**KARINE ASSIS COSTA**

# PRENATAL DEVELOPMENT AND MOLECULAR CHARACTERIZATION OF CONCEPTUSES FROM GILTS SUPPLEMENTED WITH L-ARGININE DURING EARLY GESTATION

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

Adviser: Simone Eliza Facioni Guimarães

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*Aos meus pais, José Geraldo e Marli,*

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*E a vó Toti por todo o amor.*

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*“O que vale na vida não é ponto de partida e sim a caminhada. Caminhando e semeando, no fim terás o que colher” (Cora Coralina)*

## ABSTRACT

COSTA, Karine Assis, D.Sc., Universidade Federal de Viçosa, February, 2020. **Prenatal development and molecular characterization of conceptuses from gilts supplemented with L-arginine during early gestation.** Adviser: Simone Eliza Facioni Guimarães.

Females L-arginine supplementation during gestation have been used to improve their reproductive performance through modifications of placental efficiency and conceptuses survival and development. However, the molecular and cellular mechanisms that underlie the effects of L-arginine supplementation during gestation on mother and conceptuses are not elucidated. Therefore, we aimed to evaluate the effects of gilts L-arginine supplementation during gestation on conceptuses survival and development through the phenotypic analyses of gilts and conceptuses, gene expression and protein abundance analyses of the 25 days-embryos and 35 days-fetuses from gilts not supplemented (CON) or supplemented with 1.0% L-arginine (ARG). We evaluated genes associated with developmental processes such as cell proliferation, migration, differentiation and apoptosis, conceptuses morphogenesis, growth and the epigenetics mechanisms involved in the regulation of gene expression. At the phenotypic level, we found differences between gestational ages (25 and 35 days) on gilts characteristics such as uterine weight ($P$<0.0001), left and right uterine horn length ($P$=0.001 and $P$=0.003), right and total ovaries weight ($P$=0.05 and $P$=0.04), with higher values at 35 days compared with 25 days, as expected, due to gestational advancement with the normal fetal development. We also found differences between gestational ages considering other gilts characteristics such as viable embryos number ($P$=0.05) that was higher at 25 days, which explains the higher mortality rate of fetuses at 35 days ($P$=0.01) and the lower coefficient of variation of conceptuses weight in this same gestational age ($P$=0.01). Considering diets, we found differences between CON and ARG gilts on concentrations of some metabolites, ARG gilts presented lower concentration of methionine and tyrosine ($P$=0.01 and $P$= 0.004) on the blood plasma compared to CON gilts, besides, there was a tendency to interaction between gestational age and diet for arginine blood plasma concentration in which at 25 days was a higher concentration of arginine on ARG gilts compared to CON gilts. However, at 35 days, there was a lower concentration of arginine on blood plasma from ARG gilts ($P$=0,06). Interestingly, we found a tendency to higher weight of ARG embryos and a higher liver weight of the embryos from ARG gilts at 25 days compared to CON embryos ($P$=0.07 and $P$=0.09, respectively). Besides that, we observed a higher expression of *IGF1* gene on ARG embryos ($P$=0.05). However, at 35 days, the ARG fetuses presented a smaller cephalic-caudal length compared to CON fetuses ($P$=0.05). ARG fetuses

also presented lower *MTOR* expression (*P*=0.05) as a result of higher *MLST8* gene expression (*P*=0.04) and lower concentration of arginine on ARG gilts blood plasma, since under poor-nutrient conditions mLST8 inhibits mTOR activity. Furthermore, ARG fetuses had also a lower abundance of phospho-mTOR and phospho-AMPK proteins (*P*=0.006 and *P*=0.007). ARG embryos had lower phospho-AMPK abundance related to CON embryos (*P*=0.04). Even though we found differences in expression of some genes between CON and ARG embryos and fetuses, we did not find differences between CON and ARG conceptuses in expression of epigenetic genes. We conclude that the duration of L-arginine supplementation is determinant for the biological effects on gilts and conceptuses.

Keywords: Fetal programming. Nutrigenomics. Gene expression. Protein abundance. Phenotype.

## RESUMO

COSTA, Karine Assis, D.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Caracterização molecular do desenvolvimento pré-natal de conceptos provenientes de marrãs suplementadas com L-arginina no início da gestação.** Orientadora: Simone Eliza Facioni Guimarães.

A suplementação de fêmeas com L-arginina durante a gestação tem sido utilizada para melhorar o desempenho reprodutivo através de modificações na eficiência placentária e na sobrevivência e desenvolvimento dos conceptos. No entanto, os efeitos moleculares e celulares da suplementação com L-arginina durante a gestação na mãe e nos conceptos não foram elucidados. Portanto, objetivamos avaliar os efeitos da suplementação de marrãs durante a gestação sobre a sobrevivência e o desenvolvimento de conceptos através de análises fenotípicas das marrãs, embriões e fetos, da análise de expressão gênica e abundância de proteínas nos embriões (25 dias) e fetos (35 dias) de marrãs não suplementadas (CON) ou suplementadas com 1,0% de L-arginina (ARG). Avaliamos genes associados aos processos de proliferação, migração, diferenciação e apoptose celular, além da morfogênese e crescimento dos conceptos, e mecanismos epigenéticos envolvidos na regulação da expressão gênica. A nível fenotípico, foram encontradas diferenças entre as idades gestacionais em características das marrãs como peso uterino ($P$<0,0001), comprimento do corno uterino esquerdo e direito ($P$=0,001 e $P$=0,003), peso dos ovários direito e total ($P$=0,05 e $P$=0,04), com valores mais altos aos 35 dias como esperado com o avanço gestacional e desenvolvimento normal do feto. Também encontramos diferenças entre as idades gestacionais considerando outras características das fêmeas como o número de embriões viáveis ($P$=0,05) maior aos 25 dias, o que explica a maior taxa de mortalidade observada aos 35 dias ($P$=0,01) e o menor coeficiente de variação do peso dos conceptos também aos 35 dias ($P$=0,01). Considerando as dietas, encontramos diferenças entre as marrãs CON e ARG nas concentrações de alguns metabólitos, as marrãs ARG apresentaram menor concentração de metionina e tirosina ($P$=0,01 e $P$=0,004) no plasma sanguíneo em comparação às CON, além disso, houve uma tendência à interação entre idade gestacional e dieta na concentração plasmática de arginina, na qual aos 25 dias houve uma maior concentração de arginina nas marrãs ARG em comparação às CON. No entanto, aos 35 dias, houve uma menor concentração de arginina no plasma sanguíneo das fêmeas ARG (P = 0,06). Curiosamente, encontramos uma tendência ao maior peso de embriões ARG e maior peso hepático em comparação aos embriões CON ($P$=0,07 e $P$=0,09). Além disso, observamos uma maior expressão do gene *IGF1* em embriões ARG ($P$=0,05). Entretanto, aos

35 dias, os fetos ARG apresentaram menor comprimento cefálico-caudal quando comparados aos fetos CON (*P*=0,05). Os fetos ARG também apresentaram menor expressão de *MTOR* (*P*=0,05) como resultado da maior expressão do gene *MLST8* (*P*=0,04) e menor concentração de arginina no plasma sanguíneo das marrãs ARG, uma vez que, em más condições nutricionais, o mLST8 inibe a atividade de mTOR. Além disso, os fetos ARG apresentaram menor abundância de proteínas fosfo-mTOR e fosfo-AMPK (*P*=0,006 e *P*=0,007). Os embriões ARG também apresentaram menor abundância de fosfo-AMPK (*P*=0,04). Embora tenhamos encontrado diferenças na expressão de genes entre embriões e fetos das fêmeas CON e ARG, não encontramos diferenças na expressão de genes epigenéticos. Concluímos que a duração da suplementação com L-arginina é determinante para os efeitos biológicos em marrãs e conceptos.

Palavras-chave: Abundância de proteínas. Expressão gênica. Fenótipo. Nutrigenômica. Programação fetal.

**SUMMARY**

## General Introduction

Arginine is a functional amino acid involved with the production of nitric oxide, ornithine and polyamines, creatine, agmatine, urea and other products catabolized by nitric oxide synthases, arginases, arginine: glycine amidinotransferase and arginine decarboxylase (MORRIS, 2004). Each of these products have important physiological functions (WU et al., 2004); the L-arginine metabolites are involved in many processes such as placental angiogenesis, uterine and placental synthesis of polyamines that are key regulators of gene expression, protein synthesis through activation of the mechanistic (mammalian) target of rapamycin cell signaling pathway (mTOR), embryonic implantation, regulation of steroid hormones synthesis as well as growth and development of embryos, fetuses and placenta and tissues differentiation and development (WU et al., 2004, 2010, 2013). In addition, arginine is a glycogenic amino acid used in the metabolism of energy production precursors like D-glucose and glycogen. This amino acid is conditionally essential for mammals (WU et al., 2009) since its synthesis varies according to the developmental stage and other factors such as the incidence of diseases and health status (BARBUL, 1986; MORRIS, 2004).

In the last years, L-arginine has been used to supplement mammals' females diets in different moments of gestation improving their reproductive performance. The supplementation influence on embryos viability (ZENG et al., 2008; BÉRARD; BEE, 2010; LI et al., 2014; NUNTAPAITOON et al., 2018), increasing the number of animals born (GAO et al., 2012; GREENE et al., 2012; GUO et al., 2016) and born alive (MATEO et al., 2007; GAO et al., 2012; GREENE et al., 2012; REN et al., 2012; CHE et al., 2013; GUO et al., 2016) through effects on embryo implantation, formation, vascularization and placental growth (GAO et al., 2012; GREENE et al., 2012), affecting also myogenesis (BÉRARD; BEE, 2010) and fetal growth (BÉRARD; BEE, 2010; NUNTAPAITOON et al., 2018; PALENCIA et al., 2018). Sows supplementation also reduced variation of piglets' weight at birth (QUESNEL et al., 2014; PALENCIA et al., 2018), which is important for litter uniformity. In addition, dietary L-arginine supplementation improve animals immune system (REN et al., 2012; CHE et al., 2013; WU et al., 2013).

L-arginine supplementation plays also a key role in females reproduction reducing two significant problems: embryonic losses and intrauterine growth retardation (IUGR) (WU et al., 2004). In pigs, sows supplementation reduced prenatal mortality rate, pigs suffer up to 50% of conceptuses losses during gestation (BAZER et al., 2009), and intrauterine growth restriction, most severe among livestock species (WU et al., 2006, 2010; BÉRARD; KREUZER; BEE,

2008; LI et al., 2014). However, there are no consensus about supplementation time, concentration and duration (PALENCIA et al., 2018). According to WU et al. (2010), the timing and dose of L-arginine supplementation are crucial to its positive effects in improving sows gestation. More arginine in the diet is not necessarily better for pigs conceptuses survival and in dietary formulations proper ratios of amino acids should be taken to prevent antagonism among amino acids and the toxicity of ammonia to mother and embryos/fetuses (WU et al., 2010, 2013).

In this context, the biological mechanisms in which the L-arginine supplementation influence on females and conceptuses need to be clarified. We hypothesized that gilts L-arginine supplementation would influence the expression of genes associated with cellular proliferation, migration, differentiation and apoptosis in embryos and fetuses through mechanisms such as epigenetics alterations. In addition, we hypothesized that supplementation would influence the conceptuses growth/development and hence phenotypic characteristics. Therefore, our aim was to evaluate phenotypic alterations in supplemented gilts and in their conceptuses, as well as to evaluate the expression of genes and proteins involved with conceptuses survival, growth and development and epigenetic mechanisms.

Studies like this are important to improve female's reproductive performance reducing reproductive problems such as embryonic losses and IUGR. In this case, pigs were used as a model to defining the mechanisms in which L-arginine supplementation during gestation may influence on maternal reproduction and on conceptuses development since the specie presents some metabolic and physiological similarities with other mammals, including humans.

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**CHAPTER 1**

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Review Article

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# Nutrition influence on sow reproductive performance and conceptuses development and survival: a review about L-arginine supplementation

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**Abstract**

During gestation, nutrition is the main environmental factor that influences the intrauterine environment and, consequently, development of embryos and fetuses. In this context, L-arginine supplementation of pregnant sows has been shown to be efficient to improve females' reproductive performance and conceptuses development. Among the products of arginine metabolism, nitric oxide and polyamines play important roles in placenta and pig conceptuses growth and development, through vascularization and blood flow regulation, cell proliferation, migration and differentiation, tissue formation, among other processes. In this review, we aimed to approach current knowledge about the nutrition influence on sow gestation, emphasizing how L-arginine supplementation may affect reproductive performance and conceptuses development and survival.

**Keywords:** Fetal programming, nutrigenomics, nitric oxide, pigs, polyamines, reproductive performance

## Introduction

Pig production profitability is closely related to the animals' efficiency. In this context, sows and boars reproductive performance are determinant to increase productive indexes. Litter size and uniformity are major traits when considering sows' reproductive efficiency, since

larger litters at birth are related to higher number of piglets weaned per female per year (Geisert and Schmitt, 2002; Silva et al., 2016) and uniform litters are associated with lower pre-weaning mortality rates (Milligan et al., 2002; Quesnel et al., 2012) and better post-weaning performance (Wientjes, 2013). However, these are low heritable and complex traits, influenced by many genetic and environmental factors (Quesnel et al., 2014; Wu et al., 2013).

Prenatal mortality rate is high in swine and is therefore a key limiting factor for litter size at birth (Geisert and Schmitt, 2002; Silva et al., 2016). Intrauterine growth restriction (IUGR), one of the most common reproductive problems in pigs and other mammals, occurs due to changes in the intrauterine environment structure and features, influencing prenatal mortality rates, conceptuses growth and reducing animals' production indexes in the postnatal period (Wu et al., 2013). Intrauterine environment, that is related with IUGR and prenatal losses, is mainly determined by amino acids (AA), vitamins, minerals and other nutrients in the sows diets (Barker and Clark, 1997). The products of these nutrients metabolism are related to the synthesis of hormones, AA, proteins, among other biological molecules that are important for placental growth and embryonic and fetal growth and survival (Kim et al., 2007).

Nutritional deficiencies in maternal diet lead to increased rates of IUGR and embryonic losses. These deficiencies are mainly related to the nitric oxide (NO) and polyamines synthesis impairment (Radicchi and Lobato, 2009; Wu et al., 2004). In this context, L-arginine supplementation of sows has been shown to be an efficient way to improve reproductive performance of the sow, since arginine is a versatile AA used in the synthesis of diverse products such as NO and polyamines (Wu and Morris, 1998). Among other processes, arginine metabolism products are involved in cell proliferation, migration and differentiation (Ishida et al., 2002; Meininger and Wu, 2002), vascularization and regulation of placental blood flow (Bird et al., 2003), hormone secretion (Alba-Roth et al., 1988; Chew et al., 1984; Davenport et al., 1995; Flynn et al., 2002), tissue formation (Wu et al., 2009) and embryo implantation (Zeng et al., 2013).

Therefore, further research is warranted to elucidate the cellular, genetic and epigenetic mechanisms by which nutrients, such as arginine, influence placental growth and conceptuses development. Besides that, more studies are essential to develop effective strategies of dietary supplementation with some nutrients to benefit animal reproduction and production. In this review, we aimed to approach the nutrition influence on sow gestation, emphasizing the effect of L-arginine supplementation on sows' reproductive efficiency and conceptuses development and survival.

**Nutrition and reproductive performance of the sows**

Genetic merit, environment, nutrition and management determine animal reproductive performance in the production system (Smith and Akinbamijo, 2000). Among these factors, nutrition plays a key role, since it has direct effects on animal reproduction as well as potential to influence the other factors (Smith and Akinbamijo, 2000). Therefore, adequate amounts of energy, protein, amino acids, vitamins, and minerals is an opportunity to further optimize reproductive sows efficiency during gestation (Goodband et al., 2013).

During pregnancy, protein is required for maintenance, mammary glands growth and placental and conceptuses development (Kim et al., 2009). In addition, dietary protein deficiency reduces the availability of most AA in females' blood plasma (Wu et al., 1999). Functional AA own several and crucial functions in metabolism, physiology and immunity (Wu et al., 2013, 2010) and are therefore considered for the prevention and treatment of metabolic diseases, lactation failures, infertility, intestinal and neurological dysfunctions, and infectious diseases (Wu, 2013). Requirements of functional AA in the diet is dependent on the specie, developmental physiological and pathological state, and environmental factors (Dai et al., 2013, 2012; Wu, 2013).

AA are critical for pregnant females since they play the most important role in placental growth, being required for activation of protein synthesis machinery in the cells (Li et al., 2009; Palli et al., 2009; Rhoads and Wu, 2009). It is well known for many years that commercial sows require adequate supply of dietary AA for maximum reproductive efficiency (Hoet and Hanson, 1999; Snoeck et al., 1990), since the genetic improvement for reproductive traits has achieved important progress on litter size and other traits.

Arginine is a functional and conditionally essential AA for mammals (Wu et al., 2009), once the animals' ability to synthesize it in sufficient amounts to meet their needs varies according to the age and incidence of diseases (Barbul, 1986). This amino acid is considered a glycogenic AA, after all it can be used in the metabolism of energy production precursors (D-glucose and glycogen). The supply of arginine during gestation has effects on the secretion of pancreatic (insulin and glucagon), anterior pituitary (growth hormone and prolactin) and placental lactogen hormones in animals and humans (Alba-Roth et al., 1988; Chew et al., 1984), which affect maternal and fetal metabolisms. Arginine also influences the expression of angiogenic factors, such as the vascular endothelial growth factor (VEGF) and its receptor (VEGFR2) (Greene et al., 2012). In addition, it affects the expression of microRNAs which

regulate the angiogenesis (Liu et al., 2012), potentiating placenta formation and vascularization during gestation.

Arginine is metabolically versatile and is used in urea, NO, ornithine, citrulline, creatine, agmatine, glutamate, proline and polyamines synthesis in mammalian cells (Wu and Morris, 1998). Nitric oxide is a simple gaseous molecule, a product of the arginine oxidation reaction (Flora Filho and Zilberstein, 2000), catalyzed by nitric oxide synthase. In some mammals, it is a key intercellular messenger in the most important systems of the organism (Flora Filho and Zilberstein, 2000). It also acts on the regulation of uteroplacental blood flow and, consequently, on the transfer of nutrients and oxygen from the mother to the fetus (Bird et al., 2003).

Nitric oxide and polyamines (putrescine, spermidine and spermine), in association with insulin-like growth factor (IGF), vascular endothelial growth factor and other growth factors, play important roles in placental and conceptuses growth during gestation (Wu et al., 2010), being crucial for angiogenesis, embryogenesis, and fetal development (Reynolds and Redmer, 2001; Satterfield and Dunlap, 2013; Wu et al., 2006; Wu and Meininger, 2009). In addition, both NO (Biswas et al., 1998; Chwalisz and Garfield, 2000; Manser et al., 2004; Ota et al., 1999) and polyamines (Fozard et al., 1980) are involved in embryo implantation through mechanisms that have not yet been completely elucidated, but are probably related to the mTOR signaling pathway (Martin, 2003; Zeng et al., 2013). Polyamines also regulate DNA and protein synthesis in mammals' placenta, uterus and fetuses through mTOR pathway, and are, therefore, necessary for cell proliferation, migration and differentiation (Flynn et al., 2002; Ishida et al., 2002; Kong et al., 2012) (See figure 1).

**Maternal nutrition and conceptuses survival and development**

In pigs, embryonic and fetal mortality rates are high during gestation (over 50%) (Geisert and Schmitt, 2002; Wu et al., 2013). The majority of these losses occurs in the first third of pregnancy, until 30 days (> 75%), with mortality peak between 12 and 15 days of gestation (pre-implantation period) (Ford et al., 2002; Pope et al., 1990). Prenatal losses are the main constraints for economically important traits, as litter size, which has low heritability (Rothschild, 1996). Many factors contribute to embryonic and fetal losses, such as ovulation and fertilization rates, diseases, chromosomal abnormalities, developmental asynchrony, uterine capacity and placental efficiency (Wu et al., 2006).

Maternal nutrition affects embryos and fetuses survival, growth, and development mainly through intrauterine environment modulation and placental formation during gestation (Barker and Clark, 1997; Wu et al., 2006, 2004). Prenatal growth of placental mammals is directly

controlled by maternal nutrition via supply of glucose, AA and other essential nutrients for the conceptuses (Robinson et al., 1999). This growth is regulated by genetic, epigenetic and environmental factors (Radicchi and Lobato, 2009); epigenetics modifications influence animals' efficiency through fetal programming, not only during prenatal but also in postnatal life (Wu et al., 2004).

Feed disorders, as maternal malnutrition of either protein or energy, impair embryonic/fetal growth (Radicchi and Lobato, 2009), with prenatal growth being more vulnerable to protein deficiencies during embryonic pre-implantation and placental development (Sugden and Holness, 2002; Waterland and Jirtle, 2004; Wu et al., 1998). These protein deficiencies decrease NO and polyamines synthesis, products of arginine metabolism.

The impairment of NO and polyamines synthesis could explain the occurrence of IUGR and high prenatal mortality rates in response to maternal malnutrition (Radicchi and Lobato, 2009; Vosatka et al., 1998; Wu et al., 2004). Both undernutrition (Barker and Clark, 1997; Bell and Ehrhardt, 2002) and overnutrition (Wallace et al., 2002) during gestation may result in IUGR; that is also responsible for determining many animal features in the postnatal period, compromising animal survival and creating permanent problems that negatively influence production parameters, such as feed conversion, body composition, meat quality and reproductive performance, showing important implications on profitability in any animal production system (Radicchi and Lobato, 2009). Another product of arginine metabolism, creatine, is essential for tissues development, such as skeletal muscle and nerve tissue in conceptuses (Wu et al., 2009). In creatine production process and in production of polyamines from ornithine, there is a high sequestration of methyl groups, which can lead to epigenetic changes of the conceptuses chromatin.

In this way as discussed in this topic, not only the sows reproductive efficiency, but also the conceptuses efficiency is closely related to nutrients (macro and micronutrients) offered to females during gestation. Therefore, we corroborate the need of adequates nutritional programs which are essential to maximize the exploitation of sow genetic potential and optimize conceptuses survival.

**Nutrigenomics and fetal programming**

The scientific field that uses genomics approach in nutritional sciences was entitled Nutrigenomics (Asmare and Negewo, 2019). Nutrigenomics aims to elucidate the interaction between nutrients, epigenome and gene expression, and how this interaction influences phenotypes (Kaput et al., 2005). Nutrition is the main factor in intrauterine environment that

alters fetal genome expression (Barker and Clark, 1997). Furthermore, dietary levels of nutrients, as minerals, AA, vitamins, among others, offered to pregnant females are determinant in the epigenetic regulation of conceptuses gene expression (Jaenisch and Bird, 2003; Skinner et al., 2010).

This epigenetic regulation of offspring gene expression may occur through DNA methylation and post-translational histone changes, influencing fetal programming and genomic imprinting (Wu et al., 2004). Fetal programming is the body's response to a specific challenge during a critical period of prenatal development that alters the individuals' developmental trajectory, resulting in permanent effects in the animal (Nathanielsz et al., 2007). The influence is due to the availability of nutrients, directly and indirectly affecting gene expression regulation and transcription, besides regulating intermediate metabolites of signaling pathways, with positive or negative effects in the placenta, uterus, embryo and fetal tissues (Asmelash et al., 2018; Wu et al., 2004).

Macronutrients, as protein and energy, are required for fetal development, influencing epigenetic patterns of cells (Du et al., 2013; Funston et al., 2010; Reynolds and Caton, 2012). Micronutrients are also important in this process of prenatal development, since their metabolism is involved in the availability of chemical groups (methyl, acetyl and others), consequently influencing the pattern of DNA and histone modifications and regulation of gene expression (Waterland and Jirtle, 2004).

During early development, embryo pluripotent cells have an epigenetic pattern of gene expression regulation in which genes associated with cell differentiation are silenced. Afterwards, embryonic cells change their epigenetic arrangement and genes associated with pluripotency and self-renewal of pluripotent cells are irreversibly silenced mainly through DNA methylation (Reik, 2007). At this time, genes associated with cell differentiation and, consequently, the formation of different tissues and organs (morphogenesis) become highly expressed. In this way, the intrauterine environment modulates this epigenetic process and changes the epigenetic signature in the offspring cells that later differentiate into other cell types, which is a key mechanism that links maternal nutrition in the gestational period to the performance of the offspring in the long term (Du et al., 2015).

Dietary nutrients also influence gene expression of transcription factors (Afman and Müller, 2006). In metabolically active organs, these transcription factors act as nutrient sensors, modifying the level of specific genes transcription in response to changes in nutrients (Afman and Müller, 2006). Nutrients also activate nuclear receptors, which have functions in the

regulation of process such as nutrient metabolism, embryonic development, cell proliferation and differentiation, being able to influence a extensive variety of cellular functions (Afman and Müller, 2006).

Tools used for nutrigenomic studies are the so-called omics (transcriptomic, proteomic, metabolomic and more recently, epigenomic) (Asmelash et al., 2018; Mariman, 2006; Zduńczyk and Pareek, 2009). Several dietary nutrients effects on specific tissues and organs include differential gene expression (transcriptomic), chromatin organization (epigenomic), protein expression (proteomic) and metabolite profile (metabolomic) (Afman and Müller, 2006).

Transcriptomic experiments using microarray technology with cDNA or RNA sequencing (RNA-Seq) measure the expression levels of some genes or even of all transcripts and their isoforms in response to the use of nutrients such as lipids, carbohydrates, proteins, vitamins, AA and minerals (Mariman, 2006; Wang et al., 2009; Zduńczyk and Pareek, 2009). RNA-Seq is the sequencing method that allows the entire transcriptome to be searched in a much more productive and quantitative way. As RNA-Seq is quantitative, it can be used to determine RNA expression levels in a more accurate way than microarrays (Wang et al., 2009).

The increase or decrease of mRNA in response to a change in available nutrients does not necessarily mean a change in the production of specific proteins (Mariman, 2006). In this way, there are also techniques that allow gene expression measurement at the protein level. Proteomic analyses especially use two-dimensional electrophoresis and mass spectrometry to allow the understanding of nutrients influence on protein expression in certain organs, tissues and cells (Banks et al., 2000). Metabolomic studies, through the analysis of metabolites, allow observation of dietary components influence on the metabolome of specific organs, tissues or cells in animal nutrition studies, representing the final step in understanding the function of genes and their proteins (Zduńczyk and Pareek, 2009). Different technologies are applied to analyze the profile of different metabolite classes, such as lipids, carbohydrates and AA (Mariman, 2006). The research fields of proteomic and metabolomic have made possible the understanding of the nutrient-gene interaction at the cell and individual level (Asmare and Negewo, 2019).

Diet may also silence or activate certain genes through epigenetic changes (Banerjee et al., 2015). The epigenome is the set of all epigenetic information, which can be temporarily variable according to environmental conditions (including nutrition) and is highly variable between cells. One of the most used technologies to identify the methylated regions in the

genome is the next-generation sequencing methylation analysis (Bayón et al., 2016; Suzuki and Bird, 2008). Therefore, all omics tools are useful to identify the effects of maternal dietary nutrients on gene expression pattern in embryos and fetuses, thus allowing the adaptation of diets according to the main needs of mother and offspring. Nutrigenomics application helps to properly manage dietary nutrients and understand how these components and gene expression interact and affect animals production and reproduction (Asmare and Negewo, 2019).

To our knowledge, in pigs, nutrigenomics studies still scarce despite the development of tissues such as muscle, fat and bone being regulated by nutrition through epigenetics modifications, gene transcription changes and protein synthesis (Murray et al., 2016). We can state that elucidating the effects of nutrients from the diets on genetics and epigenetics regulation, mainly during gestation, are crucial for swine production (Murray et al., 2016) being a promising field of research.

**L-arginine supplementation of sows during gestation: stage, concentration and consequences**

Dietary L-arginine supplementation during pregnancy is an alternative to increase reproductive efficiency. The ideal time to supplement is still uncertain, without consensus (Palencia et al., 2018); many studies indicate that L-arginine supplementation in the first third of gestation presents better results for sow reproductive efficiency (Palencia et al., 2018; Wu et al., 2013). Supplementation at first pregnancy stage would be determinant for embryos viability and survival, since it would benefit embryo implantation, formation, vascularization, and placental growth, thus increasing prenatal survival rates. In addition, Bérard and Bee (2010) pointed out the influence of 26 g L-arginine HCl daily supplementation between 14 and 28 days of gestation on fetal myogenesis, positively affecting the primary phase of myofiber formation, besides increasing the number of viable fetuses and the total weight of fetuses at 75 days of gestation.

On the other hand, some studies have shown that L-arginine supplementation would be efficient in later pregnancy, being determinant on fetal growth (Palencia et al., 2018), since 50% of fetal weight gain is obtained during the last 20 days of gestation (Bass et al., 2017; Che et al., 2013; Mcpherson et al., 2004). Nuntapaitoon et al. (2018) reported that dietary L-arginine HCl supplementation in late gestating sow (0.5 % L-arginine HCl supplementation) reduced stillborn (-8.3%) and increased the number of piglets born alive (+9.8%), the piglets birth weight (+6.4%) and the piglets blood oxygen saturation (+3%) at birth compared with a isonitrogenous group (1.7% L-alanine supplementation), in this study, no difference between

0.5 and 1.0 % L-arginine concentration was observed among these traits. Besides, 1.0% of L-arginine HCl supplementation enhancing immunoglobulin G (IgG) concentration in the colostrum of ARG sows. Compared to the control group, supplementation with 0.5% increased the piglets birth weight (+7.0%) and piglets blood oxygen saturation (+3%) (Nuntapaitoon et al., 2018).

In addition to possible effects of late supplementation on fetal weight gain, it would decrease the variation of piglets' weight at birth (Palencia et al., 2018; Quesnel et al., 2014), which is extremely important for the litter uniformity, another economically important trait that influences piglet postnatal mortality rate. Piglets with low birth weight have lower survival rates, since they are physiologically compromised regarding energy reserve, thermoregulation, colostrum absorption, and have disadvantage in competition with heavier piglets for sow's teats (Milligan et al., 2002; Quesnel et al., 2012; Wientjes, 2013). Lower litter uniformity and low piglets weight at birth also influence the performance of these animals in the post-weaning period, resulting in lower and more variable weaning weight and reduced post-weaning growth, which compromise meat and carcass quality (Beaulieu et al., 2010; Bérard et al., 2008; Gondret et al., 2005; Quiniou et al., 2002; Wientjes, 2013). According to Oksbjerg et al. (2019) sows dietary inclusion of 25 g/day of L-Arginine from day 30 of gestation to day 28 of lactation cause changes in offspring muscle growth, in the weaned weight and increase daily gain and growth of the offspring.

Although no studies evaluate different levels of arginine for primiparous and multiparous sows, parity order influences L-arginine effects during pregnancy, since primiparous have higher nutritional requirements than multiparous sows (Palencia et al., 2018). Nevertheless, positive effects of L-arginine supplementation have already been found in studies with primiparous and multiparous sows (Palencia et al., 2018).

Regarding the optimum dietary L-arginine concentration, the ideal AA levels required by swine females also dynamically change depending on pregnancy stage (Wu et al., 2009). Studies have pointed to the increase in number of piglets born (1.31 piglets) (Gao et al., 2012) and born alive (1.1 and 2.03 piglets, respectively) (Gao et al., 2012; Mateo et al., 2007) in the litter when sows diets have been supplemented with 1.0 % L-arginine HCl between 22 and 114 days (Gao et al., 2012) and between 30 and 114 days of gestation (Mateo et al., 2007). In other study, 0.1% L-arginine supplementation between 30 and 110 days also increased the number of total and born alive piglets (1.1 piglets per litter for both traits) (Guo et al., 2016). Such improvements in the sows reproductive performance would be related to the improvement of

intrauterine environment and to the increase of total litter placental weight in females (+16.2%; 1.0% L-arginine HCl supplementation)(Gao et al., 2012), which enhance prenatal survival (Bérard and Bee, 2010; Li et al., 2014). Li et al. (2014) reported higher embryo survival with an increase of two viable embryos per litter from females supplemented with 0.4 and 0.8% L-arginine between 14 and 25 days of gestation.

In addition to all benefits reported with dietary L-arginine supplementation, this AA also potentiates the immune system through antioxidative defense (Wu et al., 2013), increased levels of serum immunoglobulin (IgG and IgM) and specific antibodies for some pathogens (Che et al., 2013). Furthermore, according to Bronte and Zanovello (2005), large amounts of NO synthesized from arginine would be cytotoxic to pathogens. This could be a way to prevent infectious diseases associated with embryonic losses by improving the immune system of pregnant females (Li et al., 2007). Che et al. (2013) reported that 1.0 % L-arginine supplementation of the sows diet from 30 to 114 days of gestation led to an increase in the number of piglets born alive (1.6 piglets) and to an improve in the females' immune response, with the increase of serum levels of immunoglobulins and specific antibodies for a particular pathogen. In a study performed by Ren et al. (2012), supplementation with L-arginine (0.6%) reduced reproductive failures as abortion rate in mice females infected with porcine circovirus type 2 (PCV2), besides increasing the number of animals born alive and their daily weight gain and reducing newborns mortality in the postnatal period.

Studies with L-arginine supplementation of females during gestation in other species, as mice, also showed the improvement of females reproductive performance by increasing conceptuses prenatal survival (1.3% L-arginine HCl supplementation)(Zeng et al., 2008), the number of implantation sites in placenta (Greene et al., 2012; Zeng et al., 2013, 2008), and the number of total and born alive animals (+ 30% for both traits) (Greene et al., 2012). In addition, studies with cell cultures pointed to the effects of L-arginine supplementation, increasing cell proliferation and protein synthesis, besides decreasing protein degradation through mechanisms of the mTOR signaling pathway (Kong et al., 2012).

Although the majority of the L-arginine supplementation studies lead to positive results regarding the performance of females, there are others that indicate negative effects or that do not indicate differences between supplemented females and control (Palencia et al., 2018). According to Li et al. (2010), supplementation with 0.8% L-arginine of gilts between days 0 and 25 of gestation decreased uterine weight (-20%), total number of fetuses (-24%), total fetal weight (-34%), total volume of allantoic and amniotic fluids (-34 to 42%), concentrations

of progesterone in maternal plasma (-33%) and other factors decrease the supplemented females' performance.

Supplementation of gilts with L-arginine between day 25 and 80 of gestation also decreased the number of born alive piglets and increased the percentage of stillborn piglets (Dallanora et al., 2017). In addition, it did not affect piglet birth weight or the within-litter coefficient of variation of birth weight in hyper-prolific gilts. However, less prolific gilts fed with the Arginine treatment had an increase in piglets birth weight average and a reduction in the percentage of low-weight piglets born (Dallanora et al., 2017).

The findings here described related to L-arginine supplementation can be used to identify the optimum L-arginine concentration, supplementation period, and the gestational phase in which supplementation should be applied; these are critical factors for the effects of L-arginine on animals' performance (Table 1)(Bass et al., 2017; Wu et al., 2010). Besides, factors such as number of sows parities, animals genetics potential and purpose of each production system have to be considered to choose the best nutritional program.

Our research group has been working with sows L-arginine supplementation in order to observe its effects on placental and angiogenesis development, it also aimed to search for effects on conceptuses transcriptome, expression of developmental genes and proteins on embryos and fetuses, besides the effects on mTOR pathway, one of the main pathways responsible for the processes of cell proliferation, migration and differentiation. In addition, we intend to deepen our knowledge of the epigenetic mechanisms that could be altered by supplementation, thus influencing the regulation of genes at a transcriptional level. Our studies also seek to discuss the issue of supplementation time, showing different and interesting effects on this point.

**Final considerations**

Nutrition is a key factor that determines the reproductive performance of sows. Nutritional deficiencies may lead to increased prenatal mortality rates and intrauterine growth restriction, mainly due to the reduction of nitric oxide and polyamines synthesis. Studies with L-arginine supplementation are controversial, some authors indicate improvements in the reproductive performance of sows through the reduction of prenatal mortality rates and, consequently, increase in the number of piglets born and total litter weight. Other authors have shown that no effects or even negative effects are registered by the inclusion of different levels of L-arginine in the diet of pregnant sows. Further studies have to be performed to help understanding the mechanisms that modulate these supplementation effects, either positive or

negative. In addition, the concentration, period and duration of supplementation should be determined according to the purpose of the pig production system, since they influence the results obtained for sows and progeny.

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**Competing Interests**

The authors have nothing to disclose.

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**Figure 1:** Influence of dietary supplementation on sows reproductive performance and some metabolites effects of L-arginine supplementation of females during gestation.

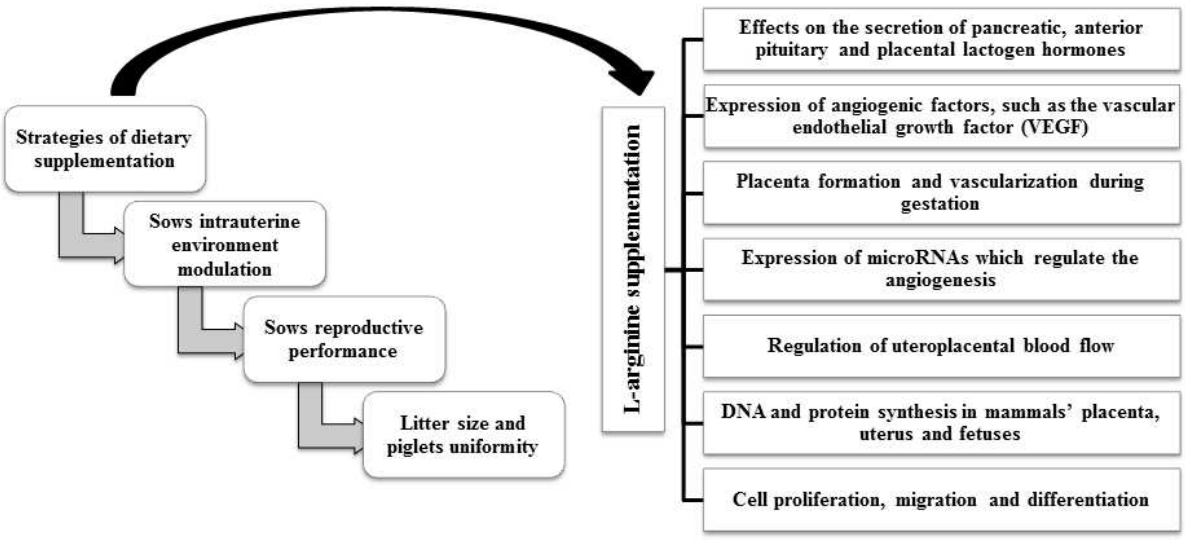

**Table 1:** L-arginine sows supplementation references, period of supplementation and main phenotypic results.

| REFERENCES | SUPPLEMENTATION PERIOD | MAIN RESULTS |
|---|---|---|
| **Mateo et al. 2007** | 30 days of gestation until farrowing | Increased the number of pigs born alive and live litter birth weight of piglets. |
| **Bérard e Bee, 2010** | 14-28 days of gestation | Positively effects on primary phase of myofiber formation, increased the number of viable fetuses and the total weight of fetuses at 75 days of gestation. |
| **Li et al. 2010** | 0-25 days of gestation | Decreased uterine weight, total number of fetuses, Corpus luteum number, total fetal weight, and others features. |
| **Gao et al. 2012** | 22 days of gestation until farrowing | Increased the total number of piglets per litter, the number of live-born piglets, the litter birth weight for live-born piglets and enhanced placental weight. |
| **Che et al. 2013** | 30-90 and 30-114 days of gestation | More pigs born alive, increased total and live litter weights. |
| **Li et al. 2014** | 14-25 days of gestation | Enhanced embryonic/fetal survival |
| **Quesnel et al. 2014** | 77 days of gestation until farrowing | Reduced within-litter variation of piglet birth weight. |
| **Guo et al. 2016** | 30-110 days of gestation | Increased the total number of piglets born and the number of live-born piglets. |
| **Bass et al. 2017** | 93 days of gestation until farrowing | No effect on number of pigs born alive, piglet birth weight, or lactation performance. |
| **Dallanora et al. 2017** | 25-80 days of gestation | Decreased born alive piglets number and increased percentage of stillborn. To less prolific gilts, increased piglets birth weight average and reduced the percentage of low-weight piglets born. |
| **Nuntapaitoon et al. 2018** | 85 days of gestation until farrowing | Increased the proportion of live-born piglets per litter, reduced stillborn and increased piglet birth weight. |
| **Oksbjerg et al. 2019** | 30 days of gestation until 28 days of lactation | Increased birth weight and daily gain and influenced on muscle area. |

**CHAPTER 2**

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# Dietary L-arginine supplementation during early gestation of gilts affects conceptuses development

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**Running title:** Effects of gilts L-arginine supplementation on conceptuses development

## ABSTRACT

L-arginine supplementation of sows has led to improvement of reproductive performance, but the mechanisms responsible for the positive effects of arginine during gestation on conceptuses survival and development are still poorly understood. Thus, we aimed to evaluate effects of 1.0% L-arginine supplementation (ARG) on phenotypic traits of commercial gilts, embryos and fetuses, concentration of gilts' blood metabolites, expression of developmental and cellular apoptosis genes in conceptuses of 25 and 35 days. At 25 days, *IGF1* gene was more expressed in embryos from ARG than in embryos from control gilts (CON) ($P$=0.05). At this same gestational age, ARG embryos tended to be heavier compared to CON ($P$=0.07) and ARG gilts showed a trend to have a greater arginine concentration in blood plasma ($P$=0.06). However, at 35 days of gestation, arginine concentration in blood plasma of ARG gilts tended to be lower compared to CON ($P$=0.06) and ARG fetuses showed smaller cephalic-caudal length ($P$=0.05).

These results indicate that duration of supplementation is determinant for arginine effects, not only on the females performance but also on the conceptuses, since supplementation upregulated *IGF1* expression at 25 days, in addition to the reduction of cephalic-caudal length of 35-day fetuses.

**Keywords**: apoptosis genes; developmental genes; mortality rate; nutrigenomics; pre-natal growth.

## 1. INTRODUCTION

Prenatal mortality is a major limitation for litter size, which is one of the most economically important traits in pigs. In this specie, the prenatal mortality rate is high, and most of these losses occur in the first third of gestation [1]. The events of this gestational period are associated with changes in the expression of genes related to nutrient transport, cell remodeling, angiogenesis, and relaxation of vascular tissues, as well as cell proliferation and migration [2]. Maternal nutrition is the main factor affecting survival, growth, and development of embryos and fetuses [3]. In this way, the regulation of gene expression by epigenetic mechanisms may be affect by dietary levels of nutrients as amino acids, reducing the incidence of diseases or defects in the conceptuses during development [4,5].

Arginine is a conditionally essential amino acid for mammals [6]. In addition to its main function in protein deposition, it participates in several important physiological events: i) synthesis and secretion of hormones, such as insulin, glucagon, prolactin, and growth hormone [7–9]; ii) synthesis and oxidation of cellular energy of other amino acids and urea (ammonia detoxification); iii) reproductive events, such as spermatogenesis, ovulation, embryo implantation, placental development and fetal growth; and iv) immune system [10]. Moreover, it aids in vasodilatation, blood pressure regulation, endothelial resilience and acid-base regulation, and it positively influences the intestinal integrity [10].

As a functional amino acid, arginine plays multiple roles in animal metabolism, besides acting as substrate for the synthesis of biological molecules, including ornithine, polyamines (putrescine, spermine and spermidine), proline, glutamine, creatine, agamatine, and nitric oxide (NO) [11]. In combination with polyamines, NO regulates DNA and protein syntheses [12]. Therefore, during gestation, NO and polyamines may regulate conceptuses survival and growth, promoting cell proliferation, migration and differentiation, angiogenesis and vessel dilatation to increase blood flow [13], as well as being involved in embryo implantation [14,15].

Several studies regarding dietary L-arginine supplementation of pregnant sows have been performed [16–24]. Most of these studies pointed out an improvement in reproductive

performance of sows supplemented with this amino acid [18,20,23], which occurs through increased prenatal survival, and, consequently, increased total number of piglets born and born alive [18,20,22]. In addition, it influences the average total litter birth weight [18,20,22]. According to Li et al.[22], the gestational period of L-arginine supplementation may be critical for its positive effects on sow reproductive efficiency.

In this way, maximization of pregnant sows performance with L-arginine supplementation is seen as one of the main breakthrough in nutrition research [17,20,24,25]. However, the mechanisms responsible for arginine effects during gestation on conceptuses survival and development are still under research [18,24,26–28]. Based on the hypothesis that L-arginine supplementation alters the expression of genes related with cell proliferation, migration and differentiation, embryos and fetuses growth, conceptuses apoptosis, and changes in prenatal survival rates and conceptuses development, we aimed to evaluate the effects of dietary 1.0% L-arginine supplementation on phenotypic traits of commercial gilts, embryos and fetuses, concentration of females' blood metabolites, expression of developmental genes and expression of cellular apoptosis genes in conceptuses of 25 and 35 days after insemination.

## 2. MATERIALS AND METHODS

### *2.1 Experimental design and female traits*

Experimental protocol has followed ethical principles in animal research (CONCEA, 2016) and was approved by the Ethical Committee on Animal Use of *Universidade Federal de Viçosa* (UFV), Minas Gerais, Brazil [protocol no. 06/2017].

From 120 days of age, 23 commercial gilts were housed in individual pens for better feed control of each animal. At 150 days of age, the management of puberty induction started by direct exposure of the female to an adult male twice daily (morning and afternoon). When the gilts expressed their third estrus, hormone synchronization was performed in groups of five females, using Regumate® (Merk Animal Health, USA). After identification of the fourth estrus, females were inseminated in two periods, 12 and 24 hours after the beginning of estrus. The first insemination day was considered day zero of gestation and the supply of experimental diets occurred 24 hours after the second insemination [24]. Semen doses were collected from two commercial boars with proven reproductive performance (semen analyses). In both insemination periods, each gilt was inseminated with semen from the same boar, and, within treatment, both situations occurred: gilts inseminated with semen from one boar and gilts inseminated with semen from the other boar. The semen parameters met the requirements for

use in pig artificial insemination (AI) programs recommended by Colégio Brasileiro de Reprodução Animal [29].

Females were weighed at 120, 150, 180 and 210 days of age and on the day of the first insemination, in order to control the weight uniformity until the beginning of the supplementation. Gilts from the group of 25 days of gestation presented in the beginning of the supplementation a body weight of 154.00 ± 3.15 kg and 152.10 ± 7.12 kg for CON and ARG, respectively. Group of 35 days of gestation presented in the same period a body weight of 143.15 ± 8.96 kg and 148.55 ± 4.71 kg for CON and ARG, respectively. Gilts were assigned to a completely randomized design and a 2x2 factorial arrangement (two diets and two gestational ages), with five replicates per treatment on average ($n$=5). Gilts were fed either a control diet (CON), mainly composed of corn, soybean meal, mineral and vitamins supplements, formulated in order to meet the nutritional requirements of gestating sows [30], or the CON diet supplemented with 1.0% ARG (Ajinomoto, Saga, Japan), associated with two gestational ages (25 and 35 days of gestation).

The supplementation of 1.0% L-arginine on gestation diet (ARG) was done by replacing clay filler by L-arginine. Nutritional levels for metabolizable energy (3148 kcal/kg), calcium (0.750%), phosphorus (0.395%) and digestible amino acids, lysine (0.535%), methionine and cysteine (0.381%), threonine (0.412%), tryptophan (0.113%) and valine (0.490%) were kept stable for both gestation diets. There were changes between CON and ARG diets for crude protein (12.16 vs. 14.46%) and digestible arginine (0.67 vs. 1.60%). In order to avoid ammonia intoxication and competition for basic amino acid transporters, it was sought not to exceed 2.0% of arginine content in both diets, neither go beyond digestible arginine to digestible lysine ratio of 3.0, as recommended for sows [13]. Hence, concentration of 1.6% of digestible arginine and 3.0 ratio of arginine to lysine in ARG diet could not result in antagonism in intestinal absorption of lysine nor histidine.

Considering the 23 commercial gilts, 11 received the diet without supplementation (CON) and 12 received basal diet for pregnant animals with 1.0% L-arginine supplementation (ARG), beginning 24 hours after the second insemination. Diets were daily provided to the gilts, in equal quantities, divided into two daily feeds (9 am and 4 pm). A total of 1.8 kg/day were offered between days 1 and 3 of gestation and 2.2 kg/day were offered between days 4 and 24 (females slaughtered at 25 days of gestation), or 4 and 34 (females slaughtered at 35 days of gestation). Animals had free access to water throughout the experimental period.

From the 23 inseminated gilts, 20 became pregnant. At 25 days of gestation, five females of CON (*n*=5) and five females of ARG (*n*=5) were rendered unconscious using head-only electrical stunning (240V, 1.3A) and immediately exsanguinated. At 35 days of gestation, four females of CON (*n*=4) and six females of ARG (*n*=6) were rendered unconscious using head-only electrical stunning (240V, 1.3A) and immediately exsanguinated. The following female traits were evaluated: slaughter weight (SW, kg), uterine weight (UW, kg), left uterine horn length (LHL, cm), right uterine horn length (RHL, cm), number of corpora lutea in the right ovary (CLR) and left ovary (CLL), total number of corpora lutea (CLT), left ovary weight (LOW, g), right ovary weight (ROW, g), total ovaries weight (TOW, g), embryos number (EN), viable embryos number (VEN), mortality rate (MR, %) calculated by the following formula: $[100 - ( \frac{\text{Viable embryos number}}{\text{Number of Corpus Luteum}} X\ 100)]$ and coefficient of variation of conceptuses weights from gilts calculated by $(\frac{\text{Standard deviation of conceptuses weights}}{\text{Mean of conceptuses weights}} X\ 100)$ (CVc, %). In addition, plasma and serum blood samples were collected from the females during slaughter, which were adequately stored until biochemical analyses of the following amino acids (nmol/mL): ornithine, aspartic acid, glutamic acid, asparagine, histidine, serine, glutamine, arginine, tyrosine, alanine, tryptophan, methionine, valine, phenylalanine, isoleucine and leucine. The levels of estradiol (pg/mL) and progesterone hormones (ng/mL) were quantified in the females' blood plasma. Urea was also quantified in the blood serum of females (mg/dL).

### *2.2 Biochemical analyses of gilts' blood parameters*

Amino acids quantification in blood plasma was performed using in-house methodology. Ornithine was quantified in blood serum by the gas chromatography methodology using a flame ionization detector (GC-FID). The quantification of estradiol in blood serum was performed using the ARCHITECT Estradiol assay (Abbott, Illinois, USA) that is a microparticle immunoassay by chemiluminescence (CMIA) and the quantitative determination of progesterone levels in serum was performed by chemiluminescent immunoassay with paramagnetic particles using the Access Progesterone test (Beckman Coulter, California, USA) that is a competitive enzyme immunoassay. Blood serum urea analysis was performed on the Cobas C311 Automation apparatus (Roche, Risch-Rotkreuz, Switzerland), using a commercial kit from Cobas C311 according to the manufacturer's instructions. All the biochemical analyses were carried out in private laboratories in Brazil: Hermes Pardini (Belo Horizonte, Minas Gerais) and Viçosa Lab (Viçosa, Minas Gerais).

### *2.3 Conceptuses experimental design, collection, and evaluated traits*

After slaughter, conceptuses (embryos: 25 days and fetuses: 35 days) were quickly collected and washed with PBS (Phosphate Buffered Saline) solution. All embryos and fetuses (from left and right uterine horns) were weighed on a precision scale for further analysis of embryo weight (EW, g) and fetal weight (FW, g). The cephalic-caudal length (CC, mm) of all fetuses was measured using a digital caliper [31]. After these measurements, conceptuses from right uterine horn were stored in liquid nitrogen for further gene expression analyses (developmental and apoptosis genes).

Phenotypic data and gene expression analyses were performed within each gestational age, considering a completely randomized design with two treatments (CON and ARG) and five replicates on average per treatment (five females in CON and five females in ARG at 25 days and four females in CON and six females in ARG at 35 days). Four conceptuses were collected per female at each gestational age (two from the cranial region and two from the caudal region of the right uterine horn), totaling in average 20 conceptuses per treatment (20 conceptuses in CON and 20 conceptuses in ARG at 25 days and 16 conceptuses in CON and 24 conceptuses in ARG at 35 days).

### *2.4 Gene expression analyses from conceptuses*

Gene expression analyses were carried out according to the routine at the Animal Biotechnology Laboratory (LABTEC) from the Animal Science Department at UFV as described previously in Costa et al. [32]. Total RNA extraction was performed from 50 mg of embryos and fetuses samples (whole conceptuses sprayed with nitrogen) using TRIzol® (InvitrogenTM) according to the manufacturer's instructions. The final precipitate was rehydrated with 30 μL of UltraPure® DNase/RNase-Free water. RNA concentration was estimated at spectrophotometer NanoVue™ Plus (GE Healthcare, Freiburg, Germany) and quality integrity was determined in 1.0% agarose gel (data not shown). The first strand of cDNA synthesis was performed using GoScript Reverse Transcriptase kit (Promega Corporation, Madison, WI, USA) and its concentration was determined by spectrophotometer NanoVue™ Plus (GE Healthcare, Freiburg, Germany). cDNA samples were stored at -20°C until their use in real-time quantitative polymerase chain reaction (RT-qPCR).

Primers for amplification of the target and endogenous gene fragments were designed using PrimerQuest software provided by Integrated DNA Technologies, Inc (Coralville, IA) using nucleotide sequences obtained from the GeneBank database (http: // www. ncbi.nlm.nih.gov) (Table 1).

As endogenous controls, β-actin (*β-ACTIN*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine guanine fosforiboxiltransferase (*HPRT1*) were tested. These genes were selected based on their amplification profile and dissociation curve (data not shown). None of the endogenous presented significant expression difference between treatments (CON and ARG) at 25 or 35 days. Due to its greater expression and stability between treatments, the *β-actin* was chosen as the best endogenous gene for embryo and fetal analyses (data not shown).

The following target genes were evaluated: i) developmental genes, embryonic and fetal growth: Sonic Hedgehog (*SHH*), Desert Hedgehog (*DHH*), Indian Hedgehog (*IHH*), WNT Family member 1 (*WNT1*), Fibroblastic Growth Factor 8 (*FGF8*), SRY-Box 6 (*SOX6*) and Insulin-Like Growth Factor 1 (*IGF1*); ii) apoptosis genes: BCL2 Associated X (*BAX*), *BCL2* and Caspase 3 (*CASP3*).

RT-qPCR analyses were performed in duplicates in ABI Prism 7300 Sequence Detection Systems thermocycler (Applied Biosystems - Foster City, CA, USA) using the Relative Quantification method and applying SYBR® Green system (Applied Biosystems - Foster City, CA, USA) and GoTaq® qPCR Master Mix kit (Promega Corporation, Madison, USA). PCR reactions were submitted to the cycles protocol according to the program: 95ºC for 3 minutes, 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. Amplification efficiencies (targets and endogenous genes) were approximately 100% in each cycle (data not shown), and the relative abundance was calculated as described by Pfaffl et al. [33]. The values of threshold cycle (Ct) obtained were later normalized (ΔCt) based on the Ct values obtained for the endogenous control gene (*β-actin*). The calculation of the relative gene expression levels was performed according to the $2^{-\Delta Ct}$ method, described by Livak & Schmittgen [34].

### *2.5 Statistical analyses*

Phenotypic and biochemical female data were submitted to the analysis of variance (ANOVA) using the MIXED procedure of SAS, version 9.0 (Statistical Analysis System Institute, Inc., Cary, NC, USA). The residue normality test was performed using the UNIVARIATE procedure of SAS (SAS Institute - Cary, NC, USA). The results were considered significant when $P \leq 0.05$. *P*-values between 0.06 and 0.10 were considered a trend.

The following statistical model was used in the analyses:

$$Y_{ijk} = \mu + D_i + A_j + (D * A)_{ij} + \varepsilon_{ijk}$$

where: $Y_{ijk}$ is the observation of the animal from the j-th gestational age which received the i-th diet; ; $\mu$ is the trait general mean; $D_i$ is the effect of the i-th diet (CON or ARG); $A_j$ is the effect

of the j-th gestational age (25 or 35 days); $(D * A)_{ij}$ is the effect of interaction between the i-th diet (D) and the j-th age (A); and $\varepsilon_{ijk}$ is the random error.

Conceptuses phenotypic and gene expression data were analyzed within each gestational age and submitted to ANOVA using the MIXED procedure of SAS, version 9.0 (Statistical Analysis System Institute, Inc., Cary, NC, USA). The residue normality test was performed using the UNIVARIATE procedure of SAS (SAS Institute - Cary, NC, USA). Conceptuses were considered false replicates of the females for each treatment, represented by a nested effect in the statistical model.

Expression data of target and endogenous genes were generated as Ct (threshold cycle) values. Data were transformed in relative expression ($2^{-\Delta Ct}$), according to Livak & Schmittgen [34], and results were considered significant when $P \leq 0.05$. *P*-values between 0.06 and 0.10 were considered a trend.

The following statistical model was used in the analyses for each gestational age:

$$Y_{ijkl} = \mu + D_i + C\{D_{(i)}\}_j + B_k + \varepsilon_{ijkl}$$

where: $Y_{ijkl}$ is the observation (phenotypic or relative expression) from the j-th concept; $\mu$ is the trait general mean; $D_i$ is the effect of i-th treatment (CON or ARG); $C\{D_{(i)}\}_j$ is the random effect of concept j nested in treatment i; $B_k$ is the random effect of k-th boar; and $\varepsilon_{ijk}$ is the random error.

## 3. RESULTS

### *3.1 Gilts phenotypic data*

The effect of the interaction between gestational age (25 and 35 days) and diet (CON and ARG) was not significant for most of the phenotypic female traits, except for LOW ($P$=0.02) (Table 2). Differences were observed for UW between females of 25 and 35 days of gestation ($P$<0.0001), as well as for LHL ($P$=0.001), RHL ($P$=0.003), ROW ($P$=0.05), TOW ($P$=0.04), VEN ($P$=0.05), MR ($P$=0.01) and CVc ($P$=0.01). Higher averages for these traits were observed at 35 days of gestation compared to 25 days as expected, except for VEN and CVc (Table 2). The LOW of the ARG females at 25 days of gestation was lower than that of CON females. However, at 35 days of gestation, the LOW of the ARG females was greater than that of CON (Table 2).

### *3.2 Biochemical data*

Table 3 shows the ANOVA results for the amino acids, hormones and urea analyzed in gilts blood samples. Gestational age vs. diet interaction was not significant for concentration of

estradiol and progesterone in the maternal blood serum, and there was no influence of the diet on the concentration of these hormones in females' blood. However, as expected, differences in estradiol and progesterone concentration were observed between 25 and 35 days of gestation (*P*=0.0003 and *P*=0.03, respectively).

There was a trend for a significant interaction between gestational age and diet for arginine plasma concentration (*P*=0.06), which showed at 25 days a greater average concentration in ARG females (169.30 nmol/mL) compared to CON females (139.96 nmol/mL). On the other hand, at 35 days of gestation, ARG females presented a lower average (119.40 nmol/mL) compared to CON females (160.28 nmol/mL). For the other amino acids, the gestational age vs. diet interaction was not significant. Differences between diets (ARG and CON) were found for tyrosine (*P*=0.004) and methionine (*P*=0.01). Tyrosine presented a higher average in CON (76.48 nmol/mL) compared to ARG (59.87 nmol/mL), as well as methionine: CON, 38.26 nmol/mL and ARG, 32.66 nmol/mL. ARG gilts also showed a trend to present a lower concentration of alanine in the blood plasma compared to CON gilts (*P*=0.07). Urea concentration in the blood serum was different between ARG and CON gilts (*P*=0.05).

### *3.3 Conceptuses phenotypic data*

Table 4 shows the ANOVA results for conceptuses phenotypic data. Regarding embryo weight, no difference was observed between treatments, however there was a trend of the embryos from ARG to present a greater average weight compared to the embryos from CON (1.05 g and 0.79 g, respectively) (*P*=0.07). On the other hand, there was no difference in fetuses weights between treatments (*P*=0.19), despite the lower average values of CC in the fetuses of ARG females (27.34 mm) compared to the fetuses of CON (28.82 mm) (*P*=0.05).

### *3.4 Gene expression*

No differences in gene expression were observed in CON and ARG embryos for the following developmental genes: *SHH* (*P*=0.21), *DHH* (*P*=0.89), *IHH* (*P*=0.30), *FGF8* (*P*=0.93) and *SOX6* (*P*=0.34). *WNT1* gene tended to be more expressed in CON embryos compared to ARG embryos (*P*=0.07) (Figure 1A). *IGF1* gene showed a significant difference in expression between treatments (*P*=0.05), with a greater expression in ARG compared to CON (Figure 1A). Fetuses did not show differences of expression between treatments for any of the developmental genes evaluated: *SHH* (*P*=0.76), *DHH* (*P*=0.26), *IHH* (*P*=0.81), *WNT1* (*P*=0.38), *FGF8* (*P*= 0.79), *IGF1* (*P*=0.81) and *SOX6* (*P*=0.47) (Figure 1B).

Apoptosis genes *BAX, BCL2* and *CASP3* did not show differences of expression between the CON and ARG embryos, presenting the following *P*-values: 0.22, 0.10 and 0.93, respectively (Supplementary table 1). Fetuses also did not show differences between treatments for these genes: *BAX* (*P*= 0.37), *BCL2* (*P*= 0.24) and *CASP3* (*P*= 0.18) (Supplementary table 1).

## 4. DISCUSSION

Several studies of L-arginine supplementation in pigs at different periods, duration and concentration indicate a decrease in the mortality rate and, consequently, increase in the number of piglets born and born alive [18,20,22,23,27], even with low concentration of this amino acid in the supplementation: 0.4% and 0.8% [23]. According to De Blasio et al. [35], the interaction between gestational phase and supplementation with different L-arginine concentrations has critical effects on embryo and fetal survival. Despite these results, in the present study no differences were observed related to EN, VEN and MR between diets (CON and ARG) at 25 or 35 days of gestation; we suggest a higher number of gilts to validate our findings considering the fact that mortality rate is high in pigs [13,18,36–39] and the high coefficient of variation of this trait; in the current study the coefficient of variation of mortality rate was 85%.

Overall, female traits such as body weight, backfat thickness, duration of gestation, among others, are not influenced by L-arginine supplementation [17–19,23]. Our results indicate that supplementation with this amino acid, in fact, did not influence the female slaughter weights at 25 and 35 days of gestation (SW). In addition, the female traits UW, LHL, RHL, CLR, CLL, CLT, ROW and TOW also did not present differences between CON and ARG.

As expected, UW, LHL and RHL presented differences between gestational ages. In the interval of 25 and 35 days of gestation, conceptuses present a fast growth, which consequently require more area to develop (transition from the embryonic stage to the fetal stage). In this period, the first wave of myogenesis in pigs begins [40], and conceptuses presented a high weight gain. Regardless of supplementation, ROW and TOW were higher at 35 days compared to 25 days of gestation. A higher progesterone concentration was also observed in the blood serum of females at 35 days compared to 25 days of gestation as consequence of the increase of the luteal mass and the activity of lutein cells. Interaction between diet and gestational age was significant for LOW, with a higher value at 25 days in CON compared to ARG. On the other hand, at 35 days of gestation, the average LOW for ARG gilts was greater compared to CON. Results for ovary weight are controversial in the present study. A deeper analysis

considering supplementation and corpora lutea weight might highlight stronger and more consistent relationships, also the local blood circulation between ovary and uterus has to be addressed.

Regarding VEN, MR and CVc, differences between 25 and 35 days of gestation were also observed. Number of viable embryos was greater at 25 days compared to 35 days. Lower VEN at 35 days is related to the greater rate of prenatal mortality observed at this gestational age (MR). Embryonic and fetal losses are cumulative and occur throughout gestation, being greater in this first third of gestation [1]. During this period, several events occur as abnormalities of embryos development [37], lethal genetic problems [37], embryo asynchrony with the uterine environment [38], competition between embryos in uterine area [38], morphological alterations of placental formation [39], among others, and can be the cause of prenatal mortality in this phase due to a decrease in the number of viable embryos. Interestingly, differences were also observed between CVc at 25 and 35 days. CVc at 25 days was higher than at 35 days; this indicates a greater uniformity between fetuses in relation to embryos; at 25 days there is greater developmental asynchrony of the conceptuses associated to higher MR. Considering litter CV and uniformity, supplementing gestation diet with L-arginine during the last third of pregnancy slightly reduced within-litter variation of birth weight [21]. Putting together all these findings, it is clear that L-arginine effects in this trait might vary according to period of supplementation.

Although we did not observe differences in embryos and fetuses weights between treatments, there was a trend for embryos from ARG gilts present higher weight compared to CON embryos. This trend is possibly explained by the greater expression of *IGF1* in embryos from the supplemented group, since genes from the IGF family present growth-promoting activity. At 25 days of gestation, there was also a trend toward a greater concentration of arginine in the blood plasma of ARG females. Arginine metabolism is related to the production of growth hormone and insulin in pigs [41], and the effects of growth hormone are largely mediated by *IGF1* [42]. According to Baker et al. [43], *IGF1* is the main responsible for embryo/fetal growth in the prenatal period, and its effect is even higher than the direct effect of growth hormone. In addition, according to Bird et al. [44], this trend in increased arginine concentration in gilts could result in higher NO production, which is involved in the transfer of nutrients and oxygen from mother to embryo, through control of vascularization and blood flow in uterus and placenta, thus influencing conceptuses growth. At 35 days of gestation, a trend was observed for a lower concentration of arginine in the blood plasma of ARG females. At

this gestational age, although there were no differences in fetal weights between treatments, the CC of ARG fetuses was lower than the CC of CON fetuses.

Effects of L-arginine supplementation on the survival and growth rates of pig embryos are mediated by mechanisms that may involve changes in progesterone production [24]. In our study, no difference was observed in progesterone concentration in blood plasma of CON and ARG females. Li et al. [24] reported that L-arginine supplementation in the early gestation period (between 0 and 25 days) led to a decrease in progesterone production, since this supplementation could lead to regression of the corpora lutea through a *PGF2α*-dependent pathway. Due to this fact, some researchers suggest that supplementation should be done from the second week of gestation [18,24].

In our study, supplementation started 24 hours after the second AI, and it was provided during 23 or 33 days of gestation. Although supplementation started in the first gestational week, there were no differences in progesterone concentration between diets, which is important, since progesterone is related to several events in mother and conceptuses for successful gestation. This hormone induces differentiation of endometrial, stromal and glandular secretions that initially nourish embryos [45,46] and placentation [47–50], among other processes, such as embryo implantation, thus being involved in conceptuses survival and development. In such way, more studies are needed to better understand the role of L-arginine in the first third of gestation in pigs.

Supplementation with 1.0% L-arginine in the diet of sows increased estradiol concentration in the sows blood plasma at 40, 70 and 90 days of gestation [18]. On the other hand, Li et al. [24] did not observe differences in estrone and estrone sulfate concentrations in the plasma of supplemented females, as in Li et al. [23], who also found no differences in the concentrations of estradiol. Differences between treatments in estrone sulfate concentration were also not observed in supplemented and not supplemented gilts [17]. In our study, differences in estradiol concentration in the blood plasma of CON and ARG gilts were not found. However, as expected, differences in estradiol concentrations between 25 and 35 days of gestation were observed, with higher concentration of this hormone at 25 days of gestation as a result of follicular cells secretion, among other factors.

Increase in arginine concentration in blood plasma of females supplemented with L-arginine is pointed out in several studies [17,18,20,23,24]. Considering the interaction between gestational age and diet, a trend was observed for a higher concentration of arginine in blood plasma of the supplemented group at 25 days of gestation. On the other hand, lower arginine

concentration in maternal blood plasma was observed in the ARG group at 35 days of gestation. Despite the increased supply of L-arginine to ARG gilts, at 35 days the additional arginine was probably catabolized in products such as nitric oxide and polyamines [51,52] according to mother and conceptuses needs or may have been transformed into urea through arginase in the cytosol [51]. In addition to arginine, the enzyme arginase also produces ornithine that goes into the mitochondria by restarting the urea cycle. The urea cycle is the main mechanism of ammonia elimination [51,52].

In our study, we observed a higher urea concentration in blood serum from ARG group. This difference between CON and ARG gilts may be a result of the higher concentration of arginine in ARG diet compared to CON diet. Because of this, some researchers formulate isonitrogenous control diets (supplemented with L-alanine). Although there was an increase in serum urea concentration of ARG gilts, it did not negatively influence our results. In addition, in a previous study, no differences in urea concentration were observed between ARG and CON treatments, in which supplementation with L-arginine was performed at the same concentration of 1.0 % [53].

These arginine concentrations findings, together with phenotypic observations of female performance and conceptuses development, support discussions about the optimal period of supplementation, in order to achieve positive results related to sows and offspring efficiences.

Among the amino acids evaluated in gilts blood, only methionine and tyrosine showed differences in concentrations between ARG and CON, regardless of gestational age. L-arginine supplementation led to a decrease in both methionine and tyrosine in plasma of supplemented females compared to the control females. The lower methionine concentration in the plasma of supplemented gilts may influence gene expression by epigenetic mechanisms, since methionine is necessary for the synthesis of S-adenosylmethionine, which is the main donor of methyl groups for DNA methylation [54,55].

Arginine is also a guanidine donor for the amino group of glycine through the enzyme arginine: glycine amidinotransferase [56] for creatine synthesis [57]. The guanidinoacetate, product of this first step, is methylated in the presence of S-adenosylmethionine produced from adenosine triphosphate (ATP) and methionine. The enzymes used for creatine synthesis are found in different concentrations in several organs, and there is cooperation between organs for the synthesis of creatine [13]. According to Wu et al. [13], the *de novo* synthesis of creatine is an important way for the use of arginine in pregnant sows and is related to neurological and skeletal muscle development in the conceptuses [2]. A study conducted by Bérard & Bee [17]

showed influence of L-arginine supplementation on development and myogenesis of swine fetuses, positively affecting the primary formation phase of myofibers. In the present study, the decrease in the concentration of methionine in the treatment with supplementation is possibly related to the production of S-adenosylmethionine for the greater synthesis of creatine during gestation, a process in which there is an extreme use of methyl groups. In addition, it is also necessary methyl groups donation by S-adenosylmethionine for the synthesis of polyamines from ornithine.

Tyrosine is often found in catalytic parts of enzymes and participates in the synthesis of thyroid hormones and catecholamines [58]. This amino acid can be synthesized from phenylalanine and methionine. Thus, in our study the reduction of tyrosine concentration in gilts' blood may be a result of the reduction in methionine concentration in blood plasma.

Regarding the expression of the developmental and apoptosis genes, some amino acids such as arginine, glutamine, glutamate, glycine and proline play important roles in the regulation of gene expression [59,60]. Several pathways related to cell proliferation, migration and differentiation, which are key events during prenatal development, are also regulated by amino acids and their metabolites [13]. Arginine catabolism products such as polyamines, NO and creatine are necessary for cell proliferation and differentiation [12], apoptosis regulation [61], and possibly mediate growth and development of muscle fibers and adipocytes in the fetuses [17,25]. In addition, these products are involved in other processes, such as embryonic implantation [14,15] and formation of other tissues [62]. Thus, in this study, genes related to the processes of cell proliferation, migration and differentiation, as well as genes related to morphogenesis, development and apoptosis of conceptuses, were analyzed.

The *FGF8* gene is involved in the regulation of embryonic development and in cell proliferation, differentiation and migration. As *FGF8*, genes from the Hedgehog pathway (*SHH, DHH* and *IHH*) control cell differentiation, and are involved in the process of morphogenesis during prenatal development. The *FGF8* and genes from the Hedgehog pathway showed no expression differences between treatments in embryos or fetuses. In association with *FGF8* and Hedgehog genes, WNT proteins are involved in a wide range of biological processes [63]. *WNT1* gene is involved in several developmental processes, including regulation of fate and cell differentiation during embryogenesis, and it has strong mitogenic activity [63]. This gene was not differentially expressed between treatments, but showed a trend to be more expressed in the conceptuses of 25 days from CON group compared to ARG ($P$=0.07). In the fetuses, there was no difference in expression between treatments. SRY-Box 6 (*SOX6*) gene,

which is associated with cell differentiation and transcriptional activation, was also not differentially expressed between CON and ARG for embryos or fetuses.

It is not well known whether the dietary L-arginine supplementation of sows leads in fact to an improvement in the conceptuses growth and which mechanisms are involved in this process [16]. In this study, *IGF1* gene presented higher expression in the conceptuses from ARG gilts compared to CON at 25 days. This gene plays an important role in the growth and prenatal development of the conceptuses, since its secretion in the uterus is independent of the direct action of growth hormone. *IGF1* can be secreted by the liver (endocrine secretion) and its autocrine and paracrine secretions are mainly responsive to nutritional regulation [64].

Apoptosis is a mechanism of damaged cells removal and cell and tissue turnover, which are extremely expressive processes during embryogenesis and fetal development [65]. In this study, apoptosis genes were evaluated, since death and cell proliferation are closely connected. The *BCL-2* gene is an example of a cell cycle regulator, influencing both cell division and programmed cell death. High levels of *BCL-2* family proteins block apoptosis (Bcl-2 and Bcl-x) and others promote apoptosis (Bax, Bad and Bak) [66]. Caspases are proteases that trigger the cascade of cell death. Caspase 3 is an effector caspase of the apoptotic process [65]. Although Tan et al. [61] observed influence of the L-arginine on cell apoptosis, we found no differences in apoptotic genes expression in embryos and fetuses from CON and ARG females.

Our results pointed out that dietary supplementation with 1.0% L-arginine in early gestation of gilts increased the expression of *IGF1* gene in embryos, which is a revelant gene responsible for conceptuses prenatal growth. In addition, embryos from supplemented females tended to have a greater weight compared to embryos from control females, which evidences the influence of the *IGF1* gene in the conceptuses prenatal growth. At 25 days of gestation, there was also a trend toward a higher arginine concentration in the blood plasma of the supplemented gilts. However, supplementation over a longer interval (33 days) resulted in fetuses with lower cephalic-caudal length, and a trend for a lower arginine concentration in the plasma of supplemented females.

Further research is needed to improve the understanding of how supplementation with amino acids such as L-arginine regulates gene and protein expression in pig embryos and fetuses [13]. Although the genes evaluated in this study are crucial for conceptuses development and apoptosis, further work addressing a global gene expression analysis in embryos and fetuses is currently in process by our team since supplementation can influence such processes through other gene groups as well. Our group also considers methylome analysis in parallel to RNA-

seq since lower methionine concentration in ARG gilts blood plasma can be related with epigenetic regulation of gene expression. In addition, the influence of supplementation on the genes studied can occur at the post-transcriptional level through the regulation of abundance or protein activity. Besides that, the duration and period of supplementation, as well as the concentration used, may have influenced the results for the evaluated genes.

## 5. CONCLUSIONS

We can conclude that supplementation duration is determinant for the effects that L-arginine exerts on the concentration of this amino acid in the plasma of gilts and on conceptuses growth. Our current results from gilts supplemented during 23 days were more interesting regarding conceptuses development since we observed increased arginine concentration in plasma of females from ARG group and increased *IGF1* gene expression in ARG 25-days embryos related to a tendency of higher embryos weight. These differences occurred through mechanisms unrelated to ovarian activity as progesterone production. On the other hand, supplementation for a longer period (33 days) did not lead to changes in the expression of developmental and apoptosis genes in 35-days fetuses, although it decreased fetuses cephalic-caudal length, which may influence phenotypic traits as litter uniformity at birth. The L-arginine supplementation also changed the concentration of methionine and tyrosine in the blood plasma of supplemented females. Thus, future studies pointing out global gene expression and epigenetic effects through methylation are on the way by our team, since the lower concentration of methionine in plasma can be related to the regulation of gene expression by epigenetic mechanisms.

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## 7. AUTHOR´S CONTRIBUTION

*Karine, A. Costa, Alysson Saraiva and Simone E. F. Guimarães* were in all stages, including conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content.

*Lívia Maria Barbosa, Faider Alberto Castaño Villadiego, José Domingos Guimarães, Mariana Machado Neves and Daniele Botelho Diniz Marques* participated in the conception, design and acquisition of data of the study.

*Letícia Fernanda de Oliveira* and *Ingrid Soares Garcia* participated of the procedures to acquisition of data. *Daniele Botelho Diniz Marques and Renata Veroneze* were important in statistical analyses and interpretation of data.

All authors participated in drafting the article and revising it critically for important intellectual content.

## 8. COMPETING INTERESTS

The authors have nothing to disclose.

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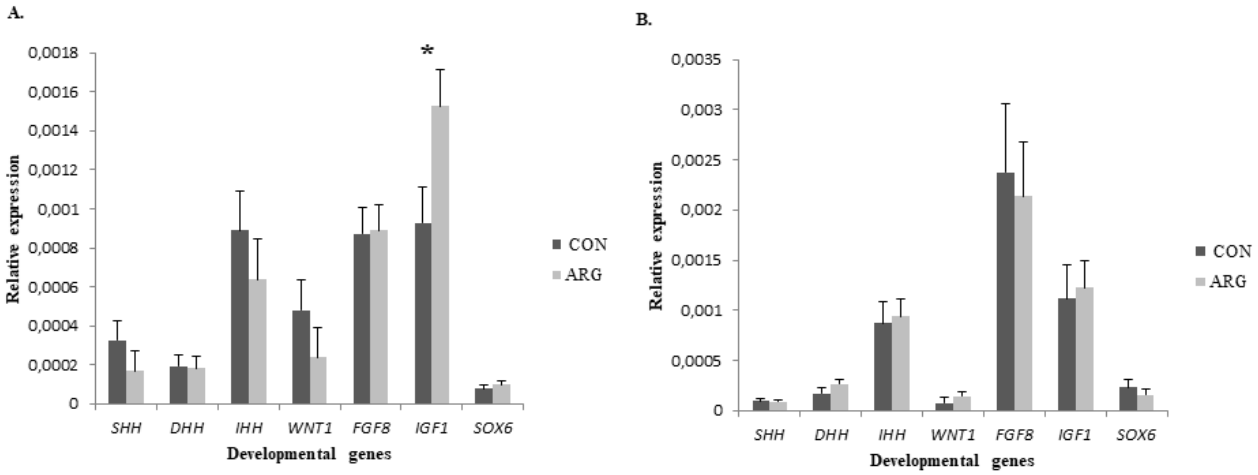
**Figure 1:** Relative expression of developmental genes in conceptuses ($2^{-\Delta Ct}$). **1.A:** 25-day embryos. **1.B:** 35-day fetuses.*$P \leq 0.05$



# TABLES

**Table 1**: *Primers* design

| GENE | Abbreviation | Sequence | NCBI Accession code |
|---|---|---|---|
| | | **ENDOGENOUS** | |
| B- actin | *BACT* | F:CTTCTAGGCGGACTGTTAGTG | XM_0031242803 |
| | | R:AGCCATGCCAATCTCATCTC | |
| Hypoxanthine Phosphoribosyltransferase 1 | *HPRT1* | F:CCAGTCAACGGGCGATATAA | NM_001032376.2 |
| | | R:GACCAAGGAAAGCAAGGTTG | |
| Glyceraldehyde-3-Phosphate Dehydrogenase | *GAPDH* | F:CAAAGTGGACATTGTCGCCATCA | NM_001206359.1 |
| | | R:AGCTTCCCATTCTCAGCCTTGACT | |
| | | **DEVELOPMENTAL** | |
| Sonic Hedgehog | *SHH* | F: TACTCGCAGCTGCTCTAC | HM803983.1 |
| | | R: CTGGACTTGACCGCCAT | |
| Indian Hedgehog | *IHH* | F: CTCGCGTACAAGCAGTTC | NM_001244470.1 |
| | | R: CGCGATCTTGCCTTCATAG | |
| Desert Hedgehog | *DHH* | F: CGTGCCTTGGACATCAC | XM_013988251.1 |
| | | R: TTGCGGGACTCGTAGTAG | |
| Wnt Family Member 1 | *WNT1* | F: GTCCTGCACATGCGACTAT | XM_003126100.5 |
| | | R: CAAACTCCCGGCCAAAGA | |
| SRY-Box 6 | *SOX6* | F: CTAGACCAGGTCACACTCATTC | XM_021085335.1 |
| | | R: CAGACAGTTGTTGCTCTCCT | |
| Fibroblast Growth Factor 8 | *FGF8* | F: ATCCGGACCTACCAACTCTA | XM_021073392.1 |
| | | R: GCTCCCAAAGGTATCTGTCTC | |
| Insulin Like Growth Factor 1 | *IGF1* | F: CATTGCCGTGTATGCTGAAC | XM_005664199.3 |
| | | R: ACCACATAGCTCCCTCAAAC | |
| | | **APOPTOSIS** | |
| BCL2 Associated X | *BAX* | F: CATGTGGTCACCCGTTTC | AY550048.1 |
| | | R: GCTATGAGGTGTTGCCATC | |
| BCL2 | *BCL2* | F: GGGTCATGTGTGTGGAGA | EF681866.1 |
| | | R: GTGCCGGTTCAGGTACT | |
| Caspase 3 | *CASP3* | F:ATGCTGCAAATCTCAGGGAGACCT | XM_005671704.2 |
| | | R:CACCATGGCTTAGAAGCACGCAAA | |

**Table 2:** Averages ± Standard errors and *P*-values of diets, gestational ages and diet vs. gestational age interaction for female phenotypic data

| Trait | CON | ARG | *P*-Value | 25 days | 35 days | *P*-value | 25 days | | 35 days | | *P*-value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | CON | ARG | CON | ARG | |
| SW (kg) | 163.97 ± 4.37 | 165.02 ± 4.81 | 0.87 | 166.88 ± 4.09 | 162.10 ± 5.05 | 0.47 | 166.76 ± 2.67 | 167.00 ± 7.73 | 161.17 ± 8.32 | 163.03 ± 5.73 | 0.90 |
| UW (kg) | 3.92 ± 0.35 | 3.94 ± 0.27 | 0.97 | 2.72 ± 0.11 | 5.15 ± 0.43 | <0.0001* | 2.61 ± 0.07 | 2.83 ± 0.22 | 5.24 ± 0.69 | 5.05 ± 0.51 | 0.66 |
| LHL (cm) | 125.31 ± 5.50 | 128.53 ± 4.97 | 0.67 | 111.95 ± 5.19 | 141.90 ± 5.30 | 0.001* | 109.00 ± 7.34 | 114.90 ± 7.34 | 141.63 ± 8.21 | 142.17 ± 6.70 | 0.72 |
| RHL (cm) | 133.57 ± 8.25 | 131.20 ± 4.77) | 0.81 | 116.08 ± 1.87 | 148.69 ± 9.34 | 0.003* | 116.26 ± 3.54 | 115.90 ± 1.21 | 150.88 ± 16.11 | 146.50 ± 9.45 | 0.84 |
| CLR (counting) | 10.12 ± 0.83 | 9.15 ± 0.75 | 0.40 | 8.90 ± 0.79 | 10.37 ± 0.80 | 0.21 | 9.00 ± 1.11 | 8.80 ± 1.11 | 11.25 ± 1.24 | 9.50 ± 1.02 | 0.50 |
| CLL (counting) | 7.80 ± 0.85 | 9.60 ± 0.77 | 0.14 | 8.90 ± 0.81 | 8.50 ± 0.82 | 0.73 | 8.60 ± 1.14 | 9.20 ± 1.14 | 7.00 ± 1.27 | 10.00 ± 1.04 | 0.31 |
| CLT (counting) | 17.93 ± 0.71 | 18.75 ± 0.64 | 0.40 | 17.80 ± 0.67 | 18.87 ± 0.68 | 0.28 | 17.60 ± 0.94 | 18.00 ± 0.94 | 18.25 ± 1.06 | 19.50 ± 0.86 | 0.66 |
| LOW (g) | 7.71 ± 0.39 | 8.52 ± 0.36 | 0.15 | 8.01 ± 0.37 | 8.22 ± 0.38 | 0.69 | 8.30 ± 0.53 | 7.72 ± 0.53 | 7.13 ± 0.59 | 9.32 ± 0.48 | 0.02* |
| ROW (g) | 9.11 ± 0.48 | 7.95 ± 0.43 | 0.06 | 7.94 ± 0.45 | 9.12 ± 0.46 | 0.05* | 8.41 ± 0.64 | 7.47 ± 0.64 | 9.81 ± 0.71 | 8.44 ± 0.58 | 0.71 |
| TOW (g) | 16.82 ± 0.47 | 16.47 ± 0.43 | 0.59 | 15.95 ± 0.45 | 17.35 ± 0.46 | 0.04* | 16.71 ± 0.63 | 15.19 ± 0.63 | 16.94 ± 0.71 | 17.76 ± 0.58 | 0.09 |
| EN (counting) | 14.63 ± 0.99 | 14.40 ± 1.02 | 0.83 | 15.26 ± 0.97 | 13.76 ± 1.04 | 0.19 | 15.39 ± 1.14 | 15.13 ±1.30 | 13.87 ± 1.38 | 13.66 ± 1.22 | 0.98 |
| VEN (counting) | 13.74 ± 0.93 | 13.77 ± 0.98 | 0.69 | 14.77 ± 0.92 | 12.74 ± 0.99 | 0.05* | 14.97± 1.03 | 14.57± 1.22 | 12.52 ± 1.28 | 12.97 ± 1.12 | 0.66 |
| MR (%) | 24.34 ± 5.07 | 28.18 ± 5.37 | 0.44 | 18.44 ± 5.02 | 34.08 ± 5.41 | 0.01* | 15.39 ± 5.50 | 21.48 ± 6.63 | 33.28 ± 6.89 | 34.89 ± 6.03 | 0.66 |
| CVc (%) | 17.12 ± 2.51 | 21.56 ± 2.26 | 0.21 | 24.71 ± 2.51 | 13.97 ± 2.53 | 0.01* | 23.27 ± 3.44 | 26.15 ± 3.44 | 10.97 ± 3.74 | 16.97 ± 3.28 | 0.65 |

SW= slaughter weight, UW= uterine weight, LHL= left uterine horn length, RHL= right uterine horn length, CLR= number of corpora lutea in right ovary, CLL= number of corpora lutea in left ovary, CLT= total number of corpora lutea, LOW= left ovary weight, ROW= right ovary weight, TOW= total ovaries weight, EN= embryos number, VEN= viable embryos number, MR= mortality rate and CVc = coefficient of variation of conceptuses from gilts.
CON=control diet; ARG=diet with 1.0% L-arginine supplementation;

*$P \leq 0.05$

**Table 3:** Averages ± Standard errors and *P*-values of diets, gestational ages and diet vs. gestational age interaction for female blood metabolites

| | | | | | | | 25 days | | 35 days | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Hormone (ng/mL)** | CON | ARG | *P*-value | 25 days | 35 days | *P*-value | CON | ARG | CON | ARG | *P*-value |
| Estradiol | 40.44 ± 11.80 | 64.94 ± 10.65 | 0.14 | 88.69 ± 11.12 | 16.69 ± 11.35 | 0.0003* | 68.96 ± 15.73 | 108.42 ± 15.73 | 11.93 ± 17.59 | 21.47 ± 14.36 | 0.36 |
| Progesterone | 34.18 ± 2.94 | 36.20 ± 2.65 | 0.62 | 30.56 ± 2.77 | 39.82 ± 2.83 | 0.03* | 29.00 ± 3.92 | 32.12 ± 3.92 | 39.37 ± 4.38 | 40.27 ± 3.58 | 0.78 |
| **Amino acids (nmol/mL)** | | | | | | | | | | | |
| Ornitine | 95.75 ± 16.49 | 89.08 ± 9.84 | 0.73 | 94.32 ± 6.64 | 90.51 ± 18.02 | 0.85 | 88.30 ± 4.57 | 100.34 ± 12.47 | 103.20 ± 32.67 | 77.82 ± 15.24 | 0.35 |
| Aspartic acid | 5.95 ± 0.90 | 6.29 ± 1.03 | 0.81 | 5.24 ± 1.05 | 7.00 ± 0.87 | 0.22 | 5.52 ± 1.78 | 4.96 ± 1.12 | 6.37 ± 0.28 | 7.62 ± 1.72 | 0.52 |
| Glutamic Acid | 115.53 ± 13.02 | 91.66 ± 11.75 | 0.19 | 88.76 ± 12.28 | 118.43 ± 12.53 | 0.11 | 105.26 ± 17.36 | 72.26 ± 17.36 | 125.80 ± 19.41 | 111.07 ± 15.85 | 0.61 |
| Asparagine | 27.13 ± 1.60 | 23.92 ± 1.45 | 0.16 | 24.91 ± 1.51) | 26.14 ± 1.54 | 0.58 | 25.28 ± 2.14 | 24.54 ± 2.14 | 28.97 ± 2.39 | 23.30 ± 1.95 | 0.27 |
| Histidine | 139.95 ± 17.79 | 140.15 ± 16.06 | 0.99 | 150.82 ± 16.77 | 129.28 ± 17.12 | 0.38 | 138.70 ± 23.72 | 162.94 ± 23.72 | 141.20 ± 26.52 | 117.35 ± 21.65 | 0.33 |
| Serine | 96.34 ± 4.98 | 87.67 ± 4.50 | 0.22 | 93.36 ± 4.70 | 90.65 ± 4.79 | 0.69 | 98.46 ± 6.64 | 88.26 ± 6.64 | 94.22 ± 7.42 | 87.08 ± 6.06 | 0.82 |
| Glutamine | 397.06 ± 20.41 | 351.00 ± 18.42 | 0.11 | 363.58 ± 19.24 | 384.48 ± 19.64 | 0.46 | 385.04 ± 27.21 | 342.12 ± 27.21 | 409.08 ± 30.42 | 359.88 ± 24.84 | 0.91 |
| Arginine | 150.12 ± 12.43 | 144.35 ± 11.72 | 0.74 | 154.63 ± 11.72 | 139.84 ± 12.43 | 0.40 | 139.96 ± 16.57 | 169.30 ± 16.57 | 160.28 ± 18.53 | 119.40 ± 16.57 | 0.06 |
| Tyrosine | 76.48 ± 3.73 | 59.87 ± 3.37 | 0.004* | 68.87 ± 3.52 | 67.48 ± 3.59 | 0.78 | 77.44 ± 4.97 | 60.30 ± 4.97 | 75.52 ± 5.56 | 59.43 ± 4.54 | 0.92 |
| Alanine | 472.49 ± 29.57 | 396.00 ± 26.69 | 0.07 | 418.22 ± 27.88 | 450.26 ± 28.46 | 0.43 | 454.60 ± 39.43 | 381.84 ± 39.43 | 490.38 ± 44.08 | 410.15 ± 35.99 | 0.93 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Tryptophan | 64.36 ± 4.10 | 57.17 ± 3.70 | 0.21 | 60.66 ± 3.87 | 60.87 ± 3.95 | 0.97 | 65.02 ± 5.47 | 56.30 ± 5.47 | 63.70 ± 6.11 | 58.03 ± 4.99 | 0.79 |
| Methionine | 38.26 ± 1.49 | 32.66 ± 1.34 | 0.01* | 36.52 ± 1.40 | 34.40 ± 1.43 | 0.31 | 38.60 ± 1.99 | 34.44 ± 1.99 | 37.92 ± 2.22 | 30.88 ± 1.81 | 0.48 |
| Valine | 228.25 ± 12.95 | 200.59 ± 11.69 | 0.13 | 213.51 ± 12.21 | 215.33 ± 12.46 | 0.92 | 226.74 ± 17.27 | 200.28 ± 17.27 | 229.75 ± 19.31 | 200.90 ± 15.77 | 0.95 |
| Phenylalanine | 75.04 ± 3.47 | 68.20 ± 3.13 | 0.16 | 71.39 ± 3.27 | 71.85 ± 3.34 | 0.92 | 74.46 ± 4.63 | 68.32 ± 4.63 | 75.62 ± 5.17 | 68.08 ± 4.22 | 0.88 |
| Isoleucine | 92.56 ± 6.67 | 85.72 ± 6.02 | 0.46 | 89.44 ± 6.29 | 88.85 ± 6.42 | 0.95 | 90.08 ± 8.89 | 88.80 ± 8.89 | 95.05 ± 9.94 | 82.65 ± 8.12 | 0.54 |
| Leucine | 173.08 ± 8.32 | 161.38 ± 7.51 | 0.31 | 162.73 ± 7.85 | 171.73 ± 8.01 | 0.43 | 164.84 ± 11.10 | 160.62 ± 11.10 | 181.33 ± 12.41 | 162.13 ± 10.13 | 0.51 |
| **Urea (mg/dL)** | | | | | | | | | | | |
| Urea | 15.99 ± 0.81 | 18.31 ± 0.73 | 0.05* | 17.16 ± 0.76 | 17.14 ± 0.78 | 0.99 | 15.64 ± 1.08 | 18.68 ± 1.08 | 16.35 ± 1.21 | 17.93 ± 0.98 | 0.51 |

CON=control diet; ARG=diet with 1.0% L-arginine supplementation;
*$P \leq 0.05$

**Table 4:** Averages ± Standard Errors and *P*-values of conceptuses phenotypic data

| Conceptuses traits | CON | ARG | *P*-value |
|---|---|---|---|
| EW (g) | 0.79±0.13 | 1.05±0.13 | 0.07 |
| FW (g) | 3.23±0.28 | 2.84±0.25 | 0.19 |
| CC (mm) | 28.82±0.87 | 27.34±0.82 | 0.05* |

EW= Embryos weight, FW= Fetuses weight, CC=Cephalic-caudal length of the fetuses.
CON=control treatment; ARG=treatment with 1.0% L-arginine supplementation
*$P \leq 0.05$

## SUPPLEMENTARY MATERIAL

**Supplementary table 1:** Relative expression of developmental and apoptosis genes in embryos ($2^{-\Delta Ct}$). Mean and Standard Error.

| Gene | Diet | Mean | Standard Errors |
|---|---|---|---|
| SHH | CON | 0.000324 | 0.000102 |
| SHH | ARG | 0.000169 | 0.000105 |
| DHH | CON | 0.000192 | 0.000060 |
| DHH | ARG | 0.000181 | 0.000061 |
| IHH | CON | 0.000889 | 0.000205 |
| IHH | ARG | 0.000637 | 0.000211 |
| WNT1 | CON | 0.000480 | 0.000153 |
| WNT1 | ARG | 0.000237 | 0.000156 |
| FGF8 | CON | 0.000873 | 0.000131 |
| FGF8 | ARG | 0.000888 | 0.000131 |
| IGF1 | CON | 0.000929 | 0.000186 |
| IGF1 | ARG | 0.001528 | 0.000186 |
| SOX6 | CON | 0.000076 | 0.000017 |
| SOX6 | ARG | 0.000099 | 0.000018 |
| BAX | CON | 0.6005 | 0.1121 |
| BAX | ARG | 0.8124 | 0.1121 |
| BCL2 | CON | 0.001526 | 0.000183 |
| BCL2 | ARG | 0.001039 | 0.000183 |
| CASP3 | CON | 0.004913 | 0.001115 |

| | | | |
|---|---|---|---|
| CASP3 | ARG | 0.005038 | 0.001132 |

**Supplementary table 2:** Relative expression of developmental and apoptosis genes in fetuses ($2^{-\Delta Ct}$). Mean and Standard Error.

| Gene | Diet | Mean | Standard Errors |
|---|---|---|---|
| SHH | CON | 0.000094 | 0.000021 |
| SHH | ARG | 0.000087 | 0.000017 |
| DHH | CON | 0.000173 | 0.000057 |
| DHH | ARG | 0.000262 | 0.000045 |
| IHH | CON | 0.000870 | 0.000216 |
| IHH | ARG | 0.000938 | 0.000171 |
| WNT1 | CON | 0.000072 | 0.000058 |
| WNT1 | ARG | 0.000140 | 0.000046 |
| FGF8 | CON | 0.002375 | 0.000687 |
| FGF8 | ARG | 0.002135 | 0.000548 |
| IGF1 | CON | 0.001115 | 0.000342 |
| IGF1 | ARG | 0.001222 | 0.000274 |
| SOX6 | CON | 0.000231 | 0.000076 |
| SOX6 | ARG | 0.000157 | 0.000060 |
| BAX | CON | 0.5764 | 0.1297 |
| BAX | ARG | 0.4190 | 0.1025 |
| BCL2 | CON | 0.007803 | 0.002765 |
| BCL2 | ARG | 0.003306 | 0.002186 |
| CASP3 | CON | 0.01250 | 0.003301 |
| CASP3 | ARG | 0.006380 | 0.002609 |

## CHAPTER 3

**[Submitted to Molecular Reproduction and Development]**

# L-Arginine supplementation during early gestation modulates the mTOR pathway in pig conceptuses

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**Running title:** Effects of L-arginine supplementation on conceptuses mTOR pathway

## ABSTRACT

There is growing evidence that L-arginine supplementation during gestation activates the mTOR signaling pathway in conceptuses, influencing cellular proliferation, migration, and differentiation. However, the underlying mechanisms have not been fully elucidated. Therefore, we aimed to investigate the expression of genes and abundance of proteins involved in the mTOR pathway in 25-day embryos and 35-day fetuses from gilts receiving either a control diet (CON) or CON diet supplemented with 1.0% L-arginine (ARG). Gene expression and protein analyses were performed within each gestational age (25 and 35 days), considering a completely randomized design with two treatments (CON and ARG). At 25 days, CON embryos showed greater phospho-AMPK protein abundance than ARG embryos ($P = 0.04$). On the other hand, at 35 days of gestation, CON fetuses had higher *MTOR* ($P = 0.05$) and lower *MLST8* ($P = 0.04$)

gene expression as well as higher phospho-mTOR ($P = 0.006$) and phospho-AMPK ($P = 0.007$) protein abundance. In addition, *AMPK* expression tended to be higher ($P = 0.07$) in CON fetuses. Overall, our results demonstrate that supplementation of gilts with 1.0% L-arginine during early gestation modulates the mTOR signaling pathway and energy metabolism in 25- and 35-day pig conceptuses.

**KEYWORDS**

amino acid supplementation, cellular proliferation, fetal programming, nutrigenomics, protein synthesis

## 1. INTRODUCTION

Arginine is a functional amino acid for mammals, influencing the production of agmatine, creatine, ornithine, nitric oxide (NO), polyamines, proline, and glutamate. These products play key roles in metabolism and molecular mechanisms (Wu & Morris, 1998), including DNA and protein synthesis (Igarashi & Kashiwagi, 2000; Wu & Morris, 1998; Zeng et al., 2013), cell and tissue differentiation (Wu et al., 2013), cell signaling (Williams, 1997), and hormone secretion (Barbul, 1986; Reyes, Karl, & Klahr, 1994). Studies have shown that L-arginine supplementation of sows during different stages of gestation improves embryo survival (Bérard & Bee, 2010; Li et al., 2014), conceptus growth, placental development (Gao et al., 2012), number of piglets born and born alive (Gao et al., 2012; J. Li et al., 2015; Mateo et al., 2007), and litter uniformity (Quesnel et al., 2014). In addition, L-arginine supplementation was shown to enhance embryonic implantation in mice (Zeng et al., 2013) and prevent intrauterine growth retardation in several mammalian species (Lassala et al., 2010; Mateo et al., 2007; Xiao & Li, 2005).

Arginine is involved in cellular proliferation, migration, and differentiation (Ishida, Hiramatsu, Masuyama, Mizutani, & Kudo, 2002; Meininger & Wu, 2002; Wu et al., 2013) through the production of polyamines and NO, which participate in DNA and protein synthesis (Igarashi & Kashiwagi, 2000; Meininger & Wu, 2002; Zeng et al., 2013). There is also increasing evidence that arginine activates the mammalian target of rapamycin (mTOR) pathway and other kinase-mediated signaling pathways in different cell types (Kong et al., 2012; J Marc Rhoads et al., 2007; Wang et al., 2017), influencing cell migration (J M Rhoads et al., 2004) and proliferation (Kong et al., 2012). Supplementation of female mice with 1.3%

L-arginine during early gestation enhanced embryo implantation through stimulation of phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB), mTOR, and NO signaling pathways, leading to greater abundance of mTOR-regulated proteins in the offspring (Zeng et al., 2013).

The mTOR pathway coordinates protein synthesis and cell metabolism, growth, and proliferation in response to growth factors, amino acids, energy levels, and stress (Sarbassov, Ali, & Sabatini, 2005) via two distinct protein complexes: mTORC1 and mTORC2 (Sarbassov et al., 2004; Saxton & Sabatini, 2017). mTORC1 triggers cell growth and proliferation, promoting protein synthesis and lipid biogenesis while reducing cell autophagy (Saxton & Sabatini, 2017). mTORC2 regulates cell survival and metabolism, cytoskeleton organization, and response to growth factors (Laplante & Sabatini, 2013). The mTORC2 complex is insensitive to amino acids. Therefore, arginine likely activates the mTOR pathway through the mTORC1 complex (González et al., 2012; Kong et al., 2012). Although the functions of the mTOR pathway have not yet been fully elucidated, there is evidence that mTOR is the central component of a complex signaling network that regulates cell growth, proliferation and possibly animal size (Sarbassov et al., 2005).

Despite the importance of mTOR pathway, the mechanisms by which L-arginine supplementation during gestation influences this pathway still needs to be better understood. Considering that there are some metabolic and physiological similarities between pigs and other mammals, we used pigs as a model to test the hypothesis that L-arginine supplementation in early gestation impacts cellular processes in conceptuses through the mTOR pathway. Therefore, we aimed to evaluate the expression of genes and the abundance of mTOR pathway proteins in 25-day embryos and 35-day fetuses from gilts supplemented with 1.0% L-arginine during early gestation.

## 2. MATERIALS AND METHODS

### 2.1 Experimental design

Experimental procedures were in accordance with Brazilian guidelines on animal experimentation (CONCEA, 2016). The study was approved by the Animal Ethics Committee of Universidade Federal de Viçosa (UFV), Minas Gerais, Brazil (protocol no. 06/2017).

The experimental design and reproductive and dietary management are described in Costa et al. (2019). Briefly, 23 commercial gilts aged 120 days were housed in individual pens. Puberty induction was started at 150 days of age by exposure of gilts to an adult male twice

daily (morning and afternoon). After gilts showed their third estrus, estrus synchronization was performed using Regumate (Merck Animal Health, USA). Females were inseminated 12 and 24 h after the beginning of the fourth estrus with semen from two commercial boars. At each insemination, each female was inseminated with semen from the same boar. Within a treatment, there were gilts inseminated with semen from boar 1 and gilts inseminated with semen from boar 2.

Gilts were randomly assigned to either a control diet (CON) or the CON diet supplemented with 1.0% L-arginine (ARG) (Ajinomoto, Saga, Japan). The CON diet consisted of a basal diet formulated to meet the nutritional requirements of gestating gilts (Rostagno et al., 2011), composed mainly of corn, soybean meal, and mineral–vitamin supplement. The supplemented diet was prepared by replacing clay filler with L-arginine in the basal diet. Eleven gilts were assigned to the CON and 12 to the ARG diet 24 h after the second insemination.

Diets provided the same amounts of metabolizable energy (3148 kcal/kg), calcium (0.750%), phosphorus (0.395%), digestible amino acids, lysine (0.535%), methionine and cysteine (0.381%), threonine (0.412%), tryptophan (0.113%), and valine (0.490%). These values represent the nutritional requirements for pregnant gilts recommended by Rostagno et al. (2011). CON and ARG diets differed in crude protein (12.16 vs. 14.46%, respectively) and digestible arginine (0.67 vs. 1.60%, respectively). Arginine concentration did not exceed 2.0% in either diet so as to prevent ammonia intoxication and competition for basic amino acid transporters (Wu et al., 2013). Diets were provided twice daily (9 am and 4 pm) in equal portions. A total of 1.8 kg/day was offered between days 1 and 3 of gestation, and 2.2 kg/day was offered between days 4 and 24 (for females slaughtered at 25 days of gestation) or days 4 and 34 (for females slaughtered at 35 days of gestation). Animals had free access to water throughout the experimental period.

Pregnancy was confirmed 20 days after insemination. Of the 23 gilts used in the experiment, 20 became pregnant. At 25 days of gestation, five females of CON ($n = 5$) and five females of ARG ($n = 5$) were killed by head-only electrical stunning (240 V, 1.3 A) and bleeding. At 35 days of gestation, four females of CON ($n = 4$) and six females of ARG ($n = 6$) were killed following the same procedures.

### 2.2 Conceptus collection

Conceptuses were obtained from CON and ARG gilts at 25 days of gestation ($n = 5$ gilts per treatment) and 35 days of gestation ($n = 4$ CON and $n = 6$ ARG gilts) using the method

described in Costa et al. (2019). Gene expression and protein analyses were performed at each gestational age (25 and 35 days of gestation).

For gene expression analysis, four conceptuses were collected from each female at each gestational age, two from the cranial region and two from the caudal region of the right uterine horn (20 CON conceptuses and 20 ARG conceptuses at 25 days and 16 CON conceptuses and 24 ARG conceptuses at 35 days). For protein analysis, one conceptus was collected from each female from the cranial region of the right uterine horn (5 CON embryos and 5 ARG embryos at 25 days and 4 CON fetuses and 6 ARG fetuses at 35 days).

**2.3 Gene expression analysis**

Total RNA was extracted from 50 mg of embryos and fetuses' samples (whole conceptus sprayed with nitrogen) with TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The resulting precipitate was rehydrated with 30 μL of UltraPure DNase/RNase-Free water. RNA concentration was estimated using a NanoVue Plus spectrophotometer (GE Healthcare, Freiburg, Germany), and RNA integrity was determined in 1.0% agarose gel (data not shown). The first cDNA strand was synthesized using the GoScript Reverse Transcriptase kit (Promega Corporation, Madison, WI, USA) and cDNA concentration was determined on a NanoVue Plus spectrophotometer. cDNA samples were stored at −20 °C until their use in real-time quantitative polymerase chain reaction (RT-qPCR).

Primers for amplification of target and endogenous gene sequences were designed using PrimerQuest software (Integrated DNA Technologies Inc., Coralville, IA, USA) and nucleotide sequences obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) (Table 1). The β-actin (*BACT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and hypoxanthine-guanine phosphoribosyltransferase 1 (*HPRT1*) genes were tested as endogenous controls. These genes were selected on the basis of their amplification profiles and dissociation curves (data provided under request). *BACT*, *GAPDH*, and *HPRT1* expression did not differ between treatments (CON and ARG) at 25 or 35 days of gestation. *BACT* was used as the reference gene for data normalization because of its higher expression and stability. The following target genes were assessed: *MTOR*, mammalian lethal with Sec13 protein 8 (*MLST8*), AMP-activated protein kinase (*AMPK*), eukaryotic translation initiation factor 4E (*EIF4E*), eukaryotic translation initiation factor 4B (*EIF4B*), eukaryotic translation initiation factor 4E-binding protein 1 (*EIF4EBP1*), and ribosomal protein S6 kinase B1 (*RPS6KB1*).

The RT-qPCR analyses were performed in duplicates using a 7500 Real Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Beverly, MA, USA), SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and GoTaq qPCR Master Mix (Promega Corporation, Madison, WI, USA). PCR reactions were submitted to the cycles protocol according to the program: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplification efficiencies of targets and endogenous genes were approximately 100% in each cycle (data provided under request). The threshold cycle (Ct) values obtained were later normalized (ΔCt) based on the Ct values of the endogenous control gene (*BACT*). The calculation of the relative gene expression levels was performed according to the $2^{-\Delta Ct}$ method, described by Livak & Schmittgen (2001).

**2.4 Protein analysis**

Total proteins were extracted from 50 mg of whole conceptus with 1 mL of lysis buffer (10 mM Tris-HCl, 100 mM NaCl, 0.5 mM of dithiothreitol (DDT), 2.5 mM $MgCl_2$, 0.5% Triton X-100, and 1% protease inhibitor cocktail; Sigma–Aldrich, San Luis, MO, USA). Protein concentration was determined by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein extracts were stored at −80 °C until separation.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% gels loaded with 80 μg protein per well. Each gel contained samples from CON and ARG conceptuses of the same gestational age as well as an internal loading control for normalization. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes. All membranes were blocked with blocking solution (3% w/v bovine serum albumin in 1× Tris-buffered saline). Membranes were incubated overnight at 4 °C with phospho-mTOR and phospho-AMPK rabbit primary antibodies (Sigma–Aldrich, Poole, Dorset, UK), diluted 1:1000 in blocking solution. After incubation, membranes were washed with Tris-buffered saline containing 0.1% Tween (TBST) and incubated with peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Sigma–Aldrich, Poole, Dorset, UK), diluted 1:5000 in blocking solution. Blots were washed three times with TBST and revealed using ECL Clarity substrate (Bio-Rad Laboratories, Hercules, CA, USA), and analyzed using a C-DiGit Blot scanner (Licor Biosciences, Lincoln, NE, USA). Proteins were quantified using Image Studio (Licor Biosciences, Lincoln, NE, USA). Values of each protein expression were normalized by the control, in which for internal control, a reference sample from each treatment was collected and both samples were mixed to make a pool that was used as a loaded on each gel. The band

intensity from the internal control (expressed by optic densitometry units) was used to normalize the remaining samples, as described by Cruzen, Paulino, Lonergan, & Huff-lonergan (2014).

### 2.5 Statistical analysis

Relative gene expression data ($2^{-\Delta Ct}$) were analyzed within each gestational age and subjected to nested analysis of variance (ANOVA) using the MIXED procedure of SAS version 9.0 (Statistical Analysis System Institute, Inc., Cary, NC, USA). Residual normality test was performed using the UNIVARIATE procedure of SAS. Conceptuses were considered false replicates of the females for each treatment, represented by a nested effect in the statistical model. Results were considered significant when $P \leq 0.05$. *P*-values between 0.06 and 0.10 were considered a trend.

The following statistical model was used for analysis of gene expression data for each gestational age:

$$Y_{ijkl} = \mu + D_i + C\{D_{(i)}\}_j + B_k + \varepsilon_{ijkl}$$

where $Y_{ijkl}$ is the observation (relative expression) of the *j*-th conceptus; $\mu$ is the trait general mean; $D_i$ is the effect of the *i*-th treatment (CON or ARG); $C\{D_{(i)}\}_j$ is the random effect of conceptus *j* nested in treatment *i*; $B_k$ is the random effect of the *k*-th boar; and $\varepsilon_{ijkl}$ is the random error.

Conceptus protein abundance data from each gestational age were also subjected to nested ANOVA using the MIXED procedure of SAS. Residual normality test was performed using the UNIVARIATE procedure of SAS.

The following statistical model was used for analysis of protein abundance data for each gestational age:

$$Y_{ijk} = \mu + D_i + B_j + \varepsilon_{ijk}$$

where $Y_{ijk}$ is the observation (protein abundance), $\mu$ is the trait general mean; $D_i$ is the effect of *i*-th treatment (CON or ARG); $B_j$ is the random effect of *j*-th boar; and $\varepsilon_{ijk}$ is the random error.

## 3. RESULTS

### 3.1 Expression of mTOR pathway genes

No differences were observed in the expression of *MTOR* ($P = 0.88$), *MLST8* ($P = 0.89$), *AMPK* ($P = 0.98$), *EIF4E* ($P = 0.44$), *EIF4B* ($P = 0.44$), *EIF4EBP1* ($P = 0.55$), and *RPS6KB1* ($P = 0.49$) between CON and ARG embryos at 25 days of gestation (Table 2).

At 35 days of gestation, no differences were observed in the expression of *EIF4E* ($P$ = 0.40), *EIF4B* ($P$ = 0.86), *EIF4EBP1* ($P$ = 0.31), and *RPS6KB1* ($P$ = 0.94) between CON and ARG fetuses. However, CON fetuses had higher *MTOR* expression ($P$ = 0.05) and lower *MLST8* expression ($P$ = 0.04) than ARG fetuses. *AMPK* tended to be more highly ($P$ = 0.07) expressed in CON fetuses (Table 3).

### 3.2 Abundance of mTOR pathway proteins

Pospho-AMPK protein abundance was higher in CON than in ARG 25-day embryos ($P$ = 0.04), but phospho-mTOR abundance did not differ between treatments ($P$ = 0.85) (Figure 1.A). At 35 days, CON fetuses had higher phospho-AMPK ($P$ = 0.007) and phospho-mTOR ($P$ = 0.006) abundances than ARG fetuses (Figure 1.B).

## 4. DISCUSSION

Supplementation of gestating sows with L-arginine has been shown to reduce two major reproductive problems: intrauterine growth retardation (Kong et al., 2012) and conceptuses prenatal mortality (Bérard, Kreuzer, & Bee, 2008; X. Li et al., 2014). The cellular and molecular mechanisms by which L-arginine regulates conceptus development are not well established; however, some authors suggested that, at the cellular level, L-arginine may influence cellular proliferation, migration, and differentiation (Ishida et al., 2002; Meininger & Wu, 2002) through mTOR pathway activation (Wu et al., 2013).

Our results indicated that L-arginine supplementation of gilts during early gestation influences the expression and abundance of genes and proteins related to the mTOR pathway in conceptuses. The pathway integrates intracellular and extracellular signals and acts as a central regulator of cell survival and growth (Laplante & Sabatini, 2009) mainly through mTORC1 (Kim et al., 2002; Sarbassov et al., 2004; Saxton & Sabatini, 2017). Amino acids such as arginine represent strong signals that upregulate mTORC1 (González et al., 2012; Guertin & Sabatini, 2007; Hara et al., 1998). The mTORC1 complex is composed of three major units: mTOR, raptor (regulatory-associated protein of mTOR), and mLST8 (Saxton & Sabatini, 2017). Activated mTORC1 induces protein synthesis via phosphorylation and activation of 4EBP1 (*EIF4EBP1*) and S6K1 (*RPS6KB1*), the major regulators of mRNA translation and ribosome synthesis (Lynch, 2001).

Phosphorylation of 4EBP1 (which occurs in response to insulin, growth factors, hormones, and other stimuli) prevents its binding to eIF4E and promotes cap-dependent translation initiation, increasing eIF4E availability (Pause et al., 1994; Saxton & Sabatini, 2017).

Association of eIF4E with the 4F complex is the limiting step in translation initiation. RPS6KB1 responds to mTOR signaling to stimulate protein synthesis through phosphorylation of other target proteins, such as eIF4B (Magnuson, Ekim, & Fingar, 2012), which is required for mRNA to bind to ribosomes. In our study, no differences in *EIF4EBP1*, *EIF4E*, *EIF4B*, and *RPS6KB1* gene expression were observed between CON and ARG treatments in embryos (25 days) or fetuses (35 days). According to González et al. (2012), arginine can activate biochemical processes in mouse embryos independently of 4EBP1 and S6K1. In line with this finding, in the present study, no differences were observed in *MTOR*, *MLST8*, and *AMPK* expression between CON and ARG embryos.

mTOR pathway activation is regulated by energy availability. For instance, during energy deficiency, the pathway is inhibited by AMPK (Howell et al., 2017; Laplante & Sabatini, 2012). AMPK detects changes in the intracellular AMP/ATP ratio and inhibits cell growth and proliferation to maintain cell homeostasis (Carling, 2004; Hardie, 2007). In response to nutrient limitations, AMPK downregulates the mTORC1 complex by raptor phosphorylation and phosphorylation and activation of tuberous sclerosis complex 2 (TSC2) to stimulate the GTPase activating protein (GAP), which inhibits mTOR (Gwinn et al., 2008).

Although no differences were observed in the expression of mTOR pathway genes between CON and ARG embryos, a greater abundance of phospho-AMPK protein was found in CON embryos. This result may be due to the increase in the intracellular AMP/ATP ratio from energy deficiency. The energy availability in conceptuses is related with maternal nutrition; in this context, the greater arginine concentration on ARG diet may explain the lower abundance of phospho-AMPK in embryos from ARG gilts. In another study by our research group, a trend for interaction between diet (CON and ARG) and treatment duration (until 25 and 35 days of gestation) was observed on arginine concentration in the blood plasma of gilts (Costa et al., 2019). We found that pregnant gilts supplemented until 25 days had higher blood plasma arginine concentration than CON gilts. At 35 days of gestation, however, the opposite was observed (Costa et al., 2019).

Differences in phospho-AMPK abundance between CON and ARG embryos did not lead to differences in the transcription of *MTOR* and other genes or phospho-mTOR abundance. In addition to influencing cell proliferation and growth, AMPK may influence the insulin pathway (Chopra, Li, Wang, & Webster, 2012), affecting the conceptuses development. The insulin signaling pathway is activated when insulin or insulin-like growth factor 1 (IGF1) binds to the

insulin receptor (IR). This leads to activation of insulin receptor kinase (IRK) and autophosphorylation of IR, promoting anabolic responses. Conversely, AMPK activation represses anabolic responses in order to conserve ATP resources (Chopra et al., 2012). In this study, the low phospho-AMPK abundance in ARG embryos might have affected the insulin pathway. This result agrees with the findings of our previous study showing that, at 25 days, *IGF1* expression was higher in ARG embryo, besides ARG embryos had a tendency to present higher weight than CON embryos (Costa et al., 2019).

At 35 days of gestation, *MTOR* expression was higher and *MLST8* expression was lower in CON than in ARG fetuses. mTOR is a kinase that acts as a sensor of nutrient availability in cells (Tavares et al., 2015). mLST8 is a subunit of mTORC1 and mTORC2 complexes that regulates cell growth and survival in response to nutritional and hormonal levels (Kim et al., 2003). In mTORC1, mLST8 either increases mTOR kinase activity or, under low nutrient conditions, stabilizes the raptor-mTOR interaction and favors raptor-mediated inhibition of mTOR activity (GeneCards, 2019).

Phospho-AMPK and phospho-mTOR abundances were highest in CON fetuses. High AMPK activity is commonly related to low expression of mTOR genes and proteins. However, we found high *MTOR* expression and phospho-mTOR abundance in CON fetuses. According to Um, D'Alessio, & Thomas (2006), in some situations, AMPK phosphorylation of TSC2 is not necessary for activation of responses to energy alterations; other mechanisms may be implicated in mTORC1 activation. The high *MLST8* and low *MTOR* expression in ARG fetuses might indicate nutritional deficiencies, aggravated by the long supplementation time such as amino acid antagonism and impairment. Therefore, in our study, the control of *MTOR* expression and phospho-mTOR protein abundance likely occurred in response to nutritional status of gilts and the low arginine concentration in the blood plasma of ARG gilts (Costa et al., 2019). In addition, the lower *MTOR* expression and phospho-mTOR abundance on ARG fetuses may have influenced fetuses development, since our previous study showed that ARG fetuses have a shorter cephalic-caudal length (Costa et al., 2019).

The differences found between embryos and fetuses were probably due to the duration of gilts L-arginine supplementation. The gestation period of supplementation is essential for its effects on female reproductive performance (Wu et al., 2013). According to Dioguardi (2011), in long-term arginine supplementation, exogenous arginine is largely destroyed by the gut. In this case, the more exogenous arginine is introduced, the more it is destroyed by arginase types

I and II (Dioguardi, 2011; Grody et al., 1989; Mori, Gotoh, Nagasaki, Takiguchi, & Sonoki, 1998). Although it is known that mTORC1 responds to arginine, it is still unclear which is the physiological arginine sensor that activates mTOR (Jung et al., 2019).

## 5. CONCLUSIONS

Supplementation of gilts with 1.0% L-arginine during early gestation affects the expression and abundance of mTOR pathway genes and proteins related to energy metabolism in 25-day embryos and 35-day fetuses.

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## AUTHOR CONTRIBUTIONS

Karine Assis Costa, Alysson Saraiva, and Simone Eliza Facioni Guimarães contributed to all stages of research development and manuscript preparation, including study conception and design; acquisition, analysis, and interpretation of data; and drafting and critical revision of the manuscript for important intellectual content. Gustavo Amorim Rodrigues, Lívia Maria Barbosa, Breno Soares Camilo, Domingos Lollobrigida de Souza Neto, and José Domingos Guimarães participated in the conception, design, and acquisition of gene expression data. Thaís Correia Costa and Márcio de Souza Duarte carried out protein analysis. Daniele Botelho Diniz Marques and Pamela Itajara Otto participated in statistical analysis and data interpretation. All authors participated in the drafting and critical revision of the manuscript for important intellectual content.

## COMPETING INTERESTS

The authors have nothing to disclose.

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**TABLE 1** Primers design

| GENE | Abbreviation | NCBI accession code | Primer sequence |
|---|---|---|---|
| β- actin | *BACT* | XM_0031242803 | F:CTTCTAGGCGGACTGTTAGTG |
| | | | R:AGCCATGCCAATCTCATCTC |
| Hypoxanthine-guanine phosphoribosyltransferase 1 | *HPRT1* | NM_001032376.2 | F:CCAGTCAACGGGCGATATAA |
| | | | R:GACCAAGGAAAGCAAGGTTG |
| Glyceraldehyde-3-phosphate dehydrogenase | *GAPDH* | NM_001206359.1 | F:CAAAGTGGACATTGTCGCCATCA |
| | | | R:AGCTTCCCATTCTCAGCCTTGACT |
| Mammalian target of rapamycin | *MTOR* | XM_003127584.6 | F: GCGATAGACACCCATCTAACC |
| | | | R: CTTCTCTCTGGTCATAGCAACC |
| Mammalian lethal with Sec13 protein 8 | *MLST8* | XM_013987830.2 | F: GGTCTTGTACAGAGTGTAACCC |
| | | | R: CTCTGGTGTTCCTTGCTCTT |
| AMP-activated protein kinase | *AMPK* | NM_001167633.1 | F: GTTTCAGGAGGCGAGCTATT |
| | | | R: ACCACCATATGCCTGTGAC |
| Eukaryotic translation initiation factor 4E | *EIF4E* | XM_005656555.3 | F: CAAACCTTCGGCTGATCTCT |
| | | | R: GTAGTCACAGCCAGGCATTA |
| Eukaryotic translation initiation factor 4B | *EIF4B* | XR_002343952.1 | F: AGAAGAGCGTTTGGTAGTGG |
| | | | R: CGGTCATCTCGTCTGTCATATC |
| Eukaryotic translation initiation factor 4E-binding protein 1 | *EIF4EBP1* | NM_001244225.1 | F: GCCAGGCCTTATGAAAGTTG |
| | | | R: GAGGTATCTGCTGGTGTTCA |
| Ribosomal protein S6 kinase B1 | *RPS6KB1* | XM_021067294.1 | F: GCTGTGGCGAAATGTAAAGG |
| | | | R: GGCAGTCGACACTACAGTTAG |

F, forward primer; R, reverse primer.

**TABLE 2** Relative expression of mTOR pathway genes in embryos at 25 days of gestation ($2^{-\Delta Ct}$)

| Gene | CON (Mean±Standard error) | ARG (Mean±Standard error) | Degrees of freedom | *F*-value | *P*-value |
|---|---|---|---|---|---|
| *MTOR* | 0.1340 ± 0.0660 | 0.1218 ± 0.0679 | 8 | 0.02 | 0.88 |
| *MLST8* | 0.0005 ± 0.0003 | 0.0004 ± 0.0003 | 8 | 0.02 | 0.90 |
| *AMPK* | 0.0227 ± 0.0126 | 0.0231 ± 0.0129 | 8 | 0.00 | 0.98 |
| *EIF4E* | 0.0016 ± 0.0005 | 0.0010 ± 0.0005 | 8 | 0.67 | 0.44 |
| *EIF4B* | 0.9989 ± 0.0003 | 0.9993 ± 0.0003 | 8 | 0.67 | 0.44 |
| *EIF4EBP1* | 0.1639 ± 0.2014 | 0.2864 ± 0.2070 | 8 | 0.40 | 0.55 |
| *RPS6KB1* | 0.0075 ± 0.0049 | 0.0027 ± 0.0050 | 8 | 0.51 | 0.49 |

CON=control diet; ARG=diet with 1.0% L-arginine supplementation.

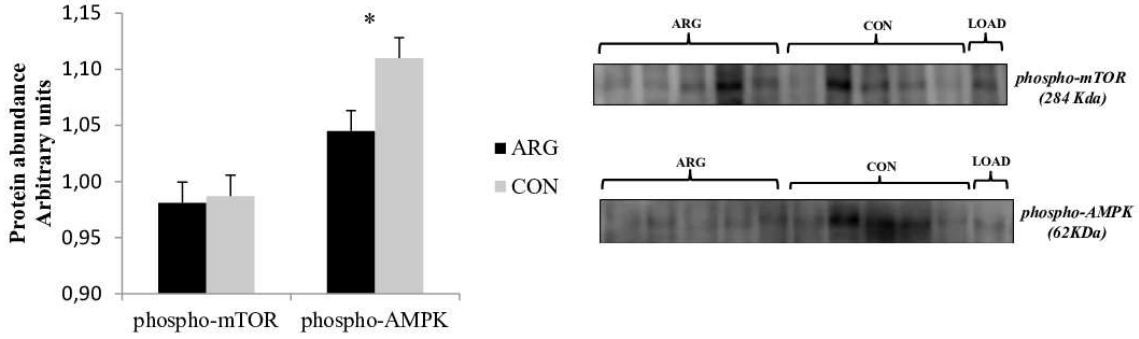
**TABLE 3:** Relative expression of mTOR pathway genes in fetuses at 35 days of gestation ($2^{-\Delta Ct}$).

| Gene | CON (Mean±Standard error) | ARG (Mean±Standard error) | Degrees of freedom | *F*-value | *P*-value |
|---|---|---|---|---|---|
| *MTOR* | 0.2283 ± 0.0384 | 0.1376 ± 0.0326 | 8 | 5.27 | 0.05* |
| *MLST8* | 0.0008 ± 0.0004 | 0.0021 ± 0.0003 | 8 | 5.79 | 0.04* |
| *AMPK* | 0.0219 ± 0.0038 | 0.0128 ± 0.0031 | 8 | 4.46 | 0.07 |
| *EIF4E* | 0.0801 ± 0.0141 | 0.0640 ± 0.0111 | 8 | 0.80 | 0.40 |
| *EIF4B* | 1.5416 ± 0.3583 | 1.6213 ± 0.2832 | 8 | 0.03 | 0.87 |
| *EIF4EBP1* | 0.2085 ± 0.0529 | 0.1348 ± 0.0424 | 8 | 1.18 | 0.31 |
| *RPS6KB1* | 0.0074 ± 0.0020 | 0.0076 ± 0.0016 | 8 | 0.01 | 0.94 |

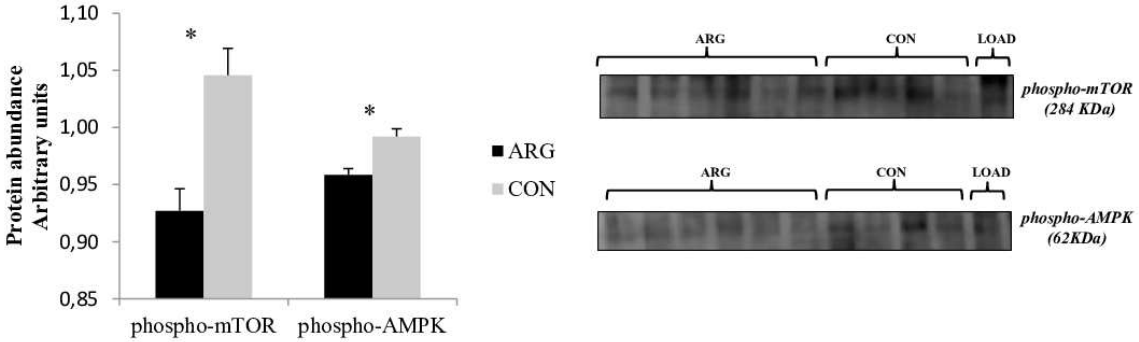
CON=control diet; ARG=diet with 1.0% L-arginine supplementation;
*$P \leq 0.05$

**FIGURE 1** Representative images of protein abundance and western blotting analysis for phospho-mTOR and phospho-AMPK (Arbitrary units); Protein abundance was measured in samples of whole 25-day embryos (1.A) and 35-day fetuses (1.B) from gilts not supplemented (CON) or supplemented with 1.0% L-arginine (ARG); Protein abundances were represented by Means±Standard errors. *$P \leq 0.05$

**1.A**




**1.B**



# CHAPTER 4

## Effects of gilts L-arginine supplementation on epigenetic mechanisms in 25-day embryos and 35-day fetuses

**ABSTRACT:** Maternal nutrition is one of the major environmental factors that regulate gene expression during fetal development through epigenetic modifications. In this context, some nutrients, such as L-arginine, have been used on maternal diets, affecting gene expression and improving females' reproductive performance and conceptuses development. Based on the hypothesis that gilts L-arginine supplementation regulates gene expression through epigenetic mechanisms, we aimed to evaluate some fetal programming phenotypic markers and the expression of epigenetic genes in 25 and 35-day conceptuses from gilts not supplemented (CON) or supplemented with 1.0% L-arginine (ARG) during gestation. At 25 days, there was only a trend for ARG embryos to present greater liver weight compared with CON embryos ($P$=0.09). On the other hand, fetuses from CON and ARG gilts presented no differences regarding phenotypic markers at 35 days. In addition, maternal L-arginine supplementation did not influence the expression of the evaluated epigenetic genes in embryos and fetuses. Therefore, in general, the 1.0% L-arginine supplementation of gilts during pregnancy did not alter the expression of epigenetic genes and phenotypic markers evaluated in conceptuses at 25 and 35 days. In this sense, L-arginine effects on conceptuses gene expression and phenotypic markers may occur through other regulation mechanisms.

**Keywords:** Development, DNA methylation, Fetal programming, Nutrigenomics, Posttranslational histone modifications.

### 1. Introduction

During prenatal development, epigenetic processes, such as DNA methylation, posttranslational histone modifications, histone variants, and non-coding RNA (ROMANI; PISTILLO; BANELLI, 2018; ELOLIMY et al., 2019), can significantly impact conceptuses chromatin structure, regulating gene expression and, consequently, cell differentiation and organogenesis influencing on conceptuses phenotypes (JAENISCH; BIRD, 2003; CHMURZYNSKA, 2009).

Changes in DNA methylation during mammalian development occur firstly during cleavage with a demethylation followed by de novo genome methylation after implantation (JAENICH, 1997). Epigenetic modifications regulate the transition of the expression of genes related to cell pluripotency in early development to genes related to cell differentiation and

fetuses morphogenesis (REIK, 2007). In addition, DNA methylation is also involved in other biological processes, such as genomic imprinting, X chromosome inactivation and suppression of repetitive elements (LI; ZHANG, 2014). Histone modifications are also implicated in chromatin structure alteration, playing a key role in the regulation of gene expression by transcription or repression (JAENISCH; BIRD, 2003; MUNSHI et al., 2009; ELOLIMY et al., 2019).

Livestock maternal nutrition during gestation can alter the fetal and postnatal epigenome and transcriptome, leading to short or long term changes on animals physiology and metabolism (ZACCHINI; TOSCHI; PTAK, 2017; ELOLIMY et al., 2019). Dietary compounds induce epigenetic changes by altering DNA methylation and histone modifications in different cells and tissues (DELAGE; DASHWOOD, 2008; CANANI et al., 2011; ELOLIMY et al., 2019). Therefore, these alterations during prenatal development as a result of modifications in the maternal diet may affect conceptuses growth and development (KITSIOU-TZELI; TZETIS, 2017) and the postnatal performance of the animals (DU et al., 2010).

The conditionally essential amino acid L-arginine has been used in dietary supplementation of mammal females during gestation to improve their reproductive performance via modifications at cellular and molecular levels on mother and offspring. The improvements are related with the expression of genes associated with processes such as placental angiogenesis (GREENE et al., 2012), cells differentiation (WU et al., 2010, 2013), conceptuses growth (COSTA et al., 2019), among others. Gene expression is mainly regulated by epigenetic mechanisms, and during production of arginine metabolites such as creatine and polyamines from ornithine, there is a large sequestration of methyl groups by S-adenosylmethionine (SAM). In addition, nitric oxide (NO), another important arginine metabolite, plays a role in histone posttranslational modifications (SOCCO et al., 2017; PALCZEWSKI; PETRAITIS; THOMAS, 2019) and in other epigenetic mechanisms (SOCCO et al., 2017). These modifications suggest that supplementation of pregnant females with L-arginine can affect gene expression through epigenetic mechanisms, and the alterations may occur in both mother and conceptuses.

Based on the hypothesis that gilts L-arginine supplementation can lead to conceptuses epigenetic modifications and affect phenotypic markers of fetal programming such as organs weights, we aimed to evaluate the effects of gilts 1.0% L-arginine supplementation on phenotypic markers and expression of epigenetic genes in 25-day embryos and 35-day fetuses.

## 2. Materials and methods

### *2.1 Experimental design*

The ethical Committee on Animal Use of Universidade Federal de Viçosa (UFV), Minas Gerais, Brazil [protocol number 06/2017] approved the experimental protocol that followed ethical principles in animal research (CONCEA, 2016).

The experimental design, reproductive and dietary managements were described in detail by COSTA et al. (2019). Briefly, gilts were inseminated 12 and 24 hours after the beginning of the fourth estrus. The first insemination day was considered day zero of gestation and the supply of experimental diets occurred 24 hours after the second insemination. Semen doses used to inseminate the gilts were collected from two commercial boars with proven reproductive performance (semen analyses). Each female was inseminated with semen from the same boar, and, within treatment, there were gilts inseminated with semen from one boar and gilts inseminated with semen from the other boar.

Considering the 23 commercial gilts inseminated, 11 received the control diet (CON), mainly composed of corn, soybean meal, mineral and vitamins supplements, formulated in order to meet the nutritional requirements of gestating gilts (ROSTAGNO et al., 2011) and 12 received the CON diet supplemented with 1.0% L-arginine (ARG) (Ajinomoto, Saga, Japan), which was done by replacing clay filler by L-arginine. For both gestation diets, nutritional levels for metabolizable energy (3148 kcal/kg), calcium (0.750%), phosphorus (0.395%) and digestible amino acids, lysine (0.535%), methionine and cysteine (0.381%), threonine (0.412%), tryptophan (0.113%) and valine (0.490%) were kept stable. There were differences between CON and ARG diets for crude protein (12.16 vs. 14.46%) and digestible arginine (0.67 vs. 1.60%). As recommended, 2.0% of arginine content in both diets was not exceeded (Wu et al., 2013) to avoid ammonia intoxication and competition for basic amino acid transporters.

Diets were daily provided to the gilts, in equal quantities, divided into two daily feeds (9 am and 4 pm). A total of 1.8 kg/day were offered between days 1 and 3 of gestation and 2.2 kg/day were offered between days 4 and 24 or 4 and 34 (females slaughtered at 25 and 35 days of gestation, respectively). Animals had free access to water throughout the experimental period. Gestation was confirmed 20 days after insemination and from the 23 inseminated gilts, 20 became pregnant. At 25 days of gestation, five females of CON ($n$=5) and five females of ARG ($n$=5) were rendered unconscious using head-only electrical stunning (240V, 1.3A) and

immediately exsanguinated. At 35 days of gestation, four females of CON (*n*=4) and six females of ARG (*n*=6) were slaughtered following the same procedures.

### *2.2 Conceptuses experimental design and collection*

Embryos and fetuses (25 and 35 days of age, respectively) from left uterine horn were collected at the time of gilts slaughter and fixed in 10% formalin for 24 hours for phenotypic analysis; afterwards, these conceptuses were stored in alcohol 70%. Conceptuses from right uterine horn were also collected and properly stored in nitrogen as described by COSTA et al. (2019) for molecular analyses. Phenotypic and gene expression analyses were performed within each gestational age, considering a completely randomized design with two treatments, CON and ARG.

For conceptuses phenotypic analysis, an average of four conceptuses were collected per female at each gestational age (two from the cranial region and two from the caudal region), totaling 20 CON and 20 ARG embryos, and 16 CON and 21 ARG fetuses. An average of four conceptuses were also collected per female at each gestational age for gene expression analysis (two from the cranial region and two from the caudal region), totaling 20 CON and 20 ARG embryos and 16 CON and 24 ARG fetuses.

### *2.3 Phenotypic measurements*

The proportion of embryos and fetuses heads (HP) was measured using a caliper (cm). In addition, the head, heart and liver (HW, HtW and LW, respectively) of conceptuses were weighed on a precision scale (g). Kidneys of 35-day fetuses (KW) were also weighed (g) for phenotypic analyses.

### *2.4 Gene expression*

Total RNA extraction was performed from 50 mg of embryos and fetuses' samples (whole conceptuses sprayed with nitrogen) using TRIzol® (InvitrogenTM), according to the manufacturer's instructions. RNA concentration was estimated at NanoVue™ Plus spectrophotometer (GE Healthcare, Freiburg, Germany) and quality integrity was determined in 1.0% agarose gel (data not shown). The first strand of cDNA synthesis was performed using GoScript Reverse Transcriptase kit (Promega Corporation, Madison, WI, USA) and cDNA concentration was determined by NanoVue™ Plus spectrophotometer.

Real time quantitative PCR (RT-qPCR) analyses were performed in duplicates in ABI Prism 7300 Sequence Detection Systems thermocycler (Applied Biosystems - Foster City, CA, USA) using the Relative Quantification method and applying SYBR® Green system (Applied

Biosystems - Foster City, CA, USA) and GoTaq® qPCR Master Mix kit (Promega Corporation, Madison, USA). RT-qPCR reactions were submitted to the cycles protocol according to the program: 95ºC for 3 minutes, 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. Amplification efficiencies (targets and endogenous genes) were approximately 100% in each cycle (data not shown). The values of threshold cycle (Ct) obtained were later normalized (ΔCt) based on the Ct values obtained for the endogenous control gene (*β-actin*). The calculation of the relative gene expression levels was performed according to the $2^{-\Delta Ct}$ method, described by LIVAK; SCHMITTGEN (2001).

Primers for amplification of the target and endogenous gene fragments were designed using PrimerQuest software provided by Integrated DNA Technologies, Inc (Coralville, IA), using nucleotide sequences obtained from the GenBank database (http: // www.ncbi.nlm.nih.gov) (Table 1). As endogenous controls, β-actin (*β-ACTIN*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine guanine fosforiboxiltransferase (*HPRT1*) were tested. These genes were selected based on their amplification profile and dissociation curve (data not shown). None of the endogenous genes presented significant differences in expression between treatments (CON and ARG) at 25 or 35 days. Due to its greater expression and stability between treatments, *β-ACTIN* was chosen as the best endogenous gene for embryos and fetuses analyses (data not shown).

The following target genes were evaluated: Lysine methyltransferase 2A (*KMT2A)*, Enhancer of zeste 2 polycomb repressive complex 2 subunit (*EZH2)*, Enhancer of zeste 1 polycomb repressive complex 2 subunit (*EZH1)*, Histone deacetylase 1 (*HDAC1)*, Histone acetyltransferase 1 *(HAT1)*, DNA methyltransferase 3 alpha *(DNMT3A)*, DNA methyltransferase 3 beta *(DNMT3B)* and DNA methyltransferase 1 (*DNMT1).*

**Table 1**: Primers design

| GENE | Abbreviation | NCBI accession code | Primer sequence |
|---|---|---|---|
| β- actin | *BACT* | XM_0031242803 | F:CTTCTAGGCGGACTGTTAGTG<br>R:AGCCATGCCAATCTCATCTC |
| Hypoxanthine-guanine phosphoribosyltransferase 1 | *HPRT1* | NM_001032376.2 | F:CCAGTCAACGGGCGATATAA<br>R:GACCAAGGAAAGCAAGGTTG |
| Glyceraldehyde-3-phosphate dehydrogenase | *GAPDH* | NM_001206359.1 | F:CAAAGTGGACATTGTCGCCATCA<br>R:AGCTTCCCATTCTCAGCCTTGACT |
| Lysine methyltransferase 2A | *KMT2A* | XM_021062911.1 | F: ACATCTGGTACTGCTGCTTC<br>R: GAGCCTCGCTTGGAGTAAAT |

| | | | |
|---|---|---|---|
| Enhancer of zeste 2 polycomb repressive complex 2 subunit | *EZH2* | XM_021102117.1 | F: CACTCCTCCCAGAAAGAAGAAG |
| | | | R: CGCAGGGCTGATAGTTGTAA |
| Enhancer of zeste 1 polycomb repressive complex 2 subunit | *EZH1* | NM_001243206.1 | F: GCGATGAGGTGAAGGAAGAA |
| | | | R: CACTAATCAGCACAGAGCCA |
| Histone deacetylase 1 | *HDAC1* | XM_013999116.2 | F: GGTGTTTGATGGCCTGTTTG |
| | | | R: GATGTCCGTCTGCTGCTTAT |
| Histone acetyltransferase 1 | *HAT1* | XM_003483674.4 | F: TGGAGGCAGATGATGTAGAG |
| | | | R: TGGACTGAGAACCGAGTATG |
| DNA methyltransferase 3 alpha | *DNMT3A* | NM_001097437.1 | F: CCCATTCGATCTGGTGATTG |
| | | | R: GGCGGTAGAACTCAAAGAAG |
| DNA methyltransferase 3 beta | *DNMT3B* | XM_013985274.2 | F: TCGAGTCTTGTCCCTGTTTG |
| | | | R: CACTTCTGAGGCGACGTATT |
| DNA methyltransferase 1 | *DNMT1* | NM_001032355.1 | F: AGAAGGATTCCACCAAGCAG |
| | | | R: CCGTGGACCCAGGATTATTT |

F, forward primer; R, reverse primer.

### *2.5 Statistical analyses*

Conceptuses phenotypic and gene expression data were analyzed within each gestational age and submitted to analysis of variance (ANOVA) using the MIXED procedure of SAS, version 9.0 (Statistical Analysis System Institute, Inc., Cary, NC, USA). The residue normality test was performed using the UNIVARIATE procedure of SAS (SAS Institute - Cary, NC, USA). Conceptuses were considered false replicates of the females for each treatment, represented by a nested effect in the statistical model.

Expression data of target and endogenous genes were generated as Ct values. Data were transformed in relative expression ($2^{-\Delta Ct}$), according to LIVAK; SCHMITTGEN (2001), and results were considered significant when $P \leq 0.05$. *P*-values between 0.06 and 0.10 were considered a trend.

The following statistical model was used in the analyses:

$Y_{ijkl} = \mu + D_i + C\{D_{(i)}\}_j + B_k + \varepsilon_{ijkl}$

wherein: $Y_{ijkl}$ is the observation (phenotypic or relative expression) from the j-th concept; $\mu$ is the trait general mean; $D_i$ is the effect of i-th treatment (CON or ARG); $C\{D_{(i)}\}_j$ is the random effect of concept j nested in treatment i; $B_k$ is the random effect of k-th boar; and $\varepsilon_{ijkl}$ is the random error.

## 3. Results

### 3.1 Phenotypic markers of 25 and 35-day conceptuses

No differences were found for HP ($P$=0.25), HW ($P$=0.22) and HtW ($P$= 0.17) between CON and ARG embryos. However, LW from ARG embryos tended to be greater compared to

CON embryos (*P*=0.09) (Table 2). For fetuses, no differences were found for HP (*P*=0.56), HW (*P*= 0.27), LW (*P*= 0.31), HtW (*P*= 0.91) and KW (*P*= 0.56) between CON and ARG treatments (Table 2).

**Table 2**: Averages ± Standard Errors, Degrees of freedom, F-values and *P*-values of conceptuses phenotypic data

| Conceptuses traits | CON (Mean±Standard error) | ARG (Mean±Standard error) | Degrees of freedom | F-value | *P*-value |
|---|---|---|---|---|---|
| | | **25-day embryos** | | | |
| HP (cm) | 0.6172±0.0377 | 0.6759±0.0387 | 8 | 1.51 | 0.25 |
| HW (g) | 0.0360±0.0026 | 0.0409±0.0026 | 8 | 1.75 | 0.22 |
| LW (g) | 0.0173±0.0014 | 0.0212±0.0014 | 8 | 3.83 | 0.09 |
| HtW (g) | 0.0147±0.0044 | 0.0082±0.0045 | 8 | 2.23 | 0.17 |
| | | **35-day fetuses** | | | |
| HP (cm) | 1.5989±0.1276 | 1.5105±0.1083 | 8 | 0.36 | 0.56 |
| HW (g) | 0.5415±0.0337 | 0.4892±0.0280 | 8 | 1.43 | 0.27 |
| LW (g) | 0.2129±0.0147 | 0.1921±0.0124 | 8 | 1.17 | 0.31 |
| HtW (g) | 0.0417±0.0023 | 0.0413±0.0020 | 8 | 0.01 | 0.91 |
| KW (g) | 0.0541±0.0044 | 0.0506±0.0037 | 8 | 0.36 | 0.56 |

HP= Head proportion, HW= Head weight, LW= Liver weight, HtW= Heart weight, KW= Kidney weight. CON=control diet; ARG=treatment with 1.0% L-arginine supplementation.

### 3.2 Expression of epigenetic genes in embryos and fetuses

At 25 days of gestation, there were no differences between CON and ARG embryos for all epigenetic genes evaluated: *KMT2A* (*P*=0.26), *EZH2* (*P*=0.46), *EZH1* (*P*=0.21), *HDAC1* (*P*=0.28), *HAT1* (*P*=0.85), *DNMT3A* (*P*=0.80), *DNMT3B* (*P*=0.38) and *DNMT1* (*P*=0.48) (Table 3). Similarly, CON and ARG fetuses showed no differences in expressions of *KMT2A* (*P*=0.25), *EZH2* (*P*=0.12), *EZH1* (*P*=0.21), *HDAC1* (*P*=0.25), *HAT1* (*P*=0.20), *DNMT3A* (*P*=0.20), *DNMT3B* (*P*=0.34) and *DNMT1* (*P*=0.65) genes (Table 3).

**Table 3:** Relative expression of epigenetic genes in embryos and fetuses at 25 and 35 days of gestation ($2^{-\Delta Ct}$).

| Gene | CON (Mean±Standard error) | ARG (Mean±Standard error) | Degrees of freedom | F-value | *P*-value |
|---|---|---|---|---|---|
| | | **25-day embryos** | | | |
| *KMT2A* | 0.0292±0.0033 | 0.0235±0.0033 | 8 | 1.50 | 0.26 |
| *EZH2* | 0.0165±0.0028 | 0.0197±0.0028 | 8 | 0.61 | 0.46 |
| *EZH1* | 0.0014±0.0002 | 0.0010±0.0002 | 8 | 1.86 | 0.21 |
| *HDAC1* | 0.0974±0.0104 | 0.0805±0.0104 | 8 | 1.34 | 0.28 |

| | | | | | |
|---|---|---|---|---|---|
| *HAT1* | 0.0105±0.0022 | 0.0111±0.0022 | 8 | 0.04 | 0.85 |
| *DNMT3A* | 0.1026±0.0125 | 0.0980±0.0125 | 8 | 0.07 | 0.80 |
| *DNMT3B* | 0.0228±0.0033 | 0.0185±0.0033 | 8 | 0.85 | 0.38 |
| *DNMT1* | 0.0640±0.0191 | 0.0839±0.0191 | 8 | 0.54 | 0.48 |
| | | **35-day fetuses** | | | |
| *KMT2A* | 0.0583± 0.0125 | 0.0384± 0.0099 | 8 | 1.57 | 0.25 |
| *EZH2* | 0.0620±0.0143 | 0.0303± 0.0113 | 8 | 3.02 | 0.12 |
| *EZH1* | 0.0060±0.0015 | 0.0033±0.0012 | 8 | 1.83 | 0.21 |
| *HDAC1* | 0.0703±0.0161 | 0.0449±0.0128 | 8 | 1.52 | 0.25 |
| *HAT1* | 0.0718±0.0182 | 0.0397±0.0144 | 8 | 1.93 | 0.20 |
| *DNMT3A* | 0.2718 ±0.0670 | 0.1514±0.0530 | 8 | 1.99 | 0.20 |
| *DNMT3B* | 0.0040±0.0009 | 0.0028±0.0007 | 8 | 1.04 | 0.34 |
| *DNMT1* | 0.1542±0.0382 | 0.1315±0.0302 | 8 | 0.22 | 0.65 |

CON=control diet; ARG=diet with 1.0% L-arginine supplementation.

## 4. Discussion

Nutritional, hormonal, and metabolic environment provided by the mother may program the differentiation of target tissues in the offspring through epigenetic changes such as DNA methylation and histone modification, contributing to fetal metabolic programming (LEE, 2019). In this context, livestock maternal nutritional management has been used to control the expression of genes related to energy metabolism, glucose homeostasis, and insulin signaling via epigenetic mechanisms (ELOLIMY et al., 2019). Nutrigenomic studies with different species provide evidences that maternal diet may modify the pattern of DNA methylation and histone modification, causing molecular changes in embryo, fetus, or neonate tissues (ELOLIMY et al., 2019; LEE, 2019); however, the effects of nutrients on epigenetic mechanisms that determine changes in livestock growth and development are still not elucidated (ELOLIMY et al., 2019).

L-arginine supplementation has been used to improve reproductive performance of females from different species (MATEO et al., 2007, 2008; GAO et al., 2012; LI et al., 2014, 2015; GUO et al., 2016). Nonetheless, there is no consensus about the optimum concentration and the period and duration of L-arginine supplementation (WU et al., 2010; PALENCIA et al., 2018; COSTA et al., 2019). In addition, the mechanisms that lead to females' reproductive improvement are also not fully understood.

Arginine is a functional amino acid involved in the production of creatine, agmatine, NO, polyamines, and other products (WU; MORRIS, 1998). There is a high sequestration of methyl

groups by SAM for creatine and polyamine productions and methionine is the SAM precursor, besides being the main methyl donor for DNA and histone methyltransferases (BROSNAN; BROSNAN, 2006; SOCCO et al., 2017). Therefore, DNA and histone methylations depend on the availability of nutrients such as methionine (CHMURZYNSKA, 2009). In this context, results from the same experiment conducted by our research group showed that gilts 1.0% L-arginine supplementation reduced the methionine concentration on the females blood plasma (COSTA et al., 2019) which could influence on epigenetic modifications and hence on gene expression regulation on conceptuses.

The NO, an important signaling molecule and product of L-arginine metabolism, is an endogenous epigenetic regulator of gene expression and cell phenotype (SOCCO et al., 2017). NO influences epigenetic regulation through histone posttranslational modifications, DNA methylation, and microRNA levels (SOCCO et al., 2017). It alters global histone posttranslational modifications and DNA adducts by directly inhibiting epigenetic enzymes or indirectly by altering enzyme expression levels or cellular localization (VASUDEVAN et al., 2015; SOCCO et al., 2017). This L-arginine product is associated with alterations in histone acetyltransferase (HAT) activity and subsequent histone lysine acetylation that commonly increases gene expression (SOCCO et al., 2017). Moreover, it regulates histone deacetylation (HDAC) enzymes that are responsible for removing acetyl groups from lysine residues on histone tails, decreasing gene expression (RUIJTER et al., 2003).

Histone methylation can also be regulated by NO; the methylation on histone lysine residue is a function of the concerted activities of both histone methyltransferases and histone demethylases (SOCCO et al., 2017). According to SOCCO et al. (2017), the signaling effects of NO reprogram gene expression profiles to dramatically alter cell phenotype (SOCCO et al., 2017). Therefore, we hypothesized that gilts L-arginine supplementation could influence epigenetic genes expression in embryos and fetuses and act on global gene regulation in conceptuses through fetal programming, influencing their phenotype.

Fetal programming can affect gene expression regulation, gene-imprinting through DNA methylation and chromatin remodeling, morphogenesis and organogenesis as alterations in organ volume and tissue composition, metabolism and hormonal production and other biological processes (KWON; KIM, 2017). Some fetal phenotypic markers are altered by epigenetic mechanisms as a result of maternal nutrition such as fetal hepatic growth (Hyatt *et al.* 2008). At 25 days, we found a trend to greater liver weight in ARG embryos compared to

CON embryos. This trend can be related with the greater concentration of arginine on blood plasma of ARG gilts and with the greater expression of insulin-like growth factor I *(IGF1)* gene on ARG embryos observed by our research group in the same experiment at 25 days of gestation (COSTA et al., 2019). *IGF1* has a key role in growth and rapidly responds to changes in maternal nutrition (KWON; KIM, 2017). According to PARADIS et al. (2017) and BRAMELD et al. (2000), low levels of nutrient resources during gestation show metabolic response in the liver of restricted fetuses, in which restricted fetuses presented lower abundance of *IGF1* mRNA.

The alterations in metabolites abundance on females blood plasma observed by our research group, i.e., the lower methionine and tyrosine and a trend to a greater arginine concentration on the blood plasma of ARG gilts compared with CON gilts at 25 days, in addition to the lower arginine concentration on ARG gilts compared with CON gilts at 35 days (COSTA et al., 2019), did not change the other phenotypic markers evaluated on conceptuses at 25 and 35 days of gestation.

Epigenetic markers with the addition and removal of specific chemical groups regulate chromatin structure and, therefore, gene expression in response to environmental factors (LEE, 2019). DNA methylation is an important epigenetic mechanism that regulates the differential expression of genes (SOCCO et al., 2017). Methylation patterns in mammals are established during embryonic development and are mainly regulated by DNA methyltransferases (DNMTs) (KWON; KIM, 2017). The main DNA methyltransferases enzymes are Dnmt3a and Dnmt3b, responsible for the de novo methylation, and Dnmt1, which maintain methylation patterns when cells divide (PORTELA; ESTELLER, 2010; KWON; KIM, 2017). According to SOCCO et al. (2017), gene-silencing can be one effect of NO through DNA methylation. On the other hand, HMADCHA et al. (1999) showed that NO exposure did not increase DNMT genes expression. We also found no differences on DNA methyltransferases gene expressions between embryos and fetuses from CON and ARG gilts.

Other epigenetic mechanisms, as histone posttranslational modifications, are essential during embryonic development and cell differentiation. *HAT1* is a gene involved in the rapid acetylation of newly synthesized cytoplasmic histones (GENECARDS, 2019). On the other hand, the protein encoded by *HDAC1* is a component of the histone deacetylase complex being an element in the control of cell proliferation and differentiation (GENECARDS, 2019). *KMT2A* encodes a transcriptional coactivator that mediates chromatin modifications associated

with epigenetic transcriptional activation (GENECARDS, 2019). *EZH2* is involved in maintaining the transcriptional repressive state of genes over successive cell generations (GENECARDS, 2019) and *EZH1* mediates methylation of histone H3 (see MIM 602812) lys27 (H3K27) (GENECARDS, 2019). In the current study, possible alteration in NO production in the supplemented females did not change the expression of genes involved in posttranslational histone modifications. Therefore, L-arginine supplementation may influence epigenetic mechanisms as DNA methylation and histone posttranslational modifications through other epigenetic genes and/or it may affect these enzymes abundance or activity in a posttranslational level. Besides the supplementation may influence gene expression through non-coding RNAs such as microRNAs.

Epigenetic regulation is a critical association between environmental stimuli, placental development and fetal growth (LEE, 2019); therefore, epigenetic gene regulation is influenced by nutrition and may be a molecular mechanism affecting fetal programming (DOLINOY; WEIDMAN; JIRTLE, 2007). However, it is important to identify the prenatal period in which the organism is susceptible to environmental stimuli leading to programming (CHMURZYNSKA, 2009), since embryogenesis is a critical period for establishment of epigenotypes and maternal dietary modifications in key nutrients needed for epigenetic processes may alter the epigenetic patterns and hence gene expression in conceptuses (CHMURZYNSKA, 2009) influencing on animals post-natal performance.

## 5. Conclusion

L-arginine supplementation of gilts until 25 and 35 days of gestation did not change the expression of the evaluated epigenetic genes and the phenotypic markers in pig embryos and fetuses. However, there was a trend for differences on liver weight between CON and ARG embryos.

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# CHAPTER 5

## Influence of gilts supplementation with 1.0% L-arginine on the expression of conceptuses *GH*, *IGF1* and *IGF2* genes and on gilts corpus luteum vascularization

## Influence of gilts 1.0% L-arginine supplementation on the expression of conceptuses *GH*, *IGF1* and *IGF2* genes[1]

**[Accept in 19th International Congress on Animal Reproduction]**

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Changes in maternal nutrition during conceptuses development may alter their genome expression, physiology and metabolism. In this context, sows L-arginine supplementation has been used during gestation to improve embryos viability and growth. However, the mechanisms in which this amino acid acts are not elucidated. Therefore, we aimed to evaluate the effects of dietary 1.0% L-arginine supplementation of gilts on expression of *GH, IGF1* and *IGF2* genes in conceptuses. Commercial pregnant gilts received either a control diet (CON) or the CON diet with 1.0% L-arginine (ARG), associated with two gestational ages (25 and 35 days). The gene expression evaluation was performed within each gestational age, considering a completely randomized design with two treatments, CON ($n$=5 gilts at 25 and $n$=4 at 35 days) and ARG ($n$=5 gilts at 25 and $n$=6 at 35 days). Four conceptuses from each gilt were collected for analyses. Total RNA extraction was performed from 50 mg of whole conceptuses samples sprayed in nitrogen; RNA concentration and quality were checked. cDNA was synthetized and real-time qPCR was performed. Differences on *GH* ($P$=0.39 and 0.79) and *IGF2* ($P$=0.86 and 0.44) were not observed between CON and ARG conceptuses at 25 and 35 days, respectively. At 25 days of gestation, greater expression of *IGF1* was observed on ARG embryos ($P$=0.05),

whereas no difference was observed between treatments for this gene at 35 days ($P$= 0.81). Greater *IGF1* expression on ARG embryos may be related to other results from our group, in which we found greater arginine concentration on blood plasma of ARG gilts compared to CON at 25 days, while at 35 days, arginine concentration was lower on ARG gilts. Greater arginine concentration on ARG gilts at 25 days of gestation may have altered the *IGF1* expression in ARG embryos. It is known that conceptuses *IGF1* concentration may be altered by maternal diet through changes in early placental growth. *IGF1* is a major regulator of prenatal development, connecting growth to maternal-placental supply of nutrients. Overall, our results show that gilts L-arginine supplementation until 25 days of gestation alters the regulation of *IGF1* in embryos, however a longer period of supplementation does not alter the expression of *IGF1*, *GH* and *IGF2* genes in fetuses.

# Efeito da suplementação de fêmeas suínas gestantes com L-arginina na vascularização do corpo lúteo[1]

**[Accept in V Simpósio de Produção Animal – ZOOTECNIA NA ERA DA TECNOLOGIA]**

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**Resumo:** A suplementação de fêmeas suínas com L-arginina durante a gestação tem sido apontada como um dos principais avanços nos estudos de nutrição, estando relacionada à melhora da performance reprodutiva da matriz. Entretanto, existem dúvidas com relação ao período ideal para suplementação, além da concentração do aminoácido utilizado na dieta. Estudos indicam que a suplementação a partir do dia zero da gestação poderia influenciar na formação do corpo lúteo (CL) e consequentemente na produção de progesterona, afetando negativamente a gestação. Assim, objetivou-se avaliar os efeitos da suplementação com 1% de L-arginina a partir do dia um da gestação de marrãs na vascularização do CL e produção de progesterona. Foi utilizado um delineamento inteiramente casualizado em esquema fatorial com duas dietas (controle - CON e suplementada - ARG) e duas idades gestacionais (25 e 35 dias), com uma média de cinco fêmeas por tratamento (n=5). A interação dieta e idade gestacional não apresentou efeito significativo na média de vasos sanguíneos no CL das fêmeas suínas, assim como a idade gestacional. Foi observada tendência a uma maior vascularização no CL das fêmeas CON. Apesar desta tendência à maior vascularização em CON, não foram

observadas diferenças na produção de progesterona entre fêmeas CON e ARG. Desta forma, a suplementação com 1% L-arginina a partir do dia um da gestação não influenciou na vascularização luteal e produção de progesterona em fêmeas suínas gestantes.
**Palavras–chave:** histologia, mortalidade pré-natal, progesterona

**Abstract:** L-arginine supplementation of swine females during pregnancy has been pointed out as one of the main advances in nutrition studies, being related to the improvement of sow reproductive performance. However, there are doubts regarding the ideal supplementation period, besides the dietary amino acid concentration. Studies have indicated that supplementation from day zero of pregnancy could influence the corpus luteum (CL) formation and consequently progesterone production, negatively affecting pregnancy. Thus, we aimed to evaluate the effects of gilts 1% L-arginine supplementation from day one of gestation on CL vascularization and progesterone production. A completely randomized design in factorial scheme with two diets (control - CON and supplemented - ARG) and two gestational ages (25 and 35 days), with an average of five females per treatment (n = 5), was used. The interaction between diet and gestational age had no significant effect on the average blood vessels in CL, as well as the gestational age. A tendency towards greater vascularization in CL of CON females was observed. Despite this tendency towards greater vascularization in CON, no differences in progesterone production were observed between CON and ARG females. Therefore, 1% L-arginine supplementation from day one of gestation did not influence luteal vascularization and progesterone production of pregnant gilts.
**Keywords:** histology, prenatal mortality, progesterone

## Introdução

A suplementação de fêmeas suínas com L-arginina durante a gestação está envolvida com o aumento da viabilidade embrionária e, consequentemente, com a redução da mortalidade pré-natal (Bérard e Bee, 2010; Li et al., 2014) e aumento do número de leitões nascidos vivos e totais (Gao et al., 2012; Nuntapaitoon et al., 2018), além de influenciar no peso placentário e da leitegada total (Gao et al., 2012). Entretanto, apesar das maiores perdas pré-natais ocorrerem no início da gestação, principalmente no período implantacional, alguns pesquisadores indicam que a suplementação tenha início após a segunda semana gestacional (Li et al., 2010). Li et al. (2010) observaram que a suplementação com 0,8% de L-arginina entre os dias 0 e 25 da

gestação diminuiu o peso uterino, o número total de fetos, o número de corpos lúteos, o peso fetal total, as concentrações de progesterona, dentre outras características. Esses resultados estariam relacionados à regressão do corpo lúteo por meio de uma via dependente de PGF2α, o que poderia ter influenciado a diminuição da produção de progesterona e, consequentemente, a performance das fêmeas durante a gestação. Assim, objetivou-se com esse estudo identificar o efeito da suplementação de fêmeas suínas com 1% de L-arginina do dia um aos dias 25 e 35 de gestação sobre a vascularização do corpo lúteo e produção de progesterona.

**Material e Métodos**

O protocolo experimental seguiu os princípios éticos em pesquisa animal (CONCEA, 2016) e foi aprovado pelo comitê ético animal da Universidade Federal de Viçosa (UFV), Minas Gerais, Brasil [protocolo nº 06/2017). Foi utilizado o delineamento inteiramente casualizado em esquema fatorial 2 x 2 (duas dietas e duas idades gestacionais). As fêmeas foram distribuídas inteiramente ao acaso em baias individuais para o maior controle alimentar. Após a identificação do quarto estro, as fêmeas foram inseminadas 12 e 24 horas após a constatação do estro. As dietas controle (CON) e suplementada com 1% de L-arginina (ARG) foram formuladas de acordo com as necessidades de fêmeas suínas gestantes (Rostagno et al., 2011) e oferecidas duas vezes ao dia (09:00 da manhã e 16:00 da tarde). As fêmeas tiverem acesso à vontade à água durante todo o período experimental. O primeiro dia da inseminação foi considerado o dia zero, e as fêmeas começaram a receber as dietas experimentais 24 horas após a segunda inseminação. Aos 25 dias de gestação, cinco fêmeas CON e cinco fêmeas ARG foram abatidas para coleta de material biológico (corpos lúteos), e momentos antes do abate o soro sanguíneo das matrizes suínas foi coletado para quantificação dos níveis de progesterona. Aos 35 dias de gestação quatro fêmeas CON e seis fêmeas ARG foram abatidas para a coleta do corpo lúteo e soro sanguíneo. Assim, foram avaliadas em média cinco fêmeas por tratamento, n=5 CON/25 dias; n=5 ARG/25 dias; n=4 CON/35 dias e n=6 ARG/35 dias. Os corpos lúteos foram fixados em paraformaldeído e transferidos para álcool 70% 24 horas após a fixação. Posteriormente, o material foi incluído em parafina, cortado em micrótomo e corado com Hematoxilina e Eosina. Foram feitas seis imagens dos corpos lúteos de cada fêmea no fotomicroscópio Olympus BX53 na ampliação de 200x, e analisados com Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD). Os resultados foram gerados em valores absolutos. Já a determinação quantitativa dos níveis de progesterona no soro sanguíneo das

fêmeas foi realizada por imunoensaio quimioluminescente com partículas paramagnéticas usando o Access Progesterone test (Beckman Coulter, California, USA). Tanto os dados da vascularização quanto da concentração de progesterona foram submetidos à análise de variância (ANOVA) utilizando o procedimento MIXED do SAS, versão 9.0 (Statistical Analysis System Institute, Inc., Cary, NC, USA). O teste de normalidade dos resíduos foi realizado pelo procedimento UNIVARIATE do SAS (SAS Institute - Cary, NC, EUA). Os resultados foram considerados significativos quando $P \leq 0,05$.

**Resultados e Discussão**

Não houve efeito da interação dieta (CON e ARG) e idade gestacional (25 e 35 dias) na vascularização do corpo lúteo ($P$= 0,75), assim como também não houve efeito da idade gestacional na média do número de vasos ($P$=0,67). Entretanto, foi observada tendência a maior vascularização no corpo lúteo das fêmeas CON em relação à ARG ($P$=0,09). Apesar do corpo lúteo das fêmeas CON apresentar tendência à maior vascularização, não foram observadas diferenças em relação à concentração de progesterona no soro sanguíneo das fêmeas suínas CON e ARG ($P$=0,62) (Tabela 1).

Em relação à concentração de progesterona, não houve efeito da interação ($P$=0,78), entretanto, a concentração deste hormônio foi maior aos 35 dias em relação aos 25 dias ($P$=0,03) como já era esperado para atender a demanda fisiológica da mãe e dos fetos. O corpo lúteo é uma glândula temporária relacionada às principais características reprodutivas em mamíferos, e em suínos é essencial para o reconhecimento e manutenção da gestação. A produção de progesterona pelo corpo lúteo em suínos durante a gestação afeta características como a mortalidade pré-natal, uma vez que esse hormônio leva à inibição da contração uterina do músculo liso, da diferenciação estromal do endométrio e secreção glandular, placentação, e outros mecanismos que envolvem a sobrevivência e desenvolvimento dos conceptos.

Li et al. (2010) observaram a redução da concentração de progesterona sanguínea de matrizes suínas, além da redução do número de corpos lúteos, com a suplementação com 0,8% de L-arginina entre os dias 0 e 25 da gestação, o que poderia estar relacionado com os efeitos fenotípicos observados, como a redução do número total de fetos. Nossos resultados apontam que a suplementação a partir do dia um da gestação não causaria os efeitos negativos na gestação das fêmeas encontrados por esses autores, e seria uma possibilidade de suplementação com a L-arginina no terço inicial da gestação. Estes resultados são extremamente importantes,

uma vez que a suplementação logo no início da gestação não afetou negativamente a formação do corpo lúteo e a sua atividade de produção de progesterona. Sendo assim, a suplementação pode ter início em um momento de altas perdas gestacionais, influenciando a viabilidade, o desenvolvimento e a sobrevivência dos conceptos suínos.

Tabela 1. Médias, erros-padrão e P-valores do número de vasos sanguíneos no corpo lúteo e da concentração de progesterona no soro sanguíneo de fêmeas suínas gestantes.

| | | | | | | | 25 dias | | 35 dias | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Característica** | CON | ARG | *P*-valor | 25 dias | 35 dias | *P*-valor | CON | ARG | CON | ARG | *P*-valor |
| **Vasos sanguíneos** | 132,09 ±5,95 | 117,06 ±5,40 | 0,09 | 126,27 ±5,53 | 122,88 ±5,64 | 0,67 | 135,14 ±8,21 | 117,41 ±8,24 | 129,05 ±8,75 | 116,71 ±7,14 | 0,75 |
| **Progesterona** | 34,18± 2,94 | 36,20± 2,65 | 0,62 | 30,56 ±2,77 | 39,82± 2,83 | 0,03* | 29,00± 3,92 | 32,12± 3,92 | 39,37± 4,38 | 40,27± 3,58 | 0,78 |

CON=dieta controle; ARG=dieta com 1% de suplementação com L-arginina.

*P-valor ≤ 0.05

## Conclusões

A suplementação de fêmeas suínas com 1% de L-arginina não influencia na vascularização do corpo lúteo aos 25 e 35 dias de gestação, assim como também não influencia na produção de progesterona. A produção de progesterona pelo corpo lúteo durante a gestação é maior aos 35 dias em relação aos 25 dias para atender a demanda fisiológica dos fetos.

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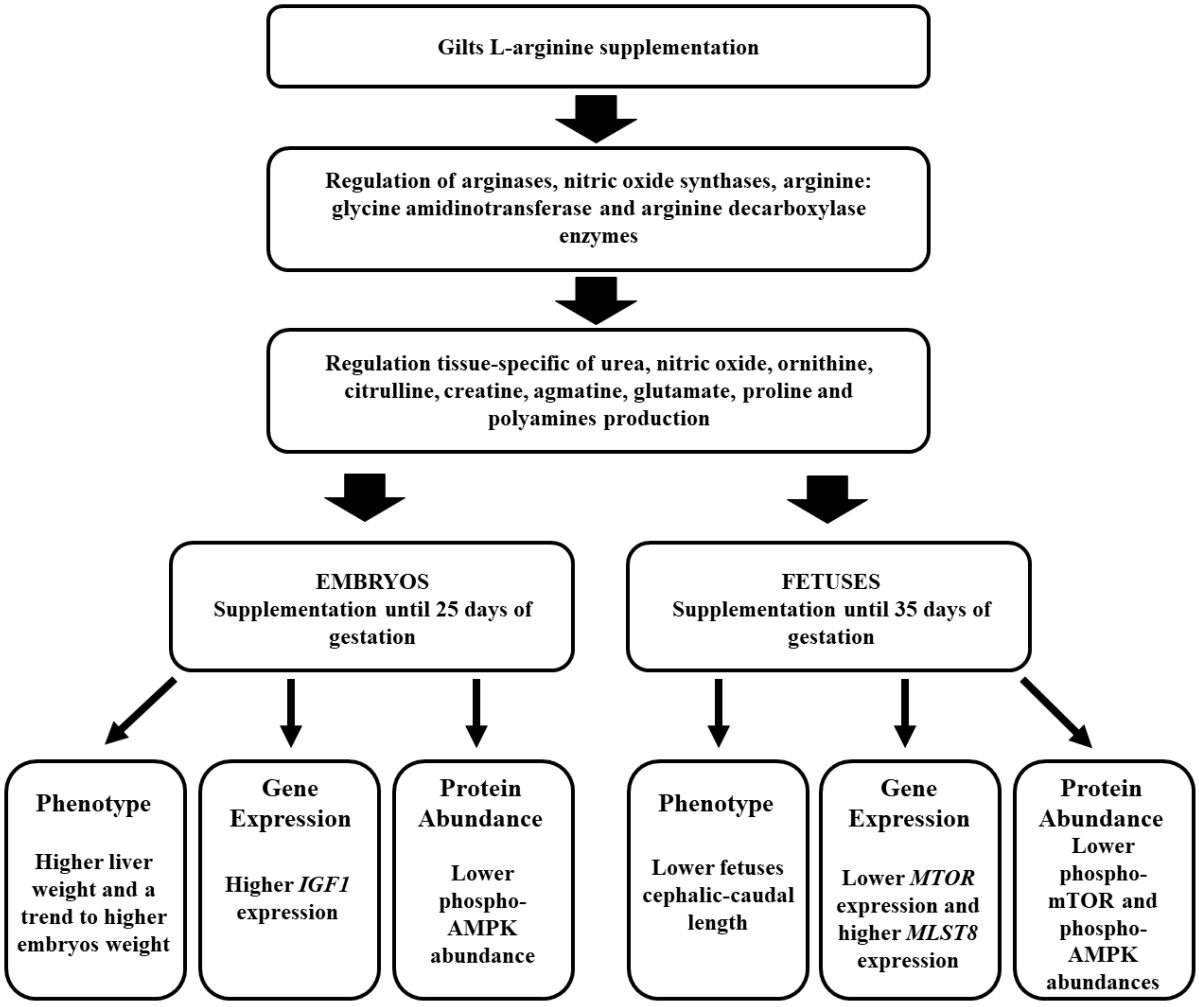
## General conclusions

In this study, we can conclude that supplementation duration is determinant for the effects that L-arginine exerts on the concentration of this amino acid in the plasma of gilts and on conceptuses growth. Our current results from gilts supplemented during 23 days were more interesting regarding conceptuses development since we observed increased arginine concentration in plasma of females from ARG group and increased *IGF1* gene expression in ARG 25-day embryos related to a tendency of higher embryos weight. In addition, we found a tendency to higher liver weight in ARG embryos compared to CON embryos. L-arginine supplementation until 25 days also influenced on energy metabolism, in which ARG embryos presented lower abundance of phospho-AMPK. Supplementation for a longer period (33 days) did not lead to changes in the expression of developmental and apoptosis genes in 35-day fetuses. However affected energy metabolism indicators in which ARG fetuses had lower *MTOR* expression and higher *MLST8* expression, besides that ARG fetuses presented lower phospho-mTOR and phospho-AMPK protein abundances. These differences can explain the lower cephalic-caudal length of ARG fetuses than CON fetuses. These differences occurred through mechanisms unrelated to ovarian activity as progesterone production. L-arginine supplementation also changed the concentration of methionine and tyrosine in the blood plasma of supplemented females. Despite the reduction of methionine in blood plasma from ARG gilts, the L-arginine supplementation of gilts until 25 and 35 days of gestation did not change the evaluated epigenetic gene expressions in embryos and fetuses (see Figure 1).

**Figure 1:** Representative figure of the effects of gilts L-arginine supplementation in 25 day-embryos and 35 day-fetuses.

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