

EFFECTS OF L-CARNITINE ON GILT GROWTH, FETAL GROWTH AND FETAL  
MUSCLE CHARACTERISTICS, AND THE IGF SYSTEM IN PIGS  
HARVESTED AT DAY 40, 55, AND 70 OF GESTATION

by

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B.S., Iowa State University, 2001  
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AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry  
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## Abstract

We used a total of fifty-nine gilts (BW=137.7 kg) from three different breeding groups were used to assess the effects of feeding L-carnitine during gestation on gilt growth characteristics, gilt and fetal blood metabolites, litter characteristics, and IGF axis components in fetal hepatic and skeletal muscle, maternal uterine and chorioallantois tissues, and porcine embryonic myoblasts collected from fetuses. Experimental treatments were arranged in a 2 × 3 factorial arrangement with main effects of L-carnitine and day of gestation. Gilts were fed a constant feed allowance of 1.75 kg/d and a top-dress containing either 0 or 50 ppm of L-carnitine starting on the first day of breeding through the allotted gestation length (40, 55, or 70). No differences ( $P > 0.16$ ) were observed for BW or estimated protein or fat mass at any gestation length. Gilts fed L-carnitine tended to have greater ( $P = 0.10$ ) backfat at d 40 and were numerically heavier at d 70 compared to control gilts. No differences ( $P > 0.77$ ) were observed in circulating total and free carnitine at breeding, but concentrations increased ( $P < 0.01$ ) as gestation length increased for the gilts fed L-carnitine compared to those fed the control diet. Fetuses from the gilts fed L-carnitine tended to be heavier ( $P = 0.06$ ) and fetal circulating IGF-II lower ( $P = 0.09$ ) at day 70 compared to the fetuses from the control gilts. Insulin-like growth factor-I (IGF-I) mRNA was lower ( $P = 0.05$ ) in fetal hepatic tissue in fetuses collected from gilts fed supplemental L-carnitine. Insulin-like growth factor binding protein-3 (IGFBP-3;  $P = 0.05$ ) and IGFBP-5 mRNA increased ( $P = 0.01$ ) and IGF-I mRNA numerically increased ( $P = 0.16$ ) in the endometrium of gilts supplemented with L-carnitine. At d 55 or 70 gestation, fetuses were removed for collection of porcine embryonic myoblasts (PEM) from the semitendinosus. There were no treatment differences ( $P > 0.10$ ) for the expression of IGF-I, IGF-II or IGFBP-5 mRNA levels. However, PEM isolated from fetuses collected from gilts fed L-carnitine had lower ( $P = 0.08$ ) IGFBP-3 mRNA levels compared to the controls. Myoblasts isolated from fetuses obtained from gilts fed L-carnitine had greater ( $P = 0.09$ ; 8.8 %) 5.1H11 monoclonal antibody attachment after 72 h in culture. Although not significant ( $P = 0.20$ ), the total number of PEM in the S phase of the cell cycle was 4.7 % greater in PEM collected from fetuses from gilts fed L-carnitine compared to the controls. This study shows L-carnitine supplementation to gestating gilts has beneficial effects on average fetal weight, due in

part to changes in the expression of the IGF axis at the fetal-maternal interface in swine. These changes in the IGF axis play a fundamental role in porcine fetal growth and development due to enhanced proliferation and delayed differentiation of PEM.

**Key Words:** insulin-like growth factor, insulin-like growth factor binding protein, L-carnitine, messenger RNA, myoblasts, pigs

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Dr. Bradley J. Johnson

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A total of fifty-nine gilts (BW=137.7 kg) from three different breeding groups were used to assess the effects of feeding L-carnitine during gestation on gilt growth characteristics, gilt and fetal blood metabolites, litter characteristics, and IGF axis components in fetal hepatic and skeletal muscle, maternal uterine and chorioallantois tissues, and porcine embryonic myoblasts collected from fetuses. Experimental treatments were arranged in a  $2 \times 3$  factorial arrangement with main effects of L-carnitine and day of gestation. Gilts were fed a constant feed allowance of 1.75 kg/d and a top-dress containing either 0 or 50 ppm of L-carnitine starting on the first day of breeding through the allotted gestation length (d 40, 55, or 70). No differences ( $P > 0.16$ ) were observed for BW or estimated protein or fat mass at any gestation length. Gilts fed L-carnitine tended to have greater ( $P = 0.10$ ) backfat at d 40 and were numerically heavier at d 70 compared to control gilts. No differences ( $P > 0.77$ ) were observed in circulating total and free carnitine at breeding, but concentrations increased ( $P < 0.01$ ) as gestation length increased for the gilts fed L-carnitine compared to those fed the control diet. Fetuses from the gilts fed L-carnitine tended to be heavier ( $P = 0.06$ ) and fetal circulating IGF-II lower ( $P = 0.09$ ) at day 70 compared to the fetuses from the control gilts. Insulin-like growth factor-I (IGF-I) mRNA was lower ( $P = 0.05$ ) in fetal hepatic tissue in fetuses collected from gilts fed supplemental L-carnitine. Insulin-like growth factor binding protein-3 (IGFBP-3;  $P = 0.05$ ) and IGFBP-5 mRNA increased ( $P = 0.01$ ) and IGF-I mRNA numerically increased ( $P = 0.16$ ) in the endometrium of gilts supplemented with L-carnitine. At d 55 or 70 of gestation, fetuses were removed for collection of porcine embryonic myoblasts (PEM) from the semitendinosus. There were no treatment differences ( $P > 0.10$ ) for the expression of IGF-I, IGF-II, or IGFBP-5 mRNA levels. However, PEM isolated from fetuses collected from gilts fed L-carnitine had lower ( $P = 0.08$ ) IGFBP-3 mRNA levels compared to the controls. Myoblasts isolated from fetuses obtained from gilts fed L-carnitine had greater ( $P = 0.09$ ; 8.8 %) 5.1H11 monoclonal antibody attachment after 72 h in culture. Although not significant ( $P = 0.31$ ), the total number of PEM in the S phase of the cell cycle was 4.7 % greater in PEM collected from fetuses from gilts fed L-carnitine compared to the controls. This study shows L-carnitine

supplementation to gestating gilts has beneficial effects on average fetal weight, due in part to changes in the expression of the IGF axis at the fetal-maternal interface in swine. These changes in the IGF axis play a fundamental role in porcine fetal growth and development due to enhanced proliferation and delayed differentiation of PEM.

**Key Words:** insulin-like growth factor, insulin-like growth factor binding protein, L-carnitine, messenger RNA, myoblasts, pigs

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# **CHAPTER 1 - Factors affecting myogenesis in the fetal pig**

## Summary

In the fetal pig, myogenesis is under the control of regulatory factors (Olson, 1990), somatomedins (Florini et al., 1991), nutrition (Pond et al., 1968), and uterine crowding (Town et al., 2004). Members of the myogenic regulatory factor family (MRF) have specific roles during myoblast proliferation and differentiation. Two members of the MRF family, Myf-5 and MyoD are expressed in proliferating myoblasts. Myogenin increases when PEM begin to differentiate and MFR4 causes the maturation of myotubes (Ludolph and Konieczny, 1995). Insulin-like growth factors and binding proteins are involved in cellular proliferation and differentiation. Specifically, IGF-I, IGF-II, IGFBP-3, and IGFBP-5 promote both of these processes (James et al., 1993; Johnson et al., 2003). In addition, injection of growth hormone increases primary muscle fiber number because of the actions of IGF axis components (Rehfeldt et al., 2001). Research has shown muscle fiber development is affected by maternal nutrition at different stages of gestation (Dwyer et al., 1994; Bee, 2004), and when sows are fed under requirement levels through the whole gestation period (Schoknecht et al., 1993). In addition, uterine crowding can decrease secondary muscle fiber development, decrease muscle weight, and cross-sectional area of muscle fibers (Town et al., 2004) L-carnitine, a cofactor involved in several biological pathways has been shown to increase the cross-sectional area and number of total muscle fibers in the semitendinosus of pigs. In addition, L-carnitine has been shown to alter the IGF axis in fetal myoblasts. The PEM have decreased IGF-II, IGFBP-3, and IGFBP-5 mRNA expression which suggest these PEM have a delayed differentiation. This will give more muscle fiber numbers at parturition. These factors are important in the understanding of fetal myogenesis. In addition, chromium tripicolinate has shown improvements in the number of total pigs born alive while maintaining individual birth weight (Lindemann et al., 1995a). The improved efficiency of insulin caused by chromium tripicolinate increases ovulation rate, therefore, an increase in litter size is observed. These factors, along with others are involved with myogenesis in the fetal pig.

## Introduction

Over the past 25 years, sow litter size within the United States has increased approximately 12.5% (USDA, 2000). The improvement in litter size has been accomplished through the application of genetic improvement focusing on ovulation rate (Foxcroft & Town,

2004), which, in turn, improves net income for producers (Johnson, 2000). But, with the benefits of increased pigs/sow/year, swine producers have observed an increased number of stillborns and lower pig birth weights.

Many factors affect fetal growth and development specifically in relation to litter size. These factors include, but are not limited to sow ovulation rate, uterine capacity, genotype, nutrition, and feeding regimens. Researchers are beginning to understand that uterine capacity, which determines the number of fetuses maintained during pregnancy (Vallet et al., 2003), is one of the greatest limitations to litter size (Ford, 1997; Foxcroft & Town, 2004). Litter size is negatively correlated with litter weight. Sows from large litters have lighter fetuses at term because of the decreased placental surface area (Pérez et al., 1997). This may be due to lower nutrient transfer from the dam to the fetus.

Mobilization of energy substrates increases in sows with larger litters. Glucose is a major energy substrate for fetuses and has been shown to decrease in sows with large litters (Pérez, 1995) due to the high energy requirement for the uterus and fetuses. Currently, the recommended feeding method is to limit feed gilts and sows in gestation. Recent research has shown secondary muscle fiber development, which is an important determinant of postnatal growth, can be improved by increasing maternal feed intake during d 25 to 50 of gestation (Dwyer et al., 1993 and 1994; Bee, 2004) or after d 70 of gestation (McPherson et al., 2004). But, high energy intake during gestation results in greater expense, decreased feed intake during lactation, and impairment of mammary gland development (KSU Swine Nutrition Guide, 1997). The question remains 'how do we supply a greater level of nutrients to the fetus without adding energy to the sow's diet?'

The addition of L-carnitine, a water-soluble vitamin-like compound, to gestation diets has demonstrated increased litter weights at birth and weaning (Musser et al., 1999). During gestation, the addition 50 ppm (100 mg/d; d 5 to 112) of L-carnitine increases sow body weight gain and last rib fat depth. In addition, sows fed supplemental L-carnitine had increased total litter (34.2 vs. 32.2 lb) and pig (3.4 vs. 3.2 lb) birth weight, increased litter weaning weight (99.2 lb vs. 91.1 lb; Musser et al., 1999), and increased litter size (15.5 vs. 10.8; Waylan et al., 2005). Other researchers have observed similar results with increased sow weight gain and improved average fetal weight at d 70 gestation in gilts fed supplemental L-carnitine (Brown et al., 2006).



Dietary additions of specific nutrients have shown positive effects on pig and litter weight at birth. Chromium is a trace mineral that is involved in glucose metabolism and improves insulin action. Recent research using chromium tripicolinate (200 ppb) in gestation diets has demonstrated an improvement in the number of pigs born alive, while maintaining individual pig birth weight (Lindemann et al., 1995a). Other research has observed supplementing chromium tripicolinate (0, 200, 600, and 1000) increases the number of pigs born alive per litter, but numerically decreases individual birth weight of total pigs born (Lindemann et al., 2004). The improved efficiency of insulin caused by chromium tripicolinate increases ovulation rate, therefore, an increase in litter size is observed. Other nutrients, such as L-carnitine show similar results. The addition of nutrient compounds, like L-carnitine and chromium tripicolinate, may be an option to improve pig and litter weights at birth.

This review focuses on some of the factors that affect myogenesis in the fetal pig, specifically, metabolic modifiers, uterine crowding, maternal nutrition, and nutrient factors.

## **Myogenesis**

Myogenesis, the formation of muscle cells and fibers, begins with the commitment of multipotent stem cells to form mononucleated myoblasts. These mononucleated myoblasts multiply, withdraw from the cell cycle, and fuse to form multinucleated myotubes which mature into muscle fibers. In the fetal pig, this process is under the control of somatomedins (Florini et al., 1991), regulatory factors (Olson, 1990), nutrition (Pond et al., 1968), and uterine crowding (Town et al., 2004). This literature review will focus on these specific areas that affect myogenesis in the fetal pig.

### **Myogenic regulatory factors**

Proliferation and terminal differentiation of myoblasts during embryogenesis is initiated by transcription factors belonging to the myogenic regulatory factor (MRF) family (Marsh et al., 1997). These transcription factors include Myf-5, MyoD, myogenin, and MRF4 (Olson, 1990). These MRF products share a basic helix-loop-helix (HLH) sequences that bind to DNA (Vissing et al., 2005). The HLH sequence allows for dimer binding with ubiquitous proteins, specifically E12 or E47. These heterodimer complexes bind to the E-Box DNA sequence (5'-CANNTG-3') found on muscle specific genes (Murre et al., 1989) and initiate transcription.

The members of the myogenic regulatory factor family are expressed at different times during myogenesis in culture suggesting MRF have specific roles. In proliferating myoblasts, Myf-5 and MyoD are co-expressed, while during the initiation of differentiation, myogenin increases. Myogenic regulatory factor-4 expression increases after differentiation causing maturation of myotubes into muscle fibers (Ludolph and Konieczny, 1995).

The function of myogenic regulatory factors during myogenesis can be inhibited by interactions with other proteins. Id proteins belong to a family of HLH transcription factors similar to the MRF, except these proteins lack the basic domain, therefore, these proteins cannot bind to DNA. High levels of the Id protein are found in proliferating cells in serum and levels are down regulated during myogenesis (Jen et al., 1992). The Id protein forms inactive heterodimers with E12 or E47, therefore are negative inhibitors of MRF (Forrest and McNamara, 2004).

### **Insulin-like growth factor system**

The insulin-like growth factor system includes three hormones, IGF-I, IGF-II, and insulin, three receptors, Type I, Type II, and insulin R, and six binding proteins, IGFBP-1 through IGFBP-6 (Florini et al., 1996). Insulin-like growth factor-I promotes the growth of postnatal animals (Daughaday and Rotwein, 1989; Mathews et al., 1988; Lee et al., 1991), while IGF-II is the prominent growth factor during fetal growth and development (Peng et al., 1996; Gerrard et al., 1998). These two growth factors are also involved in myoblast cellular proliferation (Jones and Clemmons, 1995) and differentiation (Florini and Magri, 1989; Jones and Clemmons, 1995) *in vivo* and *in vitro*.

The components of the IGF system have also been shown to potentiate or inhibit each others actions (Leal et al., 1997; Firth et al., 2002). Insulin-like growth factor binding proteins are found in association with the IGFs in serum and increase the half-life of IGFs (Florini et al., 1996). In addition, IGFBPs have been shown to exert IGF independent effects (Yi et al., 2001).

Numerous research studies have focused on endogenous and exogenous IGFBP-3 expression during myoblast proliferation and differentiation *in vitro* (Yang et al., 1999; Johnson et al., 2003; Xi et al., 2005). Recombinant IGFBP-3 suppresses porcine embryonic myoblast proliferation in PEM (Pampusch et al., 2003). In 2003, Johnson et al. discovered that IGFBP-3 mRNA expression was lower in differentiating cultures (120 h post-plating) and increased

dramatically in fused cultures. When PEM are treated with IGF-I, there is a decrease in IGFBP-3 mRNA expression and increase in myogenin mRNA (Yang et al., 1999). Treatment of PEM with transforming growth factor and myostatin suppresses proliferation and increases the production of IGFBP-3 mRNA (Kamanga-Sollo et al., 2003). In addition to the role IGFBP-3 plays on terminal differentiation, IGFBP-5 is secreted with the onset of terminal differentiation (James et al., 1993). Pampusch et al. (2005) also found recombinant IGFBP-5 aids in proliferation of PEM. Rotwein et al., (1995) also reported IGFBP-5 mRNA expression is simultaneous with the onset of myogenin gene expression. This data suggests that proliferation and differentiation of PEM is influenced by IGFBP-3 and IGFBP-5.

The IGFs bind with different affinity to each of the three receptors. Insulin-like growth factor-I binds to the Type I receptor with the highest affinity, while the Type II receptor binds to IGF-II with the highest affinity. The Type I receptor also binds insulin, while the Type II receptor does not. The insulin R binds IGFs with low affinity (Florini et al., 1996).

### **Metabolic modifiers**

It is well known that the actions of growth hormone (GH) are mediated by other agents known as somatomedins (Salmon and Duaghaday, 1957). Exogenous GH treatment has been shown to increase muscle mass in pigs (Thiel et al., 1993). Skeletal muscle fiber number in the semitendinosus can be increased 27% by injections of somatotropin from d 10 to 27 of gestation (Rehfeldt et al., 1993; Rehfeldt et al., 2006). It has been suggested injection of pST indirectly increases the number of primary muscle fibers developed, and therefore delays the differentiation of secondary muscle fibers (Rehfeldt et al., 2006). This is suggested to be due to the increased nutrient partitioning, specifically an increase in the supply to glucose transferred to the fetus (Rehfeldt et al., 1993).

### **Uterine crowding**

Differences in litter size and pig weight are observed in different genotypes. There is a negative relationship between these two response criteria (Kerr and Cameron, 1995). The most marked contrast is between the Chinese Meishan breed and the Yorkshire or Landrace breeds. The Chinese Meishan pigs are known as a highly prolific breed compared to European breeds and breeds found in the United States (Zhang et al., 1983; Jin et al., 1992; Young et al., 1993).

Numerous studies have determined increases in corpora lutea, greater number of corpora lutea, higher ovulation rate (Christenson, 1993), decreased embryo weight and length (Christenson, 1993; Ford and Youngs, 1993; Hunter et al., 1994), decreased placental weight (Christenson, 1993; Wilson et al., 1998; Biensen et al. 1999), and less amniotic fluid (Christenson, 1993) in the Meishan breed compared to white crossbred pigs. It has been reported that increased mortality is found in these larger litters with smaller pig birth weights (De Passillé et al., 1993).

Litter size and fetal development is not only influenced by ovulation rate, but also uterine capacity and function. Uterine capacity can be defined as the ability of the uterus to support embryos and fetuses through gestation (Sterle et al., 2003) and as the number of living fetuses present per uterine horn at day 105 of gestation (Vallet et al., 2002). Losses in conceptus survival and decreases in conceptus and placental weight increase uterine capacity (Vallet and Christenson, 1996), specifically when Meishan pigs are compared to European breeds (Wilson et al., 1998). Increasing the overall size of the uterus and placenta and increasing uterine and placental function are proposed methods of changing uterine capacity without losses in conceptus numbers or decreases in fetal weight (Vallet and Christenson, 1996). In addition, high ovulation rates may cause an increase in uterine crowding, therefore, affecting fetal development and the number of surviving fetuses (Town et al., 2005).

In 1996, Vallet and Christenson observed a positive correlation between hematocrits and fetal weight, suggesting uterine crowding will impair erythropoiesis. Therefore, conceptus development is affected by the diameter and numbers of blood vessels. This is because the histrotroph is transported through the blood and excreted from the endometrial glands to the conceptus (Spencer and Bazer, 2004). The histrotroph contains uterine secretions that nourish the developing conceptus and act as regulators for survival and development in the placenta (Gray et al., 2001). To maximize uterine capacity and fetal development, it has been suggested to increase endometrial gland number (Vallet and Christensen, 1996). Specific hormones and growth factors are associated with endometrial and placental growth. Estrogen, which is produced by the placenta, increases histrotroph components (Geisert et al., 1982) and acts upon the endometrium to increase specific growth factors (Spencer and Bazer, 2004). Increases in insulin-like growth factor-I (Simmen et al., 1990) and fibroblast growth factor-7 (FGF-7) are directly correlated with estrogen production (Ka et al., 2000). The role of IGF -I in cellular replication, differentiation, and development (Baserga et al., 1997) and FGF-7 in growth and

differentiation of cells (Rubin et al., 1995) is well known. In addition, the placenta expresses growth hormone (GH; LaCroix et al., 1996), which stimulates endometrial morphogenesis (Spencer and Bazer, 2004). Administration of porcine somatotropin (pST) has been shown to increase placental weight (Sterle et al., 1995) and increase uterine-placental contact area (Sterle et al., 1998). It is suspected that administration of pST increases IGF-I concentration, therefore increasing expression in the porcine uterus and affecting uterine growth and development (Sterle et al., 2003).

Other researchers have focused on increasing uterine size or placental weight to enhance uterine capacity (Christenson et al., 1987; Vallet and Christenson, 1996; Wise et al., 1997) and indicate uterine length and litter size are correlated (Chen and Dzuik, 1993; Wu and Dzuik, 1995). But, Vallet and Christenson (1996) determined the effects of prepubertal retinyl palmitate on uterine development in gilts and found different results. These researchers found an increase in uterine length, but inhibited myometrial development with administration of retinyl palmitate and no change in litter size compared to control animals. Also, when comparing the size of the uterus in gilts, the length of the uterine horns is highly variable (Chen and Dzuik, 1993), therefore, the length of the uterus may not be an indicator of uterine capacity (Vallet et al., 2002).

It is also suggested that placental function and size limits the development of the fetus (Ford, 1995; Reynolds and Redmer, 1995) dependent upon the number of fetuses in the uterus (Knight et al., 1977). Crowding of developing fetuses in the uterus reduces the weight of the placenta and the fetus due to insufficient placenta function (Knight et al., 1977). This is in agreement with Town et al. (2004) who observed detrimental effects on secondary muscle fiber development when crowding of the uterus occurred. But, Wilson et al. (1999) found smaller placentas with larger fetuses can be maintained with a high plain of placental efficiency, which contributes to enhanced uterine capacity.

## **Maternal nutrition**

Primary muscle fibers form by the diffusion of primary myoblasts to myotubes early in gestation (Kelly and Zacks, 1969) around day 38 (Wigmore and Stickland, 1983). These fibers make up 5% of the total fiber population (Dwyer et al., 1994) and are resistant to changes due to environmental factors. However, the number of secondary muscle fibers is highly susceptible to

environmental factors such as nutrition (Wigmore and Stickland, 1983). Secondary muscle fiber development occurs on the surface of primary fibers during mid-gestation (Kelly and Zacks, 1989). Maternal nutrition has been shown to affect the number of muscle fiber numbers in the porcine fetus. Specifically, when gilts and sows are fed under requirement levels, there is a reduction in birth weight, muscle fiber number, and post-natal growth (Dwyer et al., 1994; Bee, 2004; Rehfeldt and Kuhn, 2006).

Dwyer et al. (1994) observed an increase in secondary fiber development when dams were fed supplemental energy during d 25 to 50 of gestation. In addition, the offspring from these dams had improved feed efficiency up to 80 kg of BW. Protein restriction during the first or last trimester does not have a permanent effect on postnatal growth (Schoknecht et al., 1993). However, these researchers observed post-natal growth decreased in fetuses born from sows protein restricted throughout the whole gestation period. Contrary to the work of Dwyer et al. (2004), Nissen et al. (2003) found no differences in fiber number, fiber area, and concentrations and content of DNA and RNA in offspring of dams fed *ad libitum* from d 25 to 50 or d 25 to 70 of gestation. The purpose of this study was to determine effects of feed restriction on meat quality of the progeny. In addition to no effects on fiber characteristics, no differences were observed in meat quality.

## **Nutrients**

### ***L-carnitine***

L-carnitine ( $\beta$ -hydroxy- $\gamma$ -trimethyl-aminobutyrate) was discovered in 1905 and is considered a cofactor involved in different biological pathways. Unlike most cofactors, L-carnitine is not a vitamin and is derived from the amino acids lysine and methionine. (Harmeyer, 2002). The production of L-carnitine takes five specific steps. Specifically, L-carnitine synthesis begins with numerous post-translational methylations of the amino acid lysine. Then, trimethyllysine is hydroxylated and converted to carnitine (Brody, 1994). This compound is found in animals, fungi, bacteria, and plants (Gerhardt et al., 1995; Kleber, 1996).

Carnitine is required for the transport of medium and long chain fatty acids into the mitochondria (Brody, 1994). After the transport into the mitochondria, fatty acids undergo  $\beta$ -oxidation and then acetyl-CoA is later used in the Krebs's Cycle. A second function of L-carnitine is its' actions as an acetyl buffer to store acetyl-CoA in the muscle. Large amounts of

L-carnitine are required for this function (Harmeyer, 2002). In addition, L-carnitine is suggested to initiate the Krebs's Cycle and stimulate ATP transport during muscle activity (Jacobs, 2002).

The addition of L-carnitine to gestation and lactation diets shows positive effects on sow growth performance. Feeding sows supplemental L-carnitine during gestation has been shown to increase BW gain (Musser et al., 1999; Ramanau et al., 2002) and last rib backfat (Musser et al., 1999). In addition, researchers have observed the effects of L-carnitine supplementation on the IGF system. Plasma IGF-I (Musser et al., 1999; Doberenz et al., 2005) and plasma IGF-II (Doberenz et al., 2005) increase when L-carnitine is supplemented in the diet. Contradictory to this, Waylan et al. (2005) and Brown et al. (2006) observed no changes in maternal plasma IGF-I concentrations when gilts and sows were fed supplemental L-carnitine.

By supplementing L-carnitine to gestating sows, increases have been observed in the total number of pigs born and born alive (Ramanau et al., 2004; Birkenfeld et al., 2005), decreases the number of stillborn pigs (Musser et al., 1999; Doberenz et al., 2005), and increases in average pig weight (Eder et al., 2001; Ramanau et al., 2002 and 2004) and litter weight at birth (Ramanau et al., 2002 and 2004; Doberenz et al., 2005). The improvement in average fetal weight and litter weight due to L-carnitine is beneficial to the producer.

In addition to increased birth and weaning weights, researchers have observed an increase in the cross-sectional area and more total muscle fiber numbers in the semitendinosus muscle in pigs from sows fed supplemental L-carnitine (Musser et al., 2001). Specific growth factors, such as IGF-I, IGF-II, IGFBP-3 and IGFBP-5 have shown to have promoting proliferative and differentiation effects on the muscle cells of pigs (Hembree et al., 1996; Johnson et al., 1999). Researchers have shown the addition of L-carnitine lowers the expression of IGF-II and numerically increases the expression of IGF-I in porcine embryonic myoblasts or muscle cells (PEM; Waylan et al., 2005). Insulin-like growth factor-I has potent proliferative effects on PEM. This means the muscle cells keep multiplying when this growth factor is present. Insulin-like growth factor-II induces the expression of another gene, myogenin, which promotes the muscle cells to stop their proliferative capacity and differentiate into mature muscle fibers (Florini et al., 1991). Therefore, the increase in IGF-I and decrease in IGF-II due to supplementing the sow L-carnitine is allowing more muscle fiber cells to be developed, which increases birth weight. This research is supported by decreased levels of circulating IGF-II levels in fetuses at d 70 gestation (Brown et al., 2006). The significant changes in gene expression due to supplementing the sow

L-carnitine is involved in the regulation of muscle fiber development of the fetus and improved pig and litter weight at birth.

### ***Chromium tripicolinate***

Chromium, an essential trace mineral (NRC, 1998), influences the metabolism of carbohydrates, fat, and protein (Cheeke, 1999) due to its effect on insulin (Mertz, 1993; White et al., 1993). Chromium is poorly absorbed in the inorganic form and is utilized and absorbed most effectively in the organic form; therefore, it is fed to swine in the form of chromium tripicolinate. Chromium is thought to be involved in as a glucose tolerance factor (Steele et al., 1977), specifically, by potentiating the effects of insulin (Real, 2001). Contradictory results are observed on the effects of chromium on serum insulin and glucose levels. Added chromium has been shown to decrease serum insulin and glucose concentrations (Evock-Clover et al., 1993); however, other studies have observed no change in serum insulin and glucose concentrations (Lindemann et al., 1995b).

In 2002(b) van de Ligt et al. conducted a study to determine the effects of chromium supplementation to sows during gestation. The researchers found three weeks before farrowing, chromium supplementation increased total serum IgG and IgM concentrations in sows. However, no differences in immunoglobulin concentrations were observed in the remainder of the experiment. In addition, no differences were observed in immunoglobulin concentrations in neonatal or weaning pig serum (van de Ligt et al., 2002b). Growth criteria for the litters were not reported. In this study, a minimal response from chromium supplementation was observed in immunoglobulin concentrations. Additional studies focusing on chromium tripicolinate supplementation during gestation and in the nursery showed similar results on immunoglobulin concentrations (van de Ligt et al., 2002a). In 2002(a), van de Ligt et al., fed chromium tripicolinate in gestation and in the nursery phase of production. Results from this study confirm no differences in immunoglobulin response in pigs fed supplemental chromium tripicolinate. In addition to these results, growth performance of nursery pigs did not change due to supplementation of chromium tripicolinate. Research findings showed using chromium tripicolinate (200 ppb) in gestation diets has demonstrated an improvement in the number of pigs born alive (2.1 pigs/litter), while maintaining individual pig birth weight (Lindemann et al., 1995a). Other research has observed supplementing chromium tripicolinate (0, 200, 600, and



1000) increases the number of pigs born alive per litter, but numerically reduces individual birth weight of total pigs born up to 600 ppb (Lindemann et al., 2004). These authors suggested improved efficiency of insulin caused by chromium tripicolinate increased ovulation rate, therefore, an increase in litter size is observed.

## **Conclusions**

This review concludes that fetal myogenesis is under the control of the regulatory factors and the IGF axis. Strong evidence supports pST, uterine crowding, maternal nutrition and supplementation of L-carnitine and chromium tripicolinate to the gestating dam may alter fetal myogenesis and may have a negative or positive influence on muscle fiber number in offspring.

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**CHAPTER 2 - Growth characteristics, blood metabolites, and litter traits from gilts fed L-carnitine through gestation and the expression of IGF system components in fetal tissues**



## Abstract

A total of fifty-nine gilts (BW=137.7 kg) from three different breeding groups were used to assess the effects of feeding L-carnitine during gestation on gilt growth characteristics, gilt and fetal blood metabolites, litter characteristics, and IGF axis components in fetal hepatic and skeletal tissues. Experimental treatments were arranged in a 2 × 3 factorial with main effects of added L-carnitine (0 or 50 ppm) and day of gestation after initial breeding (40, 55, or 70). All gilts received a constant feed allowance of 1.75 kg/d and a top-dress containing either 0 or 50 ppm of L-carnitine starting on the first day of breeding through the allotted gestation length. No differences ( $P > 0.16$ ) were observed for gilt BW or estimated protein or fat mass at any gestation length. Gilts fed L-carnitine tended to have greater ( $P = 0.10$ ) backfat at d 40 and were numerically heavier at d 70 compared to control gilts. No differences ( $P > 0.77$ ) were observed in circulating total and free carnitine at breeding, but concentrations increased ( $P < 0.01$ ) as gestation length increased for gilts fed diets containing L-carnitine compared to those fed the control diet. Maternal IGF-I concentration decreased ( $P < 0.01$ ) from d 0 to 70 for all gilts with no differences ( $P > 0.05$ ) between treatments. As gestation length increased, total litter weight, average fetal weight, crown to rump length (CRL), and fetal circulating IGF-II increased ( $P < 0.05$ ), but total fetal number decreased ( $P < 0.05$ ). Total litter size, total litter weight, fetal number, CRL of the fetuses, and total number of corpus lutea did not differ ( $P > 0.05$ ) among treatment within gestation length. However, fetuses from the gilts fed diets with L-carnitine tended to be heavier ( $P = 0.06$ ) and fetal circulating IGF-II lower ( $P = 0.09$ ) at day 70 compared to the fetuses from the control gilts. Insulin-like growth factor-I mRNA was lower ( $P = 0.05$ ) in hepatic tissue in fetuses collected from gilts fed supplemental L-carnitine. Fetal hepatic tissue IGF-II, IGFBP-3, and IGFBP-5 mRNA expression were not influenced ( $P > 0.05$ ) by dietary treatment. As gestation length increased, IGFBP-3 mRNA increased ( $P < 0.01$ ) in the fetal hepatic tissue and IGF-I mRNA increased ( $P < 0.02$ ) in skeletal muscle. Fetal skeletal muscle IGFBP-3 mRNA decreased from d 40 to 55, but increased ( $P = 0.04$ ) from d 55 to 70. This study showed L-carnitine supplementation to gestating gilts had beneficial effects on average fetal weight, due in part to changes in the fetal IGF system.

Keywords: Fetus, Gestation, Gilts, L-carnitine, Messenger RNA, Weight

## **Introduction**

L-carnitine is a vitamin-like water soluble quaternary amine that is a derivative of the amino acids lysine and methionine. It is synthesized in the liver, kidney, and brain (Rebouche and Engel, 1980). The primary role of L-carnitine is to facilitate transport of long chain fatty acyl groups through the mitochondrial matrix for  $\beta$ -oxidation and cellular energy production (Fritz, 1955). L-carnitine also plays a role in regulating glucose disposal and carbohydrate metabolism (Grandi et al., 1997; De Gaetano et al., 1999; Woodworth, 2002).

Feeding multi-parious sows supplemental L-carnitine during gestation may increase BW gain (Musser et al., 1999; Ramanau et al., 2002), last rib backfat (Musser et al., 1999), plasma IGF-I (Musser et al., 1999; Doberenz et al., 2005) and plasma IGF-II (Doberenz et al., 2005). In addition, supplementing L-carnitine to gestating sows may increase total number of pigs born and born alive (Ramanau et al., 2004; Birkenfeld et al., 2005), decrease the number of stillborn pigs (Musser et al., 1999; Doberenz et al., 2005), and increase average pig weight (Eder et al., 2001; Ramanau et al., 2002 and 2004) and litter weight at birth (Ramanau et al., 2002 and 2004; Doberenz et al., 2005).

In the majority of data showing improvements in reproductive performance due to L-carnitine supplementation, sows or mixed parity groups have been used. Only one study has utilized gilts and they observed decreased number of stillborns when fed L-carnitine (Doberenz et al., 2005). Gilts and sows in gestation are fed for combinations of body growth, maintenance, and fetal growth, but gilts are in a greater growth phase compared to the sow. Therefore, the objective of this study was to evaluate the effects of supplementing L-carnitine through the developmental stages up to d 70 of gestation in gilts.

## **Materials and Methods**

### *Animals*

All animal procedures used in this study were reviewed and approved by the Kansas State University Animal Care and Use Committee. Fifty-nine gilts (PIC; Franklin, KY; L327  $\times$  1050; BW 137.7 kg; 190 d of age) were artificially inseminated (PIC; MQ 280) 12, 24, and 36 h after the onset of their second observed estrus. Day 1 was considered 12 h after the first insemination. Gilts were housed in individual crates (1.83  $\times$  0.55 m) in an environmentally-controlled gestation

barn at the Kansas State University Swine Teaching and Research Center from breeding until either day 39.5, 54.5 or 69.5 gestation. Gilts were allowed *ad libitum* access to water and randomly allotted to one of two dietary treatments and one of three harvesting dates based on weight at breeding. All gilts were fed a corn soybean meal gestation diet (Table 2.1) once daily (1.75 kg/d; as-fed basis) and received a 50-g ground corncob top-dress containing either none (control, n=30) or 88 mg of L-carnitine (Carniking 10; 10% L-carnitine, n=29); Lonza Group, Inc., Allendale, NJ) from 1 to d 39, 54 or 69 gestation. The gestation diet was formulated to meet or exceed NRC (1998) nutrient requirement estimates and fed at a level of 1.75 kg/d based on NRC models to be slightly above the requirements for maintenance and fetal growth. Fetal and uterine gain throughout pregnancy was predicted to be 25 kg with maternal gain predicted at an additional 13.6 kg (Aherne and Kirkwood, 1985; Williams et al., 1985). At this feeding level, gilts should have maintained backfat levels throughout gestation while increasing body weight. The total gestation energy requirement was determined by summing the daily energy requirement, energy for maternal gain, plus energy for products of fetal and uterine gain. Backfat was measured at the P2 position (last rib) on both sides of the backbone using a Lean-Meter (Renco Corporation; Minneapolis, MN) and BW were determined at breeding, d 39, 54 and 69 of gestation. Protein and fat mass were estimated using prediction equations of Dourmad et al., ((1997; protein mass =  $2.28 + 0.178 \times (\text{liveweight, kg}) - 0.333 \times (\text{backfat, mm})$ ) and ((fat mass =  $-26.40 + 0.221 \times (\text{liveweight, kg}) + 1.331 \times (\text{backfat, mm})$ )).

### ***Blood collection***

At 0, 39, 54 and 69 d of gestation, blood was collected by veni-puncture for determination of circulating IGF-I and free and total carnitine. Blood samples were collected in both heparinized and non-treated tubes and placed on ice until centrifuged ( $2500 \times g$  for 20 min at  $4^{\circ}\text{C}$ ) or refrigerated ( $4^{\circ}\text{C}$ ) 24 h before centrifugation, respectively. Plasma or sera were then separated and frozen ( $-20^{\circ}\text{C}$ ) until analysis. The concentrations of free and total plasma carnitine (Parvin and Pande, 1977) and serum IGF-I (Active IGF-I with Extraction, DSL-5600; Diagnostics Systems Laboratory Inc., Webster, TX) were determined. Assay sensitivity was 6.0 ng/mL. The intraassay CV was 6.2 % and the interassay CV was 9.0 %.

### ***Harvesting protocol and collection of samples***

Harvest was completed on gilts on either d 40, 55 or 70 gestation. Fifteen hours before harvest, gilts were transported from the Kansas State University Swine Research and Teaching Center to the Kansas State University Meat Laboratory where sample collections were performed 24-h after the last feeding. Gilts were allowed *ad libitum* access to water until harvest. Gilts were harvested by electrical stunning followed by exsanguination. A mid-lateral incision was made to gain access to the abdominal cavity. The ovarian pedicles and uterine stump, at the level of the cranial cervix were cut and the uterus removed. The number of corpus lutea on each side of the uterus was counted. Then, the number of fetuses was determined in both horns. Fetal pigs were removed by mid-ventral laparotomy under aseptic conditions and rapidly transported to our laboratory for processing.

### ***Fetal blood collection, weights, and lengths***

Individual fetuses were weighed and the crown to rump length on each fetus was measured. Total litter weight was calculated as the sum of the individual fetus weights per litter. Fetal blood was collected from the heart of each fetus and pooled with the other fetuses in the litter for determination of fetal plasma IGF-II (IGF-II RIA IGFBP Blocked, 022-IGF-R30; American Laboratory Products Company, Windham, NH).

### ***Sample preparation and RNA Isolation***

Semitendinosus from the left hind limb and hepatic tissue from the left lobe was excised from each fetus, individual identity preserved, and immediately snap frozen in liquid nitrogen and stored at -80°C in an Ultra Low Freezer. Total RNA was isolated from fetal hepatic and skeletal muscle tissue by using the RNeasy Mini Kit (Qiagen; Valencia, CA). The concentration of RNA was determined by absorbance at 260 nm. Electrophoresis of total RNA through a 1% agarose-formaldehyde gel followed by ethidium bromide staining to allow visualization of 28S and 18S ribosomal RNA (rRNA) was used to assess the integrity of RNA. One microgram of total RNA was then reverse-transcribed to produce the first-strand complementary DNA (cDNA) using TaqMan reverse transcriptase (Applied Biosystems, Foster City, CA) following the protocol recommended by the manufacturer. Random hexamers were used as primers in cDNA synthesis.

### ***Real-time quantitative PCR***

Real-time quantitative-PCR was used to measure the quantity of mRNA for IGF-I, IGF-II, IGFBP-3, and IGFBP-5 and 18S rRNA in total RNA isolated from hepatic and skeletal muscle tissues. Measurement of the relative quantity of cDNA was carried out using TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of the appropriate forward and reverse primers, 200 nM of the appropriate TaqMan detection probe, and 1  $\mu$ L (0.5  $\mu$ g cDNA) of the cDNA mixture. Sequences for primers and probes for IGF-I, IGF-II, IGFBP-3 and IGFBP-5 are presented in Table 2.2. Commercially available eukaryotic 18S rRNA primers and probes were used as an endogenous control (Applied Biosystems; Genbank Accession no. X03205). Assays were performed in an ABI Prism 7000 sequence detection system (Applied Biosystems) using thermal cycling parameters recommended by the manufacturer (50 cycles of 15 sec at 95°C and 1 min at 60°C). Relative expressions of mRNA for IGF-I, IGF-II, IGFBP-3 and IGFBP-5 were normalized to the 18S rRNA endogenous control and expressed in arbitrary units.

### ***Statistical analysis***

Statistical analyses for backfat, weight, and blood concentrations of gilts were performed with the MIXED Procedure of SAS (SAS, 2000; SAS Inst. Inc., Cary, NC). Data were analyzed as a repeated measures to include only gilts harvested at day seventy of gestation (control, n=10; L-carnitine n=10). The model included treatment as the fixed effect and day of sampling as the repeated measure. Kenward-Roger adjustment was used for the degrees of freedom.

Fetal weights, lengths, and blood concentrations and ovarian corpus lutea data were analyzed as a 2  $\times$  3 factorial arrangement with the MIXED Procedure of SAS. Fixed model effects included treatment, day of harvest and their interaction. Kenward-Roger adjustment was used for the degrees of freedom.

The Fisher's Exact method was used to determine *p*-values of a chi-square statistic between differences in the number of litters that were able to detect IGF-II for L-carnitine and control fed gilts (Higgins, 2004).

For all genes evaluated, mRNA concentrations were analyzed as a 2  $\times$  3 factorial arrangement with the MIXED Procedure of SAS. Fixed model effects included treatment, day of harvest and their interaction. Kenward-Roger adjustment was used for the degrees of freedom.

The significance was declared at  $P < 0.05$  and a trend declared at  $P = 0.10$  to  $P = 0.06$  unless noted otherwise.

## **Results**

### ***Gilt growth parameters***

No differences ( $P > 0.05$ ) between treatments were observed for body weight gain, estimated protein mass or estimated fat mass at any gestation length. As gestation length increased these response criteria differed (Table 2.3;  $P < 0.01$ ). At day forty of gestation, gilts fed L-carnitine tended to have greater ( $P = 0.10$ ) backfat compared to the gilts fed the control diet (L-carnitine = 17.9 mm; Control = 16.3 mm). No differences ( $P > 0.05$ ) were observed in backfat at day 0, 55 or 70 of gestation.

### ***Plasma and sera analysis***

No differences ( $P > 0.05$ ) were observed in maternal plasma IGF-I collected at day 0, 40, 55, and 70 of gestation between the two treatments (Figure 2.1). Plasma IGF-I concentrations were decreased ( $P < 0.01$ ) as day of gestation increased from d 0 to 40 for L-carnitine and control fed gilts.

As expected, no differences ( $P > 0.05$ ) were observed in total (Figure 2.2) and free (Figure 2.3) carnitine between the gilts fed L-carnitine or the control diet at day 0 of gestation, but these increased ( $P < 0.01$ ) on day 40, 55, and 70 of gestation for gilts fed additional L-carnitine.

### ***Litter and ovary characteristics***

Total litter size and total litter weight were not different ( $P > 0.05$ ) at day 40, 55, or 70 of gestation for the gilts fed L-carnitine or the control diet (Table 2.4). In addition, no differences ( $P > 0.05$ ) were observed in fetus number in the right uterine or left uterine horn and crown to rump length. As gestation length increased, total litter weight, average fetal weight, crown to rump length and IGF-II increased ( $P < 0.05$ ), but total fetus number and fetus number in the right and left uterine horn decreased ( $P < 0.05$ ). At day 70 of gestation, fetuses from the gilts fed L-carnitine tended to be heavier ( $P = 0.06$ ) than the fetuses from the control fed gilts (237 g vs. 218 g, respectively). Fetuses from gilts fed L-carnitine had numerically lower ( $P = 0.09$ ) plasma

IGF-II concentrations compared to the fetuses from the gilts fed the control diet (22.9 vs. 17.6 ng/mL, respectively) at day 70 of gestation. No differences ( $P > 0.05$ ) were observed in total corpus lutea, right ovarian corpus lutea or left ovarian corpus lutea at day 40, 55, and 70 of gestation for the gilts fed L-carnitine and the control diet. As gestation length increased the number of CL located on the left ovary decreased ( $P = 0.07$ ).

### ***RNA analysis***

Insulin-like growth factor-I mRNA was lower ( $P = 0.05$ ) in hepatic tissue from fetuses collected from gilts fed supplemental L-carnitine (Table 2.5). Fetal hepatic tissue IGF-II, IGFBP-3, and IGFBP-5 mRNA expression was not influenced ( $P > 0.05$ ) by dietary treatment. As day of gestation increased, fetal hepatic IGFBP-3 mRNA levels increased ( $P < 0.01$ ). No differences ( $P > 0.05$ ) in IGF-I, IGF-II, IGFBP-3, and IGFBP-5 mRNA expression were observed in fetal muscle between dietary treatments. A day effect was observed for fetal muscle IGF-I ( $P = 0.02$ ) and IGFBP-3 ( $P = 0.03$ ) mRNA. As day of gestation increased from 40 to 70, fetal muscle IGF-I mRNA expression increased. As day of gestation increased from 40 to 55, IGFBP-3 mRNA decreased and from d 55 to 70 IGFBP-3 mRNA increased similar to levels at d 40 gestation.

### **Discussion**

Previous research has shown supplementing L-carnitine increases circulating carnitine concentrations in streptozotocin-induced diabetic rats (Heo et al., 2001), finishing pigs (Owen et al., 2001), and gestating sows at day 57 (Waylan et al., 2005), day 60, and day 90 of gestation (Musser et al., 1999). The results herein agree that supplementing L-carnitine to the gestating dam increases circulating free and total carnitine concentrations at day 40, 55, and 70 of gestation compared to gilts not supplemented with L-carnitine. Increasing circulating L-carnitine concentrations via dietary supplementation has shown positive effects on carcass leanness (Owen et al., 2001), gilt growth (Musser et al., 1999; Ramanau et al., 2002), and reproductive performance (Musser et al., 1999; Eder et al., 2001; Ramanau et al. 2004). In the present study, supplementing gilts with L-carnitine increased ( $P = 0.10$ ) gilt backfat at day 40 of gestation and increased ( $P = 0.06$ ) average fetal weight at day 70 of gestation.

In newborn pigs, Kempen and Odle (1995) found that L-carnitine increases fatty acid oxidation in hepatocytes and Owen et al. (2001) suggests L-carnitine reduces backfat in finishing

pigs by the acceleration of the  $\beta$ -oxidation of fatty acids in the liver mitochondria and hepatocytes, in turn increasing the rate of acetyl CoA production. In this study, gilt backfat was greater at day 40 of gestation for the gilts fed L-carnitine compared to the gilts fed the control diet, but no differences were observed between treatments at day 55 or 70 of gestation. In addition, Musser et al. (1999) found an increase in backfat in sows fed supplemental L-carnitine at day 119 of gestation. Musser et al. (1999) suggest the increase in backfat observed in their study was due to the differences in partitioning of nutrients between finishing pigs and gestating sows, and the ability of the sow to store more adipose tissue under conditions of supplemental L-carnitine. In the present study, we only observed an increase in backfat in the gilts fed L-carnitine at day 40 gestation. We also found supplementing gestating gilts with L-carnitine numerically increased gilt body weight at d 70 of gestation with significant increases in sow body weight through gestation observed by other researchers (Musser et al., 1999; Eder et al., 2001; Ramanau et al., 2002). Fetal and uterine gain throughout pregnancy was predicted to be 25 kg with maternal gain predicted at an additional 13.6 kg (Aherne and Kirkwood, 1985; Williams et al., 1985). At this feeding level, gilts should have maintained backfat levels throughout gestation while increasing body weight. The total gestation energy requirement was determined by summing the daily energy requirement, energy for maternal gain, plus energy for products of fetal and uterine gain. The difference in backfat we observed early in gestation may have been due to excess energy and nutrients provided to the gilts by supplemental L-carnitine. As gestation length increased, backfat levels were not different between the dietary treatments. From day 0 to 55 of gestation backfat increased and from day 55 to 70 of gestation backfat decreased in gilts fed both dietary treatments. This suggests the gilts in this study were fed at a level excess of the requirement up to d 55 of gestation and at their requirement from day 55 to day 70. This is commonly observed when gilts are fed a constant feed allowance during gestation. Because gilts were gaining weight during gestation, the maintenance requirement was increasing with less energy being available for maternal weight and backfat gain.

Insulin-like growth factor-I concentrations did not differ in gilts fed supplemental L-carnitine compared to the gilts fed the control diet. This is in agreement with Waylan et al. (2005) who observed no change in circulating IGF-I levels in sows with or without supplemental L-carnitine. However, Musser et al. (1999) observed an increase in maternal circulating IGF-I at day 60 and 90 of gestation in sows fed supplemental L-carnitine compared to the sows fed no



supplemental L-carnitine. The observed increase in maternal circulating IGF-I observed by Musser et al. (1999) may be due to the sows being above their energy requirement (Thissen et al., 1994). Doberenz et al. (2005) also found increased maternal circulating levels of IGF-I and IGF-II in gilts and sows fed supplemental L-carnitine at day 80 and 95 of gestation. The gilts and sows in the study of Doberenz et al. (2005) were allowed *ad libitum* feed consumption and our gilts were limit fed.

Stronger evidence of the positive effects of L-carnitine supplementation is seen with the increase in reproductive performance from gilts and sows fed diets containing added L-carnitine. Recent studies have reported an increase in the total number of fetuses at mid-gestation (Waylan et al., 2005), an increase the total number of pigs born (Ramanau et al., 2004; Birkenfeld et al., 2005), and a reduction in the number of stillborns at birth (Musser et al., 1999; Doberenz et al., 2005) in sows fed supplemental L-carnitine. Waylan et al. (2005) found at day 55 of gestation, the number of fetuses was greater in the sows supplemented with L-carnitine in gestation compared to the sows fed the control diet. Ramanau et al. (2004) and Birkenfeld et al. (2005) found similar results when sows were fed supplemental L-carnitine during gestation. These researchers found an increase in the total number of pigs born and total number of pigs born alive (Ramanau et al. 2004; Birkenfeld et al., 2005). In addition, Musser et al. (1999) and Doberenz et al. (2005) found a reduction in the number of stillborn pigs at birth. This data suggests that L-carnitine may aid in improving conception rate or reducing embryo mortality. In contrast to these findings, the present study shows no differences in the total number of fetuses at day 40, 55, or 70 of gestation. The studies that observed an increase in the total number of pigs observed this increase in both gilts and sows. In our study, we observed no differences in the total number of fetuses in gilts. The exact mechanism behind the observed increase in total number of pigs born in other studies is unclear. L-carnitine appears to affect gilts and sows differently (Eder et al., 2001) and one could speculate that in older animals L-carnitine may be allowing greater nutrient uptake in a mature uterus which allows for enhanced fetal growth and development and increased embryo survival. In addition, one could speculate if L-carnitine was fed before breeding this may affect follicular development. Unlike our study, the studies conducted by Birkenfeld et al. (2005) and Ramanau et al. (2005) fed L-carnitine through subsequent gestation periods which allowed for L-carnitine to play a role in follicular development.

Recent studies have reported increased pig and litter birth weights when sows are supplemented with L-carnitine (Ramanau et al., 2002 and 2004). At day 70 of gestation, we observed an increase in the average fetal weight in fetuses collected from gilts fed diets containing supplemental L-carnitine compared to the fetuses collected from the gilts fed the control diet. In addition, other researchers have found increased muscle fiber numbers in offspring from sows fed diets with supplemental L-carnitine (Musser et al., 1999). Researchers have speculated that the heavier piglets born from sows fed supplemental L-carnitine is due to an increased intrauterine nutrient supply of glucose (Ramanau et al., 2002), an increase in glucose receptors (Doberenz et al., 2005), or an increased maternal IGF-I (Musser et al., 1999). The role of IGF-I in normal growth and development has been well documented and plays an important role in muscle cell proliferation (Florini et al., 1991) and increased birth weight (Hills et al., 1996). Thus, the elevated levels of IGF-I found by Musser et al. (1999) may have improved muscling in these pigs. But, our data and that of Waylan et al. (2005) suggests that the heavier average fetal weights from gilts fed L-carnitine was not due to increased maternal IGF-I. Therefore, an understanding of the changes in the IGF system in fetal tissues will aid in our comprehension of fetal muscle development and the improved fetal weight we observed at day 70 of gestation due to supplemental L-carnitine.

Insulin-like growth factors-I and -II are key regulators involved in fetal muscle development. In muscle cells, IGF-I promotes muscle proliferation (Florini et al., 1991), while IGF-II can promote muscle differentiation (Moses et al., 1980). Waylan et al. (2005) found when mononucleated porcine embryonic myoblasts collected from fetuses from dams fed supplemental L-carnitine differentiate into primary muscle fibers, IGF-II mRNA expression decreases. This would indicate that supplementation of L-carnitine to the dam allows for greater myoblast proliferation in fetuses due to lower levels of IGF-II mRNA expression. We can speculate this may allow for a greater number of porcine embryonic myoblasts to differentiate into additional muscle fibers or may allow for more DNA fusion during myoblast differentiation, therefore, increasing muscle size. The average fetus weight from the gilts fed L-carnitine was 18.9 g heavier than the average fetus weight from the gilts fed the control diet at day seventy of gestation. In our study, we found decreased levels of circulating IGF-II in fetuses collected from gilts fed supplemental L-carnitine and undetectable levels of IGF-II in half of those fetuses. These data suggests IGF-II plays a role in the increased weight we observed at day 70 of

gestation in fetuses collected from gilts fed supplemental L-carnitine. Feeding L-carnitine decreased circulating fetal IGF-II and we speculate may have increased fetal IGF-I, but this was not measured. Therefore, feeding L-carnitine may have increased cell proliferation producing heavier fetuses and caused a delay in muscle cell differentiation late in gestation. It has also been noted that levels of amino acids are greater in pigs fed supplemental L-carnitine suggesting either increased protein accretion or reduced protein break down (Bohles and Lehnert, 1984; Alverstrand et al., 1990; Owen et al., 2001). If L-carnitine increases in the fetus due to supplemental L-carnitine to the dam, an increased supply of amino acids may allow for greater protein accretion resulting in heavier fetuses at birth.

In our study we observed a decrease in hepatic IGF-I mRNA expression in fetuses collected from gilts fed supplemental L-carnitine. This is in contrast to results reported by Waylan et al. (2005) where the authors found no change in fetal hepatic IGF-I mRNA expression at d 55 of gestation. Previous studies using an ovine model show maternal under-nutrition decreases hepatic IGF-I mRNA expression (Brameld et al., 2000). Our results are unexpected because one can speculate supplementation of L-carnitine to the gilts and sows increases nutrient transfer to the fetus due to the increased pig weight observed at birth. Therefore, one would expect an increase in IGF-I mRNA expression due to increased nutrient levels. During fetal development, IGF-II is an important regulator because of its abundance in fetal tissues (Brown et al., 1986; Hill, 1990) and serum (Moses et al., 1980), but, we observed no changes in IGF-II mRNA expression in fetal hepatic tissue. These unexpected findings observed in gene expression in hepatic tissue are not in agreement with our assumed role that L-carnitine plays in fetal development.

## **Implications**

These results suggest L-carnitine supplementation to gestating gilts has beneficial effects on average fetal weight, possibly observed because of its ability to reduce fetal IGF-II concentrations. The heavier fetal weights from gilts fed L-carnitine was not due to maternal IGF-I. Therefore, the role of maternal IGF-I on impacting fetal growth and development is unclear. The decreased expression of IGF-I in fetal hepatic tissues provides support for a change in IGF system components due to supplementation of L-carnitine to the gilt. Additional research

in growth factor regulation is necessary to determine the effect of L-carnitine on biological mechanisms in the gestating and fetal pig.

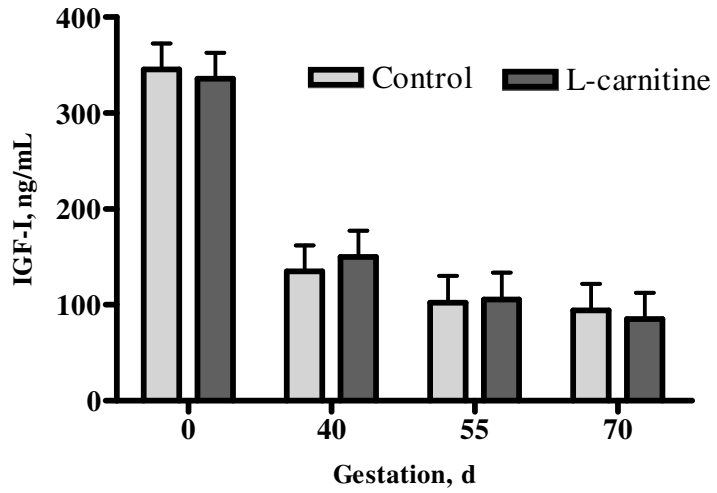
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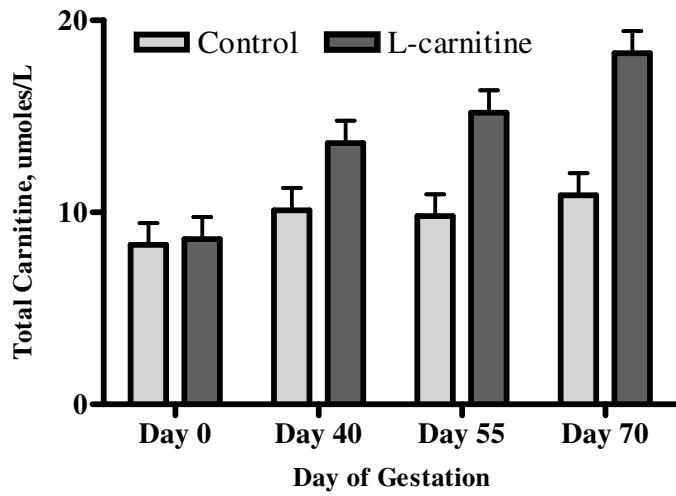
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**Figure 2.1 Plasma insulin-like growth factor-I (IGF-I) concentrations of gilts fed diets unsupplemented (control) and supplemented (50 ppm of L-carnitine) at three gestation lengths.**

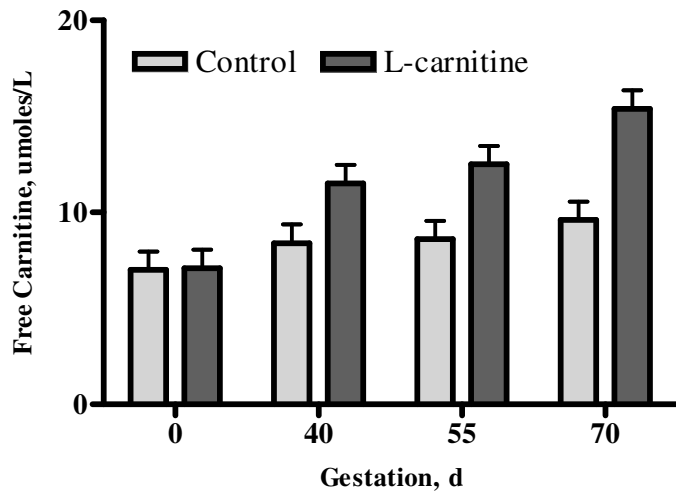




**Figure 2.2 Plasma total carnitine concentrations of gilt fed diets unsupplemented (control) and supplemented (50 ppm of L-carnitine) at three gestation lengths.**



**Figure 2.3 Plasma free carnitine concentrations of gilts fed diets unsupplemented (control) and supplemented (50 ppm of L-carnitine) at three gestation lengths.**



**Table 2.1 Diet composition fed during gestation (as-fed)<sup>a</sup>**

Item	Amount
Ingredient, %	
Corn	81.22
Soybean meal, 46.5% CP	14.55
Monocalcium phosphate, 21% P	2.03
Limestone	1.05
Salt	0.50
Vitamin premix <sup>b</sup>	0.25
Trace mineral premix <sup>c</sup>	0.15
Sow add pack <sup>d</sup>	0.25
	100.00
Calculated analysis	
Lysine, %	0.65
ME, Mcal/kg	3.27
Protein, %	13.7
Calcium, %	0.85
Phosphorus, %	0.75
Available P, %	0.48

<sup>a</sup> Gestation feeding levels of 1.75 kg/d, with a top-dress providing 0 or 50 ppm added L-carnitine.

<sup>b</sup> Supplied per kilogram of diet: 11,025 IU of vitamin A, 1,654 IU of vitamin D<sub>3</sub>, 55.1 mg of niacin, 44.1 IU of Vitamin E, 33.1 mg of pantothenic acid, 9.9 mg of riboflavin, 4.4 mg of vitamin K (menadione), and 0.04 mg of vitamin B<sub>12</sub>.

<sup>c</sup> Supplied per kilogram of diet: 165 mg of Zn (oxide), 165 mg of Fe (sulfate), 39.7 mg of Mn (oxide), 16.5 mg of Cu (sulfate), 0.30 mg of I (as Ca iodate), and 0.30 mg of Se (as Na selenite).

<sup>d</sup> Supplied per kilogram of diet: 551.3 mg of choline, 15.2 mg pyridoxine, 1.65 mg of folic acid and 0.22 mg of biotin.

**Table 2.2 Primers and probes used for real-time quantitative PCR**

Gene	Genbank accession number		Sequence
IGF-I	M31175	Forward	TCTTCTACTTGGCCCTGTGCTT
		Reverse	GCCCCACAGAGGGTCTCA
		Probe	6FAM-CCTTCACCAGCTCTGCCACGGC-TAMRA
IGF-II	X56094	Forward	CCGGACA ACTTCCCCAGATA
		Reverse	CGTTGGGCGGACTGCTT
		Probe	6FAM-CCCGTGGGCAAGTTCTTCCGC-TAMRA
IGFBP-3	AF085482	Forward	AGCACGGACACCCAGA ACTT
		Reverse	CGGCAAGGCCCGTATTC
		Probe	6FAM-TCCTCTGAGTCCAAGCGCGAGA-TAMRA
IGFBP-5	U41340	Forward	GGCAGAGGCCCGTGAAGAAG
		Reverse	CAGCTCCCCC CACGAACT
		Probe	6FAM-CCGCAGAAAGAAGCTGACCCAGTCC-TAMRA

**Table 2.3 Effects of L-carnitine on gilt growth characteristics collected from gilts harvested at day 70 of gestation<sup>a</sup>**

Item	Control	L-carnitine	<i>P</i> -value	SED
No. of gilts	10	10		
Weight, kg				
Day 0	136.4	135.5	0.86	5.08
Day 40	150.5	154.2	0.47	5.08
Day 55	158.5	162.4	0.44	5.08
Day 70	166.2	170.3	0.43	5.08
Estimated protein mass, kg <sup>b</sup>				
Day 0	23.0	22.8	0.82	0.91
Day 40	25.3	25.8	0.66	0.91
Day 55	26.7	27.3	0.50	0.91
Day 70	28.2	29.0	0.40	0.91
Backfat, mm				
Day 0	15.0	15.2	0.83	0.95
Day 40	16.3	17.9	0.10	0.95
Day 55	16.7	17.2	0.60	0.95
Day 70	15.9	15.4	0.60	0.95
Estimated fat mass, kg <sup>c</sup>				
Day 0	27.3	27.3	0.99	1.30
Day 40	31.2	33.0	0.16	1.30
Day 55	33.2	34.4	0.34	1.30
Day 70	34.4	35.1	0.61	1.30
Total carnitine, μmoles/L				
Day 0	8.3	8.6	0.77	1.15
Day 40	10.1	13.6	<0.01	1.17
Day 55	9.8	15.2	<0.01	1.15
Day 70	10.9	18.3	<0.01	1.15
Free carnitine, μmoles/L				
Day 0	7.0	7.1	0.86	0.96
Day 40	8.4	11.5	<0.01	0.98
Day 55	8.6	12.5	<0.01	0.96
Day 70	9.6	15.4	<0.01	0.96
	Trt	Day	Trt x Day	
Weight, kg	0.58	<0.01	0.11	
Estimated protein mass, kg	0.64	<0.01	0.20	
Backfat, mm	0.50	<0.01	0.31	
Estimated fat mass, kg	0.44	<0.01	0.10	
Total carnitine, μmoles/L	<0.01	<0.01	<0.01	
Free carnitine, μmoles/L	<0.01	<0.01	<0.01	

<sup>a</sup> Data was analyzed as a repeated measure to only include the gilts harvested at d 70 of gestation (Control, n=10; L-carnitine, n=10)

<sup>b</sup> Prediction equation from Dourmad et al., (1997),  $2.28 + 0.178 \times (\text{liveweight, kg}) - 0.333 \times (\text{backfat, mm})$ .

<sup>c</sup> Prediction equation from Dourmad et al., (1997),  $-26.40 + 0.221 \times (\text{liveweight, kg}) + 1.331 \times (\text{backfat, mm})$ .

**Table 2.4 Effects of L-carnitine on litter and ovary characteristics<sup>a</sup>**

Item	Day of Gestation						SED	P-value		
	40		55		70			Trt <sup>c</sup>	Day <sup>d</sup>	Trt × Day
	L-carnitine, ppm <sup>b</sup>									
	0	50	0	50	0	50				
No of gilts	9	10	10	10	10	10				
Litter										
Total weight, g	136.7	122.4	901.0	883.9	2484.5	2657.1	188.20-183.18	0.66	<0.01	0.71
Average fetal weight, g <sup>e</sup>	9.5	8.4	74.6	69.2	217.7	236.6	9.95-10.30	0.48	<0.01	0.19
Total fetus no.	14.0	14.1	12.1	12.8	11.5	11.4	1.20-1.23	0.74	0.01	0.89
Fetus no. right horn	7.4	6.9	5.7	6.1	5.8	6.2	0.75-0.77	0.83	0.05	0.63
Fetus no. left horn	6.6	7.1	6.3	6.7	5.7	5.1	0.74-0.76	0.80	0.02	0.54
Crown to rump, cm	5.3	5.2	12.0	11.6	16.8	16.8	0.32-0.33	0.44	<0.01	0.80
IGF-II, ng/mL <sup>f</sup>	*	*	16.3	14.5	22.9	17.6	3.04-3.54	0.14	0.05	0.45
CL										
Total	17.2	18.1	16.9	17.1	15.7	16.4	1.26-1.30	0.42	0.20	0.94
Right ovary	9.4	9.4	9.5	8.7	9.6	9.5	1.26-1.30	0.68	0.88	0.90
Left ovary	7.8	8.8	7.6	8.4	6.1	6.9	1.15-1.18	0.29	0.07	0.99

<sup>a</sup> Data was analyzed as a 2 × 3 factorial arrangement.

<sup>b</sup> Gestation feeding levels of 1.75 kg/d, with a top-dress providing 0 or 50 ppm added L-carnitine.

<sup>c</sup> Treatment (Trt).

<sup>d</sup> Day of gestation (Day).

<sup>e</sup> Day 70 *P* = 0.06.

<sup>f</sup> Day 70 *P* = 0.09.

**Table 2.5 Influence of L-carnitine to gestating gilts on fetal hepatic and muscle tissue mRNA relative abundance of IGF-system genes<sup>a</sup>**

Gene	Day of Gestation						SED	<i>P</i> -value		
	40		55		70			Trt <sup>e</sup>	Day <sup>f</sup>	Trt × Day
	L-carnitine, ppm <sup>d</sup>									
	0	50	0	50	0	50				
<b>Hepatic<sup>b</sup></b>										
IGF-I	1.74	0.61	0.62	0.58	0.58	0.67	0.437-0.449	0.05	0.24	0.17
IGF-II	14,504	11,748	13,930	13,776	10,408	10,316	3,055-3,139	0.67	0.51	0.82
IGFBP-3	28	38	61	63	61	68	11.5-11.8	0.34	0.0004	0.89
IGFBP-5	2.83	2.63	3.58	3.28	3.93	4.01	0.929-0.955	0.80	0.19	0.96
<b>Skeletal Muscle<sup>c</sup></b>										
IGF-I	12.8	10.9	23.8	10.0	54.7	56.8	12.71-24.36	0.74	0.02	0.89
IGF-II	6,814	8,312	14,456	3,876	40,326	76,556	27,467-28,220	0.67	0.32	0.62
IGFBP-3	149	184	71	32	132	235	77.8-80.0	0.47	0.04	0.44
IGFBP-5	393	312	257	209	213	275	105.4-108.3	0.72	0.23	0.61

<sup>a</sup> Values are least squares means and SED(standard error of difference) and data is presented as arbitrary units expressed in millions.

<sup>b</sup> Total RNA was isolated from semitendinosus muscle from the left hind limb at the median conceptus from each uterine horn.

<sup>c</sup> Total RNA was isolated from the left lobe of the liver at the median conceptus from each uterine horn.

<sup>d</sup> Gestation feeding levels of 1.75 kg/d, with a top-dress containing 0 or 50 ppm added L-carnitine.

<sup>e</sup> Treatment (Trt).

<sup>f</sup> Day of Gestation (Day).

**CHAPTER 3 - L-carnitine alters insulin-like growth factor-I (IGF-I), insulin-like growth factor binding protein-3 (IGFBP-3) and -5 mRNA in the endometrium of the gilt**



## Abstract

We investigated the influence of supplemental L-carnitine on the uterine and chorioallantois expression of IGFs and binding proteins at day 40, 55, and 70 of gestating gilts. Experimental treatments were arranged in a  $2 \times 3$  factorial with main effects of L-carnitine (0 or 50 ppm) and day of gestation (40, 55, or 70). A total of fifty-nine gilts (BW = 137.7 kg) from three different breeding groups received a constant feed allowance of 1.75 kg/day and a top-dress containing either 0 or 50 ppm of L-carnitine starting on the first day of breeding through the allotted gestation length. Insulin-like growth factor binding protein-3 (IGFBP-3;  $P = 0.05$ ) and IGFBP-5 mRNA increased ( $P = 0.01$ ) and IGF-I mRNA numerically increased ( $P = 0.16$ ) in the endometrium of gilts supplemented with L-carnitine. From d 40 to 55, IGF-I, IGFBP-3, and IGFBP-5 mRNA decreased ( $P < 0.10$ ) in the myometrium. Chorioallantois IGF-I mRNA tended to increase ( $P = 0.16$ ) as gestation length increased. These data demonstrate that L-carnitine supplementation and gestation length alter the expression of the IGF axis at the fetal-maternal interface in swine. These changes in the IGF axis at the fetal maternal interface could play a fundamental role in porcine fetal growth and development.

Keywords: Insulin-like growth factor, Insulin-like growth factor binding proteins, L-carnitine, messenger RNA, pigs

## **Introduction**

The uterus and chorioallantois are highly functional in mediating the exchange of nutrients from the dam to the fetus (1), but the exact mechanism by which growth factors and binding proteins are involved is poorly understood (2). Research has shown the IGF axis at the fetal maternal interface can be altered by maternal nutrition (3,4) and maternal body condition (5).

Previous research has shown the addition of L-carnitine to maternal gestation diets may increase BW gain (6,7), last rib backfat (6), and plasma IGF-II (8) of gestating sows. Research has also shown an increase in plasma IGF-I at d 60 and d 90 of gestation when sows were supplemented with L-carnitine in their diets (6). Contradictory to this, other researchers (9) have observed no change in plasma IGF-I when sows were supplemented L-carnitine at d 55 of gestation. Therefore, there is not an equivocal response of maternal IGF-I due to L-carnitine supplementation. In addition, supplementing L-carnitine to gestating sows has shown increased total number of pigs born and born alive (10,11), decreases the number of stillborn pigs (6,8), and increased average pig weight (7,10,12) and litter weight (7,8,10) at birth.

The addition of dietary L-carnitine to gestation diets has been shown to cause numerous changes in sow growth and reproductive performance and might be the result of an influence on the IGF system. But, the role of L-carnitine in altering insulin-like growth factors and binding proteins at the fetal-maternal interface has not been elucidated to a great extent. Therefore, the objective of this study was to evaluate the effects of supplementing L-carnitine on the IGF axis at the fetal-maternal interface in pigs. Components of the IGF axis were evaluated at day 40, 55, and 70 of gestation in the myometrium, endometrium, and chorioallantois in gilts.

## **Materials and Methods**

### *Animals*

All animal procedures used in this study were reviewed and approved by the Kansas State University Animal Care and Use Committee. Fifty-nine gilts (PIC; Franklin, KY; L327 × 1050; BW 137.7 kg) were artificially inseminated (PIC; MQ 280) 12, 24, and 36 h after the onset of the second observed estrus. Day 1 was considered 12 h after the first insemination. Gilts were housed in individual crates (1.83 × 0.55 m) in an environmentally-controlled gestation barn at the

Kansas State University Swine Teaching and Research Center from breeding to either day 39, 54 or 69 gestation. Gilts were allowed *ad libitum* access to water and randomly allotted to one of two dietary treatments and one of three harvesting dates based on weight at breeding. All gilts were fed a standard corn-soybean meal gestation diet (Table 3.1) formulated to 0.65% total lysine once daily (1.75 kg) and received a 50 g ground corncob top-dress containing either 0 (control, n=30) or 50 ppm of L-carnitine (Carniking 10 (10% L-carnitine, n=29); Lonza Inc., Allendale, NJ) from day 1 to 39, 54 or 69 of gestation. The gestation diet was formulated to meet or exceed NRC (1998) nutrient requirement estimates and fed at a level of 1.75 kg/d based on NRC models to be slightly above the requirements for maintenance and fetal growth.

### ***Harvesting protocol and collection of samples***

Harvest was completed on gilts either at day 40, 55, or 70 of gestation. Fifteen hours before harvest, gilts were transported from the Kansas State University Swine Research and Teaching Center to the Kansas State University Meat Laboratory where sample collections were performed 24-h after the last feeding. Gilts were allowed *ad libitum* access to water until harvest. Gilts were harvested by electrical stunning followed by exsanguination. A mid-lateral incision was made to gain access to the abdominal cavity. The ovarian pedicles and uterine stump, at the level of the cervix were cut for removal of the uterus. Once the uterus was removed, the number of fetuses was determined on both sides. Fetal pigs were removed by mid-ventral laparotomy under aseptic conditions and rapidly transported to the Kansas State University Growth Laboratory for additional processing.

### ***Maternal and chorioallantois tissues***

Uterus (myometrium and endometrium) and chorioallantois samples were excised at the middle conceptus from each uterine horn. The tissues samples were immediately snap frozen in liquid nitrogen and stored in a -80°C Ultra Low Freezer.

### ***Sample preparation and RNA isolation***

Total RNA was isolated from the endometrium and myometrium by using the RNeasy Mini Kit (Qiagen; Valencia, CA). The total RNA was isolated from the chorioallantois samples using TRI REAGENT (Sigma; St. Louis, MO). Samples isolated with TRI Reagent were treated with DNase to remove any contaminating genomic DNA using a commercially available kit

(DNA-free; Ambion, Austin, TX). The concentration of RNA was determined by absorbance at 260 nm. Electrophoresis of total RNA through a 1% agarose-formaldehyde gel followed by ethidium bromide staining to allow visualization of 28S and 18S ribosomal RNA (rRNA) was used to assess the integrity of RNA. One microgram of total RNA was then reverse-transcribed to produce the first-strand complementary DNA (cDNA) using TaqMan reverse transcriptase (Applied Biosystems, Foster City, CA) following the protocol recommended by the manufacturer. Random hexamers were used as primers in cDNA synthesis.

### ***Real-time quantitative PCR***

Real-time quantitative-PCR was used to measure the quantity of mRNA for IGF-I, IGF-II, IGFBP-3, and IGFBP-5 and 18S rRNA in total RNA isolated from the myometrium, endometrium, and chorioallantois. Measurement of the relative quantity of cDNA was carried out using TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of the appropriate forward and reverse primers, 200 nM of the appropriate TaqMan detection probe, and 1  $\mu$ L (0.5  $\mu$ g cDNA) of the cDNA mixture. Sequences for primers and probes for IGF-I, IGF-II, IGFBP-3 and IGFBP-5 are presented in Table 3.2. Commercially available eukaryotic 18S rRNA primers and probes were used as an endogenous control (Applied Biosystems; Genbank Accession no. X03205). Assays were performed in an ABI Prism 7000 sequence detection system (Applied Biosystems) using thermal cycling parameters recommended by the manufacturer (50 cycles of 15 sec at 95°C and 1 min at 60°C). Relative expressions of mRNA for IGF-I, IGF-II, IGFBP-3 and IGFBP-5 were normalized to the 18S rRNA endogenous control and expressed in arbitrary units.

### ***Statistical analysis***

For all genes evaluated, mRNA concentrations were analyzed as a 2  $\times$  3 factorial arrangement with the MIXED Procedure of SAS. Fixed model effects included treatment, day of harvest and their interaction. Kenward-Roger adjustment was used for the degrees of freedom. The significance was declared at  $P < 0.05$  unless noted otherwise.

## **Results**

### ***RNA analysis***

In the chorioallantois, IGF-I, IGF-II, IGFBP-3, and IGFBP-5 mRNA levels were not different between ( $P > 0.05$ ) gilts fed diets with supplemental L-carnitine and the control diet (Table 3.3). Insulin-like growth factor-I mRNA was numerically increased ( $P = 0.16$ ) as day of gestation increased in the chorioallantois. No differences ( $P > 0.05$ ) were observed in IGF-II, IGFBP-3, and IGFBP-5 mRNA expression as day of gestation increased (Table 3.3). In the endometrium, no differences ( $P > 0.05$ ) were observed in IGF-I, IGF-II, IGFBP-3, and IGFBP-5 mRNA expression as day of gestation increased. However, IGFBP-3, and IGFBP-5 mRNA expression was greater in gilts fed diets with supplemental L-carnitine (IGFBP-3,  $P = 0.05$ ; IGFBP-5,  $P = 0.01$ ) and IGF-I tended to increase (IGF-I,  $P = 0.16$ ) compared to the control-fed gilts. In the myometrium, IGF-I, IGF-II, IGFBP-3, and IGFBP-5 mRNA expression was not different ( $P > 0.05$ ) in gilts fed diets containing supplemental L-carnitine compared to the gilts fed the control diet. As day of gestation increased from d 40 to d 55, there was a decrease in IGF-I mRNA ( $P < 0.01$ ), IGFBP-3 mRNA ( $P = 0.10$ ), and IGFBP-5 mRNA ( $P < 0.01$ ) expression (Table 3.3).

### **Discussion**

We have previously reported that supplementing L-carnitine to the diets fed to gestating gilts increased circulating total and free carnitine concentrations at day 40, 55, and 70 of gestation compared to gilts not supplemented with L-carnitine (Figure 3.1 and 3.2; 14). This is consistent with other published research that confirms supplementing L-carnitine increases systemic levels of carnitine (6,9,15,16). This suggests L-carnitine provided as a dietary supplement can be absorbed and elevates the circulating levels of carnitine above that present in a non-supplemented animal. Increasing circulating L-carnitine concentrations with exogenous L-carnitine has shown positive effects on carcass characteristics (17), gilt growth (6,7), and reproductive performance (6,10,12).

In addition, we have previously reported circulating maternal plasma IGF-I concentrations did not differ at day 40, 55, or 70 in gilts fed supplemental L-carnitine compared to the gilts fed no supplemental L-carnitine (14), but found differences in fetal weight at d 70 of gestation in fetuses from dams fed diets with supplemental L-carnitine. This is in agreement with Waylan et al. (9). Waylan et al. (9) observed no change in circulating IGF-I levels in sows

diets with or without supplemental L-carnitine at mid-gestation. This suggests the heavier fetal weights we found from gilts fed L-carnitine were not due to maternal IGF-I. In fetal hepatic tissues we have also observed a decreased expression of IGF-I and reduced fetal circulating IGF-II (14) when L-carnitine was included in the diet. This provides support for a change in IGF system components due to supplementation of L-carnitine to the gilt. Therefore, additional research in growth factor regulation at the fetal-maternal interface was necessary to determine the effect of L-carnitine on biological mechanisms in the gestating gilt and fetal pig.

The placental and uterine environment play an important role in optimal fetal development (18,19), fetal weight and survival (20), and normal reproductive function, specifically, changes in these environments by autocrine, paracrine, and endocrine actions of IGFs (21). These tissues undergo rapid changes in morphology and function during pregnancy (22). The physiological changes are coordinated by changes in expression of insulin-like growth factors and binding proteins (23). In this study, growth factor expression evaluated in the myometrium and chorioallantois were not affected by maternal supplementation of L-carnitine, but IGF-I, IGFBP-3, and IGFBP-5 gene expression increased in the endometrium in gilts fed diets with supplemental L-carnitine. No changes were observed for endometrium IGF-II gene expression.

Previous studies have reported the importance of the IGF system in uterine and fetal development. *In vitro* research has shown that IGF-I stimulates DNA synthesis in uterine tissues of rats (24) and pigs (25), and expression of specific receptors for IGF-I indicate a role for IGF-I in implantation (26) uterine (27,28), and conceptus (26) development. Insulin-like growth factor-I also increases nutrient supply to the porcine conceptus (30). Previous research showed no changes in IGF-I gene expression in the porcine uterus collected from sows fed diets with supplemental L-carnitine at mid-gestation (9). In our study, we observed a numerical increase in IGF-I mRNA expression in the endometrium due to supplementation of L-carnitine in the feed, but not in the myometrium. In the current study, we excised these two uterine tissue layers and found differences in gene expression in the endometrium. Waylan et al. (9) did not find any differences, but also did not separate these two tissues. This might explain the lack of a similar response. We cannot draw specific conclusions on our contrasting results because data is non-existent on IGF system components in the pig myometrium. In addition, it is difficult to

determine whether the IGF axis is involved in the myometrium across species because some of the IGF components are not found at detectable levels in different species (30).

A number of research studies have concluded that IGFBPs inhibit the biological actions of IGFs (31). In addition to these biological effects, researchers have also suggested IGF independent effects observed from changes in IGFBPs (32). Specifically, researchers have shown human IGFBP-1 enhances the action of IGF-I in the endometrium (33). Although IGFBP-1 and IGFBP-2 are major carriers of the growth factors (27), some researchers have focused on IGFBP-3 expression in fetal development because of its importance in porcine reproductive tissues (34). We did not elucidate gene expression of IGFBP-1, IGFBP-2, IGFBP-4, or IGFBP-6 in the endometrium, only IGFBP-3 and IGFBP-5 due to their known role in fetal muscle development (9).

In this study, we observed an increase in IGFBP-3 and IGFBP-5 mRNA expression in the endometrium of gilts fed diets with supplemental L-carnitine. Maternal hormonal changes have been shown to increase levels of IGFBP-3 secretions in humans during pregnancy (35). In follicular and amniotic fluid, IGFBP-3 has been shown to have both potentiative and inhibitory effects on IGF-I and IGF-II (36). With the increase in IGFBP-3 expression observed in our study, we can conclude IGFBP-3 had potentiative effects on numerically increasing IGF-I mRNA expression. Other researchers have found IGF-I stabilizes IGFBP-3 in myometrial cells, therefore initiating a mitogenic response (37) in this cell type.

Little is known about IGFBP-5 expression in reproductive tissues in the pig (38). Insulin-like growth factor-II is co-expressed with IGFBP-5 during vascularization of the placenta (30) and increases in IGF-I have been shown to stimulate the expression of IGFBP-5 mRNA and protein expression in ovarian cultured porcine granulosa cells (39,40). Therefore, we can suggest that if L-carnitine increased IGFBP-3 mRNA expression in the endometrium, this might potentiate the effects of IGF-I mRNA expression, in turn increasing levels of IGFBP-5 mRNA. The increase in IGF-I, IGFBP-3 and IGFBP-5 mRNA expression we observed in the endometrium from gilts fed L-carnitine suggests that L-carnitine altered the IGF axis at the fetal maternal interface.

The uterus provides the conceptus with a nourishing environment in gestation through changes in hormones and other biological molecules, such as insulin-like growth factors and insulin-like growth factor binding proteins. In this study, we observed increases in IGF-I,

IGFBP-3, and IGFBP-5 mRNA expression in the endometrium of gilts fed diets with supplemental L-carnitine. In addition, we have previously observed a decrease in fetal circulating IGF-II and hepatic IGF-I expression in fetuses collected from gilts fed supplemental L-carnitine. The exact mechanism by which L-carnitine affects IGF-I and these two binding proteins in uterine development is not completely clear. One can speculate these binding proteins exert IGF-dependent and -independent effects on the uterine environment, enhancing endometrium growth similar to IGF-I, but further research is needed in this area before conclusions can be drawn.

### **Implications**

The change in insulin-like growth factors and insulin-like growth factor binding proteins in the gilt endometrium, fetal hepatic tissue, and fetal circulating IGF-II provide support for maternal-fetal exchange of IGF system components due to supplementation of L-carnitine to the gilt. In addition, our research may provide evidence of increased efficiency of nutrient utilization by the fetus due to supplementing L-carnitine to the dam. This may explain the increased weight of pigs born to dams fed supplemental L-carnitine.

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**Table 3.1 Diet composition fed during gestation (as-fed)<sup>a</sup>**

Item	Amount
Ingredient, %	
Corn	81.22
Soybean meal, 46.5% CP	14.55
Monocalcium phosphate, 21% P	2.03
Limestone	1.05
Salt	0.50
Vitamin premix <sup>b</sup>	0.25
Trace mineral premix <sup>c</sup>	0.15
Sow add pack <sup>d</sup>	0.25
	100.00
Calculated analysis	
Lysine, %	0.65
ME, Mcal/kg	3.27
Protein, %	13.7
Calcium, %	0.85
Phosphorus, %	0.75
Available P, %	0.48

<sup>a</sup> Gestation feeding levels of 1.75 kg/d, with top-dress providing 0 or 50 ppm added L-carnitine.

<sup>b</sup> Supplied per kilogram of diet: 11,025 IU of vitamin A, 1,654 IU of vitamin D<sub>3</sub>, 55.1 mg of niacin, 44.1 IU of Vitamin E, 33.1 mg of pantothenic acid, 9.9 mg of riboflavin, 4.4 mg of vitamin K (menadione), and 0.04 mg of vitamin B<sub>12</sub>.

<sup>c</sup> Supplied per kilogram of diet: 165 mg of Zn (oxide), 165 mg of Fe (sulfate), 39.7 mg of Mn (oxide), 16.5 mg of Cu (sulfate), 0.30 mg of I (as Ca iodate), and 0.30 mg of Se (as Na selenite).

<sup>d</sup> Supplied per kilogram of diet: 551.3 mg of choline, 15.2 mg pyridoxine, 1.65 mg of folic acid and 0.22 mg of biotin.

**Table 3.2 Primers and probes used for real-time quantitative PCR**

Gene	Genbank accession number	Sequence
IGF-I	M31175	Forward TCTTCTACTTGGCCCTGTGCTT
		Reverse GCCCCACAGAGGGTCTCA
		Probe 6FAM-CCTTCACCAGCTCTGCCACGGC-TAMRA
IGF-II	X56094	Forward CCGGACAACTTCCCCAGATA
		Reverse CGTTGGGCGGACTGCTT
		Probe 6FAM-CCCGTGGGCAAGTTCTTCCGC-TAMRA
IGFBP-3	AF085482	Forward AGCACGGACACCCAGAACTT
		Reverse CGGCAAGGCCCGTATTC
		Probe 6FAM-TCCTCTGAGTCCAAGCGCGAGA-TAMRA
IGFBP-5	U41340	Forward GGCAGAGGCCGTGAAGAAG
		Reverse CAGCTCCCCCAGCAACT
		Probe 6FAM-CCGCAGAAAGAAGCTGACCCAGTCC-TAMRA

**Table 3.3 Influence of L-carnitine to gestating gilts on uterine and chorioallantois mRNA concentrations on IGF-system genes<sup>a</sup>**

Gene	Day of Gestation						SED	P-value		
	40		55		70			Trt <sup>c</sup>	Day <sup>d</sup>	Trt × Day
	L-carnitine, ppm <sup>b</sup>									
	0	50	0	50	0	50				
Chorioallantois										
IGF-I	1.86	1.68	2.70	2.27	11.23	3.12	4.18-4.29	0.24	0.16	0.34
IGF-II	4,728	5,564	5,825	6,542	6,858	5,510	1,682-1,728	0.96	0.71	0.70
IGFBP-3	74.51	59.94	107.96	147.44	145.51	113.03	52.06-53.88	0.93	0.18	0.61
IGFBP-5	31.84	32.88	48.98	24.75	58.75	44.35	19.27-20.34	0.27	0.36	0.66
Endometrium										
IGF-I	3.30	5.43	4.10	4.34	2.61	3.77	1.39-1.44	0.16	0.44	0.66
IGF-II	490	1,776	1,050	1,237	655	740	713-752	0.22	0.61	0.44
IGFBP-3	358.20	722.42	540.50	742.79	592.18	1,042.18	290.05-305.78	0.05	0.41	0.84
IGFBP-5	188.06	208.45	126.76	228.80	134.19	245.97	49.51-51.19	0.01	0.85	0.38
Myometrium										
IGF-I	80.51	75.32	40.75	36.88	33.60	57.31	14.23-14.62	0.56	<0.01	0.28
IGF-II	591	527	263	266	1002	221	501.34-515.08	0.34	0.58	0.48
IGFBP-3	15.58	19.30	10.30	11.24	13.39	12.07	4.32-4.55	0.66	0.10	0.72
IGFBP-5	87.70	100.64	37.53	39.35	34.20	44.48	13.38-14.17	0.30	<0.01	0.84

<sup>a</sup> Values are least squares means and SED (standard error of difference) and data is presented as arbitrary units expressed in millions. Uterus and chorioallantois samples were excised from the middle conceptus from each uterine horn.

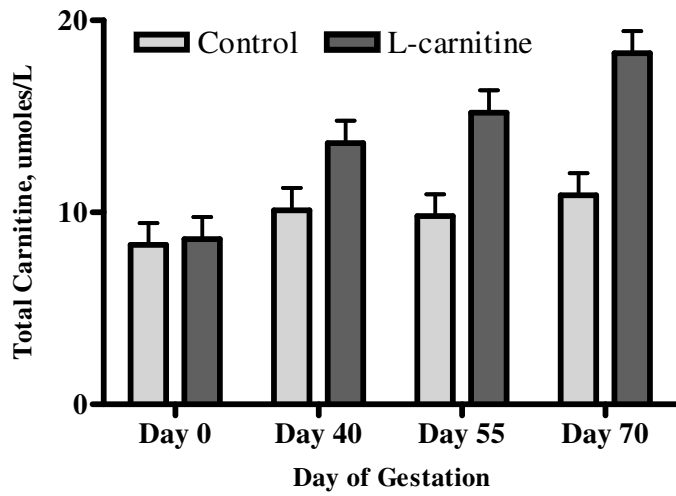
<sup>b</sup> Gestation feeding levels of 1.75 kg/d, with a top-dress providing 0 or 50 ppm added L-carnitine.

<sup>c</sup> Treatment (Trt).

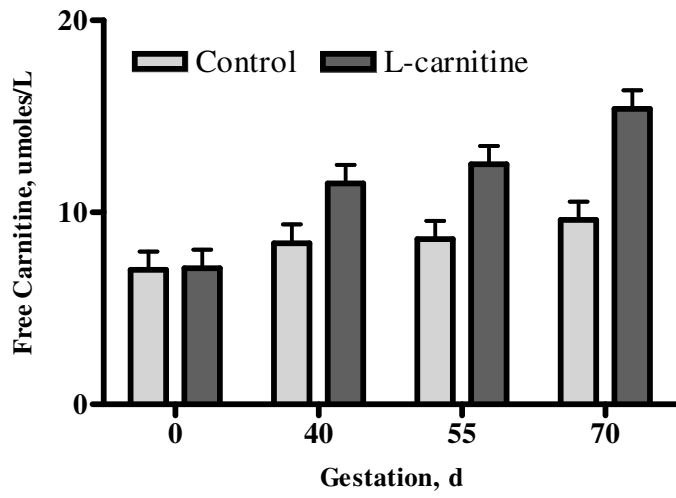
<sup>d</sup> Day of Gestation (Day).



**Figure 3.1 Plasma total carnitine concentrations of gilts fed unsupplemented (control) and supplemented (50 ppm of L-carnitine) diets at three gestation lengths.**



**Figure 3.2 Plasma free carnitine concentrations of gilts fed unsupplemented (control) and supplemented (50 ppm of L-carnitine) diets at three gestation lengths.**



**CHAPTER 4 - L-carnitine supplementation of gestating gilts alters  
insulin-like growth factor binding protein-3 (IGFBP-3) mRNA  
expression in porcine embryonic myoblasts**

## Abstract

We determined the effects of supplementing L-carnitine to gilts on the IGF system in porcine embryonic myoblasts. Forty gilts (BW=137.7 kg) were allotted to one of 4 treatments that were arranged in a 2 × 2 factorial arrangement with main effects of L-carnitine (0 or 50 ppm) and day of gestation (55 or 70). All gilts were fed a constant feed allowance of 1.75 kg/day and a top-dress containing either 0 or 50 ppm of L-carnitine starting on the first day of breeding through the allotted gestation length. At d 55 or 70 of gestation, fetuses were removed for isolation of porcine embryonic myoblasts (PEM) from the hind-limb muscles. Real-time quantitative PCR was used to determine growth factor messenger RNA (mRNA) expression in cultured PEM at 72-, 96-, 120-, and 144-h post-plating. Flow cytometry was used to analyze percentage of myogenic cells with a myoblast/myotube-specific monoclonal antibody 5.1H11 and for determination of cell cycle stage. There was no treatment differences ( $P > 0.10$ ) for the expression of IGF-I, IGF-II, or IGFBP-5 mRNA levels. However, PEM isolated from fetuses collected from gilts fed diets with L-carnitine had lower ( $P = 0.08$ ) IGFBP-3 mRNA levels compared to the controls. Myoblasts isolated from fetuses from gilts fed diets with added L-carnitine had greater ( $P = 0.09$ ; 8.8%) 5.1H11 monoclonal antibody attachment compared to the controls after 72 hours in culture (91.8% vs. 87.4%). Although not significant ( $P = 0.31$ ), the total number of PEM in the S phase of the cell cycle was 4.7% greater in PEM collected from fetuses obtained from gilts fed diets with L-carnitine compared to the control-fed gilts (37.5% vs. 34.2%). This data suggests that L-carnitine influences the IGF system, stage of the cell cycle, and recognition of muscle development, resulting in enhanced proliferation and delayed differentiation of PEM.

Keywords: Insulin-like Growth Factor, Insulin-like Growth Factor Binding Proteins, L-carnitine, Messenger RNA, Myoblasts, Pigs

## Introduction

The addition of L-carnitine, a water soluble quaternary amine (1), to gestation diets elicits positive effects on sow reproductive performance. Specifically, supplementing L-carnitine to gestating sows increases average pig weight (2,3,4) and litter weight at birth (3,4,5). This may be due to L-carnitine's role in  $\beta$ -oxidation (6), glucose disposal and carbohydrate metabolism (7,8,9). Other researchers have eluded to the effect of L-carnitine on the insulin-like growth factor system (5,10,11).

Insulin-like growth factors (IGF) and insulin-like growth factor binding proteins (IGFBP) have mitogenic effects stimulating cellular proliferation and differentiation of myogenic cells both *in vivo* (12,13) and *in vitro* (13,14), thus influencing cell cycle progression (15). These components of the IGF system are involved in several aspects of fetal growth and development (16,17) and appear to be modulators of muscle development in the pig fetus (4). The components of the IGF system have also been shown to potentiate or inhibit each others actions (14,18,19).

In previous data from this study that has already been reported we observed an increase in fetal weight at d 70 of gestation in fetuses collected from gilts fed diets with supplemental L-carnitine (20) and we have observed an increase in IGF-I, IGFBP-3, and IGFBP-5 mRNA expression in the endometrium of gilts fed diets containing supplemental L-carnitine (21). In addition, we observed a decrease in hepatic IGF-I expression in fetuses collected from gilts fed diets with supplemental L-carnitine (20). Changes in IGF-I, IGFBP-3, and -5 in the maternal endometrium and IGF-I in fetal hepatic tissue provide support for an altered maternal-fetal exchange of IGF system components due to supplementation of L-carnitine to the gilt. This may or may not explain the increased weight of pigs born to dams fed supplemental L-carnitine.

During pregnancy, growth factors and binding proteins play a role in fetal muscle development in the pig. Although data is limited, studies have observed decreased levels of IGF-II, IGFBP-3, and myogenin (11) in PEM collected from fetuses from sows fed supplemental L-carnitine up to d 55 of gestation. Insulin-like growth factor-II (15) and IGFBP-3 (22,23) and myogenin (24,25) have been shown to either suppress cellular proliferation or stimulate differentiation *in vitro*. This suggests L-carnitine may play a role in regulating proliferation and differentiation of porcine myogenic cells in culture. Further evidence to support enhanced proliferation of porcine myogenic cells comes from a study conducted by Musser et al. (26). In

2002, Musser et al. (26) found an increase in the cross sectional area of the semitendinosus, increased primary muscle fiber numbers, and a lower secondary:primary fiber ratio at birth in offspring of sows fed diets containing supplemental L-carnitine. Therefore, our experiment was designed to determine the effects of supplemental L-carnitine to gestating gilts on fetal muscle development *in vitro* using real-time quantitative PCR techniques and flow cytometry.

## **Materials and Methods**

### ***Animals***

All animal procedures used in this study were reviewed and approved by the Kansas State University Animal Care and Use Committee. Forty gilts (PIC; Franklin, KY; L327 × 1050; BW 137.7 kg) were artificially inseminated (PIC; MQ 280) 12, 24, and 36 h after the onset of their second observed estrus. Day 1 was considered 12 h after the first insemination. Gilts were housed in individual crates (1.83 × 0.55 m) in an environmentally controlled gestation barn at the Kansas State University Swine Teaching and Research Center from breeding to either day 54.5 or 69.5 gestation. Gilts were allowed *ad libitum* access to water and randomly allotted to one of two dietary treatments and one of two harvesting dates based on weight at breeding. Based on average initial weight, we calculated an allowance for moderate growth through gestation. All gilts were fed a corn-soybean meal gestation diet (Table 4.1) formulated to 0.65% total lysine once daily (1.75 kg) and received a 50 g ground corncob top-dress containing either 0 (control, n=30) or 50 ppm of L-carnitine (Carniking 10 (10% L-carnitine, n=29); Lonza Group, Inc., Allendale, NJ) from day 1 to 54 or 69 of gestation. The gestation diet was formulated to meet or exceed NRC (1998; 27) nutrient requirement estimates and fed at a level of 1.75 kg/d based on NRC models to be slightly above the requirements for maintenance and fetal growth.

### ***Harvesting protocol***

Gilts were harvested on d 55 or 70 of gestation. Fifteen hours prior to harvest, gilts were transported from the Kansas State University Swine Research and Teaching Center to the Kansas State University Meat Laboratory where sample collections were performed 24-h after the last feeding. Gilts were allowed *ad libitum* access to water until harvest. Gilts were harvested by electrical stunning followed by exsanguination. A mid-lateral incision was made to gain access to the abdominal cavity. The ovarian pedicles and uterine stump, at the level of the cranial

cervix were cut for removal of the uterus. Once the uterus was removed, the number of fetuses was determined on both sides. Fetal pigs were removed by mid-ventral laparotomy under aseptic conditions and rapidly transported to the Kansas State University Growth Laboratory for processing.

### ***Isolation of porcine embryonic myoblasts***

Fetal myogenic cells were isolated according to the procedures described previously (11,28,29). The hindlimb muscles from the right side were aseptically excised and washed with warm (37°C) Earle's balanced salt solution (EBSS, pH 7.4; Sigma, St. Louis, MO). The fetal muscle was minced with scissors and pooled for each of the gilts. The fetal muscle was digested with 10 volumes (vol/wt of minced muscle) of 0.2% (wt/vol) trypsin in Ca-Mg-free EBSS. After a 1-h incubation period, the digested tissue was pelleted by centrifugation (400 × g). The pellet was resuspended in EBSS and centrifuged (400 × g). The resultant pellet was suspended in Dulbecco's Modified Eagle medium (DMEM; Gibco) containing 10% (vol/vol) fetal calf serum (FCS; Gibco) to give 0.4 g of original tissue weight/ml of medium and then sequentially filtered through 149- and 74-µm mesh Nitex cloth. The filtrate was plated on 75-cm<sup>2</sup> tissue culture flasks and incubated for 1-h at 37°C, 5% CO<sub>2</sub>, and 95% air in a water saturated environment. The unattached cells were removed and pelleted by centrifugation (1,400 × g) and then suspended in DMEM containing 10% FCS and 10% (vol/vol) dimethylsulfoxide (Sigma, St. Louis, MO). Aliquots were placed in polypropylene cryogenic vials (12 vials of pooled fetal myogenic cells were obtained from each sow), frozen at -80°C, and stored in a liquid N<sub>2</sub> tanks, as previously described (30).

### ***Cell culture and RNA isolation***

Fetal myogenic cells were plated as previously described with minor modifications (11). To establish cultures from frozen stocks, thawed cell suspensions were diluted with 21 ml of DMEM containing 10% (vol/vol) FCS and antibiotic/antimycotic. A cell solution (5.0 mL, 3,290 cells/cm<sup>2</sup>) was plated on 4 29.26 cm<sup>2</sup> dishes coated with Basement Membrane Matrigel (diluted 1:27 [vol/vol] in DMEM; Becton Dickinson Labware, Bedford, MA). All cultures were maintained at 37°C, 5% CO<sub>2</sub>, and 95% air in a water saturated environment. After a 24-h attachment period, the plates were rinsed three times with 2.5 mL of DMEM. Cultures were re-fed with DMEM containing 10% FCS (2.5 mL/29.26 cm<sup>2</sup> plate). At 72-h post-plating cells were

rinsed three times with 2.5 mL of DMEM and cultures were re-fed with DMEM containing 3% swine serum (2.5 mL/28.26 cm<sup>2</sup> plate). At 96-h post-plating 2.5 µL cytosine furanoside (Ara-C;10<sup>-3</sup>) was added to the plates for total RNA isolation at 120- and 144-h post-plating.

At 72-, 96-, 120-, and 144 h after plating, total RNA was isolated from the cells on the 29.26 cm<sup>2</sup> plates (Absolutely RNA Microprep kit, Stratagene, La Jolla, CA). The concentration of total RNA was determined at an absorbance of 260 nm. TaqMan reverse transcription reagents and MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA) were used to produce cDNA from 1 µg of total RNA. Random hexamers were used as primers in cDNA synthesis.

### ***Real-time quantitative PCR***

Real-time quantitative-PCR was used to measure the quantity of mRNA for IGF-I, IGF-II, IGFBP-3, and IGFBP-5 and 18S rRNA in total RNA isolated from cell cultures. Measurement of the relative quantity of cDNA was carried out using TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of the appropriate forward and reverse primers, 200 nM of the appropriate TaqMan detection probe, and 1 µL of the cDNA mixture. Sequences for primers and probes for IGF-I, IGF-II, IGFBP-3 and IGFBP-5 are represented in Table 4.2. Commercially available eukaryotic 18S rRNA primers and probes were used as an endogenous control (Applied Biosystems; Genbank Accession no. X03205). Assays were performed in an ABI Prism 7000 sequence detection system (Applied Biosystems) using thermal cycling parameters recommended by the manufacturer (50 cycles of 15 sec at 95°C and 1 min at 60°C). Relative expressions of mRNA for IGF-I, IGF-II, IGFBP-3 and IGFBP-5 were normalized to the 18S rRNA endogenous control using the  $\Delta$ -CT method and expressed in arbitrary units.

### ***Cell culture for flow cytometric analysis***

To establish cultures from frozen stocks, rapidly thawed cell suspensions were diluted with 14 mL of DMEM containing 10% (vol/vol) FCS and antibiotic/antimycotic. A cell solution (2.5 mL, 3,290 cells/cm<sup>2</sup>) was plated on 1-6 well plate coated with rat collagen (Becton Dickson; Collagen I Rat Tail Multi-well Plate, #354400). All cultures were maintained at 37°C, 5% CO<sub>2</sub>, 95% air in a water saturated environment. After a 24-h attachment period, the plates were rinsed three times with 2.0 ml of DMEM. Cultures were re-fed with DMEM containing 10% FCS (2.0 mL/9.62 cm<sup>2</sup> well).



### ***Flow cytometric analysis with 5.1H11 monoclonal antibody***

Flow cytometric analysis was used according to the procedures described previously (31). Briefly, adherent cells were removed from plates via 5-min incubation with trypsin at 72-h post-plating on 4 wells of the 6-well plate. For analysis of myogenic cells, we analyzed muscle cell preparations using a myoblast/myotube-specific monoclonal antibody 5.1H11 (32,33) obtained from the Developmental Studies Hybridoma Bank (The University of Iowa, Iowa City, IA). Tissue culture tubes containing primary myogenic cultures were blocked with 5% goat serum in PBS for 20-min, followed by a 45-min incubation with 5.1H11. This was followed by a 30-min incubation with Alexa Flour 488 goat anti-mouse IgG secondary antibody (2 mg/mL; Molecular Probes). A FACSCalibur flow cytometer (Becton Dickson) with a 488-nm argon laser was used to detect fluorescent antibody tagged porcine cells.

### ***Flow cytometric analysis for cell cycle determination***

Adherent cells were removed from plates via 5-min incubation trypsin at 75-h post-plating on the remaining 2 wells of the 6 well plate. For cell cycle analysis, porcine embryonic myoblasts were washed with 1 ml PBS. Cell DNA was stained with 300  $\mu$ L propidium iodide (PI) stain and 300  $\mu$ L of Vendelov's PI stain for 1-h (34). Samples were filtered through a 30  $\mu$ m mesh before flow cytometry. A FACSCalibur flow cytometer (Becton Dickson, location) with Cell Quest software containing a 488-nm argon laser was used to detect labeled cells. The cell cycle data was modeled with ModFit *LT* (2004; Verify Software Houses, Inc.).

### ***Statistical analysis***

The statistical analysis for gene expression was performed as a repeated measure using the MIXED Procedure of SAS (SAS, 2000; SAS Inst.Inc., Cary, NC). Fixed effects of treatment, day of gestation, and hour post-plating was included in the model and hour post-plating as the repeated measure. Kenward-Roger adjustment was used for the degrees of freedom. Flow cytometric analysis was performed using the MIXED Procedure of SAS. The model contained the effects of treatment, day of gestation, and their interaction. Kenward-Roger was used to adjust the degrees of freedom.

## **Results**

Real-time quantitative PCR results are given in Table 4.3, 4.4, and 4.5. As gestation length increased, IGF-I ( $P = 0.01$ ; Figure 4.1) and IGFBP-3 ( $P = 0.07$ ; Figure 4.2) mRNA expression increased (Figure 3.1). Differences ( $P = 0.01$ ) were also observed in time post-plating for IGF-I, IGF-II and IGFBP-3 mRNA expression. As time post-plating increased from 96 to 120 h, IGF-I and IGF-II mRNA expression increased. Insulin-like growth factor-I (Figure 4.5) and -II (Figure 4.4) mRNA expression increased at 120-h post-plating. Insulin-like growth factor binding protein-3 mRNA expression decreased at 96-h post-plating, and then increased at 120- and 144-h post-plating (Figure 3.2). Treatment differences were observed for IGFBP-3 mRNA. Insulin-like growth factor binding protein-3 mRNA expression decreased ( $P = 0.08$ , 48%) in PEM collected from fetuses from gilts fed diets with supplemental L-carnitine compared to the PEM collected from the controls (Figure 4.6). At day 55 (Figure 4.7) and 70 (Figure 4.8) of gestation PEM collected from gilts fed no supplemental L-carnitine had numerically higher expression of IGFBP-3 mRNA at 96 and 120-h post-plating.

In separate experiments, porcine embryonic myoblasts isolated from fetuses from gilts fed diets containing L-carnitine had greater (8.8 %;  $P = 0.09$ ) 5.1H11 monoclonal antibody attachment at 72 h in culture compared to the PEM collected from fetuses from gilts fed the control diet implying a greater number of myogenic specific cells at 72 h in culture (91.8% vs. 87.4%; Table 4.6). Although not significant ( $P = 0.31$ ), the total number of PEM in the S phase of the cell cycle was 4.7% greater in PEM collected from fetuses from sows fed diets with L-carnitine compared to the controls (37.5% vs. 34.2%).

## Discussion

The IGF system is instrumental in skeletal muscle development, specifically cell proliferation and differentiation (15). Primary mononucleated myoblasts are destined to have three specific roles. They may differentiate into primary muscle fibers, promote secondary muscle fiber development, or may be reserved as a population of satellite cells aiding in skeletal muscle hypertrophy in the post-natal animal (11,35,36). Insulin-like growth factors are found in association with a family of insulin-like growth factor binding proteins (37,38) which inhibit or potentiate the availability of IGF-I and IGF-II for biological actions (37). Insulin-like growth factor binding protein-3 is the most abundant binding protein because it binds approximately 90% of the IGFs in circulation (39).

We found IGF-I and IGFBP-3 mRNA expression increased in PEM as day of gestation increased from day 55 to 70 (65% and 50%, respectively). Also, we observed similar results in IGF-I and IGFBP-3 mRNA expression in skeletal muscle of fetuses collected at these two gestation lengths (20). Insulin-like growth factor-I and IGFBP-3 play a role in proliferation and terminal differentiation of PEM (37,39,40,41). As day of gestation increased from day 55 to day 70 of gestation, IGF-I mRNA expression increased 70% and IGFBP-3 mRNA expression increased 72% in skeletal muscle tissue. However, IGF expression in the hepatic tissue increased earlier in gestation compared to the increase observed in skeletal muscle (20). The expression of IGFBP-3 mRNA in hepatic tissue increased from day 40 to day 55 by 47%.

Researchers suggest that IGFBP-3 plays a role in proliferation and terminal differentiation of PEM (22,23,38,42). In our experiment, we found IGFBP-3 mRNA expression was reduced at 96-h post-plating suggesting the initiation of terminal differentiation of PEM began at 96-h post-plating. In addition, IGF-I and IGF-II mRNA levels were found to be increased at 120-h post-plating. We found that levels of IGFBP-3 mRNA were increased in the control gilts at 120-h post-plating (Figure 4.4). Other researchers have found at 120-h post-plating, IGFBP-3 mRNA expression was reduced in differentiating porcine embryonic cell cultures (30). The increase in IGF-I and IGF-II mRNA levels at 120-h and reduced IGFBP-3 mRNA levels at 96-h we observed confirm the roles of IGF-I, IGF-II and IGFBP-3 mRNA in proliferation and terminal differentiation of PEM. Therefore, we conclude that the reduced levels of IGFBP-3 mRNA we observed in the L-carnitine cultures compared to the controls are a result of PEM increased proliferative capacity, but delayed differentiation. This suggests more nuclei may be available for muscle fiber development.

Data is limited on the effects of L-carnitine on IGF system changes in skeletal muscle development during fetal growth. In the current study, we found mononucleated myoblasts isolated from hindlimb muscles of fetuses had changes in IGFBP-3 gene expression when their dams were fed diets with L-carnitine. Specifically, IGFBP-3 gene expression decreased 48% when L-carnitine was supplemented to the diets, but we did not observe differences in IGF-I, IGF-II, or IGFBP-5 gene expression in these PEM. Researchers have shown IGFBP-3 reduces the expression of IGFBP-5 production in L6 myogenic cells (40), but we observed no differences. In addition to changes observed in IGF-II expression in PEM collected from fetuses

from sows fed diets with supplemental L-carnitine, Waylan et al. (11) observed a decrease in IGFBP-3 mRNA expression.

Proliferation of primary myogenic cell cultures, similar to porcine embryonic myoblast cultures is also under the mitogenic stimulation of IGF-I (43,44). This is due to IGF-I being primarily produced and expressed by muscle cells (12). Florini et al. (45) have also demonstrated that IGF-I induced the expression of the myogenin gene, which suggests IGF-I is involved in myogenic cell differentiation, and not only proliferation. In addition to the role of IGF-I in muscle proliferation and differentiation, IGF-II has been shown to stimulate terminal differentiation (14,15) greater than that of IGF-I (46). In our study, we did not observe changes in gene expression of these two growth factors in PEM. Waylan et al. (11) demonstrated IGF-II mRNA expression was 61% lower in PEM collected from fetuses from sows fed supplemental L-carnitine which corresponded with changes in myogenin expression. Because IGF-II is expressed during terminal differentiation, these researchers concluded that there is a delay in the onset of terminal differentiation in PEM isolated from fetuses from sows fed diets with supplemental L-carnitine. Insulin-like growth factor binding protein-5 has shown the potential to affect proliferation of PEM (47).

In addition to IGF system changes observed in this study, we used flow cytometry analysis to determine if L-carnitine affected the percentage of PEM positive for the monoclonal antibody 5.1H11, a myogenic cell marker (33,48,49), and stage of the cell cycle in proliferating myoblasts. The PEM isolated from fetuses obtained from gilts fed diets containing supplemental L-carnitine had 4.7% greater adhesion of 5.1H11. This indicates the PEM isolated from fetuses from gilts fed diets with L-carnitine had a more enriched population of myogenic cells (48,49). This may suggest L-carnitine has additional roles in fetal development, although direct conclusions cannot be drawn.

Similar observations were observed for flow cytometric analysis of the percentage of cells in the S phase of the cell cycle. Porcine embryonic myoblasts collected from fetuses from gilts fed diets with supplemental L-carnitine had 8.8% more PEM in the S phase of the cell cycle. This was determined by a fluorescence-activated cell sorter. Mitosis, the process of nuclear division contains four distinct cycles of which in the S phase synthesis of DNA occurs (50). At any instant, it is typical that 30% of a population of growing cells that are proliferating rapidly are in the S phase (51). In our study, 37.5% of the population of PEM collected from the

fetuses from the gilts fed supplemental L-carnitine were in the S phase, and only 34.2% of the control PEM were in the S phase.

Supplementing L-carnitine to gestating gilts altered the IGF axis in PEM collected from fetuses. Specifically, we observed a decrease in IGFBP-3 mRNA expression in PEM collected from fetuses from gilts fed L-carnitine. Insulin-like growth factor binding protein-3 plays a role in terminal differentiation of PEM. We believe L-carnitine supplementation to the dam suppresses the expression of IGFBP-3 in PEM, therefore enhancing muscle proliferation and suppressing differentiation.

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**Table 4.1 Diet composition fed during gestation (as-fed)<sup>a</sup>**

Item	Amount
Ingredient, %	
Corn	81.22
Soybean meal, 46.5% CP	14.55
Monocalcium phosphate, 21% P	2.03
Limestone	1.05
Salt	0.50
Vitamin premix <sup>b</sup>	0.25
Trace mineral premix <sup>c</sup>	0.15
Sow add pack <sup>d</sup>	0.25
	100.00
Calculated analysis	
Lysine, %	0.65
ME, Mcal/kg	3.27
Protein, %	13.7
Calcium, %	0.85
Phosphorus, %	0.75
Available P, %	0.48

<sup>a</sup> Gestation feeding levels of 1.75 kg/d, with a top-dress providing either 0 or 50 ppm added L-carnitine.

<sup>b</sup> Supplied per kilogram of diet: 11,025 IU of vitamin A, 1,654 IU of vitamin D<sub>3</sub>, 55.1 mg of niacin, 44.1 IU of Vitamin E, 33.1 mg of pantothenic acid, 9.9 mg of riboflavin, 4.4 mg of vitamin K (menadione), and 0.04 mg of vitamin B<sub>12</sub>.

<sup>c</sup> Supplied per kilogram of diet: 165 mg of Zn (oxide), 165 mg of Fe (sulfate), 39.7 mg of Mn (oxide), 16.5 mg of Cu (sulfate), 0.30 mg of I (as Ca iodate), and 0.30 mg of Se (as Na selenite).

<sup>d</sup> Supplied per kilogram of diet: 551.3 mg of choline, 15.2 mg pyridoxine, 1.65 mg of folic acid and 0.22 mg of biotin.

**Table 4.2 Primers and probes used for real-time quantitative PCR**

Gene	Genbank accession number		Sequence
IGF-I	M31175	Forward	TCTTCTACTTGGCCCTGTGCTT
		Reverse	GCCCCACAGAGGGTCTCA
		Probe	6FAM-CCTTCACCAGCTCTGCCACGGC-TAMRA
IGF-II	X56094	Forward	CCGGACAACCTCCCCAGATA
		Reverse	CGTTGGGCGGACTGCTT
		Probe	6FAM-CCCGTGGGCAAGTTCTTCCGC-TAMRA
IGFBP-3	AF085482	Forward	AGCACGGACACCCAGAACTT
		Reverse	CGGCAAGGCCCGTATTC
		Probe	6FAM-TCCTCTGAGTCCAAGCGCGAGA-TAMRA
IGFBP-5	U41340	Forward	GGCAGAGGCCGTGAAGAAG
		Reverse	CAGTCCCCCCACGAACT
		Probe	6FAM-CCGCAGAAAGAAGCTGACCCAGTCC-TAMRA

**Table 4.3 Growth factor messenger RNA levels in porcine embryonic myoblasts at d 55 of gestation<sup>a</sup>**

Gene	Hour post-plating								SED
	72		96		120		144		
	L-carnitine, ppm <sup>b</sup>								
	0	50	0	50	0	50	0	50	
IGF-I	0.13	0.21	0.17	0.05	0.30	0.35	0.18	0.30	0.277-0.330
IGF-II	882.3	769.1	779.0	723.9	1098.0	1149.0	558.8	1158.6	353.76-371.78
IGFBP-3	40.8	13.9	1.8	0.3	5.0	1.9	14.0	16.2	15.92-16.38
IGFBP-5	147.3	69.5	74.3	27.9	47.4	69.8	45.6	57.2	87.88-93.50

<sup>a</sup> Values are least squares means and SED (standard error of difference). Gene expression levels are expressed in arbitrary units (millions).

<sup>b</sup> Gestation feeding levels of 1.75 kg/d, with a top-dress providing 0 or 50 ppm added L-carnitine.

**Table 4.4 Growth factor messenger RNA levels in porcine embryonic myoblasts at d 70 of gestation<sup>a</sup>**

Gene	Hour post-plating								SED
	72		96		120		144		
	L-carnitine, ppm <sup>b</sup>								
	0	50	0	50	0	50	0	50	
IGF-I	0.42	0.29	0.22	0.41	0.87	1.01	0.65	0.98	0.277-0.298
IGF-II	1387.0	1024.6	1124.1	889.6	616.9	293.0	1159.1	277.2	353.76-394.77
IGFBP-3	43.6	5.7	6.0	3.9	32.7	18.7	41.6	35.1	15.92-16.67
IGFBP-5	146.2	62.3	52.2	82.9	376.9	121.3	53.7	55.7	87.88-101.23

<sup>a</sup> Values are least squares means and SED (standard error of difference). Gene expression levels are expressed in arbitrary units (millions).

<sup>b</sup> Gestation feeding levels of 1.75 kg/d, with a top-dress providing 0 or 50 ppm added L-carnitine.

**Table 4.5 Statistical analysis of growth factor messenger RNA levels in porcine embryonic myoblasts at d 55 and 70 of gestation<sup>a</sup>**

	<i>P</i> - value						
	TRT	Day	Hour	TRT × Day	TRT × Hour	Day × Hour	TRT × Day × Hour
IGF-I	0.58	0.01	0.01	0.73	0.84	0.29	0.83
IGF-II	0.32	0.07	0.60	0.88	0.09	0.68	0.28
IGFBP-3	0.08	0.07	0.01	0.54	0.21	0.28	0.99
IGFBP-5	0.28	0.27	0.10	0.56	0.48	0.11	0.24

<sup>a</sup> Data was analyzed as a repeated measure. Model effects include treatment, day of gestation, and hour post-plating and hour post-plating as the repeated measure.

**Table 4.6 Flow cytometric analysis<sup>a</sup>**

Item	Day of Gestation				SED	<i>P</i> -value		
	55		70			Trt <sup>c</sup>	Day <sup>d</sup>	Trt × Day
	L-carnitine, ppm <sup>b</sup>							
	0	50	0	50				
5.1H11, %	84.1	89.4	90.8	94.2	2.92-3.63	0.09	0.20	0.77
Cell cycle stage, %								
G <sub>1</sub>	60.1	55.7	56.9	55.8	3.42-3.82	0.44	0.67	0.65
S	34.0	37.7	34.4	37.4	2.84-3.60	0.31	0.99	0.91
G <sub>2</sub>	6.01	7.29	8.72	7.38	1.74-1.95	0.99	0.45	0.48

<sup>a</sup> Values are least squares means and SED (standard error of difference).

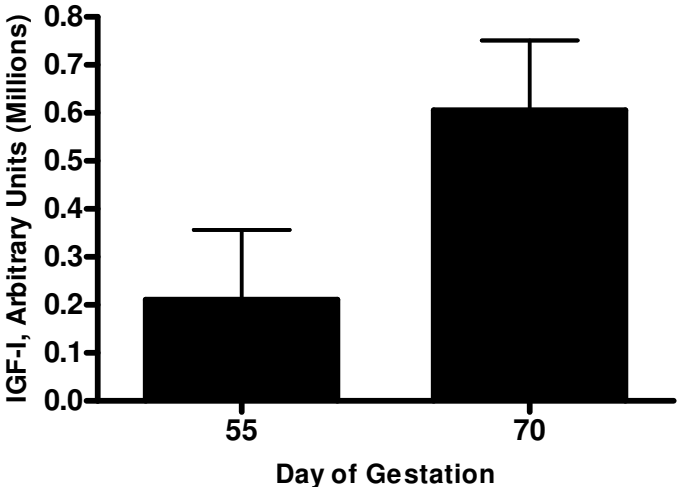
<sup>b</sup> Gestation feeding levels of 1.75 kg/d, with a top-dress providing 0 or 50 ppm added L-carnitine.

<sup>c</sup> Treatment (Trt).

<sup>d</sup> Day of Gestation (Day).



Figure 4.1 Insulin-like growth factor-I mRNA expression in PEM at two gestation lengths.



**Figure 4.2 Insulin-like growth factor binding protein-3 mRNA expression in PEM at two gestation lengths.**

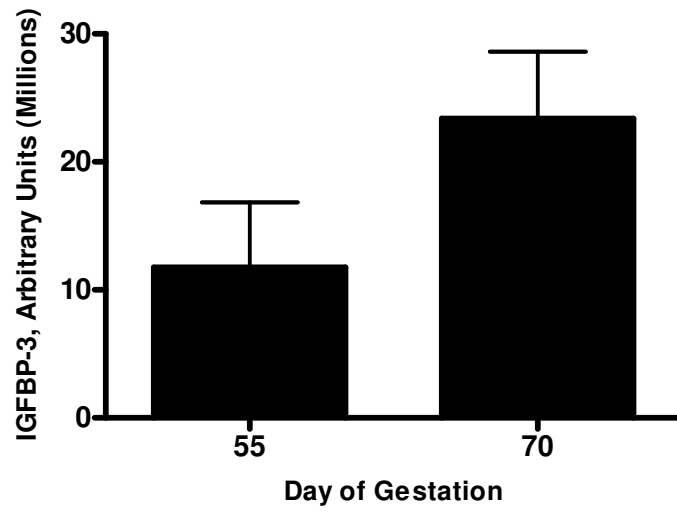
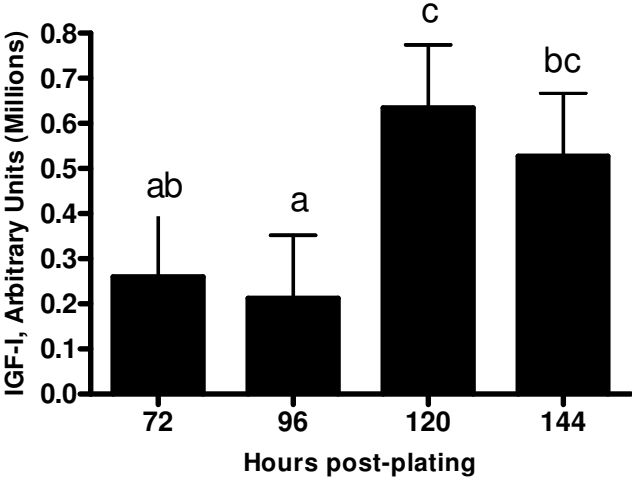
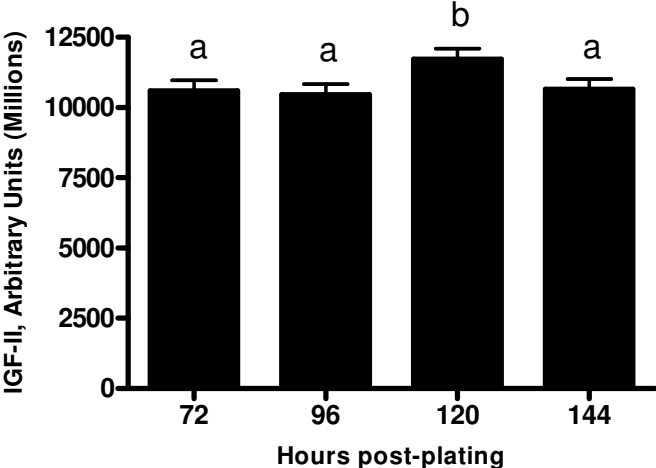


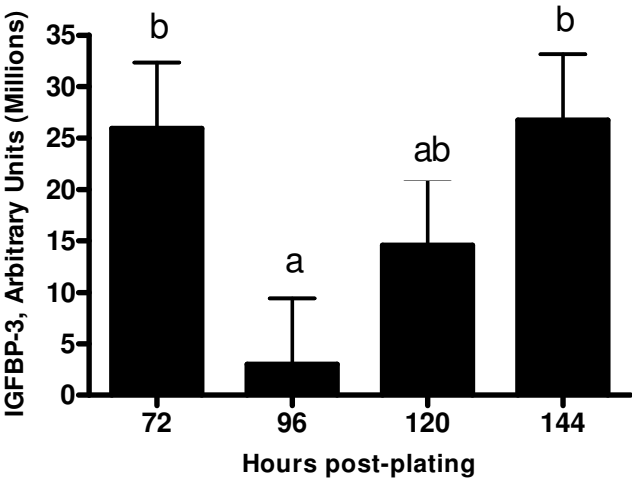
Figure 4.3 Insulin-like growth factor-I mRNA expression at form time points post-plating.



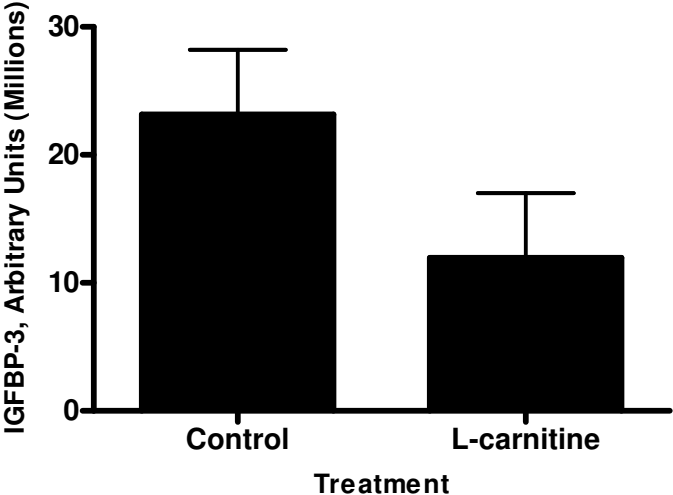
**Figure 4.4 Insulin-like growth factor-II mRNA expression at four time points post-plating.**



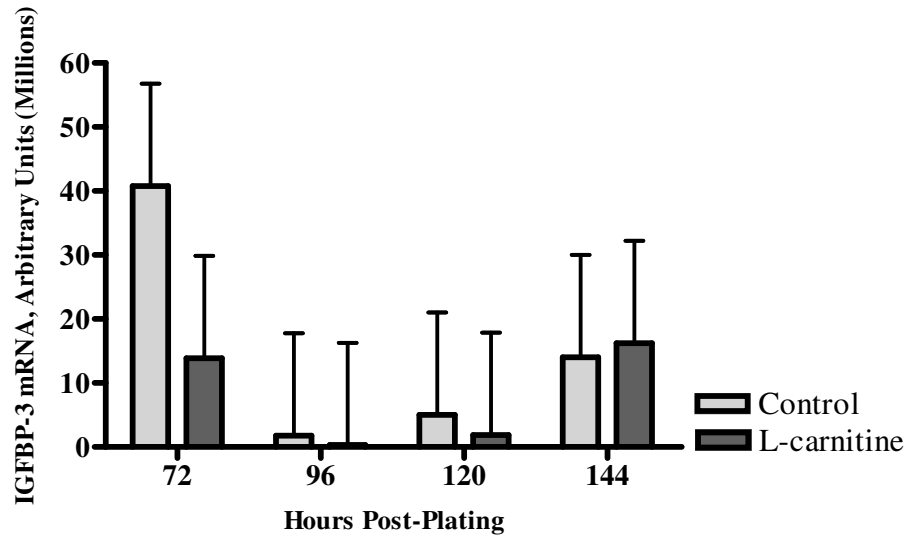
**Figure 4.5 Insulin-like growth factor binding protein-3 mRNA expression at four time points post-plating.**



**Figure 4.6 Insulin-like growth factor binding protein-3 mRNA expression in PEM collected from fetuses from gilts fed L-carnitine or the control diet.**



**Figure 4.7 Insulin-like growth factor binding protein-3 mRNA expression in PEM collected from fetuses at d 55 gestation from gilts fed L-carnitine or the control diet at four time points post-plating.**



**Figure 4.8 Insulin-like growth factor binding protein-3 mRNA expression in PEM collected from fetuses at d 70 gestation from gilts fed L-carnitine or the control diet at four time points post-plating.**

