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Transcriptional profile of Piau breed conceptuses and morphohistological characteristics of the uterus of pregnant Piau sows

Tânia Fernandes Martins
Doctor Scientiae

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Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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Assent:

Tânia Fernandes Martins
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Adviser

Dedico esta tese aos meus avós Francisco e Mirtes (in memoriam), à minha mãe e irmãs, aos meus sobrinhos e aos meus filhos de quatro patas (Lipe e Olavo), presenças constantes ao longo desta jornada. Dedico-a também a Nossa Senhora Aparecida, meu amparo nos momentos mais difíceis e auxílio presente nos momentos de angústia. A vocês, dedico este trabalho e todas as conquistas que ainda estão por vir.

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“Commit to the Lord whatever you do, and your plans will succeed” (Proverbs 16:3).

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“O sucesso na vida não é medido pelo caminho que você percorreu, mas pelas dificuldades que você superou ao longo do caminho.”
(Abraham Lincoln)

ABSTRACT

MARTINS, Tânia Fernandes, D.Sc., Universidade Federal de Viçosa, February, 2026. **Transcriptional profile of Piau breed conceptuses and morphohistological characteristics of the uterus of pregnant Piau sows**. Adviser: Simone Eliza Facioni Guimaraes. Co-advisers: Daniele Botelho Diniz Marques and Susana Amaral Teixeira.

During gestation, the uterus undergoes essential morphofunctional adaptations to ensure embryonic viability, providing an adequate environment for early conceptus development. Placental functional efficiency is a key determinant of fetal growth and survival, particularly in the early stages of pregnancy, and breed differences may be reflected in morphological variations of the uterine and placental environment, which can be assessed through morphohistological analyses. In parallel, skeletal muscle development in pigs is a complex process regulated by the coordinated expression of genes throughout the prenatal period. Myogenesis and adipogenesis involve critical events of cell proliferation and differentiation that determine muscle growth potential and intramuscular fat deposition, thereby influencing meat quality, and can be investigated through transcriptomic profiling of conceptuses using RNA-seq. Within this context, this thesis was structured into three chapters. The first chapter addresses the phenotypic and morphohistological characteristics of the endometrium and placenta of Piau sows and commercial lines at 25 and 35 days of gestation, highlighting differences in uterine, placental, and embryonic development between genetic groups. The second chapter focuses on sex determination of Piau conceptuses at early developmental stages through the identification of Y chromosome–linked transcripts using RNA-seq data, revealing genes associated with gonadal development and genetic features of this breed. The third chapter analyzes the transcriptomic profile of Piau conceptuses at 25 and 35 days of gestation in comparison with a commercial line, with the aim of identifying differentially expressed genes and biological processes related to myogenesis and adipogenesis. Overall, the results demonstrate that genetic differences between the Piau breed and commercial lines influence the uterine and placental environment, as well as transcriptomic profiles associated with skeletal muscle formation and intramuscular fat deposition. These findings contribute to a better understanding of the mechanisms involved in prenatal development in pigs and reinforce the potential of the Piau breed as a strategic genetic resource for pig production. Additionally, they expand current knowledge of the porcine Y chromosome and suggest that local breeds may exhibit relevant regulatory particularities, even in biological processes that are

highly conserved among mammals, with implications for future reproductive studies.

Keywords: adipogenesis; endometrium; gene expression; placenta; myogenesis

RESUMO

MARTINS, Tânia Fernandes, D.Sc., Universidade Federal de Viçosa, fevereiro de 2026. **Perfil transcricional de conceptos da raça Piau e características morfohistológicas do útero de porcas Piau gestantes.**. Orientadora: Simone Eliza Facioni Guimaraes. Coorientadores: Daniele Botelho Diniz Marques e Susana Amaral Teixeira.

Durante a gestação, o útero passa por adaptações morfofuncionais essenciais à viabilidade embrionária, garantindo um ambiente adequado ao desenvolvimento inicial do concepto. A eficiência funcional da placenta é determinante para o crescimento e a sobrevivência fetal, especialmente nos estágios iniciais da gestação, e diferenças entre raças podem se refletir em variações morfológicas do ambiente uterino e placentário, avaliáveis por análises morfohistológicas. Paralelamente, o desenvolvimento do músculo esquelético em suínos é um processo complexo, regulado pela expressão coordenada de genes ao longo do período pré-natal. A miogênese e a adipogênese envolvem eventos críticos de proliferação e diferenciação celular que determinam o potencial de crescimento muscular e a deposição de gordura intramuscular, influenciando a qualidade da carne, e podem ser investigadas por meio da análise do perfil transcricional de conceptos via RNA-seq. Diante desse contexto, esta tese foi estruturada em três capítulos. O primeiro aborda as características fenotípicas e morfohistológicas do endométrio e da placenta de porcas da raça Piau e de linhagens comerciais aos 25 e 35 dias de gestação, evidenciando diferenças no desenvolvimento uterino, placentário e embrionário entre os grupos genéticos. O segundo capítulo concentra-se na determinação do sexo de conceptos da raça Piau em estágios iniciais do desenvolvimento, por meio da identificação de transcritos ligados ao cromossomo Y utilizando dados de RNA-seq, revelando genes associados ao desenvolvimento gonadal e características genéticas dessa raça. O terceiro capítulo analisa o perfil transcriptômico de conceptos da raça Piau aos 25 e 35 dias de gestação, em comparação com uma linhagem comercial, com o objetivo de identificar genes diferencialmente expressos e processos biológicos associados à miogênese e à adipogênese. De modo geral, os resultados demonstram que as diferenças genéticas entre a raça Piau e as linhagens comerciais influenciam o ambiente uterino e placentário, bem como os perfis transcriptômicos associados à formação do músculo esquelético e à deposição de gordura intramuscular. Esses resultados contribuem para a compreensão dos mecanismos envolvidos no desenvolvimento pré-natal em suínos e reforçam o potencial da raça Piau como um recurso genético estratégico para a produção suína. Adicionalmente, ampliam o

conhecimento sobre o cromossomo Y em suínos e sugerem que raças locais podem apresentar particularidades regulatórias relevantes, mesmo em processos biológicos altamente conservados entre os mamíferos, com implicações para futuros estudos reprodutivos.

Palavras-chave: adipogênese; endométrio; expressão gênica; placenta; miogênese

SUMMARY

1 GENERAL INTRODUCTION

Gestation is characterized by maternal adaptations involving morphofunctional, metabolic, and hormonal changes in the uterus, particularly in the endometrium and placenta, structures essential for embryonic viability and the proper formation of the intrauterine environment (Almeida and Alvarenga Dias, 2022). During this period, the uterus undergoes intense structural and functional remodeling, primarily through angiogenesis and vasculogenesis, which are crucial for establishing the vascular network that supports conceptus growth and survival (Stenhouse *et al.,* 2018).

Despite these adaptations, limitations in placental efficiency during early gestation remain among the main causes of prenatal mortality, compromising embryonic viability (Kridli *et al*., 2016). The placenta plays a central role in mediating metabolic, gaseous, and nutritional exchanges between the maternal and fetal systems, and its functional efficiency, combined with the uterus's adaptive capacity, is a key determinant proper conceptus development (Linck *et al.,* 2024). In this context, phenotypic and reproductive variations among pig breeds tend to reflect differences in the morphology and function of the uterine environment, directly influencing fetal growth and survival during the early stages of gestation.

Beyond the morphofunctional aspects of the uterus and placenta, embryonic and fetal development is strongly influenced by genetic factors, including the sex of the conceptus. In mammals, sex is genetically determined by the XX/XY system, with the presence of the Y chromosome carrying the *SRY* gene being decisive for male development (Blanes *et al.,* 2016). During the early stages of gestation, male and female embryos and fetuses cannot be distinguished phenotypically or histologically, as the gonads remain undifferentiated (Pelliniemi *et al*., 1975; Hyttel *et al.,* 2009).

Therefore, in studies conducted at early developmental stages, in which males and females do not yet exhibit phenotypic differences, sex determination can be performed using alternative methods rather than morphological observation. In this context, molecular approaches based on the expression of sex chromosome-linked genes represent a viable strategy (Wang *et al.,* 2009). For example, in ongoing RNA-seq studies of porcine conceptuses, transcriptomic data can be used to identify sex by examining the expression of sex chromosome-specific genes, without the need for additional techniques. In pigs, this strategy has been demonstrated in commercial lines (Teixeira *et al.,* 2019), but it remains unexplored in local breeds.

Another fundamental axis of fetal programming involves the development of skeletal muscle and adipose tissue. Prenatal myogenesis occurs in two main waves, with the formation of primary muscle fibers between 30 and 60 days of gestation (Wigmore and Evans, 2002) and secondary fibers between 54 and 90 days (Wigmore and Stickland, 1983), while adipogenesis begins around day 45 of gestation (Hausman and Kauffman, 1986). The total number of muscle fibers is predominantly established prenatally and represents a key determinant of postnatal muscle growth potential (Dwyer *et al*., 1993; Rehfeldt *et al.,* 2000). Consequently, events during gestation directly influence productive traits such as carcass yield, meat quality, and intramuscular fat deposition, which in turn affect sensory attributes and consumer acceptance (Zhao *et al.,* 2015; Liu *et al.,* 2009).

Differences among pig breeds in growth rate and body composition reflect variations in the proportion of muscle fiber types, with slow-growing breeds showing a higher proportion of slow-twitch fibers and fast-growing breeds predominating in fast-twitch fibers (Lefaucheur *et al.,* 2004; Wank *et al.,* 2006). These phenotypic variations are the result of historical processes of domestication, genetic selection, and environmental adaptation (Fávero and Figueiredo, 2009; Bennewitz *et al*., 2008; Sollero *et al.,* 2009), highlighting the importance of understanding the molecular mechanisms that regulate muscle growth and fat deposition for genetic improvement and productivity in pig farming.

In this context, the Piau breed, a local Brazilian genotype, is characterized by rusticity, disease resistance, lower prolificacy, slower growth, and higher fat deposition compared to commercial lines (Sollero *et al*., 2009; Serão *et al.,* 2011; Veroneze *et al.,* 2014). Although underutilized in modern production systems, this breed represents an important genetic resource and a valuable biological model for investigating the molecular mechanisms underlying intrauterine development, muscle tissue formation, and fat deposition, as well as breed-specific biological differences.

Transcriptome analysis using RNA-seq is a powerful approach to elucidate regulatory mechanisms underlying embryonic and fetal development and to identify transcriptional differences between genetic groups (Teixeira *et al.,* 2021; Yu *et al.,* 2024). However, to date, the complete transcriptome sequencing of the Piau breed remains largely unexplored, limiting understanding of the effects of genetic selection and evolutionary differences among pig breeds.

Considering that early myogenic events occur before the visible formation of muscle fibers and are critical for establishing the total number of fibers (Wigmore and Evans, 2002; Wigmore and Stickland, 1983; Dwyer *et al.,* 1993; Rehfeldt *et al.,* 2000), gestational days 25 and 35 represent critical stages of intrauterine development in pigs. These periods are also

relevant for investigating uterine adaptation, molecular sex differentiation, and programming of muscle development.

Thus, this thesis integrates morphological and transcriptomic approaches to investigate uterine and placental adaptation, molecular sex determination, and the regulatory mechanisms associated with muscle development in Piau pigs during early gestation. Each subsequent chapter is structured as a scientific article, addressing specific aspects of these processes and providing a detailed, integrated understanding of the biological mechanisms underlying phenotypic, reproductive, and genetic differences among pig breeds.

2 GENERAL OBJECTIVE

To investigate the morphological and molecular mechanisms associated with intrauterine development in Piau breed pigs during the early stages of gestation (25 and 35 days), integrating analyses of endometrial and placental morphology and the transcriptomic profile, obtained by RNA-seq, related to sex determination, myogenesis, and adipogenesis.

2.1 Specific objectives

Compare the endometrial and placental morphology of Piau and Commercial sows at 25 and 35 days of gestation, aiming to identify structural differences associated with uterine adaptation, placental development, and fetal viability.

Determine the sex of Piau embryos and fetuses at 25 and 35 days of gestation through the expression of Y-chromosome-linked transcripts using RNA-seq data.

Characterize the gene expression profile of Piau conceptuses during two distinct periods of intrauterine development (25 and 35 days of gestation) and compare it with that of a commercial line, with emphasis on genes and biological processes related to myogenesis and adipogenesis.

3 LITERATURE REVIEW

3.1 Piau Breed

During the colonization of Brazil, Portuguese, Spanish, and Asian pig breeds were introduced into the country and subsequently dispersed throughout the Brazilian territory (Cesconeto *et al*., 2017). Over this process, these animals adapted to the environmental, climatic, management, and sanitary conditions of different habitats, giving rise to Brazilian naturalized breeds, also referred to as local breeds (Albuquerque *et al*., 2002). Among these genotypes, the Piau breed stands out for its high adaptability to Brazilian climatic conditions, rusticity, disease resistance, lower requirements for management and feeding, and greater subcutaneous and intramuscular fat deposition when compared with commercial lines (Sollero *et al.,* 2009; Serão *et al.,* 2011; Montes *et al.,* 2018; Veroneze *et al*., 2014).

Historically, some Brazilian local pig breeds, such as Piau, were raised to supply the lard industry. In this context, in 1939, Antonio Teixeira Vianna conducted a selection process based on observable phenotypic traits, without the application of formal genetic improvement methods, aiming to obtain a dual-purpose animal suitable for both fat and meat production (Gomes and D'Aulísio, 1980).

From the 1960s onward, changes in consumer preferences spurred the importation of foreign breeds with greater capacity to deposit lean meat (Fávero and Figueiredo, 2009). Throughout the domestication of pigs, artificial selection enabled the development of breeds with phenotypes better suited to market demands, encompassing behavioral, reproductive, and body composition traits (Fix *et al*., 2010; Rubin *et al*., 2012).

The population size of the Piau breed in Brazil is unknown, as there are no recent records from a national census of local pig breeds. This situation highlights the need to conserve the genetic diversity of local breeds, as population declines compromise access to their genes and, consequently, to unique traits that could be useful in the future (Bennewitz *et al.,* 2008; Bermejo *et al.,* 2019).

In this context, the Piau breed represents an important genetic resource for Brazilian pig production, since, although it has been subjected to historical phenotypic selection processes, it has not undergone modern genetic improvement programs comparable to those applied to commercial lines, which has contributed to the preservation of many of its natural adaptive characteristics. These particularities confer high potential for comparative studies with

commercial lines, as well as for conservation and sustainable use programs of animal genetic resources.

3.2 Uterine and placental morphology

The placenta is an essential organ for the exchange of nutrients, metabolites, and respiratory gases between the mother and the fetus, being a key determinant of fetal growth and development (Almeida and Alvarenga Dias, 2022). In pigs, placentation is of the epitheliochorial and diffuse type, characterized by the apposition of the maternal uterine epithelium and the fetal trophoblastic epithelium, without invasion of maternal tissues by the trophoblast (Charnock-Jones *et al*., 2001). Under these conditions, metabolic exchanges occur across two epithelial layers supported by a complex capillary network. Throughout gestation, these layers become progressively thinner and increasingly invaginated by blood vessels, thereby reducing the diffusion distance between the maternal and fetal circulatory systems (Furukawa *et al.,* 2014).

Pregnancy in pigs involves the processes of conceptus elongation, implantation, and placentation, with embryonic elongation being a determining factor for the formation of the chorioallantoic membrane and, consequently, the functional size of the placenta (Vallet *et al*., 2007). During implantation, chorionic trophoblasts adhere to the endometrial epithelium, establishing the maternal–fetal interface (Johnson *et al.,* 2001; Burghardt *et al.,* 2002; Aplin and Kimber, 2004). This process is accompanied by morphofunctional adaptations, including the development of folds at the uteroplacental interface, interdigitation of microvilli, reduction of connective tissue between vessels and epithelia, intensification of uterine and placental angiogenesis, and the formation of placental areolae responsible for the transport of histotrophic secretions to the fetus (Johnson *et al.,* 2021).

The close apposition between the maternal and fetal circulatory systems depends directly on vascular development, which occurs through vasculogenesis and angiogenesis (Charnock-Jones *et al.,* 2001). Considering that nutrient delivery to the uterus and the fetus is influenced by blood flow rate, endometrial and placental vascularization is a key determinant of fetal survival, growth, and development (Wu *et al*., 2013; Wu *et al.,* 2018; Bidarimath and Tayade, 2017). During the intrauterine period, structural alterations in the placenta may compromise its functionality, acting as mediators of environmental conditions and modulating the availability of nutrients and hormones to the fetus (Hsu and Tain, 2019; Jansson and Powell, 2013).

The fetal trophoblastic epithelium exhibits intense proliferative activity during the pre-implantation period, rapidly expanding and forming a large surface area of contact with the

endometrium (Perry and Rowlands, 1962; Geisert *et al.,* 1982; Stroband and Van der Lende, 1990). In parallel, the uterine epithelium responds to growth factors, cytokines, and ovarian steroids, modulating its structural and functional properties during the acquisition of uterine receptivity (Burghardt *et al*., 1997).

Endometrial glands play a central role in embryonic nutrition, exhibiting high secretory activity throughout gestation (Stroband and Van der Lende, 1990). The number and activity of these glands are associated with estrogen levels and the trophoblast's aromatase activity during the pre-implantation period (Gadsby *et al.,* 1980), as well as with progesterone, which promotes increased glandular complexity and the production of nutritive secretions (Hafez and Hafez, 2004).

The maintenance of pregnancy also depends on adequate uterine vascularization, which is essential for corpus luteum function and endometrial integrity (Acosta and Miyamoto, 2004). Angiogenesis is crucial for pregnancy continuity (Tamanini and Ambrogi, 2004), and endometrial development, characterized by increased glandular density and secretory activity, is a key determinant of fertility and embryonic nutrition (Vallet *et al.,* 2007). Factors such as genotype, uterine capacity, and nutrition directly influence placental development (Bauer *et al*., 1998), with uterine vascularization being more intense in the apical regions of the uterine horns, where vessels exhibit a larger caliber (Guimarães *et al.,* 2014).

Thus, the morphological evaluation of placental and endometrial tissues represents a fundamental approach for understanding the mechanisms that regulate the efficiency of the maternal–fetal interface and gestational success in pigs. The characterization of these morphological parameters provides support for the interpretation of placental structural adaptations during early embryonic development.

3.3 Transcriptome analysis

The transcriptome is a dynamic and complex entity that plays a central role in cell biology. It comprises the complete set of RNA molecules synthesized by a cell and influences various cellular processes (Tzec-Interián *et al*., 2025). The study of the transcriptome enables cataloging transcripts, characterizing gene structure, and quantifying variations in gene expression levels across different biological conditions (Wang *et al.,* 2009). Transcriptional regulation is fundamental to physiological, pathological, and developmental processes, since the mRNA expression profile determines the functional characteristics of cells and tissues (Marioni *et al*., 2008).

Among the available tools for transcriptomic analysis, RNA-Seq (RNA sequencing) stands out for enabling comprehensive identification and quantification of gene transcription (Wang *et al.,* 2009; Griffith *et al*., 2015; Hrdlickova *et al.,* 2017). According to Li et al. (2015), this approach begins by fragmenting RNA samples into short complementary DNA (cDNA) sequences, which are sequenced on a high-throughput platform. Subsequently, the generated sequences are mapped to a reference genome or transcriptome. Thereafter, expression levels of each gene or isoform are estimated. Next, the mapped data are normalized and, through statistical methods, differentially expressed genes are identified.

Based on data from differentially expressed genes, it is possible to investigate biological pathways, allowing the characterization of transcriptional activity across different tissues or cell populations and contributing to the understanding of molecular mechanisms underlying the modulation of complex phenotypes (Ritchie *et al.,* 2015; Chen *et al*., 2016). Due to these fundamental biological roles, gene expression quantification has been widely used in molecular biology and genomics studies (Conesa *et al.,* 2016).

Currently, RNA-Seq is considered one of the most efficient methodologies for global analysis of gene expression profiles and for identifying differential expression across tissues, developmental stages, or experimental conditions (Stark *et al.,* 2019). Accordingly, recent RNA-Seq applications in pigs have contributed to the understanding of molecular mechanisms associated with economically important traits, including subcutaneous fat deposition (Oliveira *et al.,* 2022), embryonic and fetal development (Teixeira *et al.,* 2021), energy metabolism in adipose tissue (Pan *et al.,* 2019), meat quality and muscle metabolism (Piórkowska *et al.,* 2018), adipogenic differentiation in subcutaneous preadipocytes (Zhao *et al*., 2019), and placental adaptation to different altitudes (Li *et al.,* 2025).

Taken together, these findings reinforce the relevance of the transcriptomic approach for elucidating the biological processes underlying productive traits in pigs, highlighting RNA-Seq as an essential tool for identifying genes and molecular pathways associated with phenotypic variation, as well as for advancing knowledge applied to animal production.

3.4 Differentially Expressed Genes

Differential expression analysis is a molecular biology technique used to compare gene expression levels between two or more groups of samples, such as healthy and diseased tissues or cells subjected to different treatments, to identify genes whose abundance shows significant changes between experimental conditions (Oshlack *et al.,* 2010; Kebschull *et al.,* 2016). This

strategy enables the identification of genes associated with specific biological processes, diseases, or responses to treatments, providing relevant information about gene regulation and the underlying molecular mechanisms (Singh *et al.,* 2018).

The first step in differential expression analysis consists of summarizing normalized reads into a count matrix, in which each row represents a gene and each column corresponds to a sample, containing read counts after filtering and quality control procedures (Dündar *et al*., 2015; Ritchie *et al.,* 2015). This matrix is subjected to statistical modeling based on multiple hypothesis testing, in which, for each gene, the existence of significant differences in expression between experimental groups is evaluated. Because this procedure involves many simultaneous tests, correction of p-values for multiple comparisons is required to control the false-positive rate, with the Benjamini-Hochberg method being widely used (Van Den Berge *et al.,* 2019; Benjamini and Hochberg, 1995).

Among the most widely used methods for differential expression analysis are: *Cuffdiff*, which models expression variance by separately considering genes with single or multiple isoforms using negative binomial distributions (Trapnell *et al.,* 2013); *edgeR*, which estimates dispersion of count data assuming a negative binomial distribution and combines common and gene-specific components (Robinson *et al.,* 2010); *DESeq*, which decomposes variance into a mean-dependent (Poisson) component and another associated with biological variability (Anders and Huber, 2010); *baySeq*, which uses an empirical Bayesian model based on negative binomial distributions, estimating prior parameters directly from the data (Hardcastle and Kelly, 2010); and *limma*, originally developed for microarrays and later adapted for RNA-seq (*limma-voom*), in which counts are transformed into log2 values weighted by precision weights and analyzed using robust linear models (Smyth, 2017; Law *et al.,* 2014; Ritchie *et al.,* 2015).

The *limma* method shows broad applicability to complex experimental designs and provides robust statistical inference even when the number of samples is limited (Ritchie *et al.,* 2015). Normalized counts are transformed to a logarithmic scale and fitted to linear models, incorporating the mean–variance relationship through an empirical Bayesian approach, either via trend (*limma-trend*) or precision weights (*limma-voom*). The former is recommended when the ratio between the largest and smallest library sizes is less than three, whereas the latter is more suitable when there is greater variation among library sizes (Law *et al.,* 2014; Ritchie *et al*., 2015).

Therefore, once differentially expressed genes have been identified, high-throughput transcriptomic analysis often yields in extensive gene lists, making a subsequent step of biological interpretation and functional analysis necessary to elucidate the molecular functions

of these genes in relation to the experimental condition under investigation.

3.5 Functional analysis

The reconstruction of genetic networks has become one of the main focuses of bioinformatics, in which algorithms and computational methods play a central role in understanding biological processes (Brugere *et al.,* 2018). However, uncovering the genetic architecture underlying variation in complex traits remains challenging, as polygenic inheritance involves multiple genes, each contributing small effects to the phenotype (Cánovas *et al*., 2016). In this context, advances in omics sciences, especially genomics, transcriptomics, and proteomics combined with the development of bioinformatics, have expanded the capacity to investigate the molecular mechanisms underlying different phenotypes (De Carvalho *et al.,* 2019; Matos *et al.,* 2023).

Functional analysis represents a fundamental step in understanding how genes act in metabolic pathways and physiological processes related to the evaluated phenotype (Wang *et al.,* 2010). A widely used strategy to facilitate this interpretation is functional annotation based on Gene Ontology (GO), which allows the standardized description of the physiological roles of genes and their products (Ashburner *et al.,* 2000).

Gene Ontology organizes this information into three main categories: molecular function, cellular component, and biological process, which describe, respectively, the biochemical activities of gene products, their cellular localization, and the processes in which they are involved (Thomas *et al.,* 2007). Thus, enrichment networks of GO terms can be used to identify biological processes significantly associated with genes of interest.

Currently, several computational tools are available for the visualization and analysis of biological networks, with Cytoscape standing out as an open-source software widely used for the analysis and visualization of molecular and genetic interaction networks (Majeed and Mukhtar, 2023). This software provides an interactive interface that allows users to import, explore, filter, group, search, and export networks. Among the available plug-ins, ClueGO stands out for enhancing the biological interpretation of large gene lists by integrating Gene Ontology terms and metabolic pathways from databases such as KEGG and BioCarta and organizing them into functionally structured networks. It can be used both for the functional visualization of a single gene list and for comparing functional annotations across different groups or clusters (Bindea *et al.,* 2009).

Thus, integrating functional analysis, Gene Ontology term enrichment, and network

visualization tools enables a more comprehensive biological interpretation of transcriptomic data, thereby facilitating the identification of biological processes and metabolic pathways associated with the phenotype of interest.

3.6. Importance of studying genes for myogenesis and adipogenesis

The development and growth of skeletal muscle and adipogenesis in pigs are complex processes, primarily regulated by myogenesis and adipogenesis, which directly influence the phenotypic traits expressed throughout postnatal life. Myogenesis and adipogenesis are the main mechanisms responsible for the formation of muscle fibers (Wigmore and Evans, 2002; Wigmore and Stickland, 1983) and adipocytes (Hausman and Kauffman, 1986), and they exert a direct influence on productive traits such as carcass yield, body composition, and meat quality (Braun; Gautel *et al.,* 2011; Hausman *et al.,* 2009). Therefore, understanding the genes and molecular pathways involved in myogenesis and adipogenesis is essential for explaining phenotypic differences between genetic groups and for integrating genetics, physiology, and nutrition, especially during the early stages of development, when cells can still differentiate into muscle or fat.

From an embryological perspective, the skeletal muscles of the trunk and limbs originate from somites, structures derived from the paraxial mesoderm that form progressively along the anteroposterior axis of the embryo (Tajbakhsh and Buckingham, 1999). During somite maturation, the dermomyotome differentiates, giving rise to myogenic progenitor cells that express muscle determination factors such as *Myf5* and *Mrf4*, which in turn form the myotome, the precursor of skeletal muscle fibers (Christ and Ordahl, 1995; Buckingham and Rigby, 2014). The activation of these genes exhibits specific temporal and spatial patterns, highlighting that gene regulation is a central component in determining cell fate and organizing muscle tissue (Buckingham, 2006; Buckingham and Rigby, 2014).

Parallel to myogenesis, adipogenesis begins around the 45th day of gestation and involves the proliferation and differentiation of precursor cells into adipocytes capable of storing lipids (Hausman and Kauffman, 1986; Hausman *et al.,* 2004; Kokta *et al.,* 2004; Fernyhough *et al.,* 2007). This process occurs asynchronously in embryonic and fetal adipose depots (Hausman and Richardson, 2004) and intensifies during the postnatal period, depending on energy availability and metabolic regulation (Azain, 2004). Adipocytes and preadipocytes play a central role in determining carcass adiposity and intramuscular fat deposition, which are essential for sensory attributes such as meat tenderness, juiciness, and flavor; however,

excessive fat accumulation in depots unrelated to marbling is energetically unfavorable and reduces production efficiency, highlighting the importance of understanding the molecular mechanisms that regulate adipogenesis (Hausman *et al.,* 2009).

In addition to the morphological and physiological aspects, differences in gene expression patterns associated with myogenesis and adipogenesis have been reported among pig genetic groups. Sollero *et al.* (2011) demonstrated that Piau fetuses exhibit a distinct transcriptional profile throughout gestation, with a greater number of differentially expressed genes between 40 and 70 days compared to commercial lines. Reis *et al*. (2006) also reported variations in gene expression during prenatal muscle development between Piau and commercial pigs at different gestational ages. Complementarily, De Brito Neto *et al.* (2020) observed differences in the expression of genes related to myogenesis and apoptosis in conceptus from these genetic groups, associating these patterns with variations in muscle development potential, cell proliferation, and the regulation of myogenic differentiation.

Thus, the study of genes associated with myogenesis and adipogenesis is essential to understand how genotype influences the formation of muscle and adipose tissues, as well as to identify mechanisms underlying variations in body composition and meat quality. Furthermore, elucidating the cellular differentiation pathways and their regulation provides a physiological basis for nutritional and management strategies that modulate these processes, thereby optimizing production efficiency and the quality of the final product.

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CHAPTER 1

Histomorphometric characterization of the uterus and placenta in Piau and Commercial sows during early gestation

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Abstract: Pregnancy induces critical physiological adaptations to support embryonic development and fetal survival. This study compared endometrial and placental phenotypic and histomorphometric characteristics of Piau and Commercial sows at two gestational ages (25 and 35 days). Twelve sows (six Piau and six Commercial) were evaluated in a randomized design, with samples collected from three regions of the right uterine horn of each animal. Histomorphometric analyses were performed using microscopy and ImageJ software. Statistical analyses employed linear mixed-effects models, with Shapiro-Wilk and Levene's tests applied to assess normality and homogeneity of variances, respectively. At 25 days of gestation, Commercial sows showed greater uterine and ovarian weights, a higher number of corpora lutea, and longer uterine horn horns, reflecting genetic selection for reproductive efficiency. Conversely, Piau sows exhibited more advanced embryonic development at this stage, with fetuses of greater size. At 35 days, the phenotypic superiority of Commercial sows persisted, while Piau fetuses maintained greater weight and length, indicating distinct temporal growth dynamics. Histomorphometric analyses at 25 days revealed that Commercial sows had increased placental connective tissue deposition and thicker endometrial epithelium, whereas Piau sows presented larger placental vascular area, as well as enhanced endometrial vascularization and glandular density across all uterine regions. At 35 days, no significant differences were observed in placental vascular area and endometrial vascularization; however, subtle trends in connective tissue development suggested ongoing placental differentiation. These findings highlight distinct reproductive strategies between Piau and Commercial sows, with potential implications for embryonic development and gestational success. Altogether, the results confirm that genetic background influences uterine and placental morphology during early gestation.

Keywords: Fetal development, endometrium, morphometry, placental vascularization, pig.

1 INTRODUCTION

Pregnancy involves maternal adaptations characterized by morphofunctional, metabolic, and hormonal changes in the uterus, particularly within the endometrium and placenta, essential for embryonic viability and proper placental formation (Almeida and Alvarenga Dias, 2022). To ensure pregnancy establishment and maintenance, the uterus undergoes significant structural and functional remodeling, mainly through angiogenesis and vasculogenesis, processes that are fundamental for vascular network development and adequate support for conceptuses growth and survival (Stenhouse et al., 2018).

Despite these adaptations, limitations in placental efficiency during early gestation remain a major cause of prenatal mortality, compromising embryonic viability (Kridli et al., 2016). In this context, the functional efficiency of the placenta, along with the adaptive capacity of the uterus, plays a crucial role in conceptuses survival and development, as the placenta mediates metabolic, gaseous, and nutritional exchanges between the maternal and fetal systems (Linck et al., 2024).

Thus, phenotypic and reproductive divergences between breeds are often reflected in morphological variations of the uterine environment, potentially influencing fetal growth and survival during early gestation. Based on these aspects, we hypothesize that genetic group differences impact uterine structure and fetal development. Therefore, this study compared the endometrial and placental morphology of Piau and Commercial sows at two gestational ages (25 and 35 days), aiming to identify structural evidence that may indicate how genetic differences influence uterine adaptation, placental development, and fetal viability.

2 METHODS

2.1Animals and experimental design

The experiment was conducted at the Universidade Federal de Viçosa, Brazil, following national animal welfare guidelines (CEUAP-UFV protocol 52-2024) and the European Union Directive 2010/63/EU on animal experimentation for the Piau genetic group. This study complied with the ARRIVE guidelines and Brazilian regulations on animal welfare. For the Commercial genetic group, the experimental protocol was approved by the Animal Research Ethics Committee of the Federal University of Viçosa (UFV), Minas Gerais, Brazil (protocol no. 06/2017), in accordance with the ethical principles in animal research established by CONCEA (2016).

A total of twelve sows were evaluated, divided equally into two genetic groups: six Piau and six Commercial sows. Each genetic group was further subdivided by gestational age, with three sows evaluated at 25 days of gestation and three sows evaluated at 35 days, resulting in n= 3 per group per gestational age. Estrus was synchronized with Regumate® (Merck Animal Health, USA) and was conducted with semen from boars of the same genetic groups.

The Piau animals were sourced from the Swine Improvement Research and Extension Unit (UEPE), at the Universidade Federal de Viçosa UFV, where the research was conducted. The Commercial animals were also obtained from the same unit. The Commercial genetic group corresponds to a hybrid lineage (Large White × Landrace × Duroc) commonly used in Brazilian swine production.

2.2 Sample collection and Histological processing

At each gestational stage, females (n = 3 per genetic group per stage) were stunned

(240V, 1.3A), and slaughtered. The uterus, ovaries and conceptuses were collected and phenotypic data recorded: weight at slaughter (SW, kg), uterine weight (UW, kg), left uterine horn length (LUHL, cm), right uterine horn length (RUHL, cm), number of corpora lutea in the left ovary (NCLL), number of corpora lutea in the right ovary (NCLR), total number of corpora lutea (TCL), left ovary weight (LOW, g), right ovary weight (ROW, g), total ovarian weight (TOW, g), number of conceptuses (NC), number of viable conceptuses (NCV), and mortality rate (MR), calculated as: 100 - $\frac{Viable\ conceptus\ number}{Number\ of\ corpus\ luteum} X\ 100$ (Costa et al., 2019).

Fetal weights (FW, g) were measured, and the coefficient of variation (CV%), was calculated as: $\frac{Standard\ deviation\ of\ conceptuses\ weights}{Mean\ of\ conceptuses\ weights} X\ 100$ (Costa et al., 2019). Fetal cranio-caudal length (FLC, mm) was assessed using a digital caliper (ZAAS Precision, Piracicaba, Brazil), following the methodology proposed by Guimarães et al. (2014).

For histomorphometric analysis, endometrial and placental samples were collected from three regions of the right uterine horn (proximal, medial and distal) of each genetic group and gestational stage (Figure 1). The samples were inserted in 4% paraformaldehyde, dehydrated, embedded in paraffin and sectioned at 5 µm . The slides were stained with hematoxylin-eosin and mounted with Entellan (Merck, Germany) for microscopic analysis. Endometrial and placental structures were evaluated together, considering the type of lining epithelium, endometrial thickness and the presence of uterine glands.

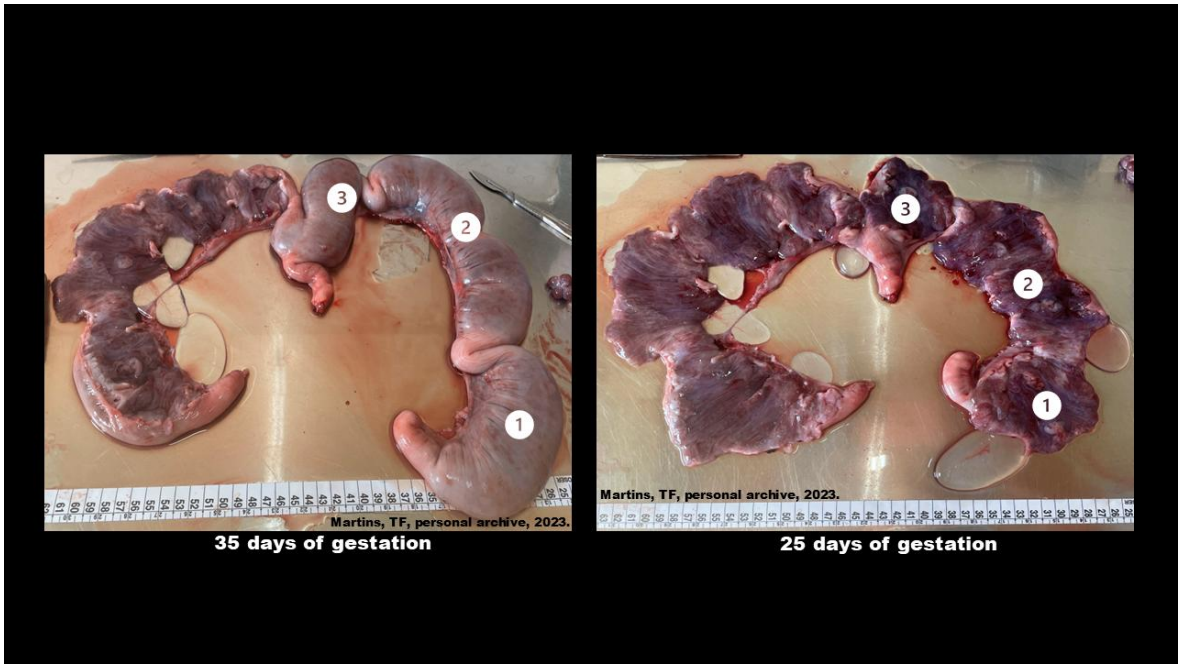

Figure 1. Representative structure of the uterine horns of a Piau sow at 25 and 35 days of gestation. Endometrial and placental samples were collected from three regions of the right uterine horn: (1)

proximal, (2) medial, and (3) distal. This image is illustrative of a single animal and does not represent the average number of conceptuses described in the tables. Images of the Commercial genetic group were unavailable because samples were collected from archived material without photographic records at the time of collection.

2.3 Histomorphometric analysis

Endometrial and placental structures were assessed using an optical microscope (Olympus; BX53; Tokyo, Japan) equipped with 1.3MP CMOS digital camera BioCAM (Takachiho, Japan), and the TCapture program. Random images per tissue were analyzed for each sow using ImageJ software (version 1.50i; National Institutes of Health, Bethesda, MD, USA). The total number of blood vessels per area in the placental and endometrial tissues at both gestational ages was determined by counting and measuring the area of each histological image.

Tissue volumetric proportions were determined using point-counting (266 points/image), evaluating placental tissues such as placental epithelial thickness (PET), placental connective tissue (PCT), placental vascularization (VB-P) and trophoblast epithelium (TRO). In this study, PET refers to the overall thickness of the placental layer visible in the histological section, measured perpendicularly from the fetal surface epithelium to the basal limit, encompassing the entire placental layer without detailing specific fetal membranes. TRO refers exclusively to the fetal trophoblastic epithelium, excluding the maternal uterine epithelium.

Although the uterine epithelium is visible in some histological sections, especially at 25 days of gestation, it was not quantified separately due to discontinuity, partial degeneration in some regions, and variability across samples. Thus, TRO represents only the fetal epithelial interface in direct contact with the maternal endometrium, while the maternal epithelium was excluded from volumetric analysis.

Considering endometrial uterine tissues: endometrial connective tissue (ECT), endometrial vascularization (VB-E) and uterine glands (EUG) were recorded (Tung et al., 2012). The percentage of points for the maternal and fetal portion, were calculated using the formula: volumetric proportion (%) = (Number of points in each structure) / (Total points in maternal or fetal tissue) ×100.

2.4 Statistical analysis

Phenotypic data of sows and conceptuses, including SW, UW, LUHL, RUHL, NCLL, NCLR, TCL, LOW, ROW, TOW, NC, NCV, MR, CVc, FW and FLC were analyzed using R software. Differences between genetic groups and gestational ages were evaluated using ANOVA with mixed linear models, as described by the equation:

$$Y_{ijk} = \mu + G_i + A_j + (G \times A)_{ij} + \varepsilon_{ijk}$$

where: Y_{ijk} is dependent variable value for the *i*-th genetic group, *j*-th gestational age, and *k*- th experimental unit; μ is the overall mean; G_i is the fixed effect of the *i*-th genetic group (Piau or Commercial); A_j is the fixed effect of the *j*-th gestational age (25 or 35 days); $(G \times A)_{ij}$ is the interaction effect between the *i*- th genetic group and the *j*-th gestational age; and ε_{ijk} is the random error.

Normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated using Levene's test. Significance was set at $P < 0.05$, whereas values between 0.05 and 0.10 were considered trends. Logarithmic or square-root transformations were applied when necessary to stabilize variance and improve normality. For FW and FLC, wich did not meet ANOVA assumptions, even after transformation, the Kruskal-Wallis non-parametric test (1952) was applied (Tables 1 and 2, Supplementary material).

Histomorphometric analyses of the placenta and endometrium were performed by calculating the percentage of each tissue type in relation to the total number of points per image, using the following formula: $\frac{Number\ of\ points\ in\ each\ structure}{Total\ points\ in\ maternal\ or\ fetal\ tissue} X\ 100$ (Souza et al., 2018).

For the histomorphometric data, the experimental unit was the individual sow. Although multiple tissue samples were collected from different regions, all samples from the same sow were considered subsamples within the experimental unit.

Linear mixed models were adjusted for each combination of genetic groups and gestational age, using the same model described above. Data were organized into subsets for placental tissues (PET, PCT, VB-P and TRO) and endometrial tissues (ECT, VB-E and EUG), allowing a detailed analysis of histomorphometric features in different genetic groups and gestational ages combinations (Figure 2).

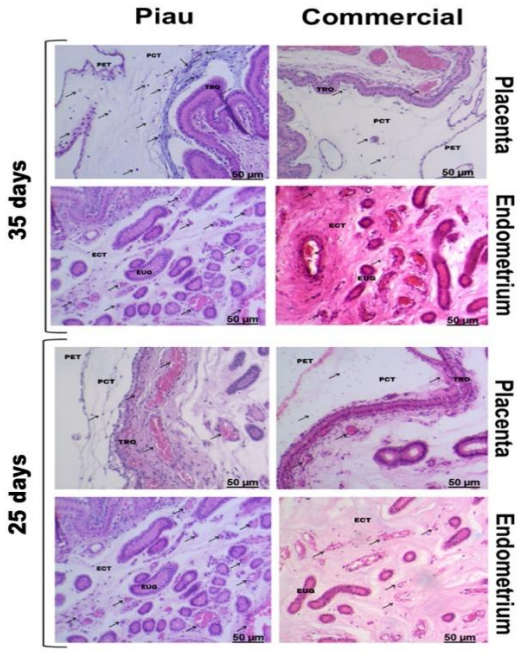

Figure 2. Histological images of placental and endometrial tissues of Piau and Commercial sows at 25 and 35 days of gestation. Placental components include PET (placental epithelial thickness), PCT (placental connective tissue), VB-P (placental vascularization), and TRO (fetal trophoblast epithelium, excluding maternal uterine epithelium). Endometrial components include ECT (endometrial connective tissue), VB-E (endometrial vascularization), and EUG (endometrial uterine glands). Note: In some 25-day images, the maternal uterine epithelium appears adjacent to the trophoblast but was not included in the quantitative analysis.

3 RESULTS AND DISCUSSION

The statistical analysis model included the interaction term between genetic group and gestational age. However, no significant interactions were detected ($P > 0.10$); therefore, only the main effects of genetic group and gestational age are presented and discussed.

3.1 Phenotypic data of sows at 25 days of gestation

Significant phenotypic differences ($P < 0.05$) were observed between genetic groups at 25 days of gestation. Commercial sows showed higher values for SW ($P < 0.0001$), UW ($P = 0.0045$), LUHL ($P = 0.0108$), RUHL ($P < 0.0001$), NCLL ($P = 0.0314$), TCL ($P = 0.0090$), and TOW ($P = 0.0248$) compared to Piau sows (Table 1). A trend toward significance ($P < 0.10$) was observed for the NCLR ($P = 0.05$). No significant differences were found for LOW, ROW, NC, NCV, MR and CVc (Table 1).

These results reflect the influence of genetic selection for reproductive efficiency in

Commercial lines. A greater number of corpora lutea and longer uterine horns may favor embryonic maintenance and fetal development (Silva et al., 2014), mainly through enhanced uterine capacity and hormonal support during early pregnancy.

Moreover, previous studies suggest that the morphofunctional superiority of Commercial sows is associated with better uterine morphology and increased placental vascularization, improving placental efficiency and nutrient transfer to the conceptuses (Foxcroft et al., 2009). Such factors can enhance embryonic survival and promote more uniform development in the initial stages of pregnancy.

Table 1. Phenotypic data for sows from the genetic groups Piau and Commercial in 25 days of gestation

Traits	Piau (25 days)	Commercial (25 days)	SEM	P-value
SW (kg)	115.67	164.13	4.72	< 0.0001*
UW (kg)	1.28	2.90	0.21	0.0045*
LUHL (cm)	55.33	101.00	1.43	0.0108*
RUHL (cm)	55.00	117.43	3.67	<0.0001*
NCLL (count)	4.67	9.00	0.12	0.0314*
NCLR (count)	6.00	10.00	0.15	0.0500
TCL (count)	10.67	19.00	0.09	0.0090*
LOW (g)	4.83	8.19	0.19	0.1400
ROW (g)	6.90	8.68	0.79	0.4000
TOW (g)	11.73	16.87	1.32	0.0248*
NC (count)	11.33	17.00	0.33	0.2000
NCV (count)	10.67	16.67	0.00	0.2200
MR (%)	6.67	11.75	2.58	0.5500
CVc (%)	30.70	23.75	9.49	0.6900

Piau (25 days of gestation) and Commercial (25 days of gestation), with three sows per group (n = 3). SW: weight at slaughter; UW: uterine weight; LUHL: length of the left uterine horn; RUHL: right uterine horn length; NCLL: number of corpora lutea in the left ovary; NCLR: number of corpora lutea in the right ovary; TCL: total number of corpora lutea; LOW: left ovary weight; ROW: right ovary weight; TOW: total ovary weight; NC: number of conceptuses; NCV: number of viable conceptuses; MR: mortality rate; CVc: coefficient of variation among conceptuses. *Significant at *P Anova* ≤ 0,05. SEM: Standard error of the mean.

3.2 Phenotypic data of sows at 35 days of gestation

At 35 days of gestation, this phenotypic superiority persisted, with significantly higher values ($P < 0.05$) for LUHL ($P = 0.003$), TCL($P < 0.0001$), TOW ($P = 0.0071$), NC ($P = 0.0226$) , and NCV ($P = 0.0198$), in Commercial sows (Table 2). Trends toward significance were also observed for RUHL ($P < 0.10$), reinforcing the superior performance of Commercial sows.

This higher reproductive efficiency may be attributed to intensive genetic selection for growth and reproductive traits, a hallmark of intensive production systems (Da Silva et al.,

2016). In contrast, Piau showed lower ovarian weight, fewer corpora lutea, and reduced uterine growth, which may compromise fetal viability.

However, Montes et al. (2018) suggest that local compensatory mechanisms, such as improved endometrial quality, maternal metabolic efficiency, and greater embryonic competence, may partially offset these structural limitations. Theses adaptations could maintain pregnancy even with lower reproductive investment.

Nonetheless, such constraints may still negatively impact fetal muscle development and overall productivity, especially considering that primary muscle fibers, crucial for determining postnatal fibers numbers, occur around 35 days of gestation (Wigmore e Stickland, 1983). Therefore, although Piau sows may rely on alternative physiological strategies to support gestation, their lower reproductive investment may ultimately result in reduced litter growth potential.

Table 2. Phenotypic data of females and fetuses of Piau and Commercial genetic groups in 35 days of gestation

Traits	Piau (35 days)	Commercial (35 days)	SEM	P-value
SW (kg)	130.23	161.87	5.51	0.0800
UW (kg)	3.03	5.48	0.57	0.0600
LUHL (cm)	94.67	143.83	4.83	0.003*
RUHL (cm)	92.67	155.83	14.1	0.0500
NCLL (count)	4.33	7.67	0.17	0.2900
NCLR (count)	7.33	11.33	0.40	0.1600
TCL (count)	11.67	19.00	0.67	< 0.0001*
LOW (g)	5.70	7.30	0.32	0.3100
ROW (g)	7.90	9.84	0.44	0.1300
TOW (g)	13.60	17.14	0.05	0.0071*
NC (count)	10.67	14.67	0.87	0.0226*
NCV (count)	10.33	13.67	0.55	0.0198*
MR (%)	10.72	28.07	2.07	0.1000
CVc (%)	17.55	11.08	3.09	0.3700
FW(g)	4.2	3.1	0.184	< 0.0001**
FLC (mm)	33	29.1	0.051	< 0.0001**

Piau (35 days of gestation) and Commercial (35 days of gestation), with three sows per group (n = 3). SW: weight at slaughter; UW: uterine weight; LUHL: length of the left uterine horn; RUHL: right uterine horn length; NCLL: number of corpora lutea in the left ovary; NCLR: number of corpora lutea in the right ovary; TCL: total number of corpora lutea; LOW: left ovary weight; ROW: right ovary weight; TOW: total ovary weight; NC: number of conceptuses; NCV: number of viable conceptuses; MR: mortality rate; CVc: coefficient of variation among conceptuses; FW: fetal weight, FLC: craniocaudal length.*Significant at *P Anova* ≤ 0,05. SEM: Standard error of the mean. ** For fetal traits (FW and FLC), values represent medians; P-values were obtained using the Kruskal–Wallis test.

3.3 *Phenotypic data of the fetuses*

Significant differences were observed between genetic groups at 35 days of gestation

for FW and FLC, with Piau fetuses showing higher values ($P < 0.0001$ for both traits) (Table 2; Figure 3). These results support previous evidence that Piau embryos exhibit accelerated growth during early gestation. Montes et al. (2018) reported that at 30 days, Piau embryos were longer than Commercial ones, but this difference disappeared by day 45, suggesting a limitation in sustaining growth throughout gestation.

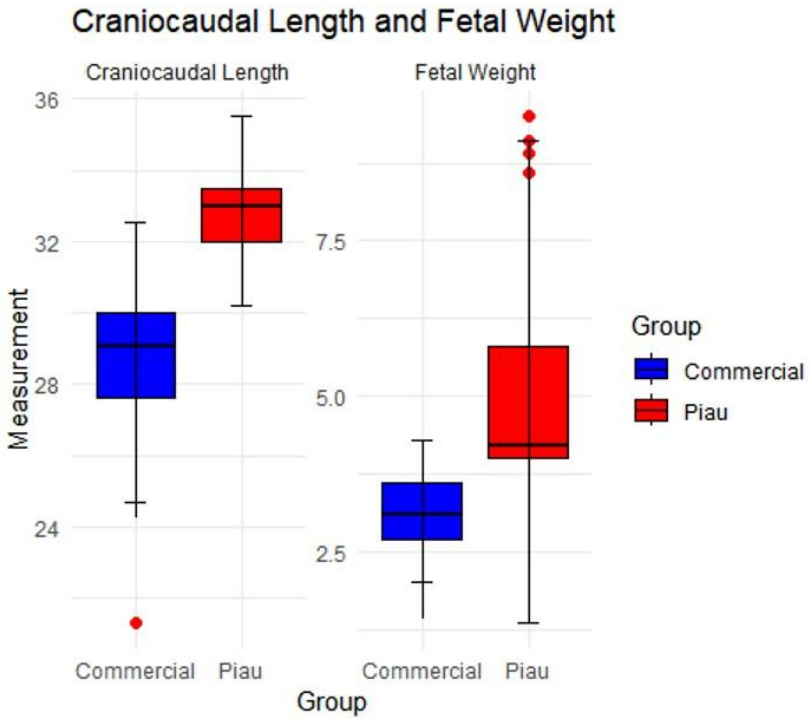

Figure 3. Boxplots for weight and craniocaudal length of fetuses at 35 days of gestation. The boxes represent the interquartile range (IQ ? R) with the median line, while the points outside the boxes indicate outliers. Statistical analysis was conducted to assess differences between treatments and/or groups.

This pattern may be linked to more intense early myogenesis in Piau fetuses, favoring primary muscle fiber formation (Sollero et al. 2011). In contrast, Commercial pigs seem to accelerate fetal growth later in gestational stages. Reis et al. (2016) detected increased expression of myogenesis-related genes, such as *MYOD1* and *MYOG*, at 40 and 70 days, indicating a delayed peak in muscle development. Similarly, Cagnazzo et al. (2006) reported that delayed myogenesis in Duroc pigs was associated with greater postnatal muscle hypertrophy, suggesting a similar developmental trajectory in Commercial pigs.

In addition, De Brito Neto et al. (2016) identified differences in apoptosis and myogenesis related gene expression. Commercial fetuses showed greater regulation of pro-apoptotic (*BAX*) genes at 30,45, and 60 days, suggesting a controlled mechanism of programmed cell death that supports secondary muscle fiber formation.

In contrast, Piau fetuses expressed these genes earlier, promoting primary fibers formation and increased cell survival during early development. Altogether, these results reveal distinct temporal patterns of fetal growth regulation and myogenesis between genetic groups. While Piau pigs prioritize early fetal growth, and myogenesis, Commercial pigs emphasize later muscle development, potentially influencing muscle fiber composition and postnatal meat quality.

3.4 Histomorphometric analyses

At 25 days of gestation, significant differences ($P < 0.05$) were observed between the genetic groups. Commercial sows showed greater PET ($P = 0.021$), while Piau showed greater VP-P ($P = 0.011$) (Table 3). These results suggest different placental development strategies, with each genetic group prioritizing distinct aspects of the maternal-fetal interface.

In the endometrium, Commercial sows displayed greater ECT ($P < 0.0001$), whereas Piau sows had greater VB-E ($P = 0.0001$) and greater EUG ($P < 0.0001$), suggesting a more vascularized uterine environment in the Piau, contrasting with the denser architecture in Commercial sows (Table 3).

Moreover, previous studies suggest that the morphofunctional superiority of Commercial sows related to better uterine morphology and placental efficiency (Foxcroft *et al*., 2009). Piau sows, with less selective pressure, may compensate through enhanced vascularization and glandular activity, optimizing the uterine environment even with lower ovulatory efficiency (Montes et al., 2018; Silva et al., 2014).

At 35 days of gestation, no significant differences ($P > 0.05$), were identified, but trends towards significance were observed for PET ($P = 0.08$) and TRO ($P = 0.06$) in Piau sows, suggesting an earlier onset of placental differentiation (Table 3). The absence of marked differences at 35 days may reflect physiological adaptations to the increasing fetal demand at this stage.

In the endometrium, greater ECT was observed in Commercial sows ($P = 0.001$), while Piau sows showed increased EUG ($P < 0.0001$) (Table 3). These findings indicate that each genetic group adopts distinct morphofunctional strategies during the initial stages of gestation.

These results align with the De Faria et al. (2019), who reported lower ovulation rate, fewer follicles, smaller follicular diameters, and lower estradiol levels in Piau compared to Commercial sows, along with differences in angiogenesis related gene expression. Such evidence indicates that genetic factors modulate reproductive physiology, reflecting divergent

strategies: structural efficiency in Commercial sows versus vascular and functional efficiency in Piau.

Montes et al. (2022) complement this interpretation, showing that although genes like *VEGFA, ANGPT1/2, TEK,* and *HIF1α* had similar expression between groups, endometrial vascular density was significantly greater in Piau sows at days 7 and 15 of gestation, indicating earlier vascular activation.

At 25 days, the predominance of blood vessels and connective tissue in the endometrium underscores their role in enhancing oxygenation, nutrient delivery, and waste removal. Vascular development, which intensifies after implantation and peaks around 70 days, is essential for an efficient maternal-fetal interface (Reynolds et al*.,* 2006).

Therefore, early uterine structural differences may directly influence fetal nutrient transfer. In Commercial sows, the increase in ECT at 35 days, along with endometrial remodeling, reflects a more advanced adaptation to support fetal demands. These findings suggest that genetic groups employ distinct physiological strategies with potential consequences for gestational success and reproductive performance.

Table 3. Comparison of the histomorphometric variables of uterine tissues of the right horns between Piau and Commercial sows at 25 and 35 days of gestation

Organ	Piau (25 days)	Commercial (25 days)	SEM	P-value
PLACENTAL TISSUES				
PET (%)	5.67	8.98	0.06	0.021*
PCT (%)	71.12	69.57	0.18	0.705
VP-P (%)	11.65	6.45	0.08	0.011*
TRO (%)	11.56	15.01	0.07	0.068
ENDOMETRIAL TISSUES				
ECT (%)	62.85	82.36	0.12	< 0.0001*
VB-E (%)	18.97	8.60	0.23	< 0.0001*
EUG (%)	18.19	9.04	0.23	< 0.0001*
Organ	Piau (35 days)	Commercial (35 days)	SEM	P-value
PLACENTAL TISSUES				
PET (%)	6.18	4.21	0.01	0.08
PCT (%)	70.86	77.79	0.18	0.17
VP-P (%)	11.08	10.43	0.26	0.85
TRO (%)	11.88	7.56	0.07	0.06
ENDOMETRIAL TISSUES				
ECT (%)	58.92	71.33	0.24	0.001*
VB-E (%)	14.27	16.34	0.12	0.26
EUG (%)	26.81	12.34	0.45	< 0.0001**

Piau and Commercial genetic groups evaluated 25 days of gestation, with three sows per group and gestational age (n = 3). Placental tissues: PET: placental epithelial thickness; PCT: placental connective tissue; VB-P: placental vascularization; TRO: fetal trophoblast epithelium, excluding maternal uterine epithelium. Endometrial tissues: ECT: endometrial connective tissue; VB-E: endometrial

vascularization; EUG = endometrial uterine glands. *Significant at *P Anova* ≤ 0,05. SEM: Standard error of the mean. Values are presented as percentages, totaling 100% per tissue group.

4 CONCLUSION

Histomorphometric differences observed in the placenta and endometrium between the genetic groups directly influence gestational development. The Commercial group demonstrated greater reproductive investment, with more developed uterine horns and a higher number of corpora lutea, supporting sustained fetal development. In contrast, Piau sows, despite a lower ovulation rate, exhibited accelerated embryonic growth and higher placental vascularization at 25 days, which was not sustained at 35 days. These findings confirm the hypothesis that genetic groups differ in uterine and placental structure as well as fetal development, suggesting distinct morphofunctional strategies for supporting gestation. Further studies are needed to clarify how these structural differences affect fetal viability and overall reproductive efficiency.

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Data availability statement: Research data is available in the body of the article.

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SUPPLEMENTARY APPENDICES

https://s3.amazonaws.com/host-client-assets/files/animreprod/1984-3143-ar-23-1-e20250070-suppl1.pdf

Table 1. Normality (Shapiro–Wilk) and homogeneity of variances (Levene's test) for raw data (pre-transformation) and transformed data (log or square-root) of the traits evaluated in Piau and Commercial genetic groups at 25 days of gestation.

Table 2. Normality (Shapiro–Wilk) and homogeneity of variances (Levene's test) for raw data (pre-transformation) and transformed data (log or square-root) of the traits evaluated in Piau and Commercial genetic groups at 35 days of gestation

CHAPTER 2

Early fetal sex identification in the Piau pig breed using Y chromosome–linked transcripts

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Abstract: Sexual differences in embryos and fetuses are traditionally attributed to the development of the reproductive system and the production of sex hormones. In this study, the sex of Piau pig conceptuses (25-day embryos and 35-day fetuses) was determined using RNA-seq data through the detection of Y chromosome–linked transcripts. After quality control, 17 conceptuses were analyzed (8 embryos and 9 fetuses). Sex classification was based on the sum of counts per million (ΣCPM_{chrY}) of twelve candidate genes, with thresholds above 200 for males and below 2 for females, wich allowed consistent separation between sexes. Among the identified transcripts, five genes (*LOC102162178, ENSSSCG00000025253, USP9Y, LOC110257938*, and *ENSSSCG00000047835*) are reported for the first time in association with sex determination in pigs. Network analysis revealed regulatory associations between these transcripts and transcription factors involved in gonadal development, including SRY, SOX9, and NOBOX. This study demonstrates that RNA-seq enables sex identification at early stages of swine development and reveals novel Y chromosome–linked transcripts in the Piau breed and their potential regulatory associations during swine sexual differentiation.

Keywords: local breed, sexing, swine, transcriptome, Y chromosome.

1 INTRODUCTION

The domestic pig is widely used as a model in developmental and genetic studies, and embryonic–fetal sex can influence prenatal developmental processes, making sex determination essential for intrauterine experiments [1]. However, at early stages of gestation, male and female embryos and fetuses cannot be distinguished phenotypically or histologically, as the gonads remain undifferentiated until more advanced stages of development [2,3].

In mammals, sex is genetically determined by the presence of the Y chromosome [4], and molecular approaches based on the expression of sex chromosome–linked genes have proven effective for early sexing [5]. In this context, RNA sequencing (RNA-seq) enables the detection of Y chromosome–linked transcripts prior to gonadal differentiation [5,6], overcoming the limitations of phenotype-based methods.

In pigs, sex identification using RNA-seq has been demonstrated in commercial lines [6] but has not yet been explored in local breeds. The Piau breed, a Brazilian local genotype characterized by rusticity, disease resistance, and high fat deposition, represents an important genetic resource that remains poorly investigated at the transcriptomic level [7,8,9]. Thus, this study aimed to determine the sex of Piau embryos and fetuses at 25 and 35 days of gestation through the expression of Y chromosome–linked transcripts using RNA-seq.

2 MATERIAL AND METHODS

2.1 Experimental population and collection of biological material

This study was approved by the Animal Use Ethics Committee of the Federal University of Viçosa (CEUA/UFV no. 52/2024) and conducted in accordance with the ARRIVE guidelines and European Directive 2010/63/EU.

Six primiparous Piau sows were used, with mean body weights of 112 ± 7.3 kg at 25 days and 122 ± 10.5 kg at 35 days of gestation and were inseminated with semen from boars of the same breed. For conceptus collection, three females from each gestational age were subjected to electrical stunning restricted to the head (240 V, 1.3 A), followed by immediate exsanguination and slaughter. In each sow, three conceptuses were collected from the proximal, medial, and distal regions of the left uterine horn, totaling 18 samples (n = 9 embryos and n = 9 fetuses). Samples were washed with phosphate-buffered saline, identified, snap-frozen in liquid nitrogen, and sent for RNA extraction and sequencing.

2.2 RNA extraction and library preparation

Total RNA was extracted using TRIzol and purified with the RNeasy Mini Kit (Qiagen), according to the manufacturers' instructions. RNA concentration was determined using a Qubit fluorimeter (Thermo Scientific), and integrity was assessed by agarose gel electrophoresis and the Agilent 2100 Bioanalyzer. Only samples with RNA integrity number (RIN > 8) were used for library preparation with the Illumina Ribo-Zero Plus kit for ribosomal RNA depletion, following the manufacturer's instructions.

2.3 Sequencing, Quality Control and Mapping

The libraries were sequenced on an Illumina HiSeq 2500 platform (Illumina, Inc., San Diego, CA, USA). FASTQ files were deposited in the Illumina BaseSpace database under project number NGS751, and RNA-seq alignment statistics are provided in Supplementary Table 1. Quality control and read mapping were performed using the BAQCOM pipeline [10]. Trimmomatic (v0.39) [11] was used to remove adapters, short reads (<70 bp), and low-quality sequences (QPhred < 20). Filtered reads were aligned to the *Sus scrofa* reference genome (version 11.1), with Ensembl annotation release 112, using STAR (v2.7.11b) [12].

After quality control, one embryo sample (Sample-01-01E) at 25 days of gestation was excluded due to low read quality, resulting in 17 conceptuses (8 embryos and 9 fetuses) used in subsequent analyses (Supplementary Table 1).

2.4 Sex identification

Sex determination of the conceptuses was performed based on differences in counts per million (CPM) of Y chromosome linked transcripts obtained from RNA-seq data. Conceptuses were classified as males when the sum of CPM values of Y chromosome transcripts (ΣCPM_{chrY}) was greater than 200 and as females when (ΣCPM_{chrY}) was lower than 2, following an adaptation of the methodology described by Teixeira et al. [6], using the R statistical environment [13].

2.5 Functional Annotation – Gene Networks

Transcripts showing markedly divergent expression between males and females at both gestational ages were selected. Promoter sequences (FASTA) were obtained from regions flanking the transcription start sites (3.000 bp upstream and 300 bp downstream) [14], based on the *Sus scrofa* 11.1 reference genome from NCBI. Promoters of candidate genes were analyzed using TFM-Explorer software (matrices from the JASPAR database) [15], and significant transcription factors (TFs) were analyzed in Cytoscape with BiNGO [16] to identify significantly enriched gene ontology terms. Based on biological processes associated with sexual differentiation and supporting literature evidence, a gene–TF network was constructed, and its topology was evaluated using the NetworkAnalyzer tool in Cytoscape [17].

3 RESULTS

3.1. RNA-Seq data

The average number of reads per sample after quality control was 26.7 million for embryos (n = 8) and 28.4 million for fetuses (n = 9) Library processing resulted in high read mapping rates to the *Sus scrofa* reference genome (*version* 11.1), enabling robust quantification of gene expression. On average, approximately 0.04% of reads were mapped exclusively to the Y chromosome. Despite the presence of reads mapped to the Y chromosome, it was not possible to distinguish male and female embryos and fetuses based on morphological characteristics, as gonadal differentiation is not yet visible at these developmental stages, as illustrated in Figure 1.

3.2. Sex determination

Of the 131 genes located on the Y chromosome, 90 protein-coding genes. Of these, 12 showed consistently higher expression in males and lower expression in females and were therefore considered candidates for early sex determination. Male conceptuses exhibited mean ΣCPM_{chrY} values (272.84 ± 12.89), whereas females showed very low mean values (1.05 ± 0.22), demonstrating a clear distinction between sexes (Tables 1 and 2). Among the 17 samples analyzed, of the 8 embryos, 3 were identified as males and 5 as females; among the 9 fetuses, 4 are males and 5 are females (Tables 1 and 2).

The transcripts identified in embryos were *EIF2S3Y* (eukaryotic translation initiation factor 2 subunit 3), *LOC102162178* (FAF-X ubiquitin carboxyl-terminal hydrolase), *USP9Y* (ubiquitin-specific peptidase 9, Y-linked), *LOC110257938* (PI-PLC X domain-like protein), and *LOC110257894* (gamma-taxilin-like protein). In fetuses, the identified transcripts were *ZFY* (Y-linked zinc finger protein), *ENSSSCG00000025253* (novel Y-linked gene), *DDX3Y* (ATP-dependent RNA helicase DDX3X), *KDM5D* (lysine demethylase 5D), *LOC110255257* (orofaciodigital syndrome-like protein), *EIF1AY* (eukaryotic translation initiation factor 1A, Y-linked), and *ENSSSCG00000047835* (novel Y-linked gene).

Among these, five genes *LOC102162178, ENSSSCG00000025253, USP9Y, LOC110257938,* and ENSSSCG00000047835 were reported for the first time in association with sexual differentiation in pigs, representing novel biomarkers for early sexing in Piau pigs.

3.3. Functional analysis

Functional analysis of candidate genes involved in sex determination revealed consistent associations with biological processes and regulatory mechanisms related to sexual differentiation. Among the twelve transcripts showing markedly divergent expression between males and females, eight genes *(ZFY, DDX3Y, KDM5D, LOC110255257* and *EIF1AY* from fetus*; e EIF2S3Y, LOC102162178* and *LOC110257894* from embryos*)* had annotated flanking regions in the NCBI database, enabling the identification of transcription factors (TFs) associated with sex-related regulatory pathways.

In total, 22 TFs were identified (Supplementary Table 2), of which three key TFs (SRY, NOBOX, and SOX9) were associated with genes expressed at both gestational ages and were selected for the construction of the gene–TFs interaction network (Supplementary Table 3). Network analysis highlighted *ZFY* and *EIF1AY* in fetuses and *LOC102162178* in embryos

as the most highly connected transcripts, indicating their central roles in processes related to male sex determination, primary sex determination in the germline, and sexual differentiation (Figure 2).

4 DISCUSSION AND CONCLUSION

The present study applies, for the first time, RNA-Seq–based sex determination in Piau breed embryos (25 days) and fetuses (35 days) without the use of additional molecular tools. This strategy enabled early sex identification and the interpretation of Y chromosome–linked (ChrY) transcripts in the context of porcine sexual differentiation.

Among the identified genes, *EIF1AY, EIF2S3Y, KDM5D*, and *DDX3Y* are well recognized for their essential roles in male germ cell proliferation and spermatogenesis [6,18,19]. Although their classical functions are associated with post pubertal spermatogenesis, the early expression of these genes suggests a possible involvement in the initial stages of male sexual differentiation. Similarly, the *ZFY* gene, initially proposed as a candidate testis-determining factor on the human Y chromosome, is expressed in fetal and adult male tissues [20], reinforcing its association with the establishment of the male phenotype.

Another identified gene was *USP9Y*, whose expression in bovine embryos begins at the 8-cell stage and persists through the blastocyst stage, coinciding with embryonic genome activation [21]. Additionally, the transcript *ENSSSCG00000025253,* which remains poorly characterized, is located near promoter regions regulated by long non-coding RNAs (lncRNAs) [22]. These elements are associated with fine transcriptional control during critical developmental periods, suggesting a potential indirect regulatory role in male sexual differentiation.

Some transcripts identified in this study, such as *LOC110257894* and *LOC110255257,* have previously been used for sex determination in pigs [6] and as auxiliary tools in transcriptomic analyses of the placenta and the maternal–fetal interface [23]. In the present study, however, these transcripts assume a central role in the investigation of early sex determination in a local pig breed.

Gene–transcription factor interaction network analysis revealed SRY, NOBOX, and SOX9 as common regulatory factors associated with the most highly connected transcripts (*ZFY* and *EIF1AY* in fetuses, and *LOC102162178* in embryos), linking them to primary sex determination, germline differentiation, and the establishment of the sexual phenotype. SRY

acts as the initiator of the male differentiation cascade by activating SOX9, promoting Sertoli cell differentiation and testis formation from undifferentiated genital ridges in XY embryos [2,24]. In contrast, NOBOX is a transcription factor specific to female germ cells and is essential for ovarian differentiation; its loss of function leads to ovarian developmental failure and sex reversal, as demonstrated in zebrafish [25].

In this context, one transcript deserves particular attention: *LOC102162178,* identified exclusively in embryos and recurrently expressed in males. This gene has not previously been described in studies of sex determination in pigs, and its association with biological processes related to male sexual development suggests that it may represent a novel functional candidate, particularly relevant for non-commercial breeds such as Piau.

When comparing our results with those of Teixeira et al. [6], conducted in commercial pigs (Large White × Landrace × Duroc), we observed both overlaps and relevant differences in ChrY transcripts identified at the same gestational ages. While both studies detected *DDX3Y, KDM5D, ZFY, EIF2S3Y, EIF1AY, LOC110257894,* and *LOC110255257*, three transcripts reported by Teixeira et al. [6], *LOC396706, LOC100625207*, and *LOC110255320* were not observed in our data. Conversely, our study identified five additional transcripts (*LOC102162178, ENSSSCG00000025253, USP9Y, LOC110257938,* and *ENSSSCG00000047835*) that have not yet been reported in pigs. These differences highlight potential breed-specific expression profiles and reinforce the need for transcriptomic analyses in native breeds such as Piau.

These findings are consistent with previous studies reporting distinct transcriptional patterns between Piau and commercial pigs throughout embryonic and fetal development [7,26], indicating that temporal and genetic variations in gene expression may contribute to phenotypic differences between genetic groups, particularly those related to developmental potential, cellular proliferation, and tissue differentiation.

In conclusion, this study demonstrates that early sex determination in pigs can be efficiently achieved using transcriptomic data from ChrY genes. By applying RNA-Seq to a threatened local breed, we identified twelve Y-linked transcripts, including five not previously associated with sex determination in pigs. These findings expand the current understanding of the porcine Y chromosome and indicate that local breeds may exhibit relevant regulatory particularities, even in a biological process that is highly conserved among mammals, with implications for reproductive, evolutionary, and genetic conservation studies.

Author Contributions: **T.F.M:** Writing – original draft, review & editing, methodology, formal analysis, data curation, investigation, project, visualization. **S.A.T:** Formal analysis, data curation and writing – review & editing. **J.D.G:** Data curation. **L.L.V:** Writing – review & editing, methodology and formal analysis. **S.E.F.G:** Supervision, writing – review & editing, funding acquisition, and project administration.

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APPENDICES

Table 1: Read counts per million of 12 genes located on the Y chromosome of male Piau pig conceptuses.

Conceptuses Sample-ID [1]	Counts Per Million (CPM) [2]												(ΣCPM_{chrY}) [3]
	***EIF2S3Y*	**ZFY*	***LOC102162178*	**ENSSSCG00000025253*	**DDX3Y*	***USP9Y*	***LOC110257938*	**KDM5D*	**LOC110255257*	**EIF1AY*	***LOC110257894*	**ENSSSCG00000047835*	
ESample-11-23E	55.19	30.04	0.88	3.04	76.58	67.85	0.88	8.89	7.21	8.65	6.09	0.00	265.30
ESample-13-21E	71.27	32.74	0.48	4.39	78.78	70.95	0.88	8.66	6.99	12.26	6.31	0.28	293.99
ESample-16-09E	60.18	31.26	0.48	4.59	78.11	68.61	0.76	12.19	7.48	9.47	6.40	0.60	280.13
FSample-02-53E	53.44	29.12	0.46	5.20	90.01	57.63	0.49	8.93	6.16	12.93	5.23	0.49	270.11
FSample-06-41E	49.00	27.77	0.37	4.41	90.91	54.22	0.37	7.17	6.36	11.75	4.88	0.30	257.51
FSample-09-32E	52.03	31.89	0.52	5.69	98.08	57.79	0.67	8.59	6.62	13.68	5.43	0.30	281.27
FSample-10-31E	46.95	28.16	0.68	4.75	91.95	55.43	0.58	9.60	5.50	12.44	5.33	0.21	261.58

[1]Identification of conceptuses samples: Three samples of males embryos, represented by samples (ESample-11-23E to ESample-16-09E) and four samples of males fetuses, represented by samples (FSample-02-53E to FSample-10-31E). [2]*EIF2S3Y* eukaryotic translation initiation factor 2 subunit 3); *ZFY* (zinc finger protein, Y-linked); *LOC102162178* (probable ubiquitin carboxyl-terminal hydrolase FAF-X); *ENSSSCG00000025253* (novel Y-linked gene); *DDX3Y* (DDX3X ATP-dependent RNA helicase); *USP9Y* (ubiquitin-specific peptidase 9, Y); *LOC110257938* (PI-PLC X domain-like protein 1); *KDM5D* (lysine demethylase 5D); *LOC110255257* (oral-facial-digital syndrome 1 protein-like); *EIF1AY* (eukaryotic translation initiation factor 1A, Y-linked); *LOC110257894* (gamma-taxilin-like); *ENSSSCG00000047835* (novel Y-linked gene). [3]ΣCPMchrY: sum of CPM readings of 07 genes linked to the Y chromosome in males conceptuses. The transcripts identified at fetuses* were *ZFY, ENSSSCG00000025253, DDX3Y, KDM5D, LOC110255257, EIF1AY* and *ENSSSCG00000047835,* while the transcripts identified at embryos** were *EIF2S3Y, LOC102162178, USP9Y, LOC110257938* and *LOC110257894.*

Table 2: Read counts per million of 12 genes located on the Y chromosome of females Piau pig conceptuses.

Conceptuses Sample-ID [1]	Counts Per Million (CPM) [2]												(ΣCPM$_{chrY)}$ [3]
	***EIF2S3Y*	**ZFY*	***LOC102162178*	**ENSSSCG00000025253*	**DDX3Y*	***USP9Y*	***LOC110257938*	**KDM5D*	**LOC110255257*	**EIF1AY*	***LOC110257894*	**ENSSSCG00000047835*	
ESample-12-22E	0.00	0.18	0.00	0.00	0.04	0.22	0.74	0.00	0.00	0.00	0.00	0.07	1.25
ESample-14-13E	0.05	0.28	0.05	0.00	0.05	0.24	0.43	0.00	0.00	0.05	0.00	0.14	1.28
ESample-15-12E	0.03	0.03	0.03	0.00	0.00	0.22	0.35	0.00	0.00	0.00	0.00	0.03	0.70
ESample-17-03E	0.03	0.29	0.03	0.00	0.09	0.03	0.43	0.00	0.03	0.00	0.00	0.03	0.95
EFSample-18-02E	0.00	0.03	0.00	0.00	0.00	0.20	0.62	0.00	0.00	0.00	0.00	0.03	0.88
FSample-03-52E	0.03	0.13	0.03	0.03	0.09	0.28	0.35	0.00	0.03	0.03	0.00	0.16	1.16
FSample-04-51E	0.11	0.11	0.00	0.00	0.11	0.06	0.45	0.00	0.06	0.00	0.00	0.06	0.96
Sample-05-42E	0.03	0.22	0.03	0.00	0.00	0.12	0.41	0.00	0.00	0.00	0.00	0.03	0.84
FSample-07-39E	0.06	0.32	0.00	0.00	0.06	0.26	0.26	0.00	0.06	0.00	0.00	0.03	1.06
FSample-08-33E	0.08	0.32	0.00	0.00	0.24	0.12	0.44	0.00	0.12	0.04	0.00	0.08	1.44

[1]Identification of conceptuses samples: Five samples of females embryos, represented by samples (ESample-12-22E to ESample-18-02E) and five samples of females fetuses, represented by samples (FSample-03-52E to FSample-08-33E). [2]*EIF2S3Y* eukaryotic translation initiation factor 2 subunit 3); *ZFY* (zinc finger protein, Y-linked); *LOC102162178* (probable ubiquitin carboxyl-terminal hydrolase FAF-X); *ENSSSCG00000025253* (novel Y-linked gene); *DDX3Y* (DDX3X ATP-dependent RNA helicase); USP9Y (ubiquitin-specific peptidase 9, Y); *LOC110257938* (PI-PLC X domain-like protein 1); *KDM5D* (lysine demethylase 5D); *LOC110255257* (oral-facial-digital syndrome 1 protein-like); *EIF1AY* (eukaryotic translation initiation factor 1A, Y-linked); *LOC110257894* (gamma-taxilin-like); *ENSSSCG00000047835* (novel Y-linked gene). [3]ΣCPMchrY: sum of CPM readings of 10 genes linked to the Y chromosome in females conceptuses. The transcripts identified at fetuses* were *ZFY, ENSSSCG00000025253, DDX3Y, KDM5D, LOC110255257, EIF1AY* and *ENSSSCG00000047835,* while the transcripts identified at embryos** were *EIF2S3Y, LOC102162178, USP9Y, LOC110257938* and *LOC110257894*

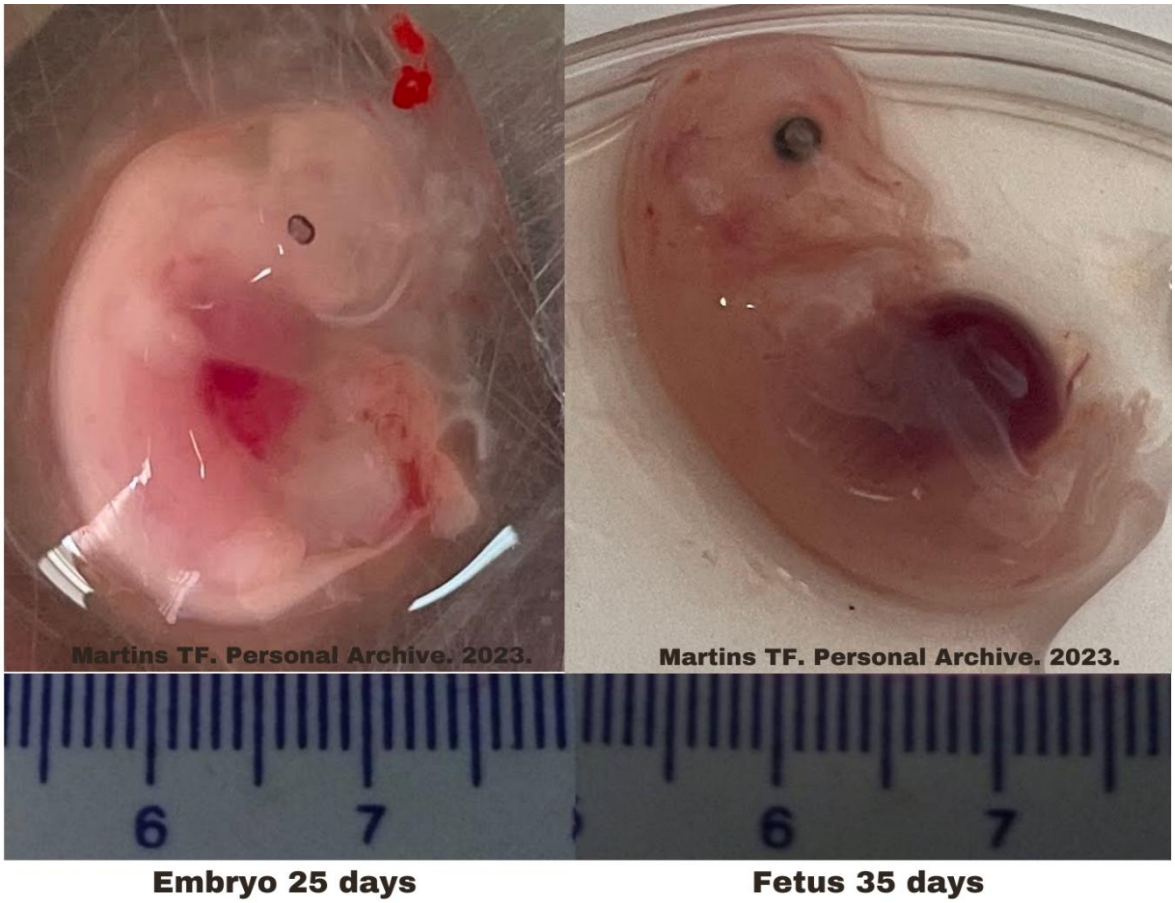

Figure 1: 25-day-old Piau sow embryo (left) and 35-day-old Piau sow fetus (right).

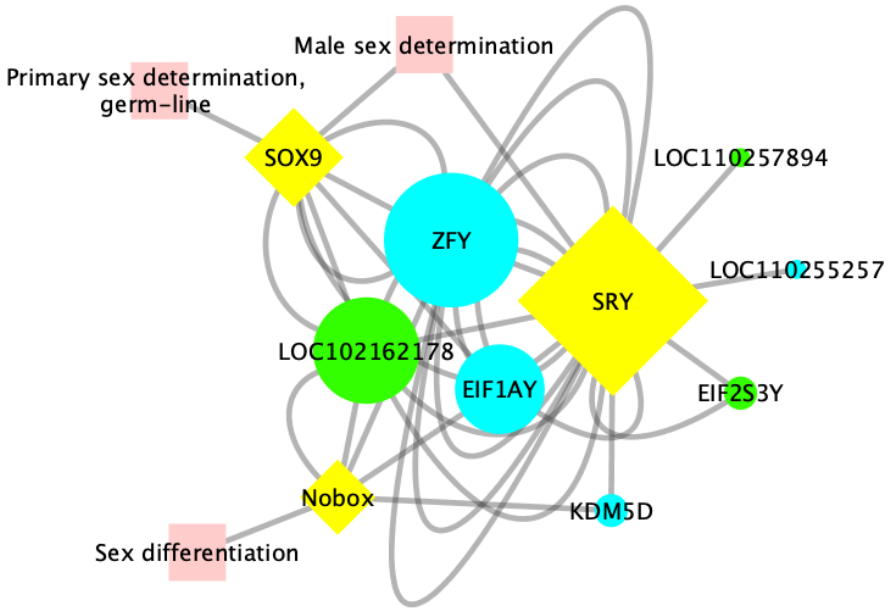

Figure 2: Transcription factor network associated with differentially expressed genes between male and female Piau conceptuses at stages embryos and fetuses. The transcription factors (yellow nodes) are connected to genes identified at fetuses (light blue circular nodes) and at embryos (green circular nodes).

The light pink square nodes represent the biological processes associated with the transcription factors. The size of the nodes reflects the degree of enrichment, proportional to the number of transcription factor binding sites.

SUPPLEMENTARY APPENDICES

Access:

https://drive.google.com/drive/folders/1FIqOQJ6DDvKUff2tqO0U5gjEWP6tOolf?usp=drive_link

CHAPTER 3

Adipogenesis and myogenesis transcriptome in Piau local breed conceptuses and its comparison with Commercial pig data

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Abstract: The growth and development of skeletal muscle, along with intramuscular fat deposition, determine the quantity and quality of pork. The Brazilian Piau breed, which has not undergone genetic improvement, exhibits slower growth, greater fat deposition, and increased disease resistance compared to Commercial lines, making it a valuable genetic resource and a relevant model for studying the molecular regulation of myogenesis and adipogenesis. This study aimed to analyze RNA sequencing (RNA-seq) data from Piau pigs to identify differentially expressed genes and biological processes during two stages of intrauterine development, at 25 and 35 days of gestation. Furthermore, gene expression profiles of Piau conceptuses were compared to those of a Commercial line to investigate transcriptional differences between genetic groups. RNA-seq data from seventeen Piau conceptuses (8 embryos and 9 fetuses) were analyzed and compared with public data from a Commercial pig line (Large White × Landrace × Duroc). Differential expression analysis was performed in R, considering FDR < 0.05 and |log2FC| ≥ 2.0, and functional enrichment was evaluated in Cytoscape. In the Piau breed, 43 gene ontology terms showed significant enrichment, mainly related to myogenesis and adipogenesis. In the Commercial breed, 65 enriched terms were identified, predominantly associated with myogenesis. In total, 41 genes showed differential expression in Piau and 55 in Commercial between gestational stages. Key genes associated with adipogenesis and myogenesis were identified, some with functions dependent on the genetic group. These findings contribute to the understanding of molecular differences between genetic groups and support future breeding strategies.

Keywords: fat, prenatal development, skeletal muscle, pig, transcriptomics.

1 INTRODUCTION

Skeletal muscle development and growth in pigs are complex processes primarily regulated by myogenesis and adipogenesis, wich directly influence phenotypes traits expressed throughout postnatal life. Prenatal myogenesis occurs in two major waves: the formation of primary muscle fibers between 30 and 60 days of gestation [1], followed by the development of secondary fibers between 54 and 90 days of gestation [2]. In parallel, adipogenesis begins around day 45 of gestation and is accompanied by stromal and vascular organization of subcutaneous tissue [3].

The total number of muscle fibers is established predominantly during the prenatal period and represents a key determinant of postnatal muscle growth potential [4, 5]. In pigs, postnatal skeletal muscle growth occurs mainly through hypertrophy of pre-existing fibers and is associated with changes in muscle fiber type composition over time [5]. Consequently, gestational events play a crucial role in shaping productive traits, as muscle development directly affects pork yield and quality [6], while intramuscular fat content influences sensory attributes such as tenderness, juiciness, and flavor, thereby impacting consumer acceptance [7].

Beyond fiber number, muscle fiber type composition constitutes an essential functional component of skeletal muscle. Comparative studies among pig breeds with different growth

rates have shown that slow-growing animals exhibit a higher proportion of slow-twitch fibers, associated with increased expression of the MYHC-I isoform, whereas fast-growing breeds present a greater proportion of fast-twitch fibers linked to MYHC-II isoforms [8]. These differences reflect functional adaptations of muscle tissue, including variations in fiber diameter and relative fiber-type proportions [9].

Such phenotypic variation among pig breeds reflects marked differences in body conformation and tissue composition [1], resulting from factors such as origin, different domestication, and genetic selection [10, 11, 12]. Therefore, understanding the molecular mechanisms regulating muscle growth and fat deposition is crucial for genetic improvement and increased production efficiency in pig farming.

In the context, the Piau pig, a Brazilian breed, is characterized by disease resistance, hardiness, low prolificacy, slower growth, and greater fat deposition compared with Commercial line pigs [12, 13, 14]. Although underutilized in modern production systems, the Piau represents a valuable genetic resource and an excellent model for investigating the molecular mechanisms underlying muscle and adipose tissue development, as well as breed-specific biological differences, contributing to advances in functional genomics.

Accordingly, transcriptome analysis represents a powerful approach for uncovering the molecular mechanism governing intrauterine development and for elucidating key regulatory differences between genetics groups at the transcription level [15,16]. To date, comprehensive transcriptome sequencing of the Piau breed has not been reported, limiting our understanding of the effects of genetic selection and evolutionary differences among pig breeds.

Given that early myogenic events occur before the visible formation of muscle fibers and are decisive for establishing the total number of fibers [1, 2, 4, 5], gestational days 25 and 35 represent critical early stages of skeletal muscle development in pigs. These developmental windows were therefore selected to investigate regulatory mechanisms involved in the molecular programming of muscle fiber development.

Thus, the objective of this study was to sequence Piau pig RNA to identify differentially expressed genes and biological processes during two distinct periods of intrauterine development (25 and 35 days of gestation). In addition, gene expression profiles of Piau conceptuses were compared with those of a Commercial line to explore transcriptional regulatory differences between breeds, particularly those related to myogenesis and adipogenesis.

2 MATERIAL AND METHODS

2.1 Animal Experiment

This research was conducted at the Federal University of Viçosa, Brazil (20º 45' 14" S, 42º 52' 55" W), in a tropical climate with a dry season in winter (Köppen classification: Aw). The study was approved by the UFV Animal Use Ethics Committee (CEUPA/UFV no. 52/2024) and conducted in accordance with the ARRIVE guidelines and European Directive 2010/63/EU.

A total of six Piau females, with an average weight of 112 ± 7.3 kg at 25 days and 122 ± 10.5 kg at 35 days, were mated with pooled semen from Piau boars. For conceptus collection, three females per gestational age were electrically stunned at the head (240 V, 1.3 A), immediately exsanguinated, and slaughtered at the Teaching, Research and Extension Unit – Experimental Slaughterhouse (Frigorifico Escola), Federal University of Viçosa, Brazil. From each sow, three fetuses were collected, totaling nine embryos at 25 days and nine fetuses at 35 days. Each fetus was individually macerated in liquid nitrogen for subsequent RNA extraction and sequencing.

2.2 RNA extraction and library preparation

The total RNA extraction from 18 conceptuses (9 embryos and 9 fetuses) was performed using TRIzol reagent (Invitrogen, San Diego, CA, USA), according to a standard protocol. Approximately 100 mg of tissue was macerated, homogenized with 1 mL of TRIzol, and incubated for 5 minutes at room temperature. Then, 200 µL of chloroform was added, shaken vigorously for 15 seconds, incubated again for 5 minutes, and centrifuged at 11,000 × g at 4°C for 15 minutes.

The aqueous phase (600 µL), containing only RNA, was carefully transferred to a new tube, mixed with 600 µL of 70% ethanol, and applied to silica columns from the RNEAsy mini kit (Qiagen, Hilden, Germany). After centrifugation (8000 x g, 15 s), the eluate was discarded and the column washed with 700 µL of buffer RW1, followed by two washes with 500 µL of buffer RPE. Finally, the RNA was eluted in 50 µL of RNase-free water.

Quantification was performed with a QUBIT fluorimeter (Thermo Scientific, Waltham, MA, USA), and integrity was assessed on a 1% agarose gel and an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with RIN >8 were used for library preparation, ensuring high quality for sequencing. Libraries were prepared with

the Illumina Ribo-Zero Plus kit for ribosomal RNA depletion, following the manufacturer's recommendations.

2.3 RNA-seq data processing and quality control

Piau breed libraries were sequenced on an Illumina HiSeq2500 (Illumina, Inc.; San Diego, CA, USA). FASTQ files were deposited in the BaseSpace database (http://basespace.illumina.com/) under project number NGS751. Read alignment statistics for RNA-seq of the Piau breed transcripts are available in Supplementary Table 1. Each library sample was classified according to gestational age and sex. The sex determination of the conceptuses resulted in three male and five female embryos, and four male and five female fetuses. The Piau conceptuses were classified as male when the sum of CPM (counts per million) mapped on chromosome Y (ΣCPM chrY) exceeded 200, and as female when the sum was bellow two. This approach was adopted from the methodology previously established for Commercial line conceptuses [17], and it adaptation for Piau conceptuses is currently under peer review.

Quality control (QC) and read mapping were conducted with the BAQCOM pipeline [18], https://github.com/hanielcedraz/BAQCOM, accessed May 7, 2024. Trimmomatic (v0.39) [19] was used to remove adapters, short reads (<70 bp), and sequences with quality lower than QPhred < 20. From these reads, a principal component analysis (PCA) was performed in R environment [20]. Filtered reads were aligned to the *Sus scrofa* genome (version 11.1) with Ensembl 112 annotation (https://may2024.archive.ensembl.org/index.html), accessed May 17, 2024, using STAR (v.2.7.11b) [21].

2.4. Differential expression gene (DEG) analysis

The DEG between Piau embryos and fetuses was performed with the Limma package [22] in the R environment [20], considering fixed effects of sex (males and females), gestational age (25 days; embryo or 35 days; fetus) and interaction between both (sex and gestational age), according to the model:

$$Y_{ijk} = \mu + S_i + GA_j + (S \times GA)_{ij} + \varepsilon_{ijk}$$

Where: Y_{ijk} is the value of the logarithmic expression (log-CPM) of gene *k* in the i^{th} sex and j^{th} gestational age; μ is the overall mean; S_i is the fixed effect of sex (*i* = male or females); GA_j is the fixed effect of gestational age ($_j$ = 25 or 35 days); $(S \times GA)_{ij}$ is the interaction

between sex and gestational age; and ε_{ijk} is the residual effect.

Because DEGs were identified by contrast between the two gestational ages, the direction of the logFC indicates the relative upregulation at each age. Genes with a positive logFC indicated higher expression in fetuses, while genes with a negative logFC indicated higher expression in embryos. DEGs were annotated based on Ensembl version 114 (https://www.ensembl.org/index.html), accessed on June 7, 2025.

The statistically significant DEGs were set on false discovery rate (FDR)-adjusted *P*-value < 0.05, according to the Benjamini-Hochberg method [23]. Furthermore, to highlight the main differences in the gene expression between gestational ages and facilitate comprehension, only DEG with $|\log2FC| \geq 2.0$ were considered for functional analysis.

2.5. Functional annotation

Gene Ontology (GO) analyses were performed in Cytoscape version 3.10.3 [24] with the ClueGO application, version 3.10 [25] (http://apps.cytoscape.org/apps/cluego), accessed on June 16, 2025, using *Sus scrofa* genome as a reference.

In order to highlight the biological processes related with myogenesis and adipogenesis, GO terms were filtered in R software [20] in two sequential steps: (1) semantic search for keywords related to myogenesis (myogenesis '*muscle', 'myo', 'myogen', 'myogenesis'*; and (2) semantic search for keywords related to adipogenesis ('*adipo', 'adipocyte', 'fat', 'lipid', 'triglyceride'*).

2.5.1. Comparative GO analysis between Piau and Commercial line conceptuses

In an effort to obtain a comprehensive analysis of the main differences in biological processes resulting from genetic improvement, a comparative Gene Ontology (GO) analysis was performed between the Piau and Commercial pig line. The RNA-sequencing data from Commercial line conceptuses used in this study were previously analyzed by Teixeira et al. [15] and it is available at the SRA repository (PRJNA576701: https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA576701).

In order to perform a comparison with the Commercial transcriptome, the statistical model incorporated the same effects, and the same filtering criteria were applied to identify DEG between prenatal ages (25 and 35 days old) ($|\log2FC| \geq 2.0$; FDR < 0.05) and between sexes ($|\log2FC| \geq 0.5$; FDR < 0.05) (Supplementary Table 2). The same procedures were also

used to perform the GO analysis. In addition, an analysis was conducted on DEGs and GO terms that were unique to Piau and commercial line conceptuses to identify the particularities of each genetic group (GG).

3 RESULTS

3.1. RNA-Seq data

The average number of reads per Piau sample, after the quality control procedure, was 26.7 million for embryos (n=8) and 28.4 million for fetuses (n=9). During quality control, sample Emb-01- (25 days of gestation) was excluded due to poor read quality (Supplementary Table 1). Library processing resulted in 100% of the reads successfully mapped to the *Sus scrofa* reference genome (*version* 11.1), allowing robust quantification of gene expression. PCA showed consistent clustering of samples by gestational age, with clear separation between embryos and fetuses (Figure 1). The complete gene expression matrix normalized to log_2-CPM is available at Supplementary Table 3.

3.2 Total Differentially Expressed Genes (DEGs) in Piau pig conceptuses

The interaction between gestational age and sex did not result in differentially expressed genes (DEGs) (FDR > 0.05), indicating that the combined effect of sex and gestational age does not significantly alter expression patterns between Piau embryos and fetuses. Consequently, the main effects of gestational age and sex were analyzed separately.

Regarding the effect of sex (males and females), only three genes in 25 days of gestation, *EIF2S3Y* (eukaryotic translation initiation factor 2, subunit 3, Y-linked structural gene), *ZFX* (X-linked zinc finger protein), and *KDM6A* (lysine demethylase 6A), were identified as DEGs (Supplementary Table 4).

The DEGs analysis identified 1.486 genes with increased expression in fetuses and 614 genes with higher expression in embryos, while 18.911 genes showed no significant differences (FDR > 0.05) (Figure 2). Considering the DEG between gestational age (25 and 35 days) with $|log2FC| \geq 2.0$, a total of 456 DEGs and were identified. Of these, 404 genes were upregulated in fetuses, while 52 genes were upregulated in embryos (Supplementary Figure 1; Supplementary Table 5).

3.3 Comparative Gene ontology (GO) functional analysis between Piau and Commercial pig conceptuses

In Piau conceptuses, 43 significantly enriched GO terms (FDR < 0.05) were identified, including 3 related to adipogenesis (*triglyceride metabolic process*, *neutral lipid metabolic process* e *acylglycerol metabolic process)* and 40 to myogenesis. Of these terms, 10 were shared between 25 and 35 days, whereas 33 were exclusive to 35 days (Supplementary Table 6). Among the GO terms identified only in this genetic group, three were exclusive to 35 days: *skeletal muscle tissue regeneration* (related to myogenesis and involving 3 genes), and *neutral lipid metabolic process* and *acylglycerol metabolic process*, both related to adipogenesis and involving 4 genes each (Table 1).

In Commercial conceptuses, 65 significantly enriched GO terms (FDR < 0.05) were identified, including one related to adipogenesis (*triglyceride metabolic process)* and 64 to myogenesis. As observed by the Piau group, 10 terms were present at both gestational ages, whereas 55 were exclusive to 35 days (Supplementary Table 7). Among these, 25 GO terms were exclusive to the Commercial line at 35 days, all related to myogenesis and involving 4 to 9 genes. These processes include *muscle cell differentiation, organization of contractile filaments*, and *muscle hypertrophy* (Table 1).

Based on the identified biological processes, we describe below the differentially expressed genes associated with these patterns in both genetic groups.

3.4. Differentially expressed genes (DEGs) associated with myogenesis and adipogenesis

Among the total number of DEGs in Piau, 41 genes were associated with myogenesis and adipogenesis, being two in embryos and 39 in fetuses (Supplementary Table 8). Among them, seven were exclusive to Piau fetuses: *GPX1* (Glutathione peroxidase 1), *LMOD3* (Leiomodin 3), *MYMK* (Myomaker, myoblast fusion factor), *MYOZ2* (Myozenin 2), *RBM38* (RNA binding motif protein 38), *TMOD1* (Tropomodulin 1) and *XK* (X-linked Kx blood group antigen, Kell and VPS13A binding protein) (Table 2).

In Commercial, 55 genes were identified, two for embryos and 53 for fetuses (Supplementary Table 9). Among them, 21 were exclusive to Commercial fetuses, including *MYL2*, *MYL4, MYL6* (Myosin light chain 2, 4 and 6), *ACTC1* (Actin alpha cardiac muscle 1), *ACTN2* (Actinin alpha 2), *SRPK3* (SRSF protein kinase 3), *MYF6* (Myogenic factor 6), *CAPN3*

(Calpain 3), *CASQ2* (Calsequestrin 2), *MEF2C* (Myocyte enhancer factor 2C), *LMOD2* (Leiomodin 2), *TNNT1* and *TNNT2* (Troponin T1 and T2), *MYH11* (Myosin light chain 11), *MYOM1* (Myomesin 1), *NMRKL2* (Nicotinamide riboside kinase 2), *RBM24* (RNA binding motif protein 24), *SMYD1* (SET and MYND domain containing 1), *SCN10A* (Sodium voltage-gated channel alpha subunit 10), *TRIM54* (Tripartite motif containing 54), *TCAP* (Titin-cap) (Table 2).

Among the identified DEGs, four genes: *C3* (Complement C3), *CAV3* (Caveolin 3), *SLC22A4* (Solute carrier family 22 member 4), and *GPX1* (Glutathione peroxidase 1) are associated with biological processes related to adipogenesis described in section 3.3.1 (Figure 3). Of these, *C3* and *SLC22A4* have a specific function in adipogenesis and were detected in both groups, while *GPX1* is exclusive to the Piau breed and functions in both adipogenesis and myogenesis. *CAV3* is present in both groups and functions in both processes (Supplementary Tables 8 and 9).

The remaining DEGs were predominantly related to myogenesis, involving muscle development processes in Piau or Commercial conceptuses (Figure 4).

4 DISCUSSIONS

4.1 DEGS of sex

Sex-specific genes were identified in our study, including *EIF2S3Y* (eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked), *ZFX* (zinc finger protein X-linked), and *KDM6A* (lysine demethylase 6A).Traditionally, these genes are associated with sexual differentiation: *KDM6A* epigenetically regulates testicular development and male germ cell differentiation [26]; *EIF2S3Y*, together with *SRY*, induces testicular differentiation and promotes spermatogonia proliferation [27]; and *ZFX* acts in the autoregulation of embryonic and hematopoietic stem cells [28].

Although these genes have classic roles in sexual differentiation, recent evidence indicates additional functions in specific tissues. Bengtsen et al. [29] identified *ZFX* as a transcription factor enriched in the nuclei of fast fibers of the *extensor digitorum longus* muscle, compared to slow fibers of the *soleus* muscle. Complementarily, Tang et al. [30] observed increased *ZFX* expression in smooth muscle cells of pulmonary arteries in mice subjected to hypoxia or pulmonary arterial hypertension and showed that manipulation of this gene

modulates the proliferation and remodeling of these cells.

The *KDM6A* gene, also known as histone demethylase *UTX*, is essential for the epigenetic regulation of embryonic stem cells and for the development of various tissues, including cardiac, mammary, and immune development [31]. Faralli et al. [32] demonstrated that the demethylase activity of *KDM6A* is crucial for satellite cell-mediated skeletal muscle regeneration, as the absence of this enzyme causes an accumulation of *H3K27me3*, a repressive epigenetic mark, in the promoters of muscle differentiation genes, such as *MYOG* (myogenin), *CKm* (creatine kinase, muscle type 2), and *TNNC2* (troponin C type 2, fast skeletal), preventing their activation and compromising muscle regeneration.

The *EIF2S3Y* gene plays an important role in maintaining pluripotency and cell proliferation in embryonic stem cells [33]. Although there is no direct evidence in other tissues, its function in fundamental cellular processes suggesting it may indirectly influence cellular activity in different tissues.

Thus, the presence of these genes in our data suggests that they may modulate expression patterns in different tissues, including muscle. However, their direct function in muscle development still needs to be clarified, especially for *EIF2S3Y*, whose activity, according to the literature, has been evidenced mainly in embryonic stem cells, with no direct reports in other tissues.

4.2. Adipogenesis

Literature reports that the development of subcutaneous fat in pigs begins only after 45 days of gestation, when the first clusters of adipose cells become visible [3]. However, in our study, evaluating gestational periods of 25 and 35 days, we were able to demonstrate, through the identified transcripts, signs of adipogenesis activation at 35 days of gestation in both Piau and commercial pig conceptuses, indicating that the process is already underway at this stage.

The genes identified in processes related to adipogenesis (*C3, CAV3, SLC22A4,* and *GPX1* in Piau conceptuses and *C3, CAV3, SLC22A4* in Commercial conceptuses) have key functions in the regulation of lipid metabolism and in the cellular dynamics associated with fat deposition. In our dataset, *C3* and *SLC22A* showed specific activity in the adipogenic pathway, while *CAV3* and *GPX1* play multifunctional roles, participating in both adipogenesis and myogenesis, reflecting the metabolic overlap between both processes during fetal development.

The *C3* gene encodes the ASP precursor protein (C3adesArg), whose absence is associated with alterations in energy expenditure and fatty acid oxidation in adipose tissue [34]. ASP acts through the C5L2/C5aR2 receptor, promoting fatty acid transport and stimulating triacylglycerol synthesis in adipocytes [35]. Previous studies have detected *C3* as a differentially expressed gene related to immunological pathways and lipid metabolism [36].

In this context, Chi et al. [37] detected *C3* expressed in subpopulations of subcutaneous adipose tissue in Mongolian cattle, suggesting a role in both fat deposition and resistance to microorganisms. In contrast, in our study, *C3* was specifically associated with adipogenic processes, indicating targeted action in this pathway during embryonic development.

The *SLC22A4* gene (also known as *OCTN1*), member 4 of Family 22 of solute transporters, is a plasma membrane carnitine carrier, essential for mitochondrial fatty acid oxidation and cellular energy metabolism [38, 39]. Evidence in sheep points to this gene as a candidate associated with fat deposition in the tail [40, 41], corroborating its relationship with lipid metabolism observed in our data.

The *CAV3* gene, classically recognized for its structural function in striated muscles [42], is also involved in lipid metabolism by interacting with cholesterol and regulating fatty acid uptake via CD36 [43, 44]. *CAV3* participates in myogenic differentiation, promoting myoblast fusion and myotube formation [45, 46, 47], and is also more highly expressed in type II fast fibers [48], a characteristic associated with differences between swine genotypes, such as Duroc and native Chinese breeds [49]. System biology studies reinforce its multifunctional role: Guo et al. [50] identified *CAV3* as a hub gene associated with oxidation and energy metabolism, and Matsunobe et al. [51] demonstrated that its reduction increases glucose and LDL uptake in myoblasts, highlighting its role in energy regulation during muscle development.

An important contribution of our study is the identification of *GPX1* as a differential gene associated with adipogenesis exclusively in the Piau breed. In the Commercial group, only the *triglyceride metabolic process* was enriched, while in Piau the *neutral lipid metabolic process* and the *acylglycerol metabolic process* were also enriched. *GPX1* transcripts were identified in the three processes, suggesting a broader role in Piau lipid metabolism.

GPX1 (cytoplasmic glutathione peroxidase) is an intracellular selenoprotein composed of four identical subunits, each containing a selenocysteine, the 21st amino acid of the genetic code [52]. This structure allows the enzyme to reduce hydrogen peroxides and lipid hydroperoxides, playing an essential role in maintaining redox balance, along with catalase and superoxide dismutase [53,54] in different cellular compartments such as cytosol, mitochondria and peroxisomes [55, 56, 57], reinforcing its role in lipid metabolism observed in the Piau breed.

In adult pigs, Ren et al. [58] demonstrated that *GPX1* exerts a strong protective effect on splenic lymphocytes, reducing oxidative damage, apoptosis and stress-induced DNA methylation alterations. On the other hand, silencing *GPX1* intensified these effects, indicating that the enzyme influences not only redox control but also epigenetic mechanisms. These findings are consistent with the expanded enrichment pattern observed in the Piau breed, suggesting that particularities in the antioxidant system may modulate differences in lipid metabolism between genetic groups.

Evidence in murine models reinforces the link between *GPX1* and energy and lipid metabolism: *GPX1*-deficient mice develop normally but exhibit lower tolerance to oxidative stress compared to wild-type mice [59]. In contrast, its overexpression increases glycolysis and lipogenesis, reduces insulin signaling, and may predispose obesity and diabetes [60], indicating that elevated enzyme levels favor lipid pathways.

Therefore, this evidence supports the broader functional pattern of *GPX1* in the Piau breed, a genotype not subjected to intensive selection and historically maintained in more rustic systems, with greater adaptive capacity and disease resistance [12, 13], contrasts with the more restricted action observed in Commercial lines, whose selection directed towards performance and efficiency [1] reduced pressures associated with rusticity and metabolic resistance. These evolutionary and zootechnical differences may influence the mechanisms of lipid deposition and mobilization, contributing to the phenotypic contrasts in adiposity between the genetics groups.

4.3. Myogenesis

Analysis of the genetic groups studied, Piau and Commercial, revealed that the first signs of myogenesis appear at 25 days of gestation, remaining active until 35 days. This period coincides with the establishment of primary fibers, since somites are formed between 14 and 22 days of gestation [61], and primary myotubes appear between 30 and 40 days, marking the first wave of fiber formation [62].

The observed myogenesis patterns, with a greater number of terms related to the myogenic pathway in Commercial compared to Piau, reflect well-established phenotypic differences. Commercial lines, selected for rapid growth and a higher percentage of lean meat, exhibit greater proliferation and maturation of muscle fibers [63], while local breeds, such as Piau, grow more slowly, accumulate a higher intramuscular fat content and have lower body

weight [13].

The presence of key genes in myogenesis suggests distinct muscle development strategies between the groups. In Piau, the skeletal muscle tissue regeneration process involves the *GPX1* gene, which, in addition to acting in adipogenesis, plays an essential role in muscle regeneration, together with *MYMK*, exclusive to Piau and responsible for myoblast fusion [64], and *MYOZ1*, present in both groups, which encodes the calsarcin-2 protein, located in the Z-line of the sarcomere and involved in fiber organization [65].

The coordinated action of the myogenic genes expressed exclusively in Piau, particularly *GPX*1 and *MYMK*, suggests a unique biological pattern that may reflect the evolutionary history of this breed. In the absence of selective pressure for growth, Piau pigs may have retained genes that favor efficient tissue regeneration, gradual myoblast fusion, and more fiber organization formation, resulting in a more controlled and progressive myogenesis, as well as potential increased resistance to diseases. Studies with mice reinforce this role, showing that the absence of *GPX1* reduces resistance to oxidants, increases susceptibility to infections, and alters the proliferation, differentiation, and survival of muscle progenitor cells, resulting in smaller fibers. In contrast, wild-type mice with functional *GPX1* exhibit normal muscle development [66].

The *RBM38* and *RBM24* genes, expressed in Piau and Commercial conceptuses, respectively, also differ in their myogenic strategies. *RBM38* regulates the cell cycle by promoting early-stage arrest, stabilizing p21 mRNA (*CDKN1A*), and modulating the translation of p53 family members (p53, p63, and p73), reducing cell proliferation [67]. In addition, it facilitates transcript elongation, allowing RNA polymerase II to proceed even under splicing defect conditions, a function not shared with *RBM24* [68]. In contrast, *RBM24* in our study was associated with the process of positive regulation of skeletal muscle tissue development. Although it shares RNA Recognition Motif domains with RBM38 and binds to mRNAs, *RBM24* acts primarily in the initiation of transcription and the stabilization of mRNAs through the C-terminal region and interactions with cofactor proteins [68]. In commercial pigs, *RBM24* shows higher expression during the formation of primary and secondary muscle fibers [69], reinforcing an active myogenic program compatible with the accelerated growth of these genetic lines. Thus, while Piau depends on *RBM38* and maintains slower and more controlled myogenesis, the Commercial line uses *RBM24* to support greater proliferation and fiber formation.

The *LMOD3* and *LMOD2* genes, which were exclusive in Piau and commercial conceptuses, respectively, are responsible for the organization of the thin filaments of the

sarcomere, an essential step for fiber stability and the formation of new myofibrils [70, 71]. Although both participate in sarcomeric architecture, *LMOD2* has a particularly important function for the performance of fast fibers and its deficiency compromises the structure of the thin filaments, reducing the strength of both fast and slow fibers [71]. This difference is consistent with the findings reported for other pig native breeds, as reported in Zlotnicka Spotted breed [72]. This breed exhibits a higher proportion of slow oxidative fibers and a lower proportion of fast glycolytic fibers in comparison to commercial pig lines [72]. Thus, the occurrence of *LMOD3* in Piau and *LMOD2* in Commercial reflects their contrasting phenotypes: Piau, more oxidative and slow-growing, and Commercial, more glycolytic and fast-growing.

Finally, in Commercial, the presence of genes such as *ACTC1*, essential for the contractility and organization of cardiac filaments [73], and myosin light chains (*MYL4* and *MYL6*), linked to the proliferation and differentiation of myoblasts [74, 75], reinforces a more active myogenesis program. In addition, *MYOM1* contributes to the sarcomeric integrity and stability of mature muscle fibers, ensuring adequate architecture for efficient contraction [76]. Together, these genes support the accelerated muscle growth and high fiber formation capacity of Commercial.

The genes and biological pathways identified in this study provide a solid foundation for future investigations, which may employ targeted techniques, such as quantitative real time PCR, CRISPR CAS9 and knockout animals, aiming to expand the functional understanding of these genes across different systems and conditions. This information is not only relevant for advancing genetic improvement and conservation of local pig breeds, such as Piau, but also to disentangle the pathways related to the transition between myogenesis and adipogenesis which may be useful for other livestock species.

5 CONCLUSION

This study is pioneering in revealing the gene expression profile during embryonic and fetal Piau pig development. Using a comparative transcriptomic analysis of the Piau breed and a commercial pig line, we identified key differences in prenatal development, particularly in pathways related to myogenesis and adipogenesis. Furthermore, the results highlight the Piau breed as a valuable Brazilian genetic resource, reinforcing its unique characteristics. These findings may provide a molecular basis for understanding and implementing strategies to optimize growth, meat quality, and genetic diversity in swine.

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APPENDICES

Table 1. Exclusive Gene Ontology (GO) terms in each genetic group (Piau and Commercial), considering gestational ages of 25 and 35 days.

Piau (exclusive GO terms)					
TermID[1]	**Description**	**Associated genes[2]**	**Myogenesis or Adipogenesis[3]**	**adj.p-value**	**Gestational age**
GO:0043403	skeletal muscle tissue regeneration	*GPX1, MYMK, MYOZ1*	Myogenesis	1.00	35 days
GO:0006638	neutral lipid metabolic process	*C3, CAV3, GPX1, SLC22A4*	Adipogenesis	1.00	35 days
GO:0006639	acylglycerol metabolic process	*C3, CAV3, GPX1, SLC22A4*	Adipogenesis	1.00	35 days
Commercial (exclusive GO terms)					
TermID[1]	**Description**	**Associated genes[2]**	**Myogenesis or adipogenesis[3]**	**adj.p-value**	**Gestational age**
GO:0033275	actin-myosin filament sliding	*ACTC1, MYL6, MYLK2, TNNT2*	Myogenesis	5.272681e-04	35 days
GO:0031034	myosin filament assembly	*MYBPC1, MYOM1, MYOM2, TCAP*	Myogenesis	1.1692e-03	35 days
GO:0071688	striated muscle myosin thick filament assembly	*MYBPC1, MYOM1, MYOM2, TCAP*	Myogenesis	1.1692e-03	35 days
GO:0048739	cardiac muscle fiber development	*MYBPC1, MYH11, MYOM1, MYOM2, TCAP*	Myogenesis	1.7046e-03	35 days

GO:0031033	myosin filament organization	*MYBPC1, MYOM1, MYOM2, TCAP*	Myogenesis	*2.2655e-03*	35 days
GO:0030049	muscle filament sliding	*MYL6, MYLK2, TNNT2*	Myogenesis	1.6297e-02	35 days
GO:0060048	cardiac muscle contraction	*CASQ2, CAV3, MYL2, MYL4, SCN10A, TCAP, TNNT1, TNNT2, TNNT3*	Myogenesis	2.0277e-02	35 days
GO:0035914	skeletal muscle cell differentiation	*EOMES, MEF2C, MYF6, MYLK2, RBM24, SMYD1*	Myogenesis	2.4883e-01	35 days
GO:0010831	positive regulation of myotube differentiation	*CAV3, MYF6, RBM24, SMYD1*	Myogenesis	2.9959e-01	35 days
GO:0051155	positive regulation of striated muscle cell differentiation	*CAV3, MEF2C, MYF6, RBM24, SMYD1*	Myogenesis	3.5186e-01	35 days
GO:0045661	regulation of myoblast differentiation	*CAPN3, MEF2C, MYF6, NMRK2, SMYD1*	Myogenesis	4.1106e-01	35 days
GO:0045445	myoblast differentiation	*CAPN3, FGF6, MEF2C, MYF6, NMRK2, SMYD1*	Myogenesis	4.8914e-01	35 days
GO:0045663	positive regulation of myoblast differentiation	*MEF2C, MYF6, SMYD1*	Myogenesis	6.8527e-01	35 days

GO:0051149	positive regulation of muscle cell differentiation	*CAV3, MEF2C, MYF6, RBM24, SMYD1*	Myogenesis	7.0195e-01	35 days
GO:0048643	positive regulation of skeletal muscle tissue development	*MEF2C, MYF6, RBM24*	Myogenesis	8.6524e-01	35 days
GO:0006942	regulation of striated muscle contraction	*CASQ2, CAV3, SCN10A, TNNT3*	Myogenesis	1.00	35 days
GO:0055117	regulation of cardiac muscle contraction	*CASQ2, CAV3, SCN10A*	Myogenesis	1.00	35 days
GO:0014896	muscle hypertrophy	*CAV3, MEF2C, TCAP*	Myogenesis	1.00	35 days
GO:0051147	regulation of muscle cell differentiation	*CAV3, MEF2C, MYF6, RBM24, SMYD1, TRIM72*	Myogenesis	1.00	
GO:1901863	positive regulation of muscle tissue development	*MEF2C, MYF6, RBM24*	Myogenesis	1.00	35 days
GO:0014897	striated muscle hypertrophy	*CAV3, MEF2C, TCAP*	Myogenesis	1.00	35 days
GO:0048636	positive regulation of muscle organ development	*MEF2C, MYF6, RBM24*	Myogenesis	1.00	35 days
GO:0003300	cardiac muscle hypertrophy	*CAV3, MEF2C, TCAP*	Myogenesis	1.00	35 days

GO:0045844	positive regulation of striated muscle tissue development	*MEF2C, MYF6, RBM24*	Myogenesis	1.00	35 days
GO:0048641	regulation of skeletal muscle tissue development	*MEF2C, MYF6, RBM24*	Myogenesis	1.00	35 days

[1] GO: Biological Process Term (BP); [2] Genes associated with the biological process; [3] GO terms related to myogenesis or adipogenesis; Gestational age: developmental stage at which the biological process is observed, according to the genes associated with the Gene Ontology (GO) terms.

Table 2. Unique gene terms in Piau and Commercial, for gestational age of 35 days.

Piau (exclusive genes)					
Gene ID	**logFC [1]**	**adj. p-value [2]**	**Gene name**	**Gene description**	**Myogenesis or Adipogenesis[3]**
ENSSSCG00000063066	2.19	3.63E-11	*TMOD1*	Tropomodulin 1	Myogenesis
ENSSSCG00000052601	2.01	4.66E-11	*MYOZ2*	Myozenin 2	Myogenesis
ENSSSCG00000052599	2.04	9.64E-09	*LMOD3*	Leiomodin 3	Myogenesis
ENSSSCG00000007504	2.71	2.71E-08	*RBM38*	RNA binding motif protein 38	Myogenesis
ENSSSCG00000022891	2.28	3.06E-08	*MYMK*	Myomaker, myoblast fusion factor	Myogenesis
ENSSSCG00000060656	2.09	1.71E-07	*GPX1*	Glutathione peroxidase 1	Myogenesis and Adipogenesis
ENSSSCG00000031868	2.37	2.96E-07	*XK*	X-linked Kx blood group antigen, Kell and VPS13A binding protein	Myogenesis
Commercial (exclusive genes)					

Gene ID	logFC [1]	adj.p-value [2]	Gene name	Gene description	Myogenesis or Adipogenesis[3]
ENSSSCG00000026533	2.81	6.34E-09	*MYF6*	Myogenic factor 6	Myogenesis
ENSSSCG00000039506	2.95	1.59E-08	*MYL6*	Myosin light chain 6	Myogenesis
ENSSSCG00000024676	2.42	1.06E-07	*SRPK3*	SRSF protein kinase 3	Myogenesis
ENSSSCG00000004728	2.47	2.82E-07	*CAPN3*	Calpain 3	Myogenesis
ENSSSCG00000010144	3.06	3.24E-07	*ACTN2*	Actinin alpha 2	Myogenesis
ENSSSCG00000014149	2.06	3.55E-07	*MEF2C*	Myocyte enhancer factor 2C	Myogenesis
ENSSSCG00000025353	2.30	3.55E-07	*TNNT1*	Troponin T1	Myogenesis
ENSSSCG00000000146	2.15	1.20E-06	*MYH11*	Myosin light chain 11	Myogenesis
ENSSSCG00000004803	2.41	8.47E-06	*ACTC1*	Actin alpha cardiac muscle 1	Myogenesis
ENSSSCG00000003693	2.38	9.27E-06	*MYOM1*	Myomesin 1	Myogenesis
ENSSSCG00000040860	2.29	9.56E-06	*NMRK2*	Nicotinamide riboside kinase 2	Myogenesis
ENSSSCG00000038455	2.02	5.60E-05	*RBM24*	RNA binding motif protein 24	Myogenesis
ENSSSCG00000061647	2.16	1.05E-04	*CASQ2*	Calsequestrin 2	Myogenesis
ENSSSCG00000017500	2.44	1.15E-04	*TCAP*	Titin-cap	Myogenesis
ENSSSCG00000008215	2.26	1.37E-04	*SMYD1*	SET and MYND domain containing 1	Myogenesis

ENSSSCG00000017307	2.18	3.18E-04	*MYL4*	Myosin light chain 4	Myogenesis
ENSSSCG00000039557	2.02	5.87E-04	*TRIM54*	Tripartite motif containing 54	Myogenesis
ENSSSCG00000011260	2.06	1.82E-03	*SCN10A*	Sodium voltage-gated channel alpha subunit 10	Myogenesis
ENSSSCG00000039710	2.41	3.08E-03	*MYL2*	Myosin light chain 2	Myogenesis
ENSSSCG00000016605	2.41	4.66E-03	*LMOD2*	Leiomodin 2	Myogenesis
ENSSSCG00000023031	2.12	5.95E-03	*TNNT2*	Troponin T2	Myogenesis

Gene Id: Identifier of the Ensembl gene in the Piau or Commercial groups. [1]LogFC: Logarithmic variation of gene expression in the conceptuse of the respective groups; [2] P-value adjusted for multiple testing; [3]Myogenesis or Adipogenesis: Indicates whether the gene is related to myogenesis or adipogenesis, according to the terms of the gene ontology.

Figures

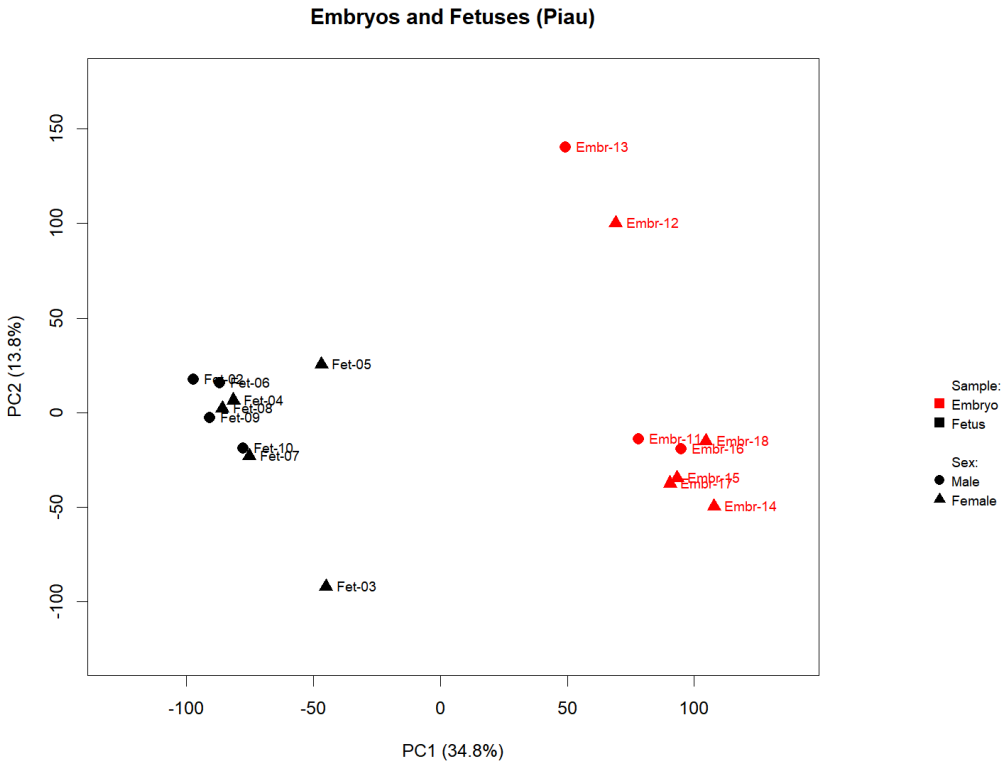

Figure 1: Principal component analysis of embryonic (25 days, *n* = 8, red) and fetal (35 days, *n* = 9, black) prenatal pig transcriptome samples from the Piau genetic group, considering all expressed genes. Symbols denote sex (circle = male, triangle = female). The distinct separation between developmental stages reflects differential gene expression, with the first two principal components accounting for 34.8% and 13.8% of the total variance, respectively.

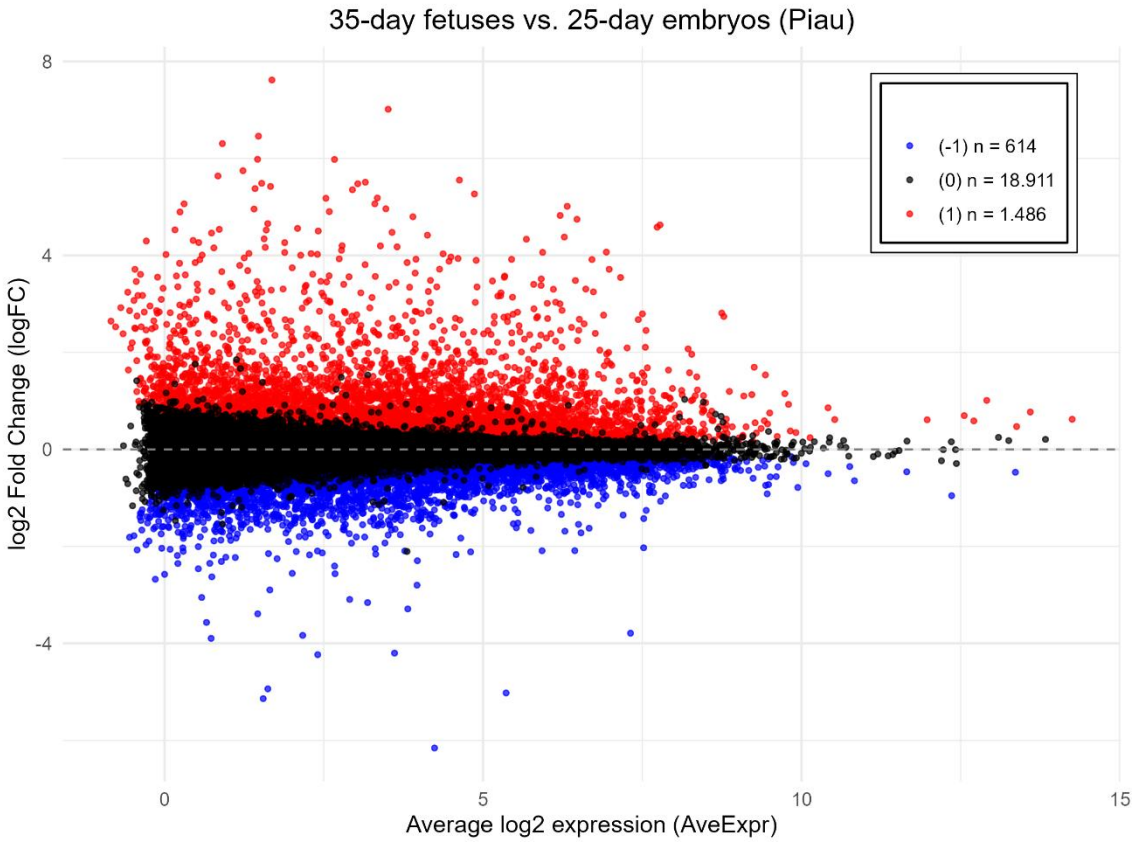

Figure 2: Mean-difference plot showing differentially expressed genes between embryos (25 days of gestation) and fetuses (35 days of gestation) in the Piau genetic group. Genes upregulated in fetuses (n = 1,486) are highlighted in red, while genes upregulated in embryos (n = 614) are shown in blue. Genes without significant expression differences (n = 18,911) are represented in black. The dashed horizontal line indicates logFC = 0, corresponding to no change in gene expression between developmental stages.

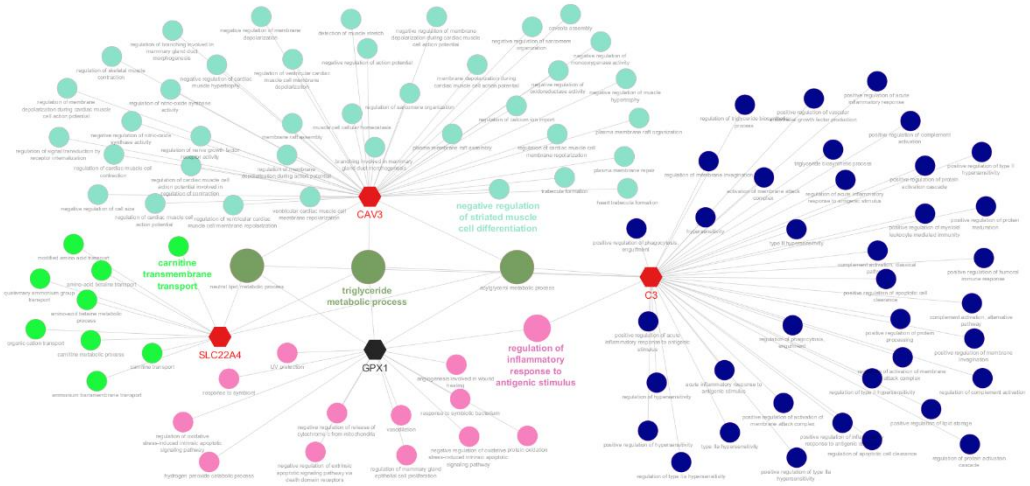

Figure 3. Functional networks between genes and biological processes associated with adipogenesis in the Piau and Comercial breeds. The figure shows the main interactions between genes (hexagonal nodes) and biological processes (circular nodes). The genes highlighted in red are shared between Piau and Commercial, while the gene in black is exclusive to the Piau breed. The colors of the circular nodes indicate the main enriched biological processes, and the dark green nodes represent processes shared

between the genes of the two groups. The size of the nodes reflects the degree of enrichment of the process, as analyzed by the ClueGO plugin of Cytoscape. The most enriched terms in each subnetwork are highlighted in bold and larger nodes.

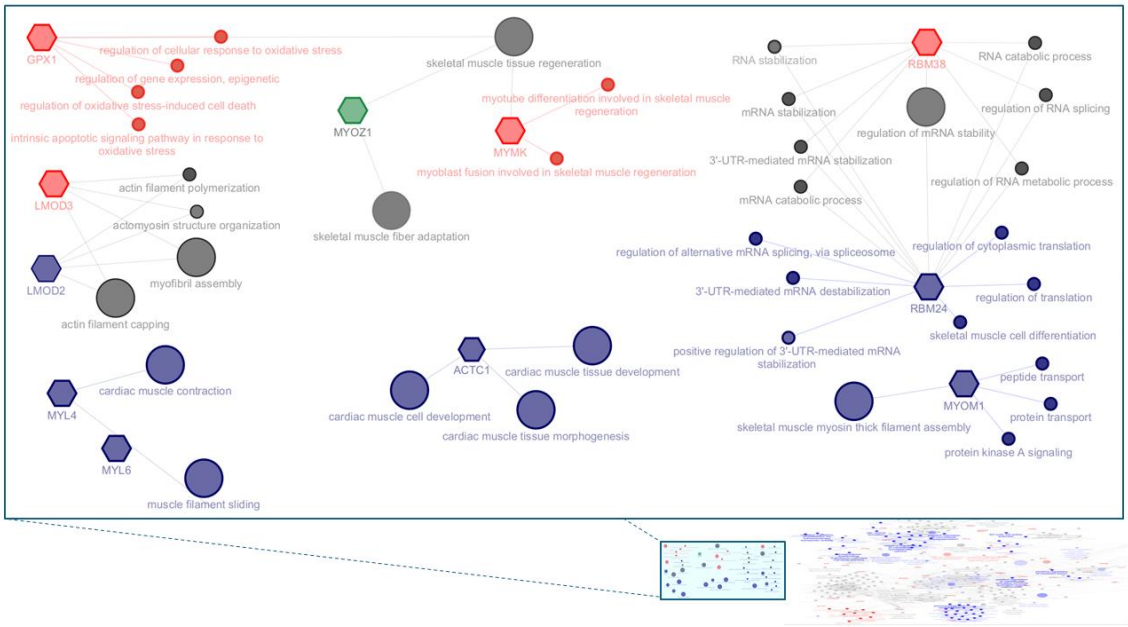

Figure 4. Functional networks between genes and biological processes associated with myogenesis in the Piau and Commercial groups. The figure illustrates the main interactions between genes (hexagonal nodes) and biological processes (circular nodes). The genes and biological processes highlighted in red correspond to Piau, while those in blue represent Comercial. The gene in green is shared between both groups. Biological processes in gray indicate terms common to both groups. The size of the nodes reflects the degree of enrichment of each process, as analyzed by the ClueGO plugin in Cytoscape. Processes with greater enrichment are represented by larger nodes.

SUPPLEMENTARY APPENDICES

Supplementary data associated with this article can be found in the online version at

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GENERAL CONCLUSIONS

This thesis demonstrates that the Piau breed represents a valuable local genetic resource, exhibiting specific morphological and transcriptomic characteristics during embryonic and fetal development, in contrast to commercial lines. Morpho-histological analyses of the uterus and placenta revealed structural differences between Piau and Commercial females, indicating distinct patterns of maternal–fetal interaction and suggesting that the uterine environment actively participates in the programming of conceptus development. These structural differences constitute the morphofunctional basis for the molecular programs observed throughout gestation.

The RNA sequencing enabled early sex determination of embryos and fetuses by identifying Y chromosome–linked transcripts, including five genes not previously associated with sexual differentiation in pigs, thereby highlighting new markers for early sexing. In addition, differential gene expression analysis between 25 and 35 days of gestation revealed genes and biological processes related to myogenesis and adipogenesis, with particular emphasis on the early activation of adipogenic pathways and the identification of Piau-specific genes associated with lipid metabolism and adaptation to rustic conditions.

Comparisons with commercial lines revealed transcriptomic and functional differences, indicating that intensive genetic selection alters the expression profiles of genes critical for muscle growth and fat deposition, whereas the Piau breed maintains a more diverse and adaptive transcriptional pattern. Taken together, these findings highlight the potential of the Piau breed for studies in developmental biology, genetic conservation, and breeding strategies, expanding our understanding of the molecular mechanisms that influence sexual differentiation and the programming of muscle and adipose tissues in pigs.