 preferentially binds IGF-II, which plays an important role in regulating the surface area and thickness of the placenta as well as stimulating proliferation, differentiation, migration and aggregation of fetal cells (Jones & Clemmons, 1995; Sibley et al., 2004; Carter et al., 2005; Sferruzzi-Perri et al., 2010). Taken

together, the decrease in fetal mRNA expression of IGFBP-2 throughout gestation in this study with its lowest relative expression during late gestation (day 135) could increase the bioavailability of fetal IGF-II, allowing for a greater influence of IGF-II on fetal growth and development during the period of rapid growth in late gestation.

## Conclusion

In conclusion, poor maternal nutrition during gestation influenced the relative mRNA expression of IGF-I, IGF-II, and IGFBP-3 in the cotyledon, but had no effect on IGF-I, IGF-II, IGFBP-2, or IGFBP-3 expression in the caruncle or IGFBP-2 expression in the cotyledon. The IGF system was altered to a greater extent in the cotyledon than in the caruncle in response to poor maternal diet, suggesting a potential mechanism by which maternal-fetal exchange may be modified to restrict placental and fetal growth. Relative mRNA expression of IGFs and IGFBPs in response to poor maternal nutrition warrants further study as there are few studies of this kind and the results of this study conflict with those found in experiments studying the effect of poor maternal nutrition on plasma concentration or *in-situ hybridization* of IGFs and IGFBPs. Future studies should also measure protein concentrations in placental tissue, fetal circulation, and maternal circulation to determine if the changes in transcription observed in this study are reflected in the protein concentrations. Further studies should investigate the effects both over- and under-feeding over the course of gestation on relative expression of IGF-I, IGF-II, and all six of the IGFBPs in both the cotyledon and the caruncle to allow for greater understanding of the interactions of the IGFs and IGFBPs in the placentome. Additional work should also focus on IGFBP-4, as it and its protease are associated with pregnancy and dysregulation of plasma IGFBP-4 in humans has been shown to contribute significantly to the development of fetal

growth restriction (Qiu et al., 2012). The results of this study indicate that the IGF system in the cotyledon was altered in reaction to poor maternal diet to maintain the bioavailable IGF-I and IGF-II concentrations in the placenta within appropriate physiological ranges to in an attempt to counteract fetal growth restriction caused by nutritional insult. Poor maternal nutrition has been correlated with restricted fetal and placental growth throughout gestation, resulting in a decrease in the productive part of the animal, the muscle. Therefore, it is important to understand the mechanisms and factors that contribute to restricted fetal growth caused by poor maternal nutrition.

## References

American Journal of Public Health. 1973. Maternal nutritional assessment. American Journal of Public Health. 63:57–63. https://doi.org/10.2105/AJPH.63.11_Suppl.57

Biedermann K., N. Dandachi, M. Trattner, G. Vogl, H. Doppelmayr, E. More, A. Staudach, O. Dietze, & C. Hauser-Kronberger. 2004. Comparison of Real-Time PCR Signal-Amplified In Situ Hybridization and Conventional PCR for Detection and Quantification of Human Papillomavirus in Archival Cervical Cancer Tissue. Journal of Clinical Microbiology. 42(8): 3758-3765. doi: 10.1128/JCM.42.8.3758-3765.2004

Carter A. M., M. J. Kingston, K. K. Han, D. M. Mazzuca, K. Nygard, & V. K. M. Han. 2005. Altered expression of IGFs and IGF-binding proteins during intrauterine growth restriction in guinea pigs. Journal of Endocrinology. 184: 179-189. doi: 10.1677/joe.1.05781

Denley A., L. J. Cosgrove, G. W. Booker, J. C. Wallace, & B. E. Forbes. 2005. Molecular interactions of the IGF system. Cytokine & Growth Factor Reviews. 16: 421-439. doi: 10.1016/j.cytogfr.2005.04.004

Firth S. M. & R. C. Baxter. 2002. Cellular Actions of the Insulin-Like Growth Factor Binding Proteins. Endocrine Reviews. 23(6): 824-854. https://doi-org.ezproxy.lib.uconn.edu/10.1210/er.2001-0033

Forbes K. & M. Westwood. 2008. The IGF Axis and Placental Function. Hormone Research. 69: 129-137. doi: 10.1159/000112585

Forbes K. & M. Westwood. 2010. Maternal growth factor regulation of human placental development and fetal growth. Journal of Endocrinology. 207: 1-16. doi: https://doi.org/10.1677/JOE-10-0174

Gadd T. S., R. P. Aitken, J. M. Wallace, & D. C. Wathes. 2000. Effect of high maternal dietary intake during mid-gestation on components of the utero-placental insulin-like growth factor (IGF) system in adolescent sheep with retarded placental development. Journal of Reproduction and Fertility. 118: 407-416.

Gallaher G. W., B. H. Breier, C. L. Keven, J. E. Harding, & P. D. Gluckman. 1998. Fetal programming of insulin-like growth factor (IGF)-I and IGF-binding protein-3: evidence for an altered response to undernutrition in late gestation following exposure to periconceptual undernutrition in sheep. Journal of Endocrinology. 159: 501-508.

Hoffman M. L, M. A. Rokosa, S. A. Zinn, T. A. Hoagland, & K. E. Govoni. 2014. Poor maternal nutrition during gestation in sheep reduces circulating concentrations of insulin-like growth factor-I and insulin-like growth factor binding protein-3 in offspring. Domestic Animal Endocrinology. 49: 39-48. http://dx.doi.org/10.1016/j.domaniend.2014.05.002

Jones J. I. & D. R. Clemmons. 1995. Insulin-Like Growth Factors and Their Binding Proteins: Biological Actions. Endocrine Reviews. 16(1): 3-34.

Kadakia R. & J. Josefson. 2016. The Relationship of Insulin-Like Growth Factor 2 to Fetal Growth and Adiposity. Hormone Research in Pediatrics. 85: 75-82. https://doi.org/10.1159/000443500

McMullen S., J. C. Osgerby, J. S. Milne, J. M. Wallace, & D. C. Wathes. 2004. The Effects of Acute Nutrient Restriction in the Mid-gestational Ewe on Maternal and Fetal Nutrient Status, the Expression of Placental Growth Factors and Fetal Growth. Placenta. 26: 25-33. doi: 10.1016/j.placenta.2004.04.010

Osgerby J. C., D. C. Wathes, D. Howard, & T. S. Gadd. 2004. The effect of maternal

undernutrition on the placental growth trajectory and the uterine insulin-like growth factor axis in the pregnant ewe. Journal of Endocrinology. 182: 89-103.

Pillai S. M., A. K. Jones, M. L. Hoffman, K. K. McFadden, S. A. Reed, S. A. Zinn, & K. E. Govoni. 2017. Fetal and organ development at gestational days 45, 90, 135, and at birth of lambs exposed to under- and over-nutrition during gestation. Translational Animal Science. 1(1): 16-25. https://doi.org/10.2527/tas2016.0002

Qiu Q., M. Bell, X. Lu, X. Yan, M. Rodger, M. Walker, S. W. Wen, S. Bainbridge, H. Wang, & A. Gruslin. 2012. Significance of IGFBP-4 in the Development of Fetal Growth Restriction. The Journal of Clinical Endocrinology and Metabolism. 97(8): E1429-E1439. doi: 10.1210/jc.2011-2511

Rajaram S., D. J. Baylink, & S. Mohan. 1997. Insulin-Like Growth Factor-Binding Proteins in Serum and Other Biological Fluids: Regulation and Functions. Endocrine Reviews. 18(6): 801-831.

Rechler M. M. & D. R. Clemmons. 1998. Regulatory Actions of Insulin-like Growth Factor binding Proteins. Trends in Endocrinology & Metabolism. 9(5): 176-183. https://doi.org/10.1016/S1043-2760(98)00047-2

Redmer D. A., J. M. Wallace, & L. P. Reynolds. 2004. Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. Domestic Animal Endocrinology. 27: 199-217. doi: 10.1016/j.domaniend.2004.06.006

Reed S. A., J. S. Raja, M. L. Hoffman, S. A. Zinn, & K. E. Govoni. 2014. Poor maternal nutrition inhibits muscle development in ovine offspring. Journal of Animal Science and Biotechnology. 5(43): 1-11. https://doi.org/10.1186/2049-1891-5-43

Reynolds T. S., K. E. Stevenson, & D. C. Wathes. 1997. Pregnancy-Specific Alterations in the

Expression of the Insulin-Like Growth Factor System during Early Placental Development in the Ewe. Endocrinology. 138(3): 886-897.

Roberts C. T., J. A. Owens, & A. N. Sferruzzi-Perri. 2008. Distinct Actions of Insulin-Like Growth Factors (IGFs) on Placental Development and Fetal Growth: Lessons from Mice and Guinea Pigs. Placenta. 29: 42-47. https://doi.org/10.1016/j.placenta.2007.12.002

Sferruzzi-Perri A. N., J. A. Owens, K. G. Pringle, & C. T. Roberts. 2010. The neglected role of insulin-like growth factors in the maternal circulation regulating fetal growth. Journal of Physiology. 589(1): 7-20

Sibley C. P., P. M. Coan, A. C. Ferguson-Smith, W. Dean, J. Hughes, W. Reik, G. J. Burton, A. L. Fowden, & M. Constancia. 2004. Placental-specific insulin-like growth factor 2 (Igf2) regulates the diffusional exchange characteristics of the mouse placenta. Proceedings of the National Academy of Sciences of the United States of America. 101(21): 8204-8208. doi: 10.1073/pnas.0402508101

Thermo Fisher Scientific Inc. 2020. Strategies for Detecting mRNA Northern blotting, Nuclease Protection Assays, In Situ hybridization, and RT-PCR. Accessed April 21, 2020. https://www.thermofisher.com/us/en/home/references/ambion-tech-support/northern-analysis/tech-notes/strategies-for-detecting-mrna.html

Xing H. J., Z. Y. Wang, B. S. Zhong, S. J. Ying, H. T. Nie, Z. R. Zhou, Y. X. Fan & F. Wang. 2014. Effects of different dietary intake on mRNA levels of MSTN, IGF-I, and IGF-II in the keletal muscle of Dorper and Hu sheep hybrid $F_1$ rams. Genetics and Molecular Research. 13(3): 5258-5268. http://dx.doi.org/10.4238/2014.July.24.4