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Molecular markers in ovaries of female pigs with different levels of welfare, in the pre-mating period

Pirassununga

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Marcadores moleculares em ovários de fêmeas suínas com níveis diferentes de bem-estar, no período pré-cópula

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Parecer Consubstanciado da CEUA FMVZ

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, na reunião de 04/02/2021, **ANALISOU** e **APROVOU** o protocolo de estudo acima referenciado. A partir desta data, é dever do pesquisador:
1. Comunicar toda e qualquer alteração do protocolo.
2. Comunicar imediatamente ao Comitê qualquer evento adverso ocorrido durante o desenvolvimento do protocolo.
3. Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 anos para possível auditoria dos órgãos competentes.
4. **Relatórios parciais** de andamento deverão ser enviados **anualmente** à CEUA até a conclusão do protocolo.

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
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Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

São Paulo, 5th February 2021

CERTIFIED

We certify that the Research "MOLECULAR MARKERS IN OVARIES OF FEMALE PIGS WITH DIFFERENT LEVELS OF WELFARE, IN THE PRE-MATING PERIOD", protocol number CEUAx 9992150121 (ID 001730), under the responsibility Adroaldo José Zanella, agree with Ethical Principles in Animal Research adopted by Ethic Committee in the Use of Animals of School of Veterinary Medicine and Animal Science (University of São Paulo), and was approved in the meeting of day February 04, 2021.

Certificamos que o protocolo do Projeto de Pesquisa intitulado "MARCADORES MOLECULARES EM OVÁRIOS DE FÊMEAS SUÍNAS COM NÍVEIS DIFERENTES DE BEM-ESTAR, NO PERÍODO PRÉ-CÓPULA.", protocolado sob o CEUAx nº 9992150121, sob a responsabilidade de Adroaldo José Zanella, está de acordo com os princípios éticos de experimentação animal da Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, e foi aprovado na reunião de 04 de fevereiro de 2021.

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For the scientist, the limitation is actually the motivation of a great investigation.

Arthur Nery da Silva

Quote inspired by João Guimarães Rosa's interview on German television, in 1962. In the dialogue, the prestigious Brazilian author said "Para o artista, toda limitação é estimulante".

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ABSTRACT

SILVA, A. N. **Molecular markers in ovaries of female pigs with different levels of welfare, in the pre-mating period.** [Marcadores moleculares em ovários de fêmeas suínas com níveis diferentes de bem-estar, no período pré-cópula]. 2021. 101 p. Dissertation (Master of Science) – School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, 2021.

The role of the environment in the trajectory of individuals has been studied for hundreds of years. From the beginning of this research field, authors have been noticed that there was an intimate relationship between the environment that individuals live in and the way they express themselves phenotypically. In this study, we reviewed the current literature concerning epi-markers to predict welfare in pigs and conducted a novel study on the role of environmental experiences on genomic factors in the porcine *corpus luteum*. In the first study, we reviewed the evidence regarding the development of a panel of epigenetic indicators associated with the negative experiences that pigs may have undergone during their lifespan. In this review of evidence collected over the last 10 years, published in international peer-reviewed journals, we identified positive perspectives regarding the consistency of epigenetic markers in the genome of farm animals, which could predict their welfare. However, we also pointed out high variability concerning genes differentially affected by these markers, which can be explained by their high diversity in terms of the experimental context. In the second manuscript of this dissertation, we presented a novel study showing that a single dose of lipopolysaccharide (LPS) was capable of down-regulating gene expression of the angiogenic gene (*VEGF*) in the *corpus luteum* of gilts housed in different welfare conditions. This study simulates one of the biggest challenges of intensive pig farming: urinary tract infections by gram-negative bacteria, which have LPS in their external membrane wall. Overall, our study revealed important findings concerning environmental factors that can compromise the productive, reproductive, and welfare aspects of pigs. Furthermore, it is reasonable to say that other fields of study can benefit from our evidence since the porcine model is recognized as one of the best species for translational research.

Keywords: swine; epigenetics; gene expression; lipopolysaccharide; *corpus luteum*.

RESUMO

SILVA, A. N. **Marcadores moleculares em ovários de fêmeas suínas com níveis diferentes de bem-estar, no período pré-cópula.** [Molecular markers in ovaries of female pigs with different levels of welfare, in the pre-mating period]. 2021. 101 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

O papel do meio ambiente na trajetória dos indivíduos é estudado há centenas de anos. Desde o início deste campo de estudos, os autores perceberam que existia uma relação íntima entre o ambiente em que os indivíduos vivem e a forma como se expressam fenotipicamente. Neste estudo, revisamos a literatura atual sobre epi-marcadores para predizer o bem-estar de suínos e conduzimos um estudo inédito sobre o papel das experiências ambientais em fatores genômicos no corpo lúteo suíno. No primeiro estudo, revisamos as evidências sobre o desenvolvimento de um painel de indicadores epigenéticos associados às experiências negativas pelas quais os suínos podem ter passado durante sua vida. Nesta revisão de evidências coletadas nos últimos 10 anos, publicadas em periódicos internacionais revisados por pares, identificamos perspectivas positivas quanto à consistência de marcadores epigenéticos no genoma de animais de produção, que poderiam predizer seu bem-estar. No entanto, também apontamos uma grande variabilidade em relação aos genes diferencialmente afetados por essas marcas, o que pode ser explicado por sua grande diversidade em termos de contextos experimentais. No segundo manuscrito desta dissertação, apresentamos um estudo inédito mostrando que uma única administração de lipopolissacarídeo (LPS) foi capaz de diminuir a expressão gênica do gene angiogênico (*VEGF*) no corpo lúteo de marrãs alojadas em diferentes condições de bem-estar. Este estudo simula um dos maiores desafios da suinocultura intensiva: infecções do trato urinário por bactérias gram-negativas, que possuem LPS em sua membrana externa. No geral, nosso estudo revelou achados importantes sobre fatores ambientais que podem comprometer os aspectos produtivos, reprodutivos e de bem-estar dos suínos. Além disso, é razoável dizer que outros campos de estudo podem se beneficiar dos nossos achados, uma vez que o modelo suíno é reconhecido como uma das melhores espécies para pesquisa translacional.

Palavras-chave: suínos; epigenética; expressão gênica; lipopolissacarídeo; *corpus luteum*.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

3βHSD – 3-beta-hydroxysteroid dehydrogenase

450scc – cytochrome 450 cholesterol side-chain

ACTH - adrenocorticotropic hormone

AM - arithmetic mean

C – crates system

CA – *corpus albicans*

cDNA – complementary DNA

CE – cholesterol ester

CK - creatine kinase

CL – *corpus luteum*

CpG - cytosine nucleotide is followed by a guanine nucleotide

Ct - cycle threshold

CYP11A1 – cytochrome P450 family 11 subfamily A member 1 gene

DMRs - differentially methylated regions

DNA – desoxyribonucleic acid

E2 – 17-β-estradiol

ESR1 – estrogen A receptor gene

ESR2 – estrogen B receptor 2 gene

F – forward

F0 – individual exposed

F1 – first generation

F2 – second generation

F3 – third generation

FLT1 – vascular endothelial growth factor receptor 1 gene

FSH – follicle-stimulating hormone

FSH-β – follicle-stimulating hormone subunit beta gene

GAPDH – glyceraldehyde-3-phosphate dehydrogenase gene

GH – group housing system

GM - geometric mean

GnRH – gonadotrophin-releasing hormone

HDL – high-density lipoprotein

HSD11B1 – hydroxysteroid 11-beta dehydrogenase 1 gene

HSD11B2 – hydroxysteroid 11-beta dehydrogenase 2 gene

HSD3B1 – hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 gene

IFNG – interferon-gamma gene

IL1B – interleukin 1 beta gene

INFγ – interferon-gamma

KDR – kinase insert domain receptor gene

KISS1R – KISS1 receptor gene

LDL – low-density lipoprotein

LH – luteinizing hormone

LHCGR – luteinizing hormone choriogonadotropin receptor gene

LH-β – luteinizing hormone subunit beta gene

LPS – lipopolysaccharide

mRNA – messenger ribonucleic acid

MT – mitochondria

NAD+ – nicotinamide adenine dinucleotide

NADP+ – nicotinamide adenine dinucleotide phosphate

NR3C1 – nuclear receptor subfamily 3 group C member 1gene

NR3C2 – nuclear receptor subfamily 3 group C member 2 gene

OD – outside system

P4 – progesterone

P5 – pregnolone

PGF2α – prostaglandin-2-alpha

PGR – progesterone receptor gene

PSE - pale soft exudative

qPCR – real-time quantitative PCR

R – reverse

RNA – ribonucleic acid

RRBS – reduced representation bisulfite sequencing

RT-qPCR – real time quantitative polymerase chain reaction

SAL -saline

SCP2 – sterol carrier 2

SD – standard deviation

SER – smooth endoplasmatic reticulum

StAR – specific StAR-related lipid transfer

STAR – steroidogenic acute regulatory protein gene

TLR4 – toll-like receptor 4 gene

TNF – tumor necrosis factor-alpha gene

TNFα – tumor necrosis factor-alpha

UBB – ubiquitin B gene

VEGF – vascular endothelial growth factor-A gene

WGBS – whole genome bisulfite sequencing

SUMMARY

1. INTRODUCTION

In recent years, ensuring the welfare of farm animals has been considered an imperative topic in the animal protein industry, whether for moral reasons or purely productive (BROWNING; VEIT, 2020; POLETTO; HÖTZEL, 2012). The pork industry, due to its production volume and revenue generation, has experienced increasing demands regarding the available housing systems and management carried out at pig farms (MAPA, 2020). In addition, this issue becomes more dramatic when pig farmers are reluctant to accept some guidelines to improve animal welfare, claiming that these demands would increase production related costs and reduce production efficiency.

The guarantee of animal welfare is not only capable of increasing the industry's turnover in terms of product volume, as it can also improve the quality of products available to consumers (MADZINGIRA, 2018). The possibility of increasing production has drawn the attention of several research groups around the world, and global efforts have been made to develop animal welfare indicators, such as the Animal Welfare Indicator (AWIN) project.

In the first manuscript of this dissertation, a literature review was performed to encourage the development of a panel of molecular markers capable of predicting the welfare of pigs. The molecular marker that we focused our efforts on was methylation. The study of methylation is part of a larger field of study called epigenetics. This field of studies seek to understand the chemical molecules that control gene expression, without altering the nitrogenous bases that form the double strand of DNA (HYDE; FRISO; CHOI, 2019). Additionally, this branch of research is concerned with understanding how these molecules behave over time in response to cellular physiology, which is directly dependent on the habits and environment in which individuals are inserted (HYDE; FRISO; CHOI, 2019).

Considering these adaptive aspects of the genome concerning environmental exposures, we conducted, as shown in the second manuscript of this dissertation, a study evaluating the gene expression with RT-qPCR. In this study, we aimed to understand how a challenge with a systemic inflammatory inducer could compromise the gene expression of important genes concerning progesterone synthesis, angiogenesis, and stress response on the corpus luteum of gilts.

Finally, this dissertation aims to show in a broad way how the environment in which animals are raised can shape their life trajectory. So, throughout the text we focus our efforts on the porcine model, but we believe that at various points in our study we can use the knowledge gained from this species for translational studies.

2. LITERATURE REVIEW

Animal welfare applied to the swine industry

Intensive systems of animal protein production require precision since the animals are dependent on human management. Recently, not only have meat producers sought housing systems that guarantee animal welfare, as well as consumers of animal protein, have also demanded products that align with their moral values (POLETTO; HÖTZEL, 2012). In this sense, the welfare of sows housed in crates has become a topic discussed in the most diverse spaces of our society. These scientific and popular discussions have even ensured the prohibition of crates for pregnant sows in the European Union and the United Kingdom (EUROPEAN COMMUNITY, 2001).

Crates are commonly used due to their lower implementation cost and the supposed increase in production rates. This system improves the utilization of floor space and facilitates the management of animals in terms of feeding and veterinary assistance (LI; GONYOU, 2013; MCGLONE, 2013). In addition, studies indicate that aggressions and hierarchical issues between the animals are less frequent in crates systems, especially at the time they are fed (JANG et al., 2017; JANSEN et al., 2007; LI; GONYOU, 2013).

However, research in the field of behaviour and animal welfare has reported several ethological damages, disorders of the reproductive/ urinary system, and claw lesions in sows housed in crates (DA SILVA; PANDORFI; PIEDADE, 2008; MCGLONE, 2013). Recently, studies have pointed out negative implications caused by housing systems in pregnant sows and their litters of pigs (BERNARDINO et al., 2016; TATEMOTO et al., 2019, 2020). Interestingly, Parada et al. (2021) reported that piglets born from lameness' sows are more likely to present lower weight at birth and to be involved in more disputes at weaning than those born from sows without lameness. The authors suggest that this issue may be related to the chronic stress suffered by lame sows during pregnancy, which may have caused foetal reprogramming and changes in the offspring's developmental trajectory.

Europe banned gestation crates for sows in 2013 (EUROPEAN COMMUNITY, 2001). After this, several countries around the world have been acting to meet this market demand, even though it is not mandatory in their countries (MCGLONE, 2013). However, in Brazil, gestation crates are still widely used, but there is a governmental resolution to replace them with group housing systems by 2045 (MAPA, 2020).

Although it seems paradoxical to return to a less intensive production system, there is a high market demand for this to take place (FAO, 2010). Research has shown that people in developed countries prioritize environmentally sustainable products that are in line with animal welfare guidelines (BLOKHUIS et al., 2008). In fact, when there are no limiting factors such as health, safety, quality, and sensory characteristics, individuals prefer to purchase products with assured animal welfare (CLARK et al., 2016). Moreover, in terms of performance, there is an advantage in collective pens housing systems. A study by SILVA et al. (2008) showed that the number of piglets born alive is higher in collective housing systems than in crated ones. In addition, they found that the weight at weaning of piglets born of sows housed in groups during pregnancy is higher.

Housing systems are decisive during the lifespan of the animals (FAO, 2010). Studies have shown that the comfort and cleanliness of the facilities are associated with success in the production chain (ALARCÓN; ALLEPUZ; MATEU, 2021). In addition, it is reported that cortisol levels can vary slightly according to the quality of the facilities. In general, animal raised in facilities that meet welfare standards maintain constant levels of cortisol, whereas animals housed in poor facilities are more likely to have their levels of cortisol fluctuate over time (REMIENCE et al., 2008).

The measurement of cortisol levels has been reported as an indicator of animal welfare in swine production (RALPH; TILBROOK, 2016). To this end, studies suggest that cortisol levels in the blood, saliva, and milk are effective indicators of acute stress, while cortisol levels in urine and faeces are suitable indicators of stress suffered a few hours before the measurement. In addition, measuring cortisol in hair is proposed to be an effective indicator of the medium-term stress (days to weeks) (CASAL et al., 2017).

Not only does cortisol perform several physiological functions in fight or flight situations, energy metabolism, immunity, cognition, and work as an essential hormone in regulating the circadian rhythm of many species of mammals (OSTER et al., 2017), it is also reported to be involved as an important issue in animal reproduction. Besides, studies have been conducted to understand the relationship between stress and reduced fertility (ASHWORTH et al., 2011; ROONEY; DOMAR, 2018; RUTHERFORD et al., 2009; TOUFEXIS et al., 2014). These studies have suggested that acute and chronically stressed individuals may be subject to lower reproductive rates.

In the following sections, we discuss the reproductive physiology of the female swine, the regulation of ovarian steroidogenesis, and how stressors may compromise hormonal pathways related to the estrous cycle of gilts in production systems.

Reproductive physiology of the swine female

Gilts are known to reach puberty between 150 and 220 days of age. After that, with all aspects of well-being guaranteed (such as good health, nutrition, housing conditions, and freedom to express their natural behaviour), gilts are expected to have an estrous cycle of approximately 21 days (which can vary between 18 and 24 days). In addition, swine females are expected to only interrupt their estrous cycle due to pregnancy and lactation or, possibly, advanced age (MCGLONE et al., 2020; SOEDE; LANGENDIJK; KEMP, 2011). After puberty, the hypothalamus, the pituitary, the ovaries, and the uterus act in synchrony to synthesize a wide variety of endogenous hormones that will be responsible for the regulation of the estrus cycle of swine females (SOEDE; LANGENDIJK; KEMP, 2011).

The estrous cycle of the swine female can be divided into four important stages to facilitate our understanding: (1) proestrus, (2) estrus, (3) metaestrus, and (4) diestrus (Fig. 1). In each of these phases we can observe specific behavioural, physiological, and molecular characteristics in female pigs. The proestrus (follicular phase) comprises the first of the four phases of the estrous cycle and extends from one to three days. This stage of the cycle takes place immediately after the female recognizes that it is not gestating. Then, the uterus releases prostaglandin-2-alpha (PGF2α), which promotes the lysis of the

corpus luteum and decreases the circulating levels of progesterone (P4). As a result of the fall in P4, increasing amounts of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) will be released from the pituitary to act on developing ovarian follicles. With the increasing activity of these gonadotropins, the ovarian follicles will grow and produce 17-β-estradiol (E2). The second phase of the estrous cycle is estrus (follicular phase), which has an average of 2.5 days and it is when the ovulation process occurs in the female. This phenomenon is possible due to the LH peak. In this process, about 15 to 30 oocytes may be released in the female's reproductive tract, as inhibin and E2 production decreases. In addition, this is exactly the period that swine female presents sexual receptivity to the boar. Some other clinical signs that are possible to be observed between the end of the proestrus and the estrus phase include immobilization or "standing", raised ears, swollen vulva, and/ or clear sticky mucous in the vulva. The third phase of the estrous cycle is the metaestrus (luteal phase) and varies from 2 to 3 days. This phase of the estrous cycle comprises the period immediately after ovulation, in which the formation of the corpus luteum and the release of progesterone starts. Importantly, the corpus luteum is responsible for maintaining pregnancy in females that effectively received male gametes in their reproductive tract and had their oocytes fertilized. The last phase of the estrous cycle is the diestrus (luteal phase). This phase is the longest in the estrous cycle, comprising between 12 to 15 days, approximately. During this period, the maximum production of P4 reaches peak concentrations by days 8 to 9 after ovulation and the recognition (or not) of the foetus is established, through the release of E2. In the absence of E2, the uterus releases PGF2α, and the cycle restarts (MCGLONE et al., 2020; SOEDE; LANGENDIJK; KEMP, 2011).

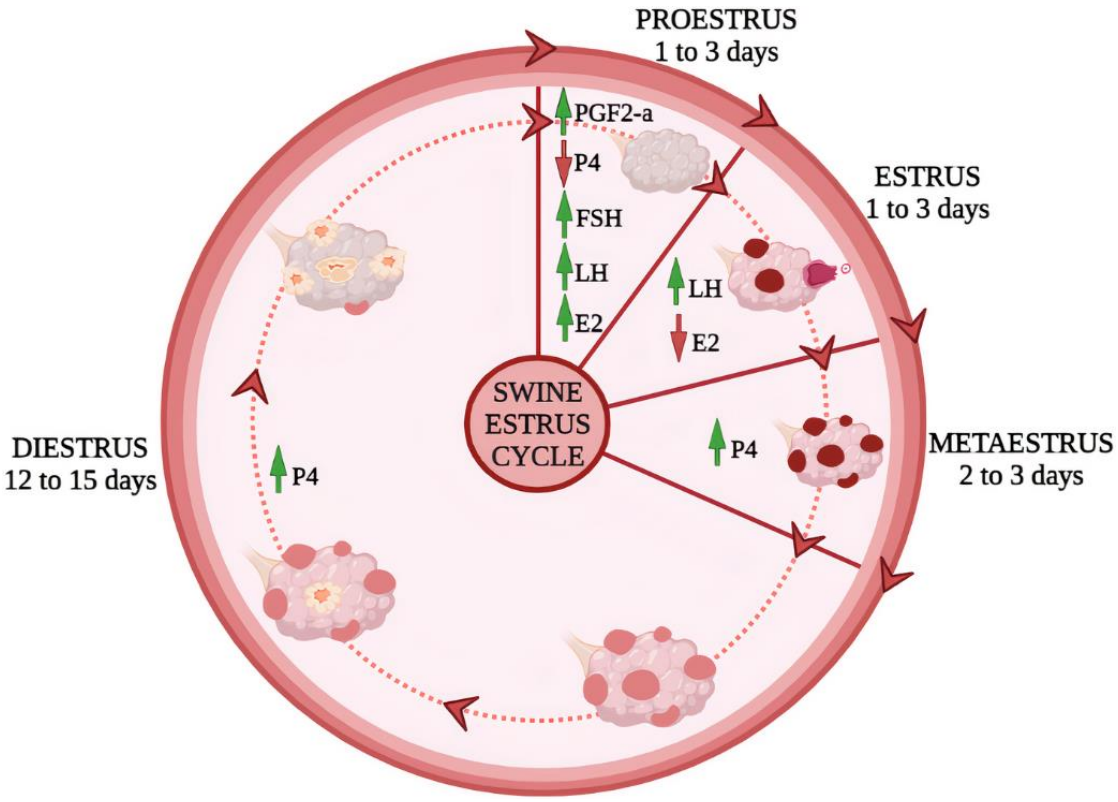

Figure 1. Main ovarian characteristics and hormones in the estrus cycle of pigs.

From this overview of the reproductive physiology of the swine female, we will explain in more detail how the current literature has suggested the molecular control of ovarian steroidogenesis might occur. Furthermore, although the focus of this review is on the synthesis of progesterone, we recognize the importance and essentiality of all other molecules involved in the process.

Regulation of the ovarian steroidogenesis

According to the current literature, ovarian steroidogenesis is regulated through positive and negative feedbacks of reproductive hormones (Fig. 2). In this regard, the gonadotropin-releasing hormone (GnRH) produced by the paraventricular nucleus of the hypothalamus is characterized to stimulating the adenohypophysis to produce FSH or LH. Then, these two glycoproteins will move from the central nervous system to act on the ovarian tissue receptors to produce specific reproductive hormones, such as E2 or P4 (SOEDE; LANGENDIJK; KEMP, 2011).

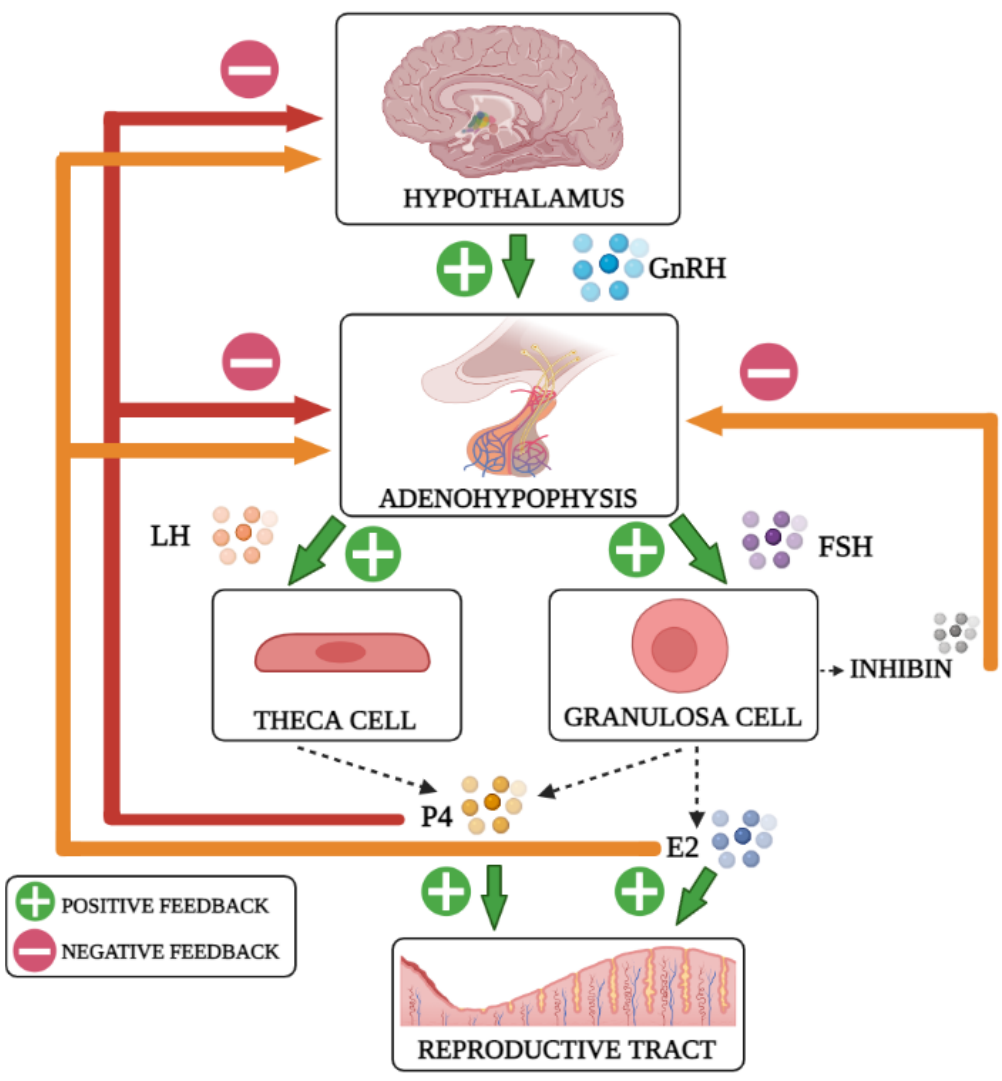

Figure 2. Simplified control of the hypothalamic-pituitary-ovarian axis in female pigs.

Recently, the kisspeptin receptor gene (*KISS1R*) was shown to play a key role in activating the pathway to produce GnRH by the neurons in many species of mammals (NEJAD; TEHRANI; ZADEH-VAKILI, 2017). After the activation of the neurons, GnRH migrates to the anterior pituitary gland, where it will trigger the LH and FSH receptors genes to activate the pathways responsible to produce these glucocorticoids. It is believed that the GnRH triggers the promoter region of the *LH-β* and *FSH-β* receptor genes to activate the molecular pathways to produce satisfactory amounts of LH and FSH, respectively (LENTS, 2019; MCNEILLY et al., 2003). Following the axis, these two hormones will trigger the receptors of the follicular or luteal cells on the ovarium. Thus, different pathways may be activated according to the demands of other chemical signs.

The hormones FSH and LH, which are produced in the central nervous system, migrate to act on the receptors of the granulosa cells and surrounding theca cells, which are located in the ovarian tissue. In theca cells, LHR catalyses the conversion of cholesterol to pregnolone (by the action of *CYP11A1*), which will later convert pregnolone to progesterone by the action of 3βHSD. Meanwhile, in granulosa cells, FSHR will catalyse the reaction to produce 17-β-estradiol using androsteridione (which is a reduced form of progesterone) as a precursor molecule (YAZAWA et al., 2019). This physiological mechanism is known as the two-cell-two-gonadotropin theory (RYAN; PETRO, 1966; RYAN; PETRO; KAISER, 1968).

In the next section of this literature review, we address the physiological mechanisms that control the life and death of the corpus luteum. This transitory gland is one of the main important factors for the reproductive success of domestic animals and humans.

Physiology and regression of the corpus luteum

The physiology of the corpus luteum (CL) is one of the most interesting and extensively studied events in the reproduction of mammals, as this transitory endocrine gland has a short and decisive lifespan for the prospection of the species (TOMAC; CEKINOVĆ; ARAPOVIĆ, 2011). The corpus luteum develops from the remaining fragments of the ovarian follicle and becomes a new transient gland, also with limited function and lifespan. For all these cell machinery to work perfectly, sophisticated endocrine mechanisms, in which cells are transformed, remodelled, or differentiate happen at the ovarium (STOCCO; TELLERIA; GIBORI, 2007). In the last times of CL functionality, it undergoes another transformation/regression process that leads to the last structure of the ovarian cycle, the corpus albicans (CA) (STOCCO; TELLERIA; GIBORI, 2007). The CA is a scar on the surface of the ovary that is a remnant of ovulation.

The product of CL is progesterone. This steroid hormone is responsible for the conceptus implantation in the uterus (LA VOIE, 2017). Moreover, a successful gestation is associated with high levels of production of progesterone by the pregnant female (SOEDE; LANGENDIJK; KEMP, 2011; STOCCO; TELLERIA; GIBORI, 2007). In this sense, several studies are investigating the role of environmental factors (non-genetic) that may be related to the modulation of the gene expression of this gland (WITEK et al., 2020).

A satisfactory production of progesterone is largely dependent on the amount of unesterified cholesterol available to be converted to pregnolone (P5) in the mitochondria (MT). This form of unesterified cholesterol can be found from exogenous plasma density lipoproteins (LDLs), high-density lipoproteins (HDLs), or hydrolysed cholesterol ester (CE) by cholesterol esterase. In larger mammals such as pigs, LDLs lipoproteins are a major source of sterol for progesterone production. The mechanisms of transport that cholesterol acts inside the ovarian cells remain poorly understood, but the current studies suggest that the sterol carrier 2 (*SCP2*) and specific StAR-related lipid transfer (*StAR*) may be overactivated for this purpose (LA VOIE, 2017). In addition, it is known that unesterified cholesterol molecules are converted by electron-transfer proteins (adrenodoxin and adrenodoxin reductase) into pregnolone at the MT by the cytochrome P450 cholesterol side-chain (*450scc/ CYP11A1*). Thus, the enzyme 3-beta-hydroxysteroid dehydrogenase (3βHSD) converts pregnolone to progesterone through an oxidation-reduction of the CH-OH group with nicotinamide adenine dinucleotide (NAD+) or nicotinamide adenine dinucleotide phosphate (NADP+) as an acceptor in the smooth endoplasmatic reticulum (SER). The gene hydroxy-delta-5-steroid dehydrogenase, 3-beta, and steroid-delta-isomerase-1 (*HSD3B1*) are responsible for codifying the 3βHSD enzyme in pigs (LA VOIE, 2017; TOMAC; CEKINOVĆ; ARAPOVIĆ, 2011). Figure 3 illustrates the process.

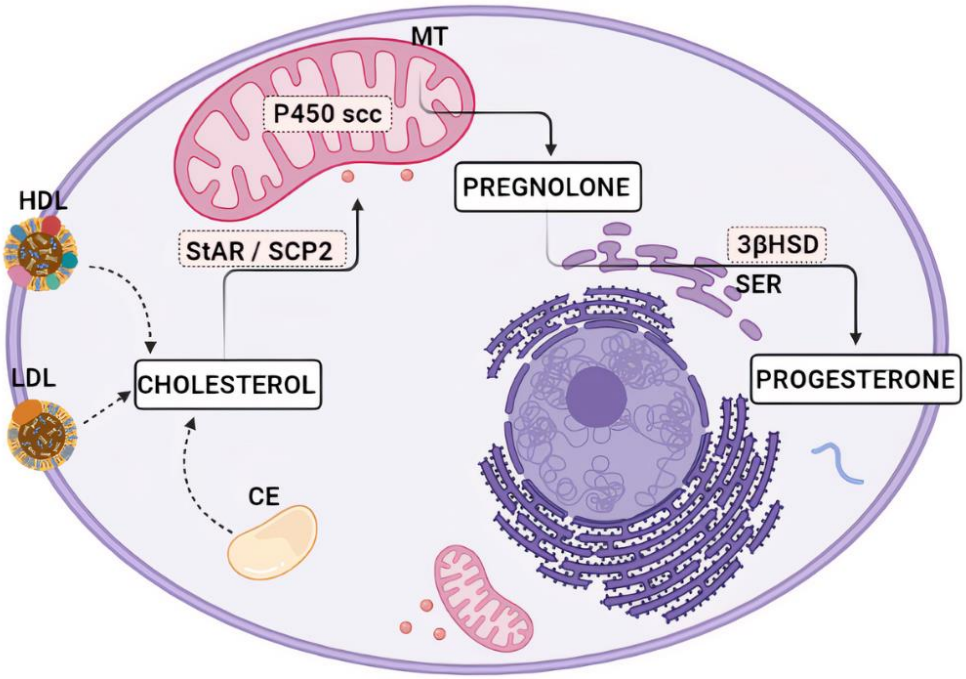

Figure 3. P4 production in luteal cells. HDL, LDL, or CE are used to start the reaction. StAR and/ or SCP2 proteins carry out the transport of the cholesterol molecules through the cytosol to the MT. In MT the 450scc converts some of the cholesterol forms to P5. Then, the 3βHSD enzyme makes the oxirreduction of P5 into P4 at the SER.

The failure or inability of the CL to express itself is associated with subfertility or embryonic loss, as P4 is responsible for both endometrial growth and embryo survival. Additionally, P4 provides direct negative feedback in the hypothalamus to suppress the

follicular wave (TOMAC; CEKINOVĆ; ARAPOVIĆ, 2011). Nevertheless, CL is not an independent gland. Likewise, its function is dependent on the pituitary gland, and endometrium tissues.

The lifespan of CL in the porcine model is dependent on pregnancy (SPENCER; BAZER, 2004). If the individual does not identify uterine and/ or embryonic signals, luteolysis happen. Lutheolysis is the phenomenon in which the CL loses its function and involute to a new structure called CA. This process is carried out by PGF2α, which reduces ovarian and luteal blood flow and induces cell death through DNA damage and apoptosis (DAVIS; RUEDA, 2002). In addition, other immune molecules are indirectly involved in the regression, such as TNFα, INFγ and IL1β, which inhibit the secretion of steroids and induce apoptosis (DAVIS; RUEDA, 2002; TOMAC; CEKINOVĆ; ARAPOVIĆ, 2011).

The relationship between the immune system and reproduction has been widely studied in pigs (ZIECIK, 2002). Research has shown that both can affect one another's physiological aspects. On the one hand, infection diseases can compromise the reproductive parameters (MAES et al., 2008). On the other hand, studies have shown the role of reproductive steroid hormones in controlling immune molecules (QIAN et al., 2018; ZIECIK, 2002).

The role of endogenous hormones in the lifespan of the CL is clear and consolidated in the scientific literature. Currently, several research groups around the world are striving to understand immunological and environmental factors that are involved in the control of ovarian steroidogenesis. This topic is briefly covered in the last session of this literature review. In addition, we introduced the model of inducing acute signs of disease, with the use of LPS, which has been used experimentally.

The link between the immune system and reproductive outcomes

Studies have shown the close relationship between the immune system and psychosocial outcomes (DANTZER et al., 2008; QIAN et al., 2018). Researchers suggest that inflammatory cytokines may act on hormonal pathways or specific neurotransmitters, compromising the HPA axis. Moreover, it is known that the cytokinetic response is not essentially bad, as long as it is physiological. Cytokines can act positively, helping the individual to adapt to the environment that it is inserted in. However, when an individual is challenged and its healthy homeostasis is broken, a pathological scenario is established. In these scenarios, it is suggested that cytokines play a harmful role in the organism (NORDGREEN et al., 2018).

Lipopolysaccharide (LPS) is a structural component extracted from the wall of gram-negative bacteria. This molecule is an important agent for inducing a systemic inflammatory response. Studies suggest that the LPS molecule binds to the CD14 and Toll-like receptor 4 (TLR4) of lymphocytes, which, in turn, activates the transcription of NF-Kb factors from all other cells in the body (WRIGHT et al., 1990). In pigs, studies have shown that the effects of this exogenous agent can compromise noradrenaline levels for at least 72 h in the hippocampus, in the hypothalamus, and the frontal cortex. In addition, cortisol levels are compromised (elevated) for about 4 h after the injection, as

well as food intake is reduced for about 24 h, which compromises the individual's psychological aspects (NORDGREEN et al., 2018). Lastly, LPS was recently characterized as one of the most stable non-infectious models to study reduced growth performance in different categories of pigs, for inducing a very concise inflammatory response, especially in female pigs (RODRIGUES et al., 2021).

Recently, it has been shown that several disease conditions have the potential to increase the circulation of cytokines in the female reproductive tract. Also, there are direct effects on the function of reproductive tissues, as well as systemic elevation of circulating pro-inflammatory agents (ROBERTSON et al., 2018). Moreover, the study by Dantzer et al. (2008) characterized that the challenge with LPS is capable to increase pro-inflammatory cytokines, such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6), which are associated with manifestations of sickness behavior. Some of the clinical signs of this challenge include anorexia, lethargy, and decreased social motivation. Thus, the study suggests that animals that exhibit this behavior are much more susceptible to becoming involved in conflicts with their co-specifics, or simply being irresponsible to the environment in which they are inserted (DANTZER et al., 2008).

In this brief literature review we introduced the main important aspects of female swine reproductive physiology and the role of swine welfare to industry. From this overview, we aimed to introduce the themes we will cover in the next two manuscripts: (1) the role of the environment on the epigenome of the porcine model; and (2) how an acute and systemic challenge with LPS on the day of estrus can compromise the gene expression of the corpus luteum.

3. HYPOTHESIS

We hypothesize that the experiences lived by the swine female during the estrous cycle are not only capable of being segregated for future generations through generational epigenetic mechanisms linked to oocytes, but are also likely to compromise the quality and efficiency of the corpus luteum that will maintain the gestation. In this sense, the conceptus is doubly dependent on the mother's estrous cycle, as she will be responsible for providing:

(1) A good oocyte to be fertilized;

(2) And a functional corpus luteum to maintain a healthy and effective pregnancy.

4. GENERAL OBJECTIVE

Identification of molecular markers related to the impact of environmental exposure in the porcine model.

SPECIFIC OBJECTIVES

(1) Review on the potential epigenetic biomarkers related to pig welfare in studies already published.
(2) Evaluation of the gene expression of the corpus luteum of gilts housed under different welfare conditions, submitted to a health challenge on the estrus day.

5. MANUSCRIPT 1

The article was formatted according to the guidelines for publication in International Journal of Molecular Sciences (https://www.mdpi.com/journal/ijms).

Well-being in pigs: how epigenetics can enhance animal welfare

Abstract: Swine, in addition to providing the most consumed animal protein worldwide, it is also recognized as one of the most important animal models for biological studies in humans. This species not only have high genetic similarity with humans, but also have a wide variety of behaviors and physiological outcomes like humans. In this review, we reported the scientific concerns in the swine production chain, the management carried out on the farms, and the potential bottlenecks of these practices for the animals' epigenome. In addition, we selected potentially stress-related genes surrounding epi-biomarkers. For that, we carried out functional enrichment analysis of differentially methylated regions (DMRs) of the DNA of swine subjected to different stress-related conditions. These are conditions which simulate the production challenges that animals are constantly subjected to. Lastly, our study provides evidence of potential epi-biomarkers that could be useful as a molecular-level means of assessing animal welfare in the swine industry. We presented here potential epi-biomarkers to be added into the current guidelines and certification schemes to guarantee and certify animal wellbeing on farms. More-over, animal welfare is currently a hot topic for consumers who are increasingly demanding that products meet their moral expectations.

Keywords: swine; stress; biomarkers; DNA methylation; epigenetics; welfare certification.

1. Introduction

Animal welfare has become a public concern in developed countries [1]. This spans livestock industries, laboratory experimentation, sporting events and companion animals. There is a growing demand for high animal welfare products, which the industry is strongly committed to meeting [2]. Consequently, the biggest worldwide pork exporters have adjusted their production chain to align with animal welfare demands [3]. For instance, in the UK and in the European Union, gestation crates have been banned [4], and a similar trend has been observed in Brazil with the recent directive to establish good animal management and welfare practices on commercially-raised pigs [5].

An important topic which has been attracting attention of the animal protein industry is the animal welfare certification, which is demanded by importers. This stems from the fact that consumers want to be aware of the origin of the meat they pay for, and the animal welfare conditions within the production systems in which these animals are reared [6]. In this regard, biotechnological approaches have been applied to ensure with great accuracy that this demand is met. Therefore, companies that go to farms to check behavior, management, biosecurity, and animal welfare play a fundamental role in accomplishing the current goals of ensuring adequate animal welfare within the industry. Moreover, these companies provide certification for pig farms that comply with all animal welfare guidelines. However, once the technician/ auditor has left the farm, it is difficult to guarantee that appropriate procedures will be constantly applied. Even though practices like improper handling may cause variations in the organoleptic characteristics (such as color, brightness, odor, texture, and taste) and/ or chemical composition (such as pH, water holding capacity or color) of the meat [7,8], these changes are seldom noticed by the consumer. Moreover, it directly infers on the moral values of the final consumer who is purchasing the product certified for animal welfare. Thus, the establishment of the

animal´s physiological and molecular information, which ensures consumers that the animals have been bred and raised in compliance with a set of pre-established welfare standards, not only benefits customers by providing the tools to make informed choices about their purchases, but also allows the market to aggregate more value to their products. This information could be certified, for example, by a stamp that translates physio-logical and molecular parameters of the animals into a "handling score", for example.

One of the areas of study that has offered interesting contributions, linking the effects of the environment and intrinsic factors on individuals is epigenetics [9]. Epigenetic studies have been used in many fields of research, such as pharmacology [10], nutrition [11], and welfare [12] across species. Among the numerous fields that epigenetics permeates, efforts to identify epigenetic markers of long-term stress in production animals is currently a hot-topic within the animal welfare field [12,13].

In this article, we provide an overview of some of the investigations already carried out, as well as challenges and potentials associated with this approach. This compilation of peer-review articles explored epigenetic markers in animal welfare research. Our aim is to encourage researchers to extensively investigate potential new paths for the development of a robust molecular tool for animal welfare certification. This tool, together with a careful human inspection, may have the power to greatly increase the precision of current welfare indicators. Consequently, it boosts the credibility of pig producers that comply with welfare guidelines and empowers meat consumers concerned about animal welfare and food quality.

2. Livestock demands

Livestock production is expected to continue to increase to meet growing demand for

animal products [14]. However, this is expected to result in poorer animal welfare [15]. Recently, more and more consumers have raised concerns about the systems and conditions in which their food is produced, potentially driving new trends focused on ethical production [16]. Furthermore, in 2015, the United Nations implicitly set animal welfare as a point of synergy for the sustainable development of food production at the global level [17,18].

Farming activities are no longer seen simply as for the production of food [19]. Farming is increasingly viewed through the lens of "one welfare" where farmers are influencing the health of the environment, of the farm animals under their care, and consumers who buy their products [20]. This new demand came from consumers who dictate what kinds of products they want to eat based on their concepts of quality and safety [21]. In addition, these reflections on "one welfare" generated the possibility of increasing monetization for the farmer [22], because some certified products are more expensive for the final consumer.

Considering these demands and market opportunities, the management and housing systems of the animals play a fundamental role to guarantee animal welfare. To illustrate the importance of housing systems, we present in the next section some of the implications observed in the field that are of relevance to pig welfare.

3. Housing systems

Meeting minimum necessary housing requirements may not be a major challenge in extensive production and for small pig farmers who normally target their product to the local market or for self-consumption. However, in intensive systems, even the minimum requirements can be challenging as they must follow international rules to meet all the animals' needs and market expectations, which includes animal welfare [23,24]. In this

regard, one of the most important factors to provide adequate welfare is the housing system.

Currently, there are different setups of conventional housing systems for pigs, which include crates, indoor group housing, and outdoor systems – each of which carries advantages and disadvantages for pigs and farmers. For example, indoor group housing system is commonly characterized as posing physical challenges to veterinary assistance and to animal feeding, in comparison to crates [25,26]. However, in terms of behavior indicators, indoor group housing tends to result in better welfare [25,27,28]. Another relevant issue involved in the livestock industry are the management of organic and pharmaceutical residues [29], sustainable use of the land [30], and financial costs, which are also vary by housing system.

Despite the variety of housing system possibilities for pigs, each with its respective pros and cons, the welfare conditions currently found in some systems are considered critical and requiring immediate change [31]. For example, the welfare of crated sows in several countries is deemed very poor. It was reported that sows kept in crates have limited expression of natural behavior, which leads to neurological dysfunctions and lame-ness [31–34]. Alternatively, group housing allows animals to express social behavior, which is associated with decreased agonistic interactions, reduced stereotype and improved cognition [32]. However, a frequent concern reported by pig farmers is the innate social aspect of hierarchy, which can be a challenge when housing sows in groups, as hostile behavior may arise from social disputes and result in compromised welfare and production outcomes [35].

In this scenario, even though indoor group housing and outdoor systems may represent challenges of their own and the transition may be difficult or costly for farmers, pressures by legislators [4] and demands by consumers are decisive [36,37]. Therefore,

in the next topics of our review we point out potentially useful approaches to certify animal welfare.

4. Animal welfare indicators

Broom [38] suggested two ways to access behavioral indicators of poor animal welfare. The first focuses on individual failure to cope with the environment. This is an easily identifiable indicator by the pig farmers, since it aligns with increases in mortality and productivity declines. An example of this situation is when the environment in which the individual is raised is poor and leads animals to develop abnormal behaviors or diseases. Therefore, it is impossible for the animal to express its full potential and, consequently, significant economic losses are inevitable. The second type of indicators focuses on how individuals cope with environmental adversity. These indicators are usually more difficult for farmers to assess because they involve physiological outcomes in the animals, such as cardiac, respiratory, gastro-intestinal, and hormonal changes. In general, these indicators do not lead to death, but they may reduce the welfare and performance of the animals [38].

In order to use quantitative measures to assess the level of animal welfare, biochemical markers have been employed at the experimental level. However, these approaches remain insufficient, because they can only identify the animal's biochemical profile in a limited time frame, compromising its applicability in the industry [39]. For example, these indicators may reflect the poor welfare experienced within just a few hours or days before the measurement, depending on its half-life, and not a reliable of the animal’s life trajectory, like the effects of weaning or housing in gestating in crates. Moreover, these markers are usually limited to specific tissues or fluids limiting their applicability to a broad suite of welfare problems. For example, creatine kinase (CK) is an enzyme involved in the citric acid cycle producing energy in the mitochondria. Many

studies have shown its consistency as a biomarker in the tissue of farm animals raised under different levels of welfare [40–43]. These studies suggested that animals with high CK at the time of slaughter were previously subjected to stressful situations [40–43]. Likewise, the measurement of serum lactate concentrations has also shown to be a promising biomarker, mainly for measuring pre-slaughter stress, despite its short half-life [42,44].

Hormones are another known indicators of animal welfare [45]. Cortisol, for example, is a glucocorticoid hormone released under stressful situations, capable of affecting several physiological pathways, including the hypothalamic-pituitary-gonadal axis [46,47]. This hormone has a recognized importance in the evolution and physiological adjustment of many species [48]. However, cortisol also plays a fundamental role as a biochemical marker of acute or medium-term stress in animal production [45]. Currently, different research groups have been striving to develop consistent endocrine profiles for chronically stressed animals and its outcomes in the organism [49]. However, cortisol measurement techniques remain insufficient for the purpose of welfare assessment [49,50]. Sampling techniques usually provide cortisol concentrations only from a few days or weeks [45,51], which limit its practical use. Lastly, cortisol level variations are not fully understood as a stress indicator because both positive and negative exposures can affect its fluctuation [52]. Furthermore, its use as an indicator of stress has been questioned [52, 53].

Studies have shown that chronic stress alters the expression of key enzymes involved in stress susceptibility [54,55] and spine plasticity [56], which can cause specific epigenetic marks in the genome and behavioral changes [54,56–58]. These triggered modifications around the genome do not produce genetic changes, however, can alter gene expression and protein transcription. An attempt to predict the proteomic profile of

animals raised under good or poor welfare conditions was reviewed by Mouzo et al. [39]. They high-lighted the advantages of using proteomic approaches to predict animal welfare according to the animal protein biochemical composition. For example, the study made important considerations about the protein profile of pale soft exudative (PSE) meat, which is one of the main depreciation factors of pork meat, and how proteomic approaches can be used to predict it. However, proteomic approaches have provided a landscape view of the gene expression and its consistency is quite variable because there is a wide variety of mechanisms describing post-translational mechanisms shaping the protein production [39]. As a consequence, challenges can arise for the long-term stress assessment using proteomic approaches [59]. By comparing epigenetics and proteomics, proteomics was recently shown to be a preferable approach to assess short-term stress, whereas epigenetics might be a better forecaster for early prediction of stress susceptibility and a suited approach to be added into the animal breeding schemes [59]. Taking this into consideration, we hypothesize that the investigation of epigenetic mechanisms may offer a valuable at-tempt to identify signatures above the genome of animals raised under different welfare conditions, which may have a greater power to predict its life-long welfare.

5. Epigenetics

The term "epigenetics" was first coined by Waddington in 1942, who suggested the interaction of external factors with the genome as “epigenotype”, and that this interaction could affect the development of the individuals [60]. Recently, epigenetics was recognized as an interface in the transition from the genetic code into a functional mRNA that may be traduced into a protein [9]. Epigenetics consists of a heritable pattern of chemical alterations on DNA that can modulate gene expression without alterations in the base pairs structure of the DNA double strand [61]. In addition, these specific patterns are

maintained and inherited during the mitotic, and possibly meiotic, divisions of the cells [61].

The importance of epigenetics is not limited to a cellular perspective only. Epigenetics has a huge and decisive role in evolution and speciation [62]. Moreover, epigenetic mechanisms can affect the gene expression of one or more generations and influence the adaptation of individuals to a specific environment [62]. Therefore, Hyde et al. [9] suggested that epigenetics works as the interface between the individual and its environment to provide phenotypic plasticity to increase their adaptation capabilities [9]. Some of the epigenetic mechanisms that act in modulating gene expression are DNA methylation [13], DNA acetylation [63], histone modifications [64], nucleosome repositioning [65], and small interfering RNAs [66]. These biological mechanisms help cells to differentiate not only morphologically, but also functionally, controlling the genomic regions that will be accessed and/ or expressed [9]. DNA methylation is the most investigated mechanism in epigenetics [9]. This mechanism involves an addition of 5' methyl to the cytosine followed by guanine (CpG) in the DNA chain, and this reaction is generally associated with repression in gene expression [9]. However, several studies reported an enormous dependence on the methylation location regarding the gene to predict their potential effect on the gene expression and/ or regulation. For example, if the methyl marks are in a promoter, intronic or coding region of the genome. In addition, the level of methylation of one CpG is also important, because it can also infer on the modulation of the gene expression [67]. Then, to examine this amount of information, bioinformatics analysis using differential methylation regions (DMR) are used to compare hypo or hypermethylated patterns among individuals [68].

The interaction among animals and their environments is an issue of discussion even before the theory of evolution through natural selection [69]. However, the role of genetic

factors at the molecular level and their environmental interactions are still premature [70–74]. When the individual is exposed to an environmental experience, it is possible to determine what are the epigenetic effects generated [75], and methylation is a promising biomarker to detect and evaluate these effects [76]. In this regard, in the last decades, valuable efforts have been done to understand the epigenetic differences in the genome of experimental animals, which is the topic of our next session.

6. Epigenetic assessment in mammals

Weaver et al. [77] provided the first evidence that maternal care could produce persistent changes in epigenetic patterns in rats, which included DNA methylation and chromatin remodeling analyses. They revealed a mechanism for the long-term effects of maternal care in the progeny, caused by a stressful challenge during early life. The authors showed that the descendants who received more maternal care demonstrated low reactions to stress in adulthood, while offspring, which received less maternal care were more susceptible to stress. This happened because the epigenomes of the adult rats exhibited different patterns, specifically the glucocorticoid receptor gene promoter in the hippo-campus, which possibly affected the hypothalamic-pituitary-adrenal axis and its responses to stressful situations [77]. In addition, Champagne [78] elucidated the evidence for the generational transmission of maternal care and mechanisms underlying transmission [78].

Intergenerational and transgenerational epigenetics is one of the most discussed topics in the field of epigenetics inheritance. Current literature supports the understanding that the transmission of epigenetics marks from one generation (F0) to the next (F1) represents an intergenerational event; while the transmission acquired in F0 that is transmitted to the third or fourth generation (F2 for males or F3 for females) can be considered as transgenerational epigenetic event [79]. In other words, different number

of generations are directly affected by environmental insults in males and non-pregnant females when compared to pregnant females. This happens because the majority of female mammals have their oocytes produced during their fetal development [80,81]. For example, the por-cine female fetuses have their gonads differentiates into an ovary containing gamete cells by day 30 of pregnancy and by mid-gestation primordial follicles are already recognizable [82]. Consequently, if a pregnant female suffers a potential epigenotoxic environmental exposure, not only the fetus will be directly affected by this environmental insult, but also the germ cells of the fetus. In summary, three generations can be epigenetically affected by an environmental insult: F0 (somatic and germ cells of the pregnant female), F1 (fetus), F2 (germ cells of the fetus) [79,83,84]. Figure 1 was provided for exemplification, using the porcine model.

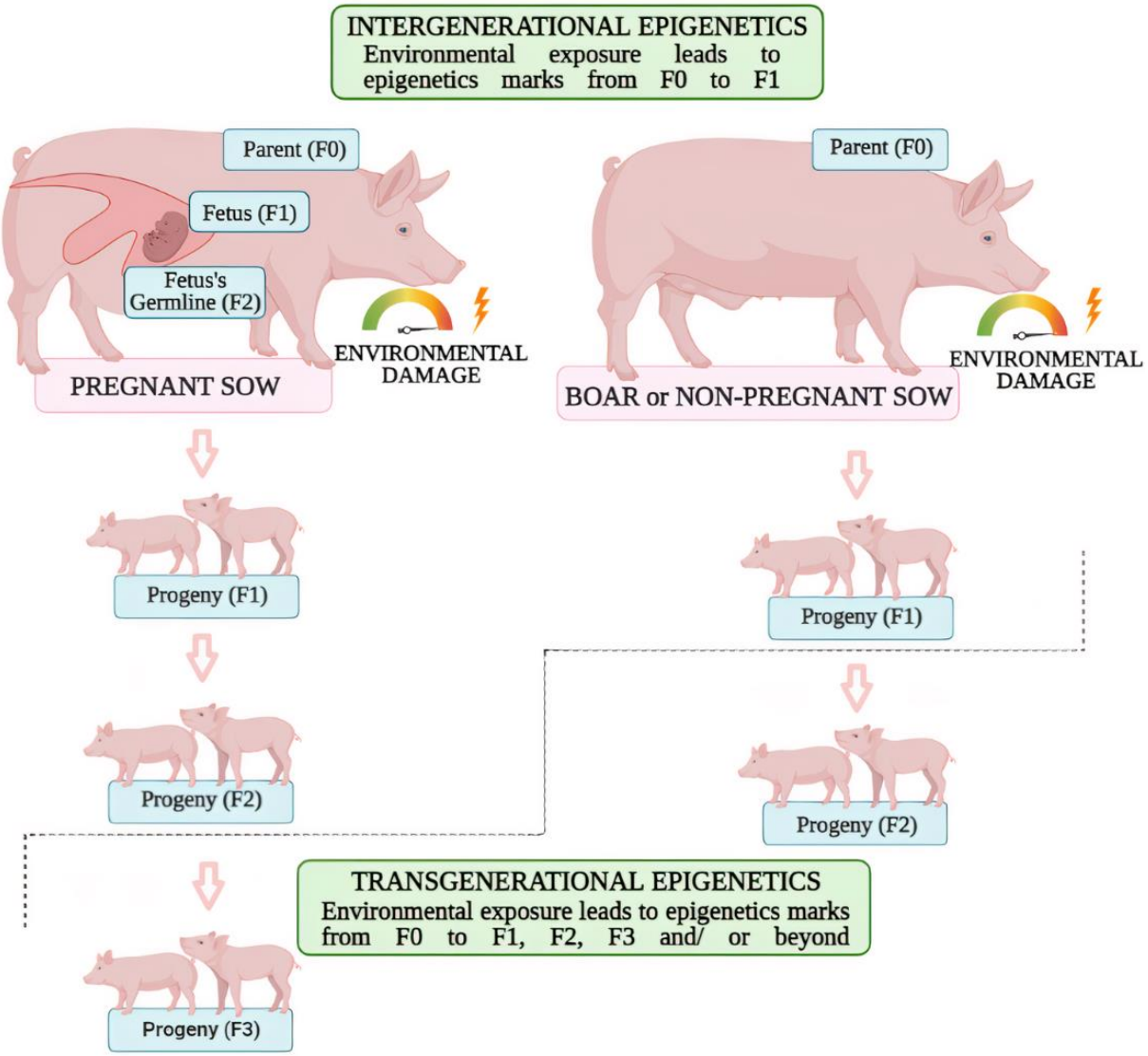

Figure 4. Intergenerational and transgenerational epigenetic inheritance in porcine models. When a swine (F0) is exposed to an environmental insult, somatic and germ cells will potentially affect their epigenome. In addition, if it is a pregnant sow, the fetus (F1) and its germ cells - which will give rise to a next generation - will be directly affected (F2). So, if these epige-netic marks contained in the fetus's germ cells remains for subsequent generations (F3 and beyond), there will be a transgenerational epigenetic event.

Although the most characterized epigenetic studies have been conducted in small experimental animals, such as rodents [77,85,86], insects [11], and worms [87]; a representative number of studies have been done on domestic animals, such as pigs [88–93]. In the following section, we will discuss in detail studies focused on pigs as models in epi-genetic investigations and draw attention to the role of stress as an epigenetic modulator.

7. Epigenetics studies in the porcine model

It has been proposed an important role of epigenetics on productive [13,66,94–96] and reproductive traits [97–99], as age at puberty in swine models [100]. Moreover, publications on swine epigenetics, including the influence of nutrition on the pregnant sow epigenome [88,92,101], the impact of the exposure of pregnant sows to chemicals on its offspring epigenome [89,91], and also epigenetic marks in boars and their ejaculate [90,102–104] is a current hot topic.

Studies have indicated that the management of pigs during gestation promotes changes in their offspring behavior [70,72,74,105]. However, to the best of our knowledge, stressful events in pig farming, such as the effect of gestation crates, lameness, and social isolation have not been explored by epigenetic studies in pigs [106,107]. These studies would be valuable for both animal welfare and the industry. Likewise, these epigenetic investigations would also be valuable for translational studies, once the porcine model is a well-known standard for human studies.

A possible mechanism through epigenetics may act is shown in figure 2. We hypothesize that negative situations where pigs are exposed to at challenging production systems can affect somatic and germ cells. Thereby, epigenetic patterns could be transmitted from parents to their offspring, but for that, it need to be present somehow in their germline strain (sperm or oocytes) epigenome. So, not only does the individual accumulate epigenetic marks during its life, but it also inherits different patterns from its parents.

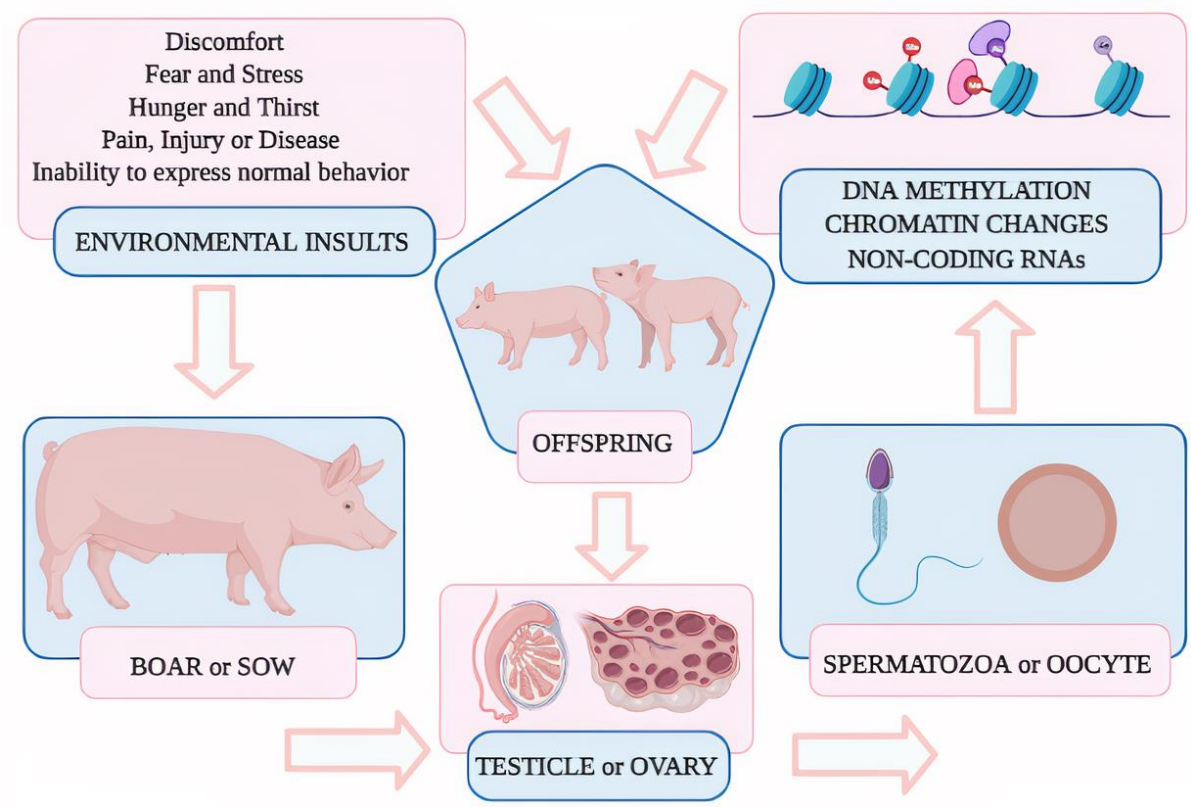

Figure 5. A possible pathway of epigenetic transmission in industrial pig production systems.

Valuable efforts have been made using the porcine model in epigenetic studies (Table 1). However, to the best of our knowledge, there are few epigenetic studies related to the daily challenges of pig farming, such as inadequate housing, painful procedures, heat stress, or other stressful situations. First, Collier et al. [108] highlighted the role of maternal stress and its potential for the inheritance of epigenetic changes by future generations [108]. Nevertheless, they did not use an epigenetic approach to support their suggested mechanism. Their study was focused on measuring cortisol, interleukins, cytokines and others physiological biomarkers. Then, Schachtschneider et al. [109] demonstrated that early-life challenges, such as iron deficiency and porcine reproductive and respiratory syndrome virus (PRRS), were able to alter DNA methylation and expression of key genes related to hippocampal plasticity, which can cause several long-term cognitive damages [109]. Recently, Kasper et al. [59] provided a literature review

showing the potential of omics approaches in the development of biomarkers in pig production, including epigenetics, and how it could positively impact the issue of tail biting.

Table 1. Studies related to swine epigenetics in different experimental contexts.

Mechanism	Generation	Swine model	Context	Reference
Direct exposure	F0	Boars	Investigation of methylation patterns of testis samples and their relationship with the boar taint flavour.	[103]
		Boar's semen	Correlation between different parameters of sperm DNA integrity and their methylation patterns.	[102]
			DMRs are more efficient at discerning the fertility of boars' ejaculate than single nucleotide polymorphisms (SNPs) using reduced representation of the methylated DNA.	[104]
		Gilts	The epigenetic dynamic in hypothalamus-pituitary-ovary axis and its tissue-specific manner to establish the biological functions.	[115]
			The dynamics of hypothalamic methylation at puberty.	[116]
			Long-term effects of endocrine-active compounds on corpus luteum of swine females exposed during early life period.	[117]
		Porcine embryos	Investigation of the effects of histone deacetylase inhibitors on the in vitro development of porcine embryos derived from somatic cell nuclear transfer.	[98]
		Porcine oocytes	The effects of vitamin C in the regulation of global epigenetic modifications at DNA, RNA and histones levels and its potential for oocyte maturation and developmental competence.	[97]
		Porcine ovary	Epigenetic mechanisms of ovarian development during the transition from puberty and sexual maturation.	[100]
Intergenerational epigenetics	F0 - F1	Pregnant sows and its offspring	Effects of exposure to low or high doses of estrogen during pregnancy and its role in female reproductive organs.	[89]
			The immediate and long-term effects of maternal dietary protein affecting gene expression of offspring.	[88]
			Restriction and excess dietary protein during pregnancy alters the offspring's epigenetic marks and influences gene expression.	[92]
		Boar's semen and sow's placenta	The role of breeding season in altering epigenetic components of the placenta and its consequences to foetal development.	[99]
Transgenerational epigenetics	F0 - F2	Boars	Transgenerational response of a methyl-enriched diet to boars and its responses on carcass traits, gene expression and DNA methylation.	[90]

The absence of disease is a welfare demand, and epigenetic studies have also revealed interesting contributions to explain pathophysiological mechanisms of infections by microorganisms. The study by Sajjanar et al. [110] showed the role of the DMRs in regulating genomic regions of porcine mammary epithelial cells infected by Escherichia coli strains when compared with non-infected cells. They identified significant DMRs, hypo-methylated in the cells infected with E. coli, in the promoter region of the *SDF4, SRXN1, CSF1* and *CXCL14* genes. Using functional network analysis, the authors also reported that these genes are related to innate and adaptative immune response pathways. In addition, the study by Simões et al. [111] clarified how an African swine fever infection can impair the subnuclear domains and chromatin architecture of infected cells, compromising gene expression, and favoring viral dissemination. This mechanism is part of an emergent field of studies, which have shown that some viruses subvert cellular epigenetic mechanisms and recruit host transcription factors to their benefit by changing chromatin structure [112].

Using muscle tissue samples from a heat stress-exposed group and an unexposed group of pigs, the study by Hao et al. [13] showed that the methylation level of the heat-stressed group was significantly lower than what was found among the control group for some genomic regions. Moreover, they showed that the DMRs were located around important genes related to cell development, which may have a play in muscle performance and function. Moreover, in another study, Hao et al. [66] evidenced that even the microRNA expression profile of the heat stress exposed group was affected by this chronic source of stress, possibly compromising gene expression at a post-transcriptional level. Lastly, the study conducted by Ponsuksili et al. [53] was able to show the

differences in epigenetic patterns of muscle cells from different pig breeds and their role in the development of the muscle phenotype.

Table 1 summarize some important findings and advances using pigs as an experimental model in epigenetic related studies. The criteria used to include studies in the table were original research published in peer-reviewed journals that addressed relevant information on epigenetics over the past 10 years. To classify the studies according to epigenetic mechanisms in the table, we used as basis the concepts recommended by Lacal and Ventura [79]; Tuscher and Day [83]; and John and Rougeulle [113]. They suggested that only changes in F3 generation in females, and F2 in males [79,83], can be defined as a "transgenerational epigenetic inheritance" event. In addition, the concept of "intergenerational epigenetics" was used to define studies that addressed exposures that led to epigenetic changes in the somatic tissues of the F1 offspring but did not persist/ or was not tested in the F2 or F3 generations [79,83]. Finally, the term "direct exposure" was used to define studies that investigated epigenetic marks in a single generation (F0) [113].

8. Gene network analysis on stress in pigs

Although there is substantial evidence regarding the suitability of methylation as a molecular marker to predict pig welfare [59], the findings are still premature and further research will be needed. To the best of our knowledge, the number of articles reporting DMRs in contexts of compromised welfare is limited [13,95,101,109,110]. Also, the results are quite variable in terms of specific affected genes. However, this is expected, since the experimental contexts are different, which include intrinsic and extrinsic variations of individuals, such as genotypic variability and tested stress models, for example. In addition, the laboratorial and statistical approaches performed for DMR identification across the experiments are also variable among the previous published studies.

Therefore, to explore the common biological functions performed by the previously identified stress-related genes, we performed an integrative analysis considering these previous identified epi-markers. A functional enrichment analysis was carried out with the DMR-related genes identified as significant from each one of the previous studies when subjected to different stress conditions (Tab. 2). The 28 affected genes identified in these studies were affected by DMRs, so we analyzed by gene enrichment in the category of co-expression, physical interactions, predicted network, co-localization, and pathway analysis using GeneMania web environment [114]. The integration of the genes and its most cited molecular functions can be seen in figure 3. We summarized the main identified functions of the genes using a word cloud software (https://www.wordclouds.com/), which takes in consideration the number of times a word is identified in order to output this word with its proportional font size.

Table 2. Effect of stress on pigs' genome subjected to different environmental insults. The approaches used to access the methylated DNA was whole genome bisulfite sequencing (WGBS) or reduced representation bisulfite sequencing (RRBS).

Stress source	Analysed sample	Approach	Effect of stress	Biomarker	Reference
Heat stress	*Longissimus dorsi* muscle	WGBS	DMRs in important genes involved in muscle development, metabolism, immunity, and stress response.	*RYR3, PGK1, CRYAB* and *FHL1C*	[13]
Intrauterine insult	Small intestine	RRBS	DMRs in several genes involved in cell development and immunity.	*IRAK1, AIFM1, PIM2, BCAP31, MTMR1, SOX3, TWIST2* and *HAUS7*	[95]
Sanitary challenge	Mammary epithelial cell	RRBS	DMRs in functional genes of the innate and adaptive immune response.	*SDF4*, *SRXN1*, *CSF1* and *CXCL14*	[110]
	Mid intestine	RRBS	DMRS in genes involved in structural pathways of the cells with outcomes in the immature prenatal intestine.	*CYP2W1, GPR146, TOP1MT* and *CEND1*	[101]
	Hippocampus	RRBS	DMRs in genes associated with blood brain barrier permeability and regulatory T-cell activation, which are reported to cause reductions in cognitive development.	*VWF, LRRC32, NGF, GNG13, PIK3R5, KCNJ6, KCNJ5* and *AKT2*	[109]

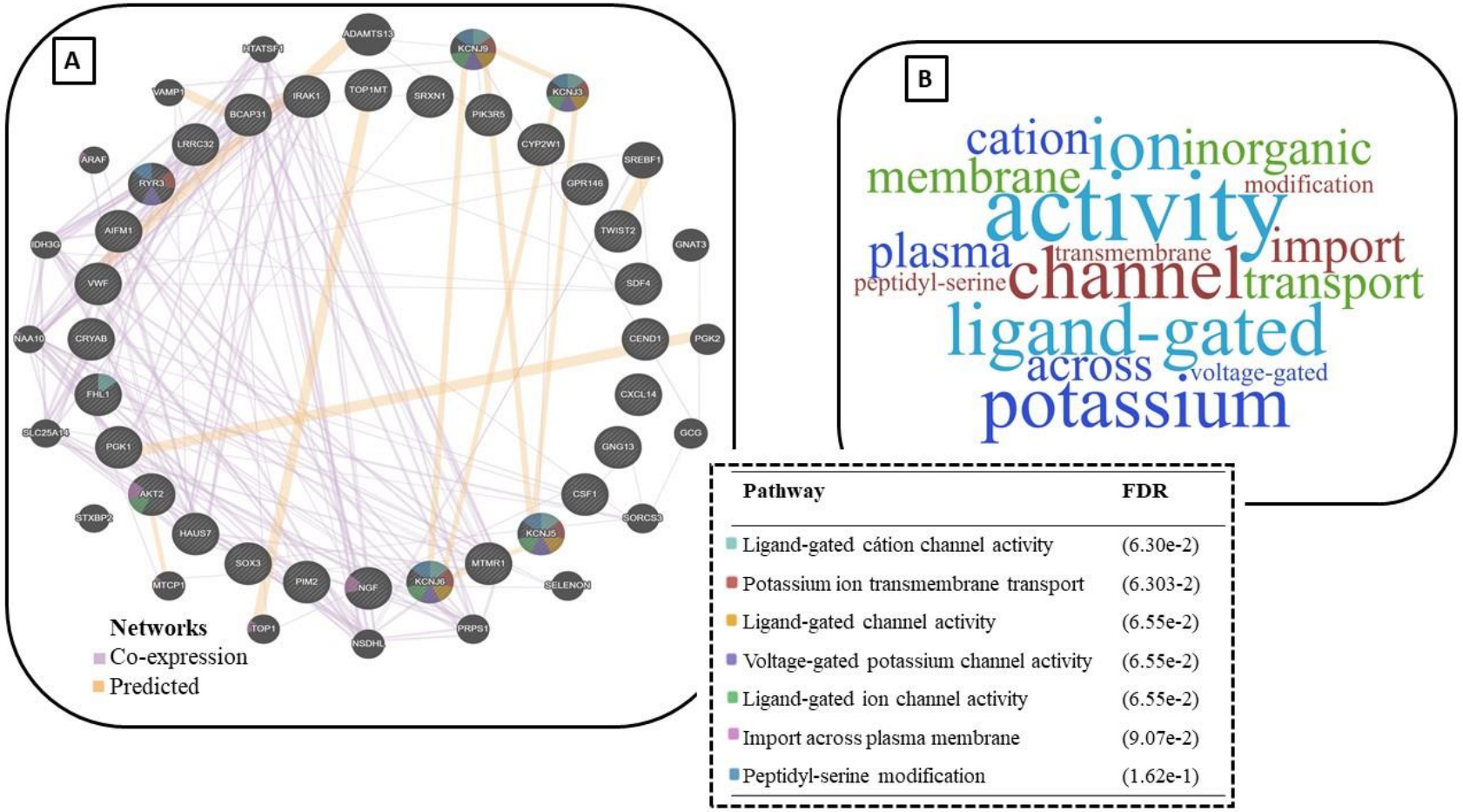

Figure 6. Prediction of the gene network (A) and most cited pathways (B) in which the genes are enrolled using the human genome as reference.

From the gene network (Figure 3 a), most of the pathways (Figure 3 b) identified (FDR ≤ 0,19) by the enriched genes play a role in the regulation of transmembrane transport and basic cell signaling processes. Notably, the regulation of potassium channel activity was the top pathway in our analysis. Interestingly, after inducing a group of mice to acute stress and assessing their behavior response to this situation, Guo et al. [118] identified that acute stress-induced a significant reduction in calcium-potassium channels in the amygdala of the stressed mice. This molecular pathway and this source of tissue may be of great importance to assess long-term information of pigs exposed to stressful situations.

Moreover, from the 28 potential genes connected in our stress-related gene network, another 20 genes were outputted as connected with this network. In addition, the genes *KCNJ6, KCNJ5, FHL1, AKT2, NGF* and *RYR3* from the main core of the network were enriched for the regulation of potassium channel activity pathway. Moreover, they were previously re-ported to be relevant when analyzing heat stress [13], and animal exposition to sanitary challenges [109], which are two of the hot topics in animal welfare in swine field [13,109].

9. Conclusion

In the last decade, the interest in the epigenetic field has exponentially increased and has shown enormous potential in answering scientific questions in different areas. In the animal welfare field applied for livestock animals, valuable attempts have been made to identify putative epigenetic biomarkers of stress. Furthermore, the potential of applying epigenetic markers for productivity, health, and meat quality improvements has been described so far. Thus, considering the latest evidence, using epigenetics as a tool to certify animal welfare may be one of the new trends in the pig industry. In this study, we brought together the latest in the area of epigenetic markers in studies of well-being in pigs. In

addition, we provided a list of potential genes for target analysis, which can enhance the ap-plication of this technology in animal breeding schemes. In the future, the link between epigenetics, physiological parameters, and animal management should be investigated to provide some insights into its applications to improve housing systems, food quality, and the production system in general. However, at this moment, findings remain quite premature to assure the development of an epigenetic panel of biomarkers capable to predict life-long welfare in commercially raised pigs. Future studies not only will elucidate mechanisms in the stress response but are also likely to increase the number of publications to foment a panel of biomarkers capable of predicting animal welfare

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6. MANUSCRIPT 2

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A challenge with lipopolysaccharide (LPS) on the day of estrus can compromise gene expression of the corpus luteum of gilts

Abstract

The corpus luteum (CL) is a temporary endocrine gland that plays a decisive role in the reproductive physiology of gilts. Recently, it has been suggested that exogenous factors may compromise the normal functioning of the CL through epigenetic mechanisms. In the present study, we aimed to understand to what extent an acute and systemic challenge with lipopolysaccharide (LPS) on the day of ovulation could compromise gene expression of gilts' CLs housed in different welfare conditions. For this, we housed 42 gilts in three different housing systems: group housing (14), outdoor (14), and crates (14). Then, we challenged 6 females from each group with LPS and 8 with saline (SAL) on the day of estrus. After slaughtering gilts on the 5th day after the challenge, ovaries were collected for gene expression analysis, using RT-qPCR. Employing the Student t-test, we identified significant ($p=0.039$) down regulation of the angiogenic gene *VEGF*, which is responsible for inducing vascular endothelial cell proliferation and migration, in the LPS-challenged group. Notably, the crated group, which is associated with poorer welfare, was the most compromised ($p=0.110$). Our results indicate that not only an acute health challenge on the day of ovulation can compromise CL gene expression, but it can be more challenging in animals housed in poor conditions.

Keywords: Acute stress; Gilts; RT-qPCR; Angiogenesis; Development.

Introduction

In the swine species, the corpus luteum is a transient endocrine gland that has a short lifespan, from 12 to 15 days, approximately [1]. The main secreted product by this temporary gland is progesterone [2,3], which reaches its maximum plasma concentration between days 8 and 9 after the day of ovulation [1,4]. Progesterone not only plays an essential role in the maintenance and success of pregnancy [5], as it also acts as a direct negative feedback mechanism in the hypothalamus to suppress follicular development, regulating the timing of ovulation [6]. Considering these factors, it has been suggested that the inadequate performance of the corpus luteum is one of the main causes of subfertility and embryonic loss in mammals [2].

There is increasing evidence that the functionality of the corpus luteum can be affected by environmental factors and stress, through physiological impairments that involve inflammatory cytokines and androgen excess [6,7]. In addition, valuable efforts have been made to understand what are the main factors that affect the development of the corpus luteum over pregnancy [8,9], as well as the nutritional factors that can improve the early development in pig foetuses [10,11]. Therefore, we hypothesized that the environment in which the swine females are housed in the pre-mating period may interfere with the early-developmental gene expression on the corpora lutea, which can compromise its maximum progesterone production and systemic presence throughout the body. Housing systems are not only able to improve the welfare of the individuals, as they can also interfere in the resilience of animals when facing a health challenge [12,13].

Urinary tract infections of female pigs caused by environmental bacteria are among the most important challenges of intensive pig farming [14]. These infections, which can cause systemic diseases, are often caused by gram-negative bacteria [15]. This class of bacteria has lipopolysaccharides (LPS) in its external membrane, which is

responsible for promoting a systemic inflammatory response, which includes fever, vasodilation, and eicosanoid secretion in their hosts [16]. Remarkably, the day of insemination of the female pig can be a day susceptible to infections, as not only will the semen be deposited in the female's cervix or uterus body, but because there is manipulation with materials and the possibility of introducing environmental bacteria into the reproductive tract of the female pig [15]. Thus, we hypothesize that this breakdown of homeostasis may be associated with reproductive problems, including the establishment of the newly formed corpus luteum.

The study of gene expression by real-time qPCR is recognized as one of the best methods for determining to what extent a gene is being expressed during tissue development and in the face of a health challenge [17]. This assessment is important because there are molecular mechanisms that can modulate gene expression, causing substantial changes in the number of transcripts generated by cells, and dramatic systemic consequences in the individuals' physiology [18]. Furthermore, it has been suggested that the modulation of these epigenetic markers is highly dependent on the environment and challenges that individuals have been subjected to in previous experiences [19].

Therefore, our objective was to evaluate the gene expression of the corpora lutea of swine females housed in three housing systems (crates, group housing, and outdoor system) that were challenged with LPS – or saline (SAL) – on the day of ovulation. We hypothesize that the housing system may interfere with the female's resilience in dealing with a health challenge on the day of ovulation, which may compromise the expression of genes related to progesterone synthesis (*STAR*, *CYP11A*, *HSD3B1*, *LHCGR*, and *PGR)*, angiogenesis (*VEGF*, *FLT1*, and *KDR)*, apoptosis regulation (*IL1B*, *TNF*, and *IFNG)*, and stress response (*HSD11B2*, *NR3C1*, and *NR3C2)* on the corpora lutea.

Materials and Methods

Animal experiments were designed and conducted in accordance with the Ethic Principle in Animal Research adopted by Ethic Committee in the Use of Animals of the School of Veterinary Medicine and Animal Science of the University of São Paulo (CEUAx 9992150121).

Animals and experimental design

To determine the effect of LPS on porcine corpora lutea and the role of the environment to cope with this challenge, we used the same animals described in our previous study [20]. Briefly, forty-two gilts from commercial crossbreed lineages participated in this study (Fig. 7). All females received water *ad libitum* and the same commercial diet, even when females were housed in groups because they had access to individual feeding boxes. In addition, all of them were identified as sexually receptive using a boar before the experiment, all of them presented at least once clinical signs of heat. The animals had their estrus cycle synchronized with Altrenogest (Regumate, MSD Saúde Animal, São Paulo, Brazil) at a dose of 5 mL per animal per day for 18 days, as recommended by the supplier. Five days before the expected estrous, the 42 gilts were divided into three groups of 14 animals each: crates (C), outside group (OD), and group housing (GH). The animals were kept throughout the estrous cycle in the specific housing system they were housed in. On the estrus day, 6 gilts from each housing system received a single dose of 2 µg/kg of LPS (E. coli O111:B4, Sigma Aldrich, Missouri, USA) intravenously, while the other 8 received SAL as a control. On the 5th day (~120 h) after the estrus day, all the gilts were slaughtered, and the right and left ovaries of each gilt were collected. The ovaries were immediately frozen in liquid nitrogen and stored at −80 °C.

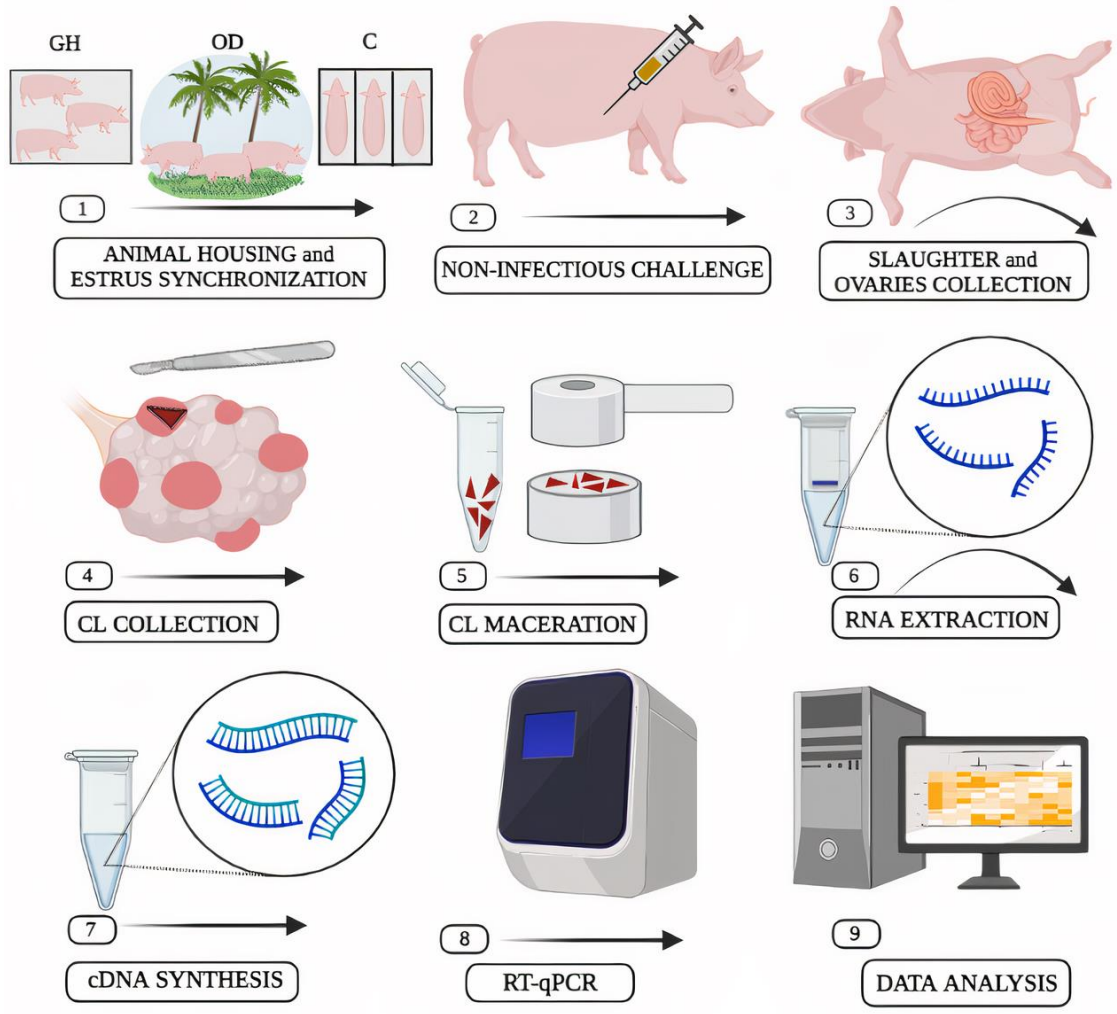

Figure 7. Summarized experimental design.

Corpus luteum collection

For macroscopic evaluation and tissue collection, a systematic procedure was organized with liquid nitrogen, which preserved the samples always frozen. Moreover, we used sterile materials for each one of the samples collected, strictly controlling contamination between samples and by materials. The CLs collection was performed collecting fragments with a stab incision with scalpel blade 24 to extract a cone of tissue of the 5 largest CLs from each ovary. Thus, soon after collecting the biopsies, approximately 0,1 g in total, the fragments were macerated and mixed using a metallic

apparatus. During the maceration process, liquid nitrogen was used to preserve the 5 biopsies of the CLs, frozen, which facilitated the tissue maceration procedure. The resulting macerated tissue was stored in cryotubes of 2 mL at -80 °C until RNA extraction.

RNA extraction and cDNA synthesis

Approximately 50 ng of macerated CL was used for total RNA extraction, using a standard protocol with TRIzol (Thermo Fisher Scientific, Massachusetts, USA) [21]. To check the concentration of the total RNA extracted (A260) and purity (A260 / A280), spectrophotometric absorbance was measured in the NanoDrop 2000 (Thermo Fisher Scientific, Massachusetts, USA). Then, total RNA was treated with DNase I (Life Technologies, California, USA) to eliminate eventual contamination with genomic DNA. To finish, the cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, California, USA) according to the manufacturer's instructions.

The cDNA of each sample was stored at -20 °C until qPCR analysis. Besides, the final transcriptase reverse reaction was standardized at 1:80 and this cDNA concentration was used as a template for each one of the qPCRs reactions.

Oligonucleotide's synthesis

The oligonucleotides *PGR, VEGF, FLT1, KDR, STAR, CYP11A, HSD3B1, LHCGR, HSD11B2, NR3C1, NR3C2, IL1B, TNF, IFNG, GAPDH, and UBB* were designed according to gene sequences from Ensembl (http://www.ensembl.org/index.html) and mRNA sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov), avoiding genomic DNA amplification (Tab. 3). In addition, the specificity was confirmed through in silico analysis by blasting the sequences of primers against the NCBI database (https://blast.ncbi.nlm.nih.gov/).

Table 3. Swine specific oligonucleotide forward (F) and reverse (R) primer sequence (5'-3'), amplicon length of the evaluated genes, and primer efficiency in the standard curve on qPCR.

Target name	GenBank ID	Primer (5'-3')	Amplicon	Efficiency (%)
PGR	NM_001166488.1	F: 5´AACACCAAACCCGACACTTC 3´	107 bp	89,80
		R: 5´ CGAAAACCTGGCAGTGACTT 3´		
VEGF	X81380	F: 5´ CGAAGTGGTGAAGTTCATGG 3´	120 bp	99,07
		R: 5´ ACACAGGACGGCTTGAAGAT 3´		
FLT1	AJ245445.1	F: 5´ ACCCCGGAAATCTATCAGATCA 3´	94 bp	87,97
		R: 5´ GGTCGCCTAGTTTTTCCACAAG 3´		
KDR	AJ245446.1	F: 5´ CTCAGCAGGATGGCAAAGACTA 3´	128 bp	86,90
		R: 5´ GGGGTCACACACTTCCTCTTCT 3´		
STAR	NM213755	F: 5´CAGACTTTGGAGAGATGCCTGA 3´	138 bp	90,10
		R: 5´ ATCCCTTGAGGTCAATGCTGAG 3´		
CYP11A1	NM_214427.1	F: 5´ CCTGCCAAGACATTGGTACAAG 3´	113 bp	86,41
		R: 3´ AGGTCCCTTTCTTTACCCAACC 3´		
HSD3B1	NM_001004049.1	F: 5´ TGGTCATCCACACTGCCTCTAT 3´	90 bp	91,11
		R: 5´ GGAGCTGGGTACCTTTCACATT 3´		
LHCGR	XM_021085888.1	F: 5' CATAACCACCGTACCAGCAA 3'	135 bp	98,80
		R: 5' TTCAGCTCCAGGGAAATCAG 3'		
HSD11B2	AF414125	F:5' GCGAAAGCTTCCCACTGAAC 3'	59 bp	102,63
		R: 5' AGGGTCTGTTTGGGCTCATG 3'		
NR3C1	AF141371	F: 5' GATCATGACCGCACTCAACATG 3'	68 bp	97,11
		R: 5' TTGCCTTTGCCCATTTCAC 3'		
NR3C2	XM_013978840.2	F: 5' TTGCCTTGAGCTGGAGATCG 3'	143 bp	106,17
		R: 5' GAACTGCAGGCTGATCTGGT 3'		
TNF	NM_214022.1	F: 5' GCCCTTCCACCAACGTTTTC 3'	97 bp	–
		R: 5' CAAGGGCTCTTGATGGCAGA 3'		
IFNG	NM_213948.1	F: 5' GCGCAAAGCCATCAGTGAAC 3'	105 bp	–
		R: 5' GCTCTCTGGCCTTGGAACAT 3'		
IL1B	XM_021085847.1	F: 5' TTTGAAGAAGAGCCCATCATCC 3'	119 bp	97,98
		R: 5' CCAGCCAGCACTAGAGATTTG 3'		
GAPDH	NM_001206359.1	F: 5´ TCCTGGGCTACACTGAGGAC 3´	123 bp	109,59
		R: 5´ ACCAGGAAATGAGCTTGACG 3´		
UBB	U72496.1	F: 5´ ACCAGCAGCGTCTGATTTTT 3´	92 bp	100,03
		R: 5´ CAAGTGCAGGGTGGACTCTT 3´		

Quantitative Real-Time PCR

Quantification of specific transcripts was performed by real-time polymerase chain reaction (RT-qPCR) using PowerUp SYBR Green Master Mix (Life Technologies, California, USA) with a final volume of 10 µL per reaction, including a cDNA amount of 2 µL, and a primer concentration of 400 nM. The reactions were run in triplicate on a 96-well plate, which was sealed with a MicroAmp optical adhesive cover (Life Technologies, California, USA) before its reading in a Step-One Plus Real-Time PCR System (Applied Biosystems, California, USA). The thermocycling profile consisted of 40 cycles of 15 s at 95 °C for denaturation and 12 s at 60 °C for annealing and extension, including a previous activation step of 95 °C for 10 min. The final stage included an analysis of the melting curve verifying the presence of a single peak in the different PCRs.

Selection of the reference genes and data normalization

The amplification data were extracted from the Step-One Plus Real-Time PCR System (Applied Biosystems, California, USA) and each sample was analyzed through LinRegPCR (version 2020.2) software [22] for baseline correction, determination of qPCR efficiency, and cycle quantification values per sample. The election of the reference genes was determined through the findings provided by Okino et al. [23]. Geometric mean (GM) of GAPDH and UBB Ct values were used for relative analysis. Thus, gene expression of each target gene relative to the housekeeping genes was normalized using the comparative ΔCt and the fold change due to treatment $2^{-\Delta\Delta Ct}$ [17], using the arithmetic mean (AM) of the ΔCt values of the SAL challenged group, independently of the housing system. The formula used for normalization was: $2^{-\Delta\Delta Ct}$, where, $\Delta\Delta Ct = [Ct\ (\text{target gene mRNA}) - Ct\ (\text{GM mRNA})]\ \text{experimental groups} - [AM\ (Ct\ (\text{target gene mRNA}) - Ct\ (\text{GM mRNA})]\ \text{SAL group}$.

Statistical analysis

Descriptive analysis of the individual variables was performed: mean, median, standard deviation (SD), minimum/maximum, confidence interval (CI) (95%), and Shapiro-Wilk test to verify normality. For expression pattern analysis and comparison (heatmap), relative expression of each gene was used (ΔCt). In addition, for the comparison between LPS and SAL groups, data was assessed by fold-change estimates ($2^{-\Delta\Delta Ct}$), using the Student t-test. Unpaired analysis was considered in our study and the analyzes were performed in Python (version 3.8.3). Results were considered significant when $p \leq 0.05$. P-values between 0.06 and 0.10 were considered a trend.

Results

Morphological measures of the ovaries

During the macroscopic evaluation of the 42 ovaries, two ovaries did not present CLs on their surface and one ovary had only one CL. The ovaries that did not present CLs on their surface were from gilts kept in the crates system (one treated with LPS and the other one with SAL). The ovary that had only one CL was from a gilt kept in the outdoor system and was treated with LPS. These three samples were removed from our study of gene expression because they did not meet our minimum standards of 5 CLs.

Gene expression evaluation on the corpus luteum

To start, the *TNF* and *IFNG* oligonucleotides were not amplified in RT-qPCR, nor were they included in the analysis of our study.

The descriptive analysis between the LPS and SAL groups on the gene expression of each of the 12 genes was performed (Fig. 8 and Sup. Fil. 1). In general, for all genes

studied, the expression of the LPS-challenged group was reduced compared to the SAL-challenged group.

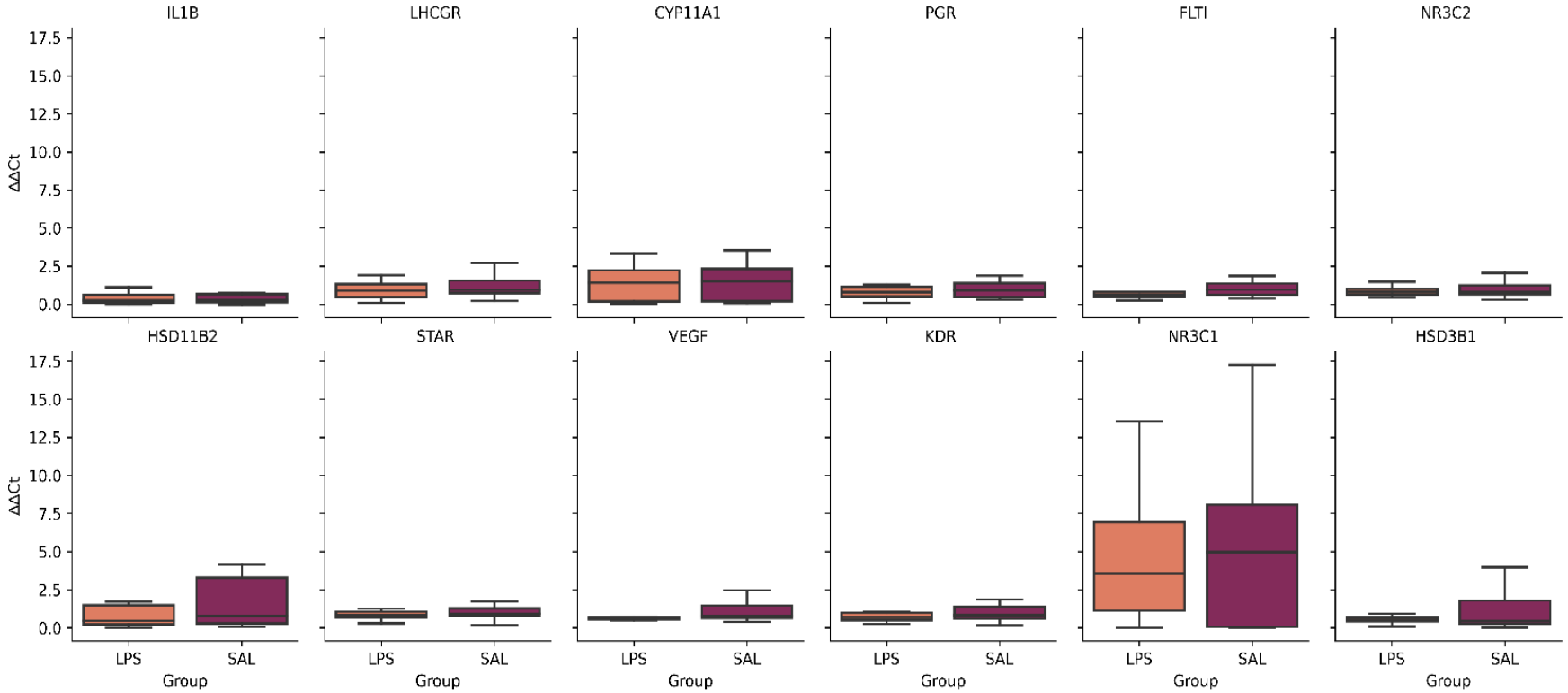

Figure 8. Descriptive analysis of the gene expression of the 12 evaluated genes, considering $2^{-\Delta\Delta Ct}$ values. Comparison between LPS and SAL groups, disregarding the housing system of the animals. Box plot without outliers for better illustration. Data are presented as mean ± SD.

Using the Student t-test, a significant interaction (p=0.039) was identified only for the *VEGF* gene (Fig. 9 and Tab. 4) comparing LPS or SAL treated animals. Interestingly, we identified the smallest p-value (p=0.110) among the animals housed in the crates system - and challenged with LPS - to have their gene expression reduced (Fig. 10 and Sup. Fil. 2). Moreover, it is also important to be emphasized that the lower variability in terms of gene expression for the *VEGF* gene was found among the animals housed in the outdoor system, independently of the source of challenge (LPS/ SAL). Lastly, a trend was observed among LPS or SAL challenged animals for the *IL1B* gene expression (p=0,090).

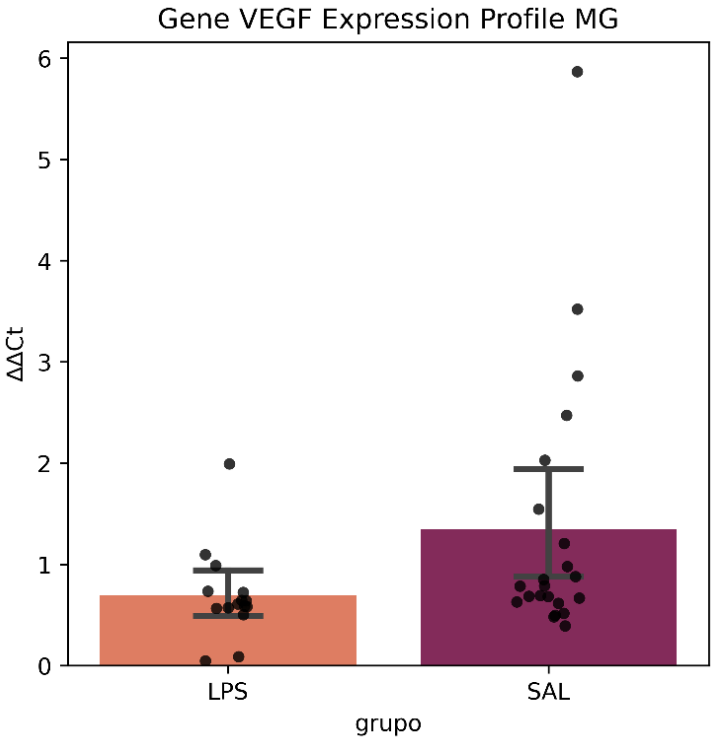

Figure 9. Relative gene expression level of *VEGF* gene between the treatment LPS or SAL, disregarding the housing system of the animals. Box plot without outliers for better illustration. Data presented as mean ±SD.

Table 4. Comparison analysis by t-test.

Group*	t-test	p-value	CI (95%)
IL1B	-1.78	0.090**	(-406.682, 32.721)
LHCGR	-0.78	0.439	(-0.693, 1.307)
CYP11A1	-0.69	0.495	(-1.760, 0.867)
PGR	-1.01	0.321	(-10.795, 3.716)
FLTI	-1.50	0.141	(-1.160, 0.173)
NR3C2	-1.19	0.247	(-2.262, 0.619)
HSD11B2	-1.47	0.154	(-7.284, 1.227)
STAR	-1.51	0.139	(-1.092, 0.160)
VEGF	-2.16	0.039***	(-1.278, 0.133)
KDR	-1.35	0.190	(-3.054, 0.644)
NR3C1	-1.01	0.320	(-17.270, 5.945)
HSD3B1	-1.05	0.304	(-620.907, 203.251)

* LPS x SAL group; ** p-value between 0.10 and 0.05; ***p-value <0.05

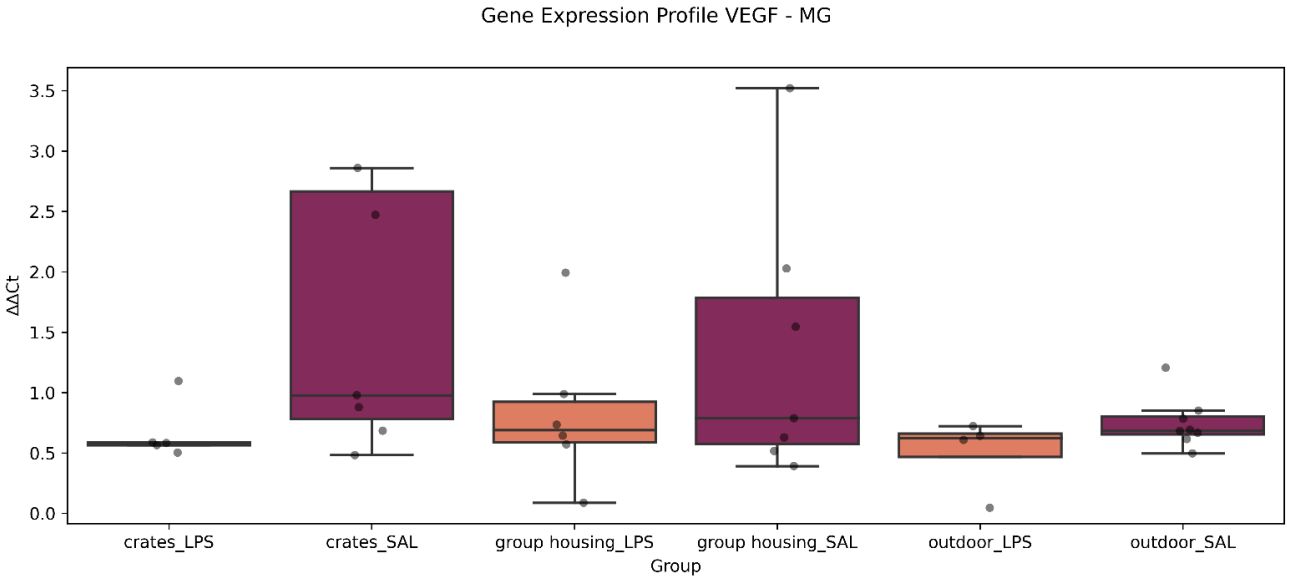

Figure 10. Descriptive analysis of the gene expression of *VEGF* gene among the treatments and housing systems. Box plot without outliers for better illustration. Data are presented as means ±SD.

Furthermore, when we performed an individualized gene analysis, also segregating the six experimental groups (C-SAL, C-LPS, GH-SAL, GH-LPS, OD-SAL, and OD-LPS) we identified two trends. Animals from the group housing system showed a trend when contrasting the different expression between exposed to LPS or SAL ($p=0.074$) for *NR3C2* gene expression. Likewise, when contrasting the different expression of the *KDR* gene ($p=0.098$) was observed. For both these genes, a down expression among the LPS-challenged animals were identified.

Overall, the heatmap shows that genes related to the control of progesterone synthesis (*STAR, CYP11A, HSD3B1, LHCGR,* and *PGR*) presented a similar expression. Likewise, genes related to angiogenesis (*FTL1, VEGF,* and *KDR*) and stress response (*IL1B, HSD11B2, NR3C1,* and *NR3C2*) also responded in a modestly similar responses, regardless of the housing system or LPS challenge (LPS/SAL) (Fig. 11).

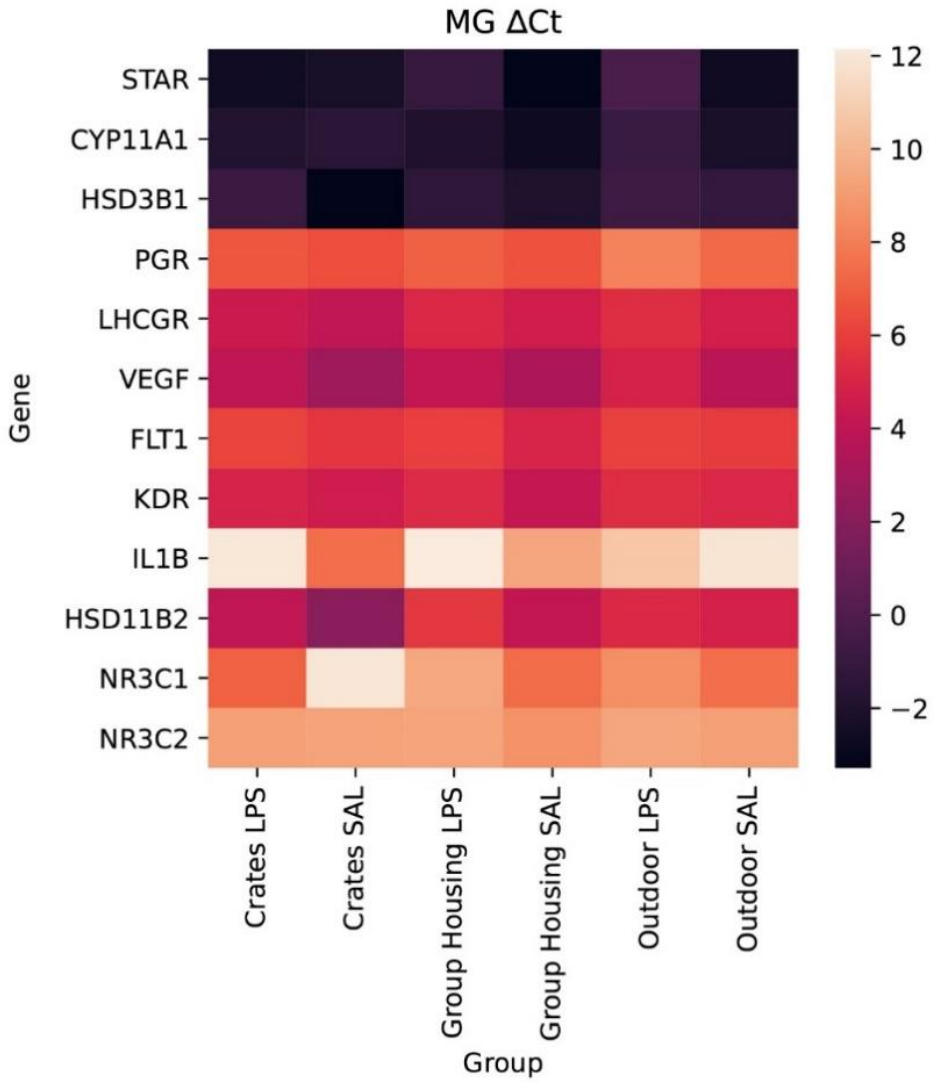

Figure 11. Heatmap of the evaluated genes contrasting with the treatments. The expression values equal to six was designated in red; black means reduced expression and white increased expression. The heatmap was generated by a log transformation of the RT-qPCR data as ΔCt (Ct (target gene mRNA) – Ct (GM mRNA)).

Discussion

Lipopolysaccharide was used for inducing acute inflammatory symptoms in 18 animals of our experiment. The choice of this systemic inflammatory inducer is justified by its recognized role in terms of breaking the homeostasis and impairment of the welfare of female pigs [24], in addition to simulates one of the biggest medical challenges for females: urinary tract infection by gram-negative bacteria [25]. It is reported that LPS

binds in toll-like receptors 4 (TLR-4) across different cells types [16]. Moreover, it is also suggested that the activation of these receptors initiates a complex cellular response, resulting in pro-inflammatory mediators such as inflammatory cytokines, reactive oxygen species, and steroid hormones [16]. This broken in the homeostasis of the organism generates different outcomes, which include reduced performance [24], neurologic dysfunctions [16], and changes in gene expression [26].

To the best of our knowledge, this report is the first evidence that an in vivo systemic challenge using LPS on the day of ovulation can compromise the gene expression of the newly formed corpus luteum of female pigs. In our study, we identified significant (p=0.039) down expression of the *VEGF* gene on the group challenged with LPS. Notably, the lowest expression in the LPS challenged group (p=0.110) was identified among gilts housed in the crates system. Interestingly, none of the other genes showed significantly different gene expression among the groups evaluated, except a trend that was observed in the gene expression of the *IL1B* gene (p=0.090), when comparing SAL versus LPS groups. Furthermore, we observed a clear difference between the expression pattern of each of the genes, regardless of the treatment (Fig. 5). In general, depending on the biological function of the gene and the physiological period that the luteal tissue was in, different patterns were observed.

There has been previous work suggesting that the immune and endocrine systems coordinate the development of the follicle and the CL lifespan, as well as the modulation in the face of an adverse metabolic or environmental challenge [27]. Furthermore, it is also reported that this modulation in the face of an environmental insult can compromise the quality of the oocyte and the function of the CL [6,27]. Thus, using the porcine model, which is recognized as one of the best species for human translational research, we attempted to assess how the environment can help the female pig to cope with a health

challenge, represented by LPS challenge, on day of ovulation. To verify its impacts on the CL tissue, we measured gene expression of 12 genes that have been reported in the literature as part of the progesterone cascade (*PGR, STAR, CYP11A, HSD3B1*, and *LHCGR*), angiogenesis (*VEGF, FLT1*, and *KDR*), control of the CL apoptosis (*IL1B*), and stress response (*HSD11B2, NR3C1*, and *NR3C2*).

The mRNA transcribed by *VEGF* in the CL has been considered the main mitogenic factor for endothelial cells [28]. In addition, studies have identified differences in its expression when evaluating animals with different genetic backgrounds [8], or submitted to high doses of steroids [7]. These studies suggest that *VEGF* plays a central role in inducing neovascularization [7], as well as in the differentiation, maturation, and stabilization of blood vessels in the luteal tissue [8]. Furthermore, it was suggested that animals stressed with the exogenous adrenocorticotropic hormone (ACTH), not only have down expression of this gene, as they also have genes related to progesterone biosynthesis compromised [7]. Considering these factors, we suggest that the reduced presence of transcripts from this gene may be involved in CL reduced nutrition and failure to release progesterone from the luteal tissue. This is because, according to Bacci et al. [29], there is a relationship between the reduction of blood vessels, the fading of progesterone, and CL regression.

Unlike the findings by Qian et al. [7], which identified down regulation of the *VEGF, CYP11A1*, and *HSD3B* in the CLs of stress induced sows by ACTH administration before estrus, our study was not able to identify differential expression in genes related to progesterone synthesis cascade. Neither *CYP11A1* nor *HSD3B* was differently expressed between animals challenged with LPS or SAL, in our study. However, it is important to clarify that the source of stress that Qian et al. [7] used was different from ours. The author used repeated acute stress for the stimulation of the adrenal and cortisol

secretion. They administered ACTH for 7 days every 8 hours prior the estrus day. Whereas, in our study, we used a single dose of LPS on the estrus day. So, from these findings, we hypothesize that *VEGF* expression may be more susceptible to downregulation than that of *CYP11A1* and *HSD3B* genes under stress conditions. However, more studies using LPS as a source of chronic stress are needed to elucidate this mechanism.

In a study with stem cells, isoforms of VEGF had already been shown to be low secreted when cell cultures were exposed to LPS, compared to a control group exposed to saline. In that study, the researchers identified time dependence concerning exposure to LPS [26]. In addition, authors argue that a possible mechanism that may be involved in the control of secretion of VEGF isoforms is through the TLR4, when the stem cells cultures were exposed to LPS. We also hypothesize that porcine luteal cells, as evidenced in sheep [30] and cattle [31] luteal cells, may have this receptor on their surface. Furthermore, we suggest that activation of this receptor in pigs - if present - might indirectly compromise the gene expression of other genes involved in the maintenance of the corpus luteum. However, to precisely elucidate this mechanism, we suggest that characterization studies of TLR4 be carried out in the CL of the porcine model. These studies would be valuable for research in swine reproduction because it has been perceived a high relevance of this gene for CL maintenance, CL vascularization, and successful maintenance of pregnancy in other species of mammals [30,31].

Another novel evidence of our research is that acute stress on the day of ovulation can have consequences that last long up to ~120 h. Previously, the study by Nordgreen et al. [16] had shown that pigs had pro-inflammatory cytokines altered in the central nervous system for about 72 h after challenge, in addition to lower levels of noradrenaline in their hypothalamus, hippocampus, and frontal cortex compared to saline-injected pigs. Thus,

our findings suggest that the systemic impairment, in the current experiment, affected the biological functioning of pigs for longer periods than it has been reported previously. Remarkably, the findings that the LPS challenge can compromise both the hypothalamus and ovaries emphasize its importance as a consistent stressor agent of the hypothalamic–pituitary–gonadal axis in pigs.

Recently, studies in the field of animal welfare and behaviour have reported that piglets born from sows that suffered chronic stress during pregnancy [32], presented stereotypes [33], or were subjected to restrictive diets [34] had litters with aggressive behaviour or with lower productive performance. The study by Parada et al. (2021) suggests that lameness in sows during pregnancy may be associated with foetal reprogramming in-uterus, caused by intergenerational epigenetic mechanisms. Our hypothesis is that somehow the genetic modulation of CL development may also have influence intrinsic aspects of pregnancy, and may also be playing a role in the intrauterine foetal experience. Moreover, we suggest that the segregation of the environmental effect is not only transmitted by epigenetic mechanisms in the germ cells but that somehow there are also molecular mechanisms that control the gene expression of the parents' glands that support the pregnancy. In other words, we hypothesized that the inefficiency of the CL can compromise the foetus. This hypothesis becomes clearer when we look from the perspective that the group-housed in crates – which is associated with poorer welfare – had the greatest impairment of gene expression ($p=0.110$), followed by the outside group ($p=0.220$), and the group housing group ($p=0.333$). However, this hypothesis requires further study to be better elucidated.

In conclusion, our study was able to identify that a single dose of LPS on the estrus' day can cause down expression of the angiogenic gene (*VEGF*) in the corpus luteum of gilts up to 120 h post challenge. Moreover, we were able to identify a trend

regarding the housing system: animals raised under conditions that allow them to express their social behaviour are more likely to suffer less repression in their luteal gene expression profile. Finally, future studies are necessary to investigate if there is dose-dependence of LPS in *in vivo* models, and if chronic stress also plays a harmful role in the CL gene expression.

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Institutional Review Board Statement: Animal experiments were designed and conducted in accordance with the Ethic Principle in Animal Research adopted by Ethic Committee in the Use of Animals of the School of Veterinary Medicine and Animal Science of the University of São Paulo (CEUAx 9992150121).

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary files

Supplementary file 1. Descriptive analysis of the ΔΔCt data.

Gene	Grupo	n	Mean	Std[a]	Min	25%	50%	75%	Max	Shapiro[b]
IL1B	LPS	15	0,53	0,71	0,01	0,09	0,25	0,63	2,75	>0.0001
	SAL	20	187,51	469,43	0,01	0,14	0,27	0,67	1764,87	>0.0001
LHCGR	LPS	16	1,02	0,73	0,1	0,48	0,89	1,33	2,79	0.313*
	SAL	22	1,21	0,77	0,21	0,73	0,94	1,57	2,99	0.007
CYP11A1	LPS	16	1,58	1,71	0,02	0,19	1,42	2,22	6,45	0.006
	SAL	23	2,03	2,34	0,08	0,2	1,52	2,34	10,8	>0.0001
PGR	LPS	16	0,88	0,52	0,1	0,52	0,8	1,15	2,31	0.121*
	SAL	22	4,42	16,36	0,32	0,5	0,94	1,39	77,62	>0.0001
FLTI	LPS	16	0,81	0,67	0,26	0,53	0,63	0,81	3,19	>0.0001
	SAL	22	1,3	1,32	0,4	0,64	0,97	1,38	6,52	>0.0001
NR3C2	LPS	16	0,87	0,29	0,45	0,66	0,83	1,01	1,48	0.574*
	SAL	20	1,69	3,07	0,3	0,68	0,84	1,25	14,48	>0.0001
HSD11B2	LPS	16	1,06	1,35	0,01	0,22	0,45	1,48	4,51	>0.0001
	SAL	23	4,09	9,74	0,05	0,29	0,78	3,29	45	>0.0001
STAR	LPS	16	0,83	0,51	0	0,68	0,83	1,06	2,13	0.145*
	SAL	23	1,29	1,34	0,17	0,82	0,92	1,29	6,98	>0.0001
VEGF	LPS	15	0,69	0,45	0,05	0,57	0,61	0,73	1,99	0.006
	SAL	22	1,35	1,32	0,39	0,64	0,79	1,46	5,87	>0.0001
KDR	LPS	16	0,76	0,37	0,26	0,5	0,71	0,98	1,74	0.098*
	SAL	22	1,96	4,15	0,15	0,59	0,82	1,38	20,18	>0.0001
NR3C1	LPS	15	4,66	4,37	0	1,12	3,57	6,93	13,55	0.078*
	SAL	19	10,32	23,71	0	0,06	4,98	8,07	106,04	>0.0001
HSD3B1	LPS	16	0,59	0,34	0,08	0,44	0,63	0,7	1,27	0.420*
	SAL	23	209,42	952,93	0,01	0,25	0,44	1,78	4577,61	>0.0001

a Standard deviation; b Shapiro-Wilk Test; * $p > 0.05$ normal distribution.

Supplementary file 2. Comparison analysis using t-test. Contrast between animals challenged with LPS or SAL within each of the housing systems, for each of the genes.

Gene	**Group***	**t-test**	***p-value***	***CI (95%)***
IL1B	Crates	-1.5	0.184	(-1320.650, 330.809)
	Group Housing	-1.5	0.19	(-253.936, 60.864)
	Outdoor	1.72	0.133	(-0.131, 0.778)
LHCGR	Crates	1.26	0.227	(-1.073, 0.294)
	Group Housing	-1.3	0.941	(-1.100, 1.176)
	Outdoor	1.17	0.471	(-1.287, 0.638)
CYP11A1	Crates	0.14	0.969	(-2.156, 2.081)
	Group Housing	2.08	0.736	(-3.873, 2.816)
	Outdoor	2.81	0.126	(-2.010, 0.288)
PGR	Crates	-1	0.381	(-45.283, 20.665)
	Group Housing	-1.2	0.239	(-0.711, 0.196)
	Outdoor	-1.7	0.117	(-0.766, 0.098)
FLTI	Crates	-1.3	0.259	(-1.657, 0.556)
	Group Housing	-1.1	0.305	(-2.653, 0.908)
	Outdoor	-0.4	0.692	(-0.495, 0.352)
NR3C2	Crates	-0.8	0.462	(-10.071, 5.510)
	Group Housing	-2	0.074	(-1.180, 0.069)
	Outdoor	-0.9	0.384	(-0.589, 0.247)
HSD11B2	Crates	-1.5	0.17	(-9.525, 2.028)
	Group Housing	-1	0.336	(-18.817, 7.388)
	Outdoor	0.39	0.704	(-1.684, 2.355)
STAR	Crates	-0.3	0.775	(-0.915, 0.713)
	Group Housing	-1.2	0.278	(-2.693, 0.873)
	Outdoor	-1.9	0.11	(-0.886, 0.123)
VEGF	Crates	-1.9	0.11	(-3.147, 0.416)
	Group Housing	-1	0.333	(-1.628, 0.610)
	Outdoor	-1.4	0.22	(-0.702, 0.313)
KDR	Crates	-1	0.342	(-9.639, 3.921)
	Group Housing	-1.8	0.098	(-1.613, 0.158)
	Outdoor	-0.7	0.499	(-0.416, 1.221)
NR3C1	Crates	-0.2	0.824	(10.202, 8.464)
	Group Housing	-1	0.351	(60.916, 25.988)
	Outdoor	0.17	0.867	(-7.170, 8.164)
HSD3B1	Crates	-1	0.35	(-2258.367,935.649)
	Group Housing	-1	0.351	(-71.937,29.206)
	Outdoor	-0.7	0.486	(-1.421, 0.735)

* LPS x SAL group. *p-value <0.05

7. FINAL CONSIDERATIONS AND FUTURE REMARKS

After reporting our main findings in the articles, we would like to report here some personal lessons of our study. In this session, our main focus will be the article that involved animal experimentation, as there were more points open for discussion.

Regarding the literature review, more articles would be valuable to perform a meta-analysis with a wide-brand of studies regarding the theme of epigenetics in the pig welfare field. Although we already have a variety of studies in the field, the different contexts and experimental designs available make it hard to perform a complete analysis and reveal accurate information for its consistence as a potential biomarker.

An important factor in our experimental study is the choice of the experimental model and the type of challenge to generate the inflammatory response. First, gilts are the animals responsible for the future of the pig farm. This category of animals ensures successful breeding, the number of healthy piglets born, and the long-term maintenance of these animals within the system is important for economic reasons. Second, the choice of LPS mimics one of the biggest challenges in pig farming: female urinary tract infections caused by environmental bacteria. This health problem negatively compromises the health, well-being and economy of the production systems. Furthermore, one way of entry of these bacteria into the female body is through the vagina, on the day of insemination.

The findings by COSTA et al. (2017) suggested that there are no substantial differences in the expression of genes involved in vascularization and control of apoptosis in the CL of gilts when evaluating animals with different genetic backgrounds. However, for future research, the molecular approach proposed by PÉRTILLE et al. (2016, 2020), which assesses the genetic, single-nucleotide polymorphisms, SNPs, and epigenetic, DNA methylation, material, would have been a valuable contribution to our study. This technique would measure the extent to which the individual genetic variations may compromise the gene expression of the evaluated genes. In other words, with this approach, we could have shown more accurately the effects of the housing systems and LPS challenge on gene expression on the CLs of pig females.

A second important issue of our study, which is also a challenge when studying CL, is its multi-dependence on other tissues and cells. This gland is not only dependent on the individual's endocrine and immune microenvironment at present, as it depends on past experiences. For example, the CL depends on the follicular microenvironment and oocyte nests, which have been formed in-uterus. Therefore, all experiences that the female has undergone during its lifespan have, to a greater or lesser extent, some impact on aspects of gene expression regulation. As discussed by MADEJ et al. (2005), there is evidence that acute or acute-repeated stress can affect female swine reproductive factors in different ways. For example, they reported that elevated levels of cortisol or PGF2-alpha can compromise post-weaning estrus, ovulation, and development of the new corpora lutea.

A third variable that may have compromised the assessment of corpus luteum gene expression was the lack of information on the precise estrous cycle synchronization interval among the experimental animals. In female pigs, the interval between the onset and the end of the ovulation of the follicles is reported to occur in approximately 40 h

(TUMMARUK; DE RENSIS, 2011). Moreover, it has been suggested that this period compromise 70% of the total timing of estrous in commercial pigs (TUMMARUK; DE RENSIS, 2011). However, it has been also reported that environmental factors can compromise the interval of ovulation if the female's homeostasis is broken (PEARODWONG et al., 2019). The ovulation interval is relevant for the initial development of the corpus luteum and early adaptation of the female porcine reproductive tract, which can lead to variations in the number of transcripts by the CLs. In our study, although we did not ultrasonographically control the ovulation trajectory to define the corporea lutea that would be evaluated, we selected the 5 largest CLs on the surface of the ovary to collect the biopsies. We hypothesized that collecting the largest CL may have reduced possible biases. Moreover, for future studies, it would be relevant to use an ovulation inducer or a better way to monitor the process, to minimize variability in the interval of ovulation.

In conclusion, in this dissertation we collected a wide variety of essential information regarding the role of the environment on the biological processes of pigs. In the future, more studies simulating the challenges experienced in of pig farms are necessary to better elucidate the role of the stress for the pig epigenome. These studies will advance the science of animal welfare in a molecular level, which may have the potential to increase the consumers trust and assure good animal welfare.

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