VIVIAN ANGÉLICO PEREIRA ALFRADIQUE

IMPACT OF AGE AND FSH TREATMENT ON FOLLICULAR ENVIRONMENT, FOLLICULOGENESIS AND ENDOMETRIAL HISTOMORPHOMETRY IN PREPUBERTAL GILTS

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

Adviser: Simone Eliza Facioni Guimarães

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"*Parece que a vida não é fácil para nenhum de nós. Mas, e daí? Precisamos ter perseverança e principalmente confiança em nós mesmos. Precisamos acreditar que somos dotados para algo, e que alcançaremos esse objetivo, custe o que custar*".

(Marie Curie)

ABSTRACT

ALFRADIQUE, Vivian Angélico Pereira, D.Sc., Universidade Federal de Viçosa, August, 2022. **Impact of age and FSH treatment on follicular environment, folliculogenesis and endometrial histomorphometry in prepubertal gilts.** Adviser: Simone Eliza Facioni Guimarães.

This study investigated the effect of age and FSH treatment on plasma levels of IGF-1 and E2, folliculogenesis, endometrial histomorphometry, biochemical composition of follicular fluid (FF), and on nuclear maturation, gene expression and quality of cumulus-oocytes complexes (COCs) of prepubertal gilts. For this, thirty-five prepubertal gilts were separated according to the age (140 or 160 days), and within each age, gilts were allotted to receive 100 mg of FSH [treated; G140+FSH (n = 10) and G160+FSH (n = 7)] or saline solution [control; G140+control (n = 10) and G160+control (n = 8)]. Before and after FSH treatment, blood was collected for IGF1 and E2 assay (radioimmunoassay), and reproductive tract ultrasound were performed. After 24 h of the last FSH injection, animals were slaughtered and their ovaries and uterus were collected for biometrical and histomorphometrical analysis. The biochemical profile of FF was analyzed in automatic biochemistry analyzer. COCs recovered were morphologically classified and submitted to brilliant cresyl blue (BCB) staining, gene expression (RT-qPCR) and *in vitro* maturation for subsequent evaluation of nuclear oocyte maturation. The percentage of medium follicles increased (P<0.05) as the same proportion that the percentage of small follicles reduced (P<0.05) in FSH-treated and younger gilts. In addition, the concentration of glucose in FF increased (P<0.05) in FSH-treated and older gilts; in contrast, the concentration of triglycerides decreased (P<0.05) in these same groups of gilts. Increasing of age and FSH stimulation improved (P<0.05) the metaphase II and BCB+ COCs rates and decreased (P<0.05) number of early atretic follicles. Also, changes (P<0.05) on endometrial histomorphometric are caused by FSH treatment and age. However, age and FSH treatment had no impact (P>0.05) on most transcripts' abundance of selected target genes in COCs. Regarding the effect of age, biometrical data of uterus, plasma levels of IGF-1 and E2 increased (P<0.05) and changes (P<0.05) in uterine and ovaries ultrasound images occurs from 140 to 160 days of age. In conclusion, the increasing of the age and FSH treatment improved oocyte nuclear maturation and stimulated the endometrium epithelium in prepubertal gilts. Also, uterine growth still occurs during 140 and 160 days of age in prepubertal gilts.

Keywords: Follicular development. Oocyte. Ovarian stimulation. Pig

RESUMO

ALFRADIQUE, Vivian Angélico Pereira, D.Sc., Universidade Federal de Viçosa, agosto de 2022. **Impacto da idade e do tratamento com FSH no ambiente folicular, foliculogênese e histomorfometria endometrial em marrãs pré-púberes.** Orientadora: Simone Eliza Facioni Guimarães.

Este estudo investigou o efeito da idade e do tratamento com FSH nos níveis plasmáticos de IGF-1 e E2, na foliculogênese, histomorfometria endometrial, composição bioquímica do fluido folicular (FF), e na maturação nuclear, expressão gênica e qualidade dos complexos *cumulus*-oócitos (CCOs) de marrãs pré-púberes. Para isso, 35 marrãs pré-púberes foram separadas de acordo com a idade (140 ou 160 dias), e dentro de cada idade, as marrãs foram tratadas com 100 mg de FSH [tratado; G140+FSH (n = 10) e G160+FSH (n = 7)] ou solução salina [controle; G140+controle (n = 10) e G160+controle (n = 8)]. Antes e após o tratamento com FSH, o sangue foi coletado para dosagem plasmática de IGF1 e E2 (radioimunoensaio) e a ultrassonografia do trato reprodutivo foi realizada. Após 24 h da última injeção de FSH, os animais foram abatidos e os ovários e o útero foram coletados para análise biométrica e histomorfométrica. O perfil bioquímico do FF foi avaliado em analisador bioquímico automático. Os CCOs foram classificados morfologicamente e submetidos à coloração com azul cresil brilhante (BCB), expressão gênica (RT-qPCR) e maturação *in vitro* - para posterior avaliação da maturação nuclear. A porcentagem de folículos médios aumentou ($P<0,05$) na mesma proporção que a porcentagem de folículos pequenos diminuiu ($P<0,05$) em leitoas mais jovens e tratadas com FSH. Ainda, a concentração de glicose no FF aumentou ($P<0,05$) em marrãs mais velhas e tratadas com FSH; em contraste, a concentração de triglicerídeos diminuiu ($P<0,05$) nestes mesmos grupos de marrãs. O aumento da idade e o tratamento com FSH aumentaram ($P<0,05$) as taxas de metáfase II e CCOs BCB+ sem impactar ($P>0,05$) a expressão dos genes alvos nos COCs. Além disso, o FSH exógeno e a idade alteraram ($P<0,05$) a histomorfometria endometrial e diminuíram ($P<0,05$) o número de folículos atrésicos iniciais. Os dados biométricos do útero, os níveis plasmáticos de IGF-1 e E2 aumentaram ($P<0,05$), e alterações ($P<0,05$) nas imagens ultrassonográficas do útero e dos ovários ocorreram entre 140 e 160 dias de idade. Dessa forma, o aumento da idade e o tratamento com FSH melhoraram a maturação nuclear do oócito e estimularam o epitélio endometrial em marrãs pré-púberes. Ainda, o crescimento uterino continua ocorrendo entre os 140 e 160 dias de idade em marrãs pré-púberes.

Palavras-chave: Desenvolvimento folicular. Estimulação ovariana. Oócito. Suíno

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LIST OF ACRONYMS AND ABBREVIATIONS

ACSL3	acyl-CoA synthetase long chain family member 3
ALDOA	aldolase
ANOVA	analysis of variance
BAX	Bcl-2 associated X-protein
BCB	brilliant cresyl blue
Bcl-2	B-cell lymphoma 2
BMP15	bone morphogenetic protein 15
BSA	bovine serum albumin
CAPES	Coordination for the Improvement of Higher Education Personnel
cDNA	complementary DNA
CNPq	Council for Scientific and Technological Development
CO_2	carbon dioxide
COCs	cumulus-oocytes complexes
Cq	cycle quantification
eCG	equine chorionic gonadotropin
EDTA	ethylenediaminetetraacetic acid
ERβ	estrogen receptor beta
ET	endometrial thickness
E2	17β-estradiol
FA	fatty acid
FF	follicular fluid
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GD	glandular diameter
GDF9	growth and differentiation factor 9
GEH	glandular epithelium height
GH	growth hormone
GLM	general linear model
GSI	gonadosomatic index
GT	“grape” type

GV	germinal vesicle
GVBD	germinal vesicle breakdown
GI-IV	grade I-IV
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
HT	“honeycomb”
IGF-1	insulin-like growth factor 1
IL-6	interleukin-6
IL-7	interleukin-7
IVM	in vitro maturation
JIVET	juvenile in vitro embryo transfer
LEH	luminal epithelium height
LH	luteinizing hormone
MATER	maternal antigen that embryos require
MI-II	metaphase I-II
N_2	nitrogen
OPU	ultrasound-guided ovum pick-up
O_2	oxygen
PBS	phosphate-buffered saline
PVA	polyvinylalcohol
qPCR	quantitative polymerase chain reaction
SAS	statistical analysis system
SAsono	area of the cross-sectioned uterine horns
SEM	standard error of the mean
TCM 199	tissue culture medium 199
UFV	Universidade Federal de Viçosa

SUMMARY

1. GENERAL INTRODUCTION

A carne suína representa em torno de 40% da carne consumida mundialmente. Isto faz com que seja necessária alta produtividade para atender esta demanda, que é realizada por meio da rápida seleção para conversão alimentar, resistência a doenças e fertilidade. Neste ponto de vista, as tecnologias da reprodução assistida podem ser favoráveis para a indústria suinícola, visto que são capazes de aumentar a eficiência reprodutiva associada ao melhoramento genético. Além disso, a utilização precoce das marrãs em atividades reprodutivas é de grande interesse financeiro na indústria suinícola, estando relacionada a redução dos dias não produtivos e à formação antecipada de estoque de marrãs de reposição (DO LAGO et al., 2005; FOWLER et al., 2018).

A utilização de fêmeas pré-púberes como doadoras de oócitos é conhecida como *juvenile in vitro embryo transfer* (*JIVET*), e permite a aceleração do progresso genético pela redução do intervalo de geração, sendo dessa forma uma tecnologia promissora a ser utilizada na produção animal (ARMSTRONG; KOTARAS; EARL, 1997). No entanto, tem sido demonstrado que os oócitos obtidos de marrãs pré-púberes apresentam menor capacidade de desenvolvimento embrionário comparado aos obtidos de porcas multíparas (IKEDA; TAKAHASHI, 2003; MARCHAL et al., 2001; SHERRER; RATHBUN; DAVIS, 2004). Isto se explica pelo fato do oócito obtido de uma fêmea pré-púbere apresentar menor tamanho oocitário e número de mitocôndrias, e maior número de vesículas endocíticas, gotas lipídicas nas células do *cumulus* e microvilos na membrana plasmática (DE PAZ et al., 2001). Além disso, o oócito proveniente de fêmeas pré-púberes apresenta uma anormalidade no padrão de proteínas (LÉVESQUE; SIRARD, 1994) e maior quantidade de espécies reativas de oxigênio quando comparado ao de fêmeas adultas, devido a sua menor síntese de glutationa (JIAO et al., 2013). Dessa forma, a redução na competência de desenvolvimento desses oócitos está associada à falta ou anormalidade desses fatores importantes para o processo de maturação oocitária, resultando em inabilidade ou falha nesse processo (SALOMONE et al., 2001).

Diversas estratégias têm sido adotadas na tentativa de aumentar a competência oocitária dos oócitos provenientes de fêmeas pré-púberes em diversas espécies. As estratégias que antecedem o momento de aspiração têm tido maior foco de estudo, visto que o ambiente folicular influencia o desenvolvimento oocitário e a remoção do oócito do ambiente folicular limita a capacidade de alterar o potencial do desenvolvimento oocitário (LONERGAN; FAIR, 2008). Dentre estas, se destaca a administração das gonadotrofinas em doadoras de oócitos previamente à aspiração folicular (CURRIN et al., 2017; KRAUSE et al., 2020; MORTON et

al., 2005; TECHAKUMPHU; LOHACHIT; TANTASUPARAK, 2000). O uso do hormônio folículo estimulante (FSH) em protocolos de estimulação ovariana promove o crescimento folicular homogêneo e permite que o oócito complete as modificações pós-transcricionais, aumentando dessa forma a competência oocitária (LABRECQUE et al., 2013; VIEIRA et al., 2014). Inúmeros estudos mostraram o efeito benéfico do tratamento com FSH na capacidade do desenvolvimento embrionário e na resposta folicular ovariana em fêmeas pré-púberes (MORTON et al., 2005; PRESICCE et al., 1997; ZACARIAS et al., 2018). No entanto, a literatura é escassa a respeito das alterações provocadas pelo efeito da idade e pelo tratamento com FSH no microambiente folicular na espécie suína.

O FSH desempenha papel importante na foliculogênese, estimulando o crescimento de folículos antrais por meio da proliferação das células da granulosa, além de aumentar a expressão de fatores anti-apoptóticos. Além disso, o FSH tem papel na esteroidogênese regulando a biossíntese de estradiol (RIMON-DAHARI et al., 2016; VEGETTI; ALAGNA, 2006). Embora os folículos pré-antrais em estágios iniciais sejam capazes de se desenvolver na ausência do estímulo das gonadotrofinas (ELVIN; MATZUK, 1998), evidências indicam que o FSH tem papel no desenvolvimento de folículos ovarianos murinos em estágios pré-antrais (WANG; GREENWALD, 1993). Em estudo de Candelaria et al. (2020) foi demonstrado a presença dos receptores de FSH (FSHR) nos folículos pré-antrais bovinos, além disso os autores reportaram que estes folículos são responsíveis ao FSH, e a ação do FSH nestes folículos está envolvida com a estimulação das vias de sinalização da MAPK e genes relacionados ao metabolismo energético. No entanto, mais estudos são necessários para elucidar o papel regulatório do FSH na foliculogênese pré-antral em estágios iniciais em outras espécies de animais.

Foliculogênese é um processo dinâmico e complexo referente ao desenvolvimento do folículo primordial até o estágio de folículo pré-ovulatório (HANNON; CURRY, 2018). Durante a vida fetal em suínos, as oogonias se proliferam por divisões mitóticas e se diferenciam nos oócitos (BIELAŃSKA-OSUCHOWSKA, 2006). Posteriormente, os oócitos ficam circundados por uma única camada de células da granulosa achatadas, e a partir disso são conhecidos como folículo primordial. No início do crescimento folicular, alguns folículos sofrem um processo não reversivo de ativação folicular. Neste processo, as células da granulosa que circundam o oócito sofrem modificações morfológicas e se tornam células cubóides e os receptores de FSH aparecem, e os folículos se tornam primários (ARAÚJO et al., 2014). A progressão para o estágio de folículo secundário se caracteriza pelo aparecimento de uma segunda camada de células da granulosa, em resposta a ação do FSH, e um baixo número de

células da teca. O FSH estimula a expressão do receptor de LH nas células da teca dos folículos antrais iniciais (folículo terciário; KISHI et al., 2018). No estágio terciário, o crescimento dos folículos é dependente da ação do FSH e LH, que estimulam o desenvolvimento de uma cavidade repleta de líquido folicular entre as camadas de células da granulosa, denominada antro folicular. Além disso, nesse estágio ocorre diferenciação das células da teca em interna (camada de células mais próxima das células da granulosa responsáveis pela síntese dos hormônios esteroides) e externa (camada de tecido conjuntivo responsável pela produção de fatores de matriz extracelular; RICHARDS, 2018).

O desenvolvimento folicular em marrãs durante o período pré-puberal consiste em mudanças nos aspectos morfológicos dos ovários do tipo "favo de mel" (ovários com inúmeros folículos pequenos com 1-3 mm de diâmetro) para o tipo "cacho de uva" (ovários com vários folículos grandes com ≥6 mm de diâmetro) em torno de 5-10 dias. Os ovários tipo "favo de mel" indicam o crescimento dos folículos antes do momento da fase de seleção folicular. Após a fase de seleção, a morfologia ovariana muda e os ovários tornam-se do tipo intermediário, que apresenta uma curta duração. Com o aumento do diâmetro folicular devido ao estimulo das gonadotrofinas durante a fase de seleção, os ovários passam a ser do tipo "cacho de uva". Posteriormente, os ovários podem retornar para o tipo "favo de mel", indicando que os folículos grandes sofreram atresia, levando ao surgimento dos folículos pequenos (BOLAMBA et al., 1994).

As interações entre os ovários e o útero autorregulam as funções e o desenvolvimento uterino-ovariano, através de secreções de vários fatores, incluindo os hormônios esteroides (BOOTH, 1990). Além do conhecido papel descrito dos esteroides no útero (O'BRIEN et al., 2006; WALTER; ROGERS; GIRLING, 2005; YOUNG, 2013) foi demonstrado um possível efeito do FSH nas funções uterinas, tais como a receptividade endometrial e proliferação das células uterinas (BRUNMA et al., 2021; JARVELA et al., 2005; VALDEZ-MORALES et al., 2015). O efeito do FSH no útero pode ser mediado pelo estímulo ao estradiol ou até mesmo pela ação direta do FSH no endométrio. Foi descrita a presença do FSHR no endométrio de mulheres, indicando um possível papel do FSH na regulação da interação embrião-endométrio (LA MARCA et al., 2005). Recentemente foi descrita a presença do FSHR no miométrio de marrãs indicando a ação extragonadal do FSH nesta espécie (WASZKIEWICZ et al., 2020). No entanto, os efeitos mediados pelo FSH na função uterina suína precisam de mais investigações.

O trato reprodutivo de marrãs continua se desenvolvendo no período após nascimento até a puberdade. Neste contexto, os folículos ovarianos se tornam responsivos a ação das gonadotrofinas a partir dos 60 dias de idade, levando ao aparecimento dos folículos terciários com a intensificação do crescimento folicular ovariano ocorrendo entre os 60-100 dias de idade (SCHWARZ; KOPYRA; NOWICKI, 2008). Em relação ao útero, foi reportado que o crescimento uterino ocorre sob influência do ovário a partir dos 60 dias de idade (WU; DZIUK, 1988), e que as glândulas endometriais se desenvolvem do nascimento aos 70-85 dias (DYCK; SWIERSTRA, 1983). O desenvolvimento e o crescimento dos órgãos reprodutivos no período pós-natal foram elucidados em estudos citados anteriormente, no entanto ainda não é conhecido se ocorre mudanças nas características morfométricas do útero e dos ovários em escala macroscópica e microscópica no período peripuberal. Em síntese, a idade e o estímulo com FSH podem afetar os componentes do ambiente folicular. Além disso, esses fatores podem ter impacto na foliculogênese e nas funções uterinas em diversas espécies animais. Sendo assim, o objetivo desta tese foi avaliar o efeito da idade e do tratamento com FSH no microambiente folicular (artigo 1), e na morfologia e na histomorfometria ovariana e uterina (artigo 2) de marrãs pré-púberes com 140 ou 160 dias de idade.

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2. MANUSCRIPT 1

Effect of age and FSH stimulation on ovarian follicular response, nuclear maturation and gene expression of cumulus-oocyte complexes in prepubertal gilts*

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ABSTRACT

This study aimed to investigate the effect of age and FSH treatment on ovarian response, biochemical composition of follicular fluid (FF), nuclear maturation and molecular profile of cumulus-oocytes complexes (COCs) recovered from prepubertal gilts. Thirty-five prepubertal gilts were separated according to the age (140 or 160 days), and within each age, gilts were allotted to receive 100 mg of FSH [treated; G140+FSH (n = 10) and G160+FSH (n = 7)] or saline solution [control; G140+control (n = 10) and G160+control (n = 8)]. Percentage of medium follicles increased (P<0.0001) in the same proportion that percentage of small follicles reduced (P<0.0001) in FSH-treated and younger gilts. In addition, FF concentration of glucose increased (P<0.05) in FSH-treated and older gilts; in contrast, FF concentration of triglycerides decreased (P<0.05) in these same groups of gilts. FSH stimulation improved (P<0.05) the number of grade I COCs, meiotic maturation and BCB+ rates. As prepubertal gilts got older, the metaphase II and BCB+ rates and plasma IGF-1 level increased (P<0.05). Age had no effect (P>0.05) on transcripts abundance of the target genes. FSH treatment only upregulated (P<0.05) *HMGCR* expression in COCs from prepubertal gilts at 140 or 160 days of age. In conclusion, oocytes obtained from 140 days prepubertal gilts apperared less meiotically competent than 160 days old prepubertal gilts-derived oocytes. Our study suggests a possible strategy using FSH treatment to improve oocyte quantity, quality and nuclear maturation in 140 and 160 days old prepubertal gilts.

Keywords: ovarian stimulation; follicular environment; oocyte; pig; peri-pubertal;

2.1 Introduction

Oocyte competence refers to the ability of the oocyte to resume meiosis, cleave following fertilization and develop into a viable embryo (Sirard *et al*. 2006). *In vitro* studies showed that oocytes obtained from prepubertal females has a lower embryo development capacity compared to adult females in several domestic species (Leoni *et al*. 2009; Bernal-Ulloa *et al*. 2016), including pigs (Marchal *et al*. 2001; Ikeda and Takahashi, 2003; Sherrer *et al*. 2004). Changes in protein synthesis of cumulus-oocyte complex (COC), oocyte energy metabolism and ultrastructural and cytochemical modifications of prepubertal oocytes have been related with the inability or failure to undergo nuclear and ooplasmic maturation and explain the reduced developmental potential of prepubertal oocyte (Gandolfi *et al*. 1998; de Paz *et al*. 2001; Salomone *et al*. 2001). In pigs, puberty is regulate by multiple factors, such as age, breed, nutritional status, environment, body weight and backfat thickness; however it is acceptable to assume that gilts reach puberty in around 150-180 days of age (Evans and O'Doherty, 2001; McGlone *et al*., 2021). In vivo derived oocytes increase the developmental competence progressively after the occurrence of puberty (Archibong *et al*. 1987). However, the precise age in which oocytes obtained from peripuberal gilts become competent remains largely unknown.

Strategies have been adopted to improve oocyte quality and/or developmental competence of prepubertal oocytes, some of these are supplementation of cytokines and others factors in *in vitro* maturation (IVM) medium (Córdova *et al*. 2010; Hammami *et al*. 2014; Tian *et al*. 2021), and hormonal treatment in the oocyte donor prior follicular aspiration (Oropeza *et al*. 2004). In this regard, gonadotropin stimulation (e.g. FSH, eCG) has been widely used in ruminant species (Techakumphu *et al*. 2000; Leoni *et al*. 2009; Zacarias *et al*. 2018). Studies showed that FSH treatment increase the number of viable oocytes (Zacarias *et al*. 2018) and the *in vitro* embryo development of oocytes recovered from prepubertal lambs (Morton *et al*. 2005), this indicates that exogenous hormonal stimulation can be an alternative to improve oocyte competence in prepubertal oocyte donors. Recently, Yoshioka *et al*. (2020) reported the birth of piglets from *in vitro*-produced blastocyst from oocytes recovered by ultrasound-guided ovum pick-up (OPU). In this study, an increase in the efficiency of blastocyst production from oocytes collected from cyclic donor sows previous treated with FSH compared with oocytes obtained from gilts ovaries collected in slaughterhouse was demonstrated. This shows that FSH treatment prior to oocyte collection could improve the oocyte competence of the oocytes obtained from donor sows (Yoshioka *et al*. 2020).

In summary, it is unknown the effect of age and hormonal treatment in the components of follicular microenvironment of prepubertal gilts. Thus, the propose of this study was investigate the potential impact of age (140 *vs* 160 days) and FSH treatment on: i) density of preantral follicles; ii) oocyte quality and nuclear maturation; iii) metabolic and hormonal status of follicular fluid (FF), and ii) mRNA expression levels of target genes in COCs collected from prepubertal gilts.

2.2 Material and methods

2.2.1 Ethics, location and animal conditions

The Ethics Committee in the use of Farm Animals from Universidade Federal de Viçosa approved the current experimental design (CEUAP/UFV: 0118/2018). This experiment was conducted during December 2020 to May 2021 in the Pig Breeding Farm from Department of Animal Science at Universidade Federal de Viçosa, Viçosa, MG, Brazil.

Commercial line TN70 (Topigs Norsvin, Netherlands) gilts were housed in collective pens (2 animals/pen) and fed with soy/corn based diet and *ad libitum* water. Gilts were selected based on following selection criteria (mean ± SD): birth weight (≥1.4 kg; 1.5 ± 0.2), teat number (≥14 teats; 16.0 ± 0.9), weaning weight (≥5.4 kg; 7.1 ± 1.1), 60 days weight (≥24 kg; 25.0 ± 2.8), 100 days weight (≥42 kg; 49.2 ± 4.1), adequate body and vulva conformation, and absence of physical defects such as hernias or lameness. To assess the effect of age, gilts used in this study were at 140 or 160 days of age and had a mean body weight of 100.3 ± 8.7 or 75.0 ± 4.4 kg (mean ± SD), respectively.

2.2.2 Experimental design and hormonal treatment

Experimental design is illustrated in Fig. 1. Previous data from our laboratory led us to conclude that puberty occurs at 160 days of age in commercial maternal line TN70 (data not shown). Therefore we chose to evaluate the effect of age during the peripuberal period (140-160 days). Thirty-five prepubertal gilts were separated according to the age (140 ± 4 days and 160 ± 4 days) and within each age, gilts were allotted to receive FSH [treated; 100 mg of FSH; G140+FSH (n = 10) and G160+FSH (n = 10)] or saline [control; 0.9% sterile saline solution; G140+control (n = 10) and G160+control (n = 10)] treatment. Total dose of 100 mg of FSH (Folltropin-V; Vetoquinol Saúde Animal, São Paulo, Brazil) was divided into six similar doses administered every 8 hours by intramuscular injection (Days 0-2; Breen and Knox 2012). After 24 h of last FSH injection (coasting period; Day 3), gilts were slaughtered and ovaries were collected. Medium follicles were aspirated, recovered COCs were morphologically classified

(grade I-IV) and COCs grades I-II were used to gene expression analysis, brilliant cresyl blue staining, and in vitro oocyte maturation. Moreover, recovered FF were submitted to biochemical analysis (glucose, total protein, cholesterol and triglycerides). Pubertal gilts that presented corpora haemorrhagica or corpora lutea in ovaries at post-mortem examination were excluded from the study.

2.2.3 Blood samples

Blood collection was performed at the first (0 h) and last (40 h after the first FSH injection) FSH/saline injection for the analyses of plasma insulin-like growth factor 1 (IGF-1) and glucose levels. Blood samples were collected from the orbital venous sinus by insertion of needle (16 G - 1,6x40 mm; BD, Juiz de Fora, Brazil) in medial eye canthus. Vacuum tubes containing EDTA were used for IGF-1 assay and those containing sodium fluoride were used for measurement of plasma glucose levels. Plasma was immediately separated by centrifugation at 1500 × *g* for 15 min and stored in microtubes at -20ºC for subsequent analysis.

2.2.4 IGF-1 assay

IGF-1 plasma concentration was analyzed in an automatic gamma counter (model: 2470 Wizard; PerkinElmer, Waltham, MA, USA) using a solid-phase radioimmunoassay kit (IRMA IGF1 – Beckman Coulter; Prague, Czech Republic), according to the manufacturer's instructions. The intra-assay coefficient of variation was 8%.

2.2.5 Plasma glucose concentration

The analysis of plasma glucose concentration was performed in an automatic biochemistry analyzer (Bioclin 2200; Bioclin Quibasa, Belo Horizonte, Minas Gerais, Brazil) using a commercial kit (BioClin Quibasa, Belo Horizonte, Minas Gerais, Brazil) based on colorimetric enzymatic method.

2.2.6 Biochemical analysis of follicular fluid

Pooled FF obtain from all medium follicle in both ovaries of each animal was used for the analysis of biochemical parameters (glucose, total protein, cholesterol and triglycerides). FF samples were centrifuged (1000 × g at 4 ºC for 30 min) to eliminate cells, and the supernatants were stored at -80ºC before analysis (Sun *et al*. 2011). FF samples that were contaminated with blood were discarded and were not used for analysis (Alves *et al*. 2014). All evaluations were performed in an automatic biochemistry analyzer (Bioclin 2200; Bioclin Quibasa, Belo Horizonte, Minas Gerais, Brazil) according to manufacturer's recommendation

using commercial kits (Bioclin Quibasa, Belo Horizonte, Minas Gerais, Brazil) based on colorimetric enzymatic or Biuret method (total protein).

2.2.7 Collection of COCs and brilliant cresyl blue (BCB) staining

The reproductive tract was removed from each gilt, the ovaries were excised and placed in separate plastic bags containing pre-warmed (37ºC) 0.9% NaCl for transport to the laboratory. Small (1-3 mm), medium (3-6.49 mm) and large (≥6.5 mm) follicles were counted and COCs were aspirated from medium follicles with an 18-gauge needle attached to a 3-mL disposable syringe. Then, the COCs recovered were morphologically classified as grade I (homogeneous cytoplasm oocyte and compact cumulus oophorus with more than three layers), grade II (homogeneous cytoplasm oocyte with small irregular pigmentation areas, and compact cumulus oophorus with 1-3 layers), grade III (oocyte with incomplete compact cumulus oophorus layer or irregular cytoplasm) or grade IV (naked oocytes or oocyte with heterogeneous cytoplasm or expanded cumulus cells; Stringfellow and Givens 2010). COCs graded I or II were washed twice in phosphate-buffered saline (PBS) supplemented with 0.4% bovine serum albumin (BSA) and incubated in BCB medium (PBS supplemented with 0.4% BSA and 13 µM of BCB dye) for 90 min at 38.5ºC in a humidified atmosphere of 5% CO_2, 10% O_2 and 85% N_2. Then, COCs were washed twice in PBS supplemented with 0.4% BSA and classified under a stereomicroscope as: 1) BCB+ (fully grown and developmental competent) – COC with the ooplasma stained blue or 2) BCB- (growing and non-developmental competent) – COC with a colorless ooplasma (Pawlak *et al*. 2014).

2.2.8 In vitro maturation

After BCB staining, COCs were washed twice in TL-HEPES-PVA medium, consisting of 114 mM NaCl, 3.2 mM KCl, 2 mM $CaCl_2.2H_2O$, 0.34 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 10 mM sodium lactate, 10 mM Hepes, 0.2 mM sodium pyruvate, 12 mM sorbitol, 2 mM $NaHCO_3$, 0.1 mg/mL polyvinylalcohol (PVA), 75 µg/mL penicillin G and 50 µg/mL streptomycin sulfate (Li *et al*. 2016). Subsequently, COCs were washed twice in IVM medium, consisting of TCM 199 medium, supplemented with 3.05 mM D-glucose, 0.57 mM cysteine, 0.91 mM sodium pyruvate, 10 IU/mL eCG (Folligon; MSD Animal Health), 10 IU/mL hCG (Vetecor; Hertape Calier Animal Health), 10 ng/mL epidermal growth factor, 0.1% PVA, 20 ng/mL human leukemia inhibitory factor, 20 ng/mL IGF1, 40 ng/mL human fibroblast growth factor 2, 75 µg/mL penicillin G and 50 µg/mL streptomycin sulfate (Yuan *et al*. 2017). Groups of 20 COCs were transfer into a drop (150 µL) of IVM medium covered with mineral oil on a 35 mm dish for 22 hours at 38.5ºC, in a humidified atmosphere of 5% CO_2, 10% O_2 and 85%

N_2. COCs were then incubated for another 20-22 h in IVM medium without hormones (Funahashi and Day, 1993).

2.2.9 Assessment of nuclear maturation

To evaluate oocyte nuclear maturation status, mature COCs were denuded of their surrounding cumulus cells by repeated pipetting. Briefly, oocytes were placed in a hypotonic solution containing 0.045 M KCl for 5 minutes. Groups of 5-10 oocytes were then placed on microscope slide and fixed in acetic acid:ethanol (1:3) for 72 h at room temperature. After that, the oocytes were stained with aceto-orcein (1% orcein in 45% acetic acid), and examined for meiotic stage using a light microscope at 400x magnification. Oocyte nuclear maturation status was classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII; Park *et al*. 2018).

2.2.10 Gene expression analysis

Groups of five COCs graded as I or II of each gilt were loaded in 2 µL of PBS supplemented with 0.1% PVA, placed into a 2 mL sterile DNase/RNase-free cryotube (Corning, New York, USA), snap frozen and stored in liquid nitrogen (Bartolac *et al*. 2018). RNA extraction, DNase treatment and reverse transcription for cDNA synthesis were performed using a single step kit (cells-to-cDNA™ II Kit, Invitrogen™, Waltham, MA, USA) according to manufacturer's recommendations. For RNA extraction, 50 µL of the cell lysis buffer was added to each cryotube containing the COCs samples and incubated at 75ºC for 10 min. After that, the samples were treated with DNase I (0.04 U/µL) and incubated at 37ºC for 15 min to degrade the genomic DNA. Immediately after that, each sample was heated at 75ºC for 5 min to inactivate the DNase. For cDNA synthesis, 10 µL cell lysate, 4 µL dNTP Mix and 2 µL oligo (dT) primers were transferred to a 0.2 mL microtube, cooled on ice for 1 min and heated at 70ºC for 3 min. Then, the reaction microtubes were cooled on ice and the following components were added to the reaction microtubes: 2µL 10 × RT buffer, 1 µL M-MLV-Reverse Transcriptase and 1 µL RNase inhibitor. Reverse transcription occurred at 42ºC for 60 min, followed by incubation at 92ºC for 10 min. After that, cDNA synthetized of each sample was stored at -20ºC until real time PCR procedure.

Real-time PCR (qPCR) was performed using a CFX96 Touch Real-time PCR Detection System (Bio-Rad, CA, USA). Amplifications were carried out under the following conditions: 2 min at 50 ºC, 2 min at 95 °C followed by 40 cycles of 95 °C for 15 s, and finished by cooling for 60 s at 60 °C. The reaction system was composed by qPCR PowerUp™ SYBR™

Green Master Mix (Invitrogen™, Waltham, MA, USA), 100 ng cDNA, 100, 200 or 400 nM of specific primers for each gene analyzed (*BMP15*, *GDF9*, *IL-6*, *IL-7*, *FSHr*, *ERβ*, *ALDOA*, *HMGCR*, *ACSL3*, *MATER*, *BAX* and *Bcl-2*; Table S1) and nuclease-free water. For each biological sample, two technical replicates were performed and the average cycle quantification (Cq) value calculated. Primers were designed using the PrimerQuest™ assay tool (https://eu.idtdna.com/PrimerQuest/Home/Index; Integrated DNA Technologies, Coralville, IA, USA) using sequences obtained from GenBank database (https://ncbi.nlm.nih.gov). Primer efficiencies were verified for each gene using the equation: $E = 10^{[-1/slope]} - 1$ (Rasmussen 2001). The final quantification was calculated using the $2^{-\Delta\Delta Ct}$ (fold-change) and $2^{-\Delta Ct}$ methods (relative expression; Livak and Schmittgen 2001) and normalized with the endogenous *GAPDH* gene. The validation of *GAPDH* gene as an endogenous reference gene was established using the RefFinder software (https://www.heartcure.com.au/reffin der/?type=reference; Xie *et al*. 2012) and there was no statistically significant effect of FSH treatment and age on *GAPDH* transcript levels. In addition, *GAPDH* was previously validated as good reference gene candidate for pig embryos and oocytes due to their stable expression in these biological samples (Kuijk *et al*. 2007).

2.2.11 Statistical analysis

Statistical analysis was performed using Statistical Analysis System (SAS OnDemand; SAS Inst., Cary, NC, USA) software. The experiment was conducted in a completely randomized design in a 2 (treatment; FSH or saline) × 2 (age; 140 or 160 days) factorial scheme.

Quantitative variables were analyzed according to the following model:

$$Y_{ijk} = \mu + T_i + A_j + (TA)_{ij} + e_{ijk} \quad (1)$$

Where: Y_{ijk}, observed response; μ, constant; T_i, effect of the treatment; A_j, effect of the age; $(TA)_{ij}$, interaction; and $e_{ijk} \sim N(0, \sigma_e^2)$, error. All quantitative variables were subjected to normality (Kolmogorov-Smirnov test) and homoscedasticity test (Bartlett test). Whenever necessary, the data were submitted to arcsine transformation prior to a general linear model (GLM) factorial design analysis. If the main effects (FSH treatment and age) or their interaction were significant, mean values were compared using the Tukey-Kramer's *post-hoc* test. Categorical variables (metaphase II, BCB+ and BCB-rates) were arranged in contingency tables and analyzed by Chi-square test. Significance level was settle at $\alpha = 0.05$. Results are presented as mean and standard error of the mean (SEM).

For gene expression analysis, data were analyzed by using the following mixed linear model proposed by Steibel *et al*. (2009):

$$Y_{ijkl} = \mu + A_{(ij)l} + D_{ijk} + (RIG)_{ijk} + e_{ijkl} \quad (2)$$

Where: $Y_{ijkl:}$ is the expression level of gene *k*, in animal *l*, treatment *i* and age *j*, in which *i* = 1 or 2 (FSH or saline, respectively) and *j* = 140 or 160 days of age; μ is the general constant; $A_{(ij)l}$ is the random effect of animal *l* in treatment *i* and age *j*. $A_{(ij)l} \sim N(0, \sigma^2_e)$; D_{ijk} is the sample-specific random effect, $D_{ijk} \sim N(0, \sigma^2_D)$; $(RIG)_{ijk}$ is the interaction effect between treatment *i* and age *j* in gene *k*; and $e_{ijkl:}$ is the random error. $e_{ijkl} \sim N(0, \sigma^2_e)$.

Statistical analyses were performed by using the macro QPCR_MIXED (Steibel *et al* 2009). Effects of treatment, age, and interaction were evaluated by F-test and pairwise comparisons were performed by Student t-test. Significance level adopted was $\alpha = 0.05$. The BAX/Bcl-2 gene expression ratio was based on average relative expression level of each gene ($2^{-\Delta Ct}$); the values were submitted to square root transformation and then tested by ANOVA according to the model in (1). Differences between means were analyzed using *t* test significance level of 5%. For clarity, data were presented as non-transformed values.

2.3 Results

Puberal gilts at 160 days of age were excluded from the study. Thus, two gilts were excluded from control group [G160+control; final sample size (*n*) = 8] and three gilts were excluded from treated group [G160+FSH; final sample size (*n*) = 7].

2.3.1 Effect of age and FSH treatment on ovarian follicular response of prepubertal gilts

Fig. 2 shows the ovarian follicle population observed in ovaries obtained from prepubertal gilts at 140 or 160 days of age treated or not with FSH. The results showed effect ($P < 0.05$) of FSH and age in the ovarian follicle population. Prepubertal gilts aged 160 days old presented lower ($P < 0.05$) percentage of medium follicles (3-6.49 mm) and higher ($P < 0.01$) percentage of small follicles (1-3 mm) compared with gilts at 140 days of age. In addition, the percentage of small follicles was lower ($P < 0.0001$) and the percentage of medium follicles was greater ($P < 0.0001$) in FSH-treated prepubertal gilts. The percentage of large follicles (≥ 6.5 mm) were not affected by age ($P = 0.14$) and FSH treatment ($P = 0.63$).

2.3.2 Effect of age and FSH treatment on oocyte quality and nuclear maturation of prepubertal gilt oocytes

Morphological COCs classification, BCB test and nuclear maturation of COCs obtained from prepubertal gilts at 140 or 160 days of age treated or not with FSH are presented in Table 1. The number of total COCs and nonviable COCs were greater ($P < 0.05$) in younger prepubertal and FSH-treated gilts; however, no effect ($P > 0.05$) of age was observed considering the viable COCs population. Moreover, older prepubertal gilts (160 days) presented a lower ($P < 0.05$) amount of denuded COCs (GIV) compared with prepubertal gilts at 140 days of age.

FSH treatment increased ($P < 0.01$) the number of COCs with better (GI) and good morphology (viable COCs; GI/II), regardless of the age of prepubertal gilts. In addition, the percentage of BCB+ and nuclear maturation rate (MII stage) were higher ($P < 0.0001$) in COCs derived from older (160 days *vs* 140 days of age) and FSH-treated (FSH *vs* saline) prepubertal gilts. FSH treatment affected ($P < 0.05$) the percentage of COCs categories based on the morphological evaluation in prepubertal gilts at 140 and 160 days of age. Stimulation with FSH in prepubertal gilts resulted in a higher ($P < 0.05$) percentage of grade I COCs and lower ($P < 0.05$) percentage of grade II COCs (Fig. S1). There was no effect ($P > 0.05$) of age and FSH treatment in the percentage of grade III, grade IV, viable and nonviable COCs populations (Fig. S1 and S2).

2.3.3 Effect of age and FSH treatment on gene expression of immature COCs obtained from prepubertal gilts

Transcripts levels of 12 genes were compared between immature COCs derived from prepubertal gilts at 140 and 160 days of age (Fig. 3; Fig. S4 and S5) stimulated or not with FSH (Fig. 4; Fig. S4 and S5). No effect of interaction between age and FSH treatment was observed ($P > 0.05$) for all genes expression levels evaluated in this study. Additionally, there was no significant difference ($P > 0.05$) in the *BAX*/*Bcl-2* mRNA ratio (Fig. 5) and mRNA abundances of *ACSL3*, *ALDOA*, *HMGCR*, *BMP15*, *GDF9*, *BAX*, *Bcl-2*, *FSHr*, *ERβ*, *MATER*, *IL-6* and *IL-7* genes between immature COCs retrieved from prepubertal gilts at 140 and 160 days of age. Regarding the effect of FSH treatment, no differences ($P > 0.05$) were observed in the *BAX*/*Bcl-2* mRNA ratio and mRNA abundances of *ACSL3*, *ALDOA*, *BMP15*, *GDF9*, *BAX*, *Bcl-2*, *FSHr*, *ERβ*, *MATER*, *IL-6* and *IL-7* genes between immature CCOs derived from unstimulated and FSH-stimulated prepubertal gilts. Only *HMGCR* was upregulated ($P < 0.05$) in COCs derived from FSH-stimulated prepubertal gilts.

2.3.4 Effect of age and FSH treatment on the concentrations of biochemical constituents of follicular fluid obtained from prepubertal gilts

The concentrations of different biochemical parameters in the FF are shown in Fig. 7. No effect of interaction between age and FSH treatment was observed ($P > 0.05$) for all biochemical analysis of FF. The concentration of glucose in FF increased ($P < 0.05$) in FSH-treated (FSH *vs* saline) and older prepubertal gilts (160 *vs* 140 days of age). In contrast, the concentration of triglycerides decreased ($P < 0.05$) in these same groups of animals. In addition, the FF obtained from prepubertal gilts at 160 days of age presented higher ($P < 0.05$) concentrations of total protein than FF obtained from prepubertal gilts at 140 days of age. No effect of age and FSH treatment was observed ($P > 0.05$) in FF cholesterol levels.

2.3.5 Effect of age and FSH treatment on plasma IGF-1 and glucose concentrations of prepubertal gilts

The plasma glucose levels were not affected ($P > 0.05$) by FSH treatment or age (Fig. 6). In addition, FSH treatment did not affect ($P > 0.05$) the plasma concentrations of IGF-1 in prepubertal gilts at 160 or 140 days of age (Fig. 6). However, plasma concentration of IGF-1 were greater ($P < 0.05$) in older than younger prepubertal gilts (160 *vs* 140 days of age).

2.4 Discussion

In vitro embryo production from oocyte retrieved from prepubertal gilts have the potential to be a cutting edge technology in swine industry. In attempt to increase the developmental competence of prepubertal oocyte, gonadotropin stimulation is used in other species, however, it is unknown whether or not FSH treatment increases the quantitative and qualitative aspects of COCs obtained from prepubertal gilts, and if FSH treatment effects are related with the donor age. This study investigated the effects of FSH treatment and age on ovarian follicular response, biochemical profile of FF, oocyte quality and the expression pattern of specific genes in COCs recovered from prepubertal gilts. The main findings that can be drawn from our study are: i) FSH stimulation enhance the ovarian follicular response, morphological quality (grade I) and meiotic maturation rate of COCs obtained from prepubertal gilts at 140 or 160 days of age; ii) oocytes derived from prepubertal gilts at 160 days of age present higher meiotic maturation and BCB+ rate than 140 days aged prepubertal gilts; iii) increasing the age of prepubertal gilts plus FSH treatment benefits glucose metabolism and suppresses triglycerides/fatty acid metabolism in FF; iv) age and FSH stimulation does not seem to affect the molecular status of specific genes of immature COCs retrieved from prepubertal gilts.

The effect of age on follicular response of prepubertal gilts was evaluated in this study and it was showed that younger gilts (140 days of age) are better ovarian responders to FSH treatment than older gilts, since these females provided a higher number of COCs than the older ones. Previous studies in ruminant species showed that younger females provide a greater number of aspirated follicles (Baldassare *et al*. 2002; Landry *et al*. 2016). This might happen since younger females have a larger ovarian reserve resulting in a higher number of COCs (Currin *et al*. 2021). Stimulation with 100 mg of FSH allows growing of small antral follicles, leading to a greater proportion of medium-sized follicles in prepubertal gilts regardless of the age evaluated in this study. This finding indicates that prepubertal gilts at 140 or 160 days of age respond to exogenous gonadotrophin injections and that stimulation with FSH in a total dose of 100 mg can be applied to increase ovarian follicular response.

BCB test is a useful non-invasive method to select fully-grown, large and more competent immature prepubertal oocytes for in vitro embryo production programs (Catalá *et al*., 2011). Our results indicate that FSH treatment in prepubertal gilts at 140 or 160 days of age resulted in a higher percentage of grade I oocyte and number of viable COCs, along with significantly higher rate of BCB+ and oocyte nuclear maturation rate. These results corroborate findings in ruminant species in which an improvement in oocyte quality and competence was observed after FSH treatment in prepubertal calves (Techakumphu *et al*. 2000; Currin *et al*. 2017; Zacarias *et al*. 2018). FSH is an essential hormone for the proliferation of granulosa and cumulus cells, and directly acts in folliculogenesis (Khan *et al*. 2015; Turathum *et al*. 2021). Cumulus cells have an important role in the bidirectional communication with oocyte, allowing nutrient transfer and paracrine signaling, which is essential for oocyte maturation and competence acquisition (Da Broi *et a*l. 2018). Moreover, the direct contact between cumulus cells and oocyte mediates the organization of meiotic spindle through actin-dependent mechanisms improving meiotic oocyte maturation (Barret and Albertini 2010).

Regarding the effect of age on oocyte maturation, increasing of the age in prepubertal gilts improved nuclear maturation and BCB+ rate of oocytes. Although our results indicates that younger prepubertal gilts were considered better responders to gonadotropin stimulation, the ability to resume meiosis and arrest in metaphase II was impaired in oocytes from these females. This suggests that oocyte competence ability increases near the onset of puberty in pig. This agrees with previously reported by Presicce *et al*. (1997), in which calf oocytes gradually acquire developmental competence around puberty. Additionally, in vivo-derived

oocytes from gilts at first estrus have lower embryonic development ability than gilts at third estrus (Menino *et al*. 1989).

The FF plays an essential role providing conditions for oocyte development and maturation, and consequently, to attain developmental competency (Revelli *et al*. 2009). Fatty acids (FAs) and glucose are the major energy subtracts used by the components of ovarian follicle. FF is composed of proteins, metabolites, ionic compounds, polysaccharides, antioxidant enzymes and regulatory factors derived from blood and secreted by granulosa cells (Freitas *et al*. 2017; Wu *et al*. 2018). Considering this, we hypothesized that age and gonadotrophins stimulation might have a concomitant effect on oocyte quality by changing FF composition.

It has been demonstrated that concentrations of glucose and FAs in FF differ between prepubertal and adult females (Pawlak *et al*. 2012; Warzych *et al*. 2017; Izquierdo *et al*. 2022). In agreement with these studies, we observed an effect of age on composition of FF obtained from both groups of prepubertal gilts. Older prepubertal gilts presented a higher concentration of glucose and total protein, and lower concentration of triglycerides in FF, which indicates a favoring of glucose metabolism in relation to triglycerides/fatty acid metabolism in FF. Recently, Izquierdo *et al*. (2022) reported that FF of adult goats have a greater amount of glucose and lower total fatty acids than FF obtained from prepubertal goats. One possible explanation is that as the gilt approaches the age of puberty, the biochemical composition of follicular fluid is modified with the increases in the concentrations of glucose and total protein attending the oocyte demand to reach the full maturation potential (Dumesic *et al*. 2015; Warzych *et al*. 2017). Besides that, we also show that FSH treatment promoted a shift of fatty acid metabolism to glucose metabolism in follicular fluid of prepubertal gilts. Intra-follicular concentration of glucose is regulated locally by hormonal environment of follicle (such as FSH, LH and E2) and has a direct impact on IVF success (Dupont and Scaramuzzi 2016). Glucose is required for energy production, and also provide substrates for nucleic acid synthesis, nuclear and cytoplasmic maturation; therefore, this is a crucial subtract for oocyte quality and competence (Sutton-McDowall *et al*. 2010).

Triglycerides represents an esterified form to store and transport FAs in FF (Valckx *et al*. 2014). Although FAs has a distinct role in oocyte maturation and supply energy to support oocyte development (Zarezadeh *et al*. 2021), the elevated FA concentration in FF has a lipotoxicity effect that leads to apoptosis of cumulus cells and reactive oxygen production causing a negative impact on oocyte development (Van Hoeck *et al*. 2013). Indeed, triglycerides

are considered the storage form of FAs to protect cumulus cells against fatty acid-induced lipotoxicity (Listenberger *et al*. 2003) and then triglycerides can be used as an indicative of imbalance between lipid influx and utilization. Our results indicates that age and FSH treatment has impact on FF composition of prepubertal gilts, increasing glucose availability to be used as the main energy substrate by COC while decreasing triglyceride/fatty acid cycle. This can be beneficial to COCs since elevated concentration of FAs increases oxidative stress resulting in negative impact on COC (Paczkowski *et al*. 2014).

The insulin-glucose system has important roles on ovarian steroidogenesis and folliculogenesis (Dupont and Scaramuzzi, 2016), and therefore, the increase in plasma concentrations of glucose and IGF-1 can regulate follicular development through a direct effect on the ovaries (Booth *et al*. 1996). Moreover, IGF-1 seems to have an impact in the responsiveness of the ovary to FSH action (Mazerbourg *et al*. 2003) indicating that FSH can act synergistically with the IGF-1 in the follicular microenvironment. Our data showed an increase in the plasma concentration of IGF-1 when gilts got older. This can be explained by the anabolic effects occurring near puberty mediated by IGF-1. In gilts approaching puberty, GH-IGFs growth axis is activated resulting in increase of plasma IGF-1 secreted by liver, regulating growth and body composition (Patterson *et al*. 2010; Ipsa *et al*. 2019). FSH treatment had no effect in the systemic concentrations of IGF-1 and glucose of prepubertal gilts at 140 or 160 days of age. However, since plasma IGF-1 is primarily from liver (Stratikopoulos *et al*. 2008), FSH treatment might have impact on IGF-1 locally synthesized within the follicle, as observed in glucose levels in which increases occurred only at local level (FF).

Gene expression of COCs is a molecular approach to identify potential markers for oocyte competence and quality (Aguila *et a*l. 2020). Some studies have reported a correlation between stored maternal mRNAs during oocyte growth and embryo preimplantation development (Lonergan *et al*. 2003; Wrenzycki *et al*. 2007; Li *et al*. 2014). In our study, we evaluated the impact of age and FSH treatment on several target genes related to apoptosis, energy metabolism, oocyte growth, inflammatory response and steroidogenic pathways (Fig. S3). Only *HMGCR* mRNA expression was affected by FSH treatment, suggesting that age and FSH treatment do not alter the transcriptional pattern for the analyzed pathways in immature CCOs obtained from prepubertal gilts at 140 or 160 days of age. It is known that during oocyte maturation an increase in transcription activities occurs (Mamo *et al*. 2011) as well as a dramatic degradation of unnecessary transcripts for final MII oocyte growth (Ntostis *et al*. 2021). Perhaps, the effect of age and FSH treatment on the target genes selected in this

study can be observed in MII CCOs, instead of GV CCOs from prepubertal gilts since the genes analyzed are involved in oocyte maturation.

HMGCR is a rate-limiting key enzyme that regulates *de novo* cholesterol synthesis (Reverchon *et al*. 2014), and therefore, provides the substrate for steroidogenesis, cell signaling and proliferation (Hu *et al*. 2010; Lima-Valassi *et al*. 2021). Several studies reported that FSH stimulates cholesterol biosynthesis via upregulation of *HMGCR* expression in hepatocyte and granulosa cells (Lino *et al*. 1986; Yamashita *et al*. 2005; Liu *et al*. 2009; Guo *et al*. 2019). FSH activates the PIK3/AKT/FOXO1 pathway leading to an increase of mature SREBP2 promoting the transcription and expression of *HMGCR* (Guo *et al*. 2019). In porcine, increasing the expression of genes related with cholesterogenesis results in increased local concentration of progesterone in cumulus cells that affects meiotic resumption (Shimada *et al*. 2005). Our results agree with these previous studies and indicates that the use of exogenous FSH in prepubertal gilts alters the transcription of *HMGCR* gene that might be related with the intracellular increase of cholesterol in cumulus cells and/or oocyte.

In conclusion, the administration of 100 mg of FSH equally divided into 6 injections was capable to promote the development of higher percentage of medium-sized follicles resulting in a higher number of better quality and fully grown oocytes (BCB+) in prepubertal gilts. Moreover, it was found that oocyte meiotic status and glucose concentration in FF increased with FSH stimulation and increasing age of prepubertal gilts. We demonstrated that FSH stimulation is an efficient strategy to produce large number of oocytes with better quality and high maturational competence in prepubertal gilts at 140 or 160 days of age.

DECLARATION OF INTEREST

The authors have no conflict of interest to declare.

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Table 1
Morphological classification, brilliant cresyl blue test and metaphase II rate of cumulus-oocyte complexes (COCs) from gilts at 140 or 160 days of age submitted or not (control) to FSH stimulation (mean ± SEM)

Parameter		Treatment				*P value*		
		140 days of age		160 days of age				
		Saline (control)	FSH	Saline (control)	FSH	Age	FSH	Age*FSH
Total of COCs		56.2 ± 5.2	88.5 ± 10.1	36.5 ± 9.3	64.9 ± 16.3	0.0419	0.0057	0.8482
Morphological classification of COCs	GI (n)	7.9 ± 1.3	19.6 ± 3.4	4.5 ± 1.7	17.3 ± 4.8	0.1289	0.0001	0.5534
	GII (n)	18.6 ± 3.0	23.9 ± 4.3	11.7 ± 3.1	17.0 ± 5.2	0.0928	0.1930	0.9950
	GIII (n)	16.9 ± 2.5	25.1 ± 3.9	11.6 ± 3.0	17.8 ± 6.9	0.1362	0.0876	0.8115
	GIV (n)	12.8 ± 2.4	19.9 ± 2.4	8.6 ± 2.5	12.7 ± 3.7	0.0470	0.0502	0.5874
Viable COCs[1]		26.5 ± 3.4	43.5 ± 6.3	16.2 ± 4.5	34.3 ± 9.7	0.0929	0.0062	0.8510
Nonviable COCs[2]		29.7 ± 3.2	45.0 ± 4.9	20.2 ± 5.2	30.6 ± 9.2	0.0399	0.0283	0.6579
BCB + % (n)*		20.8 (42)	52.2 (165)	39.1 (43)	87.9 (174)	<0.0001	<0.0001	-
Metaphase II % (n)*		42.9 (79)	62.8 (179)	59.2 (61)	73.4 (138)	0.0004	<0.0001	-

Tukey-Kramer or Chi-square* test.
[1]Viable COCs were considered those classified as GI and GII. [2]Nonviable COCs were considered those classified as GIII and GIV.

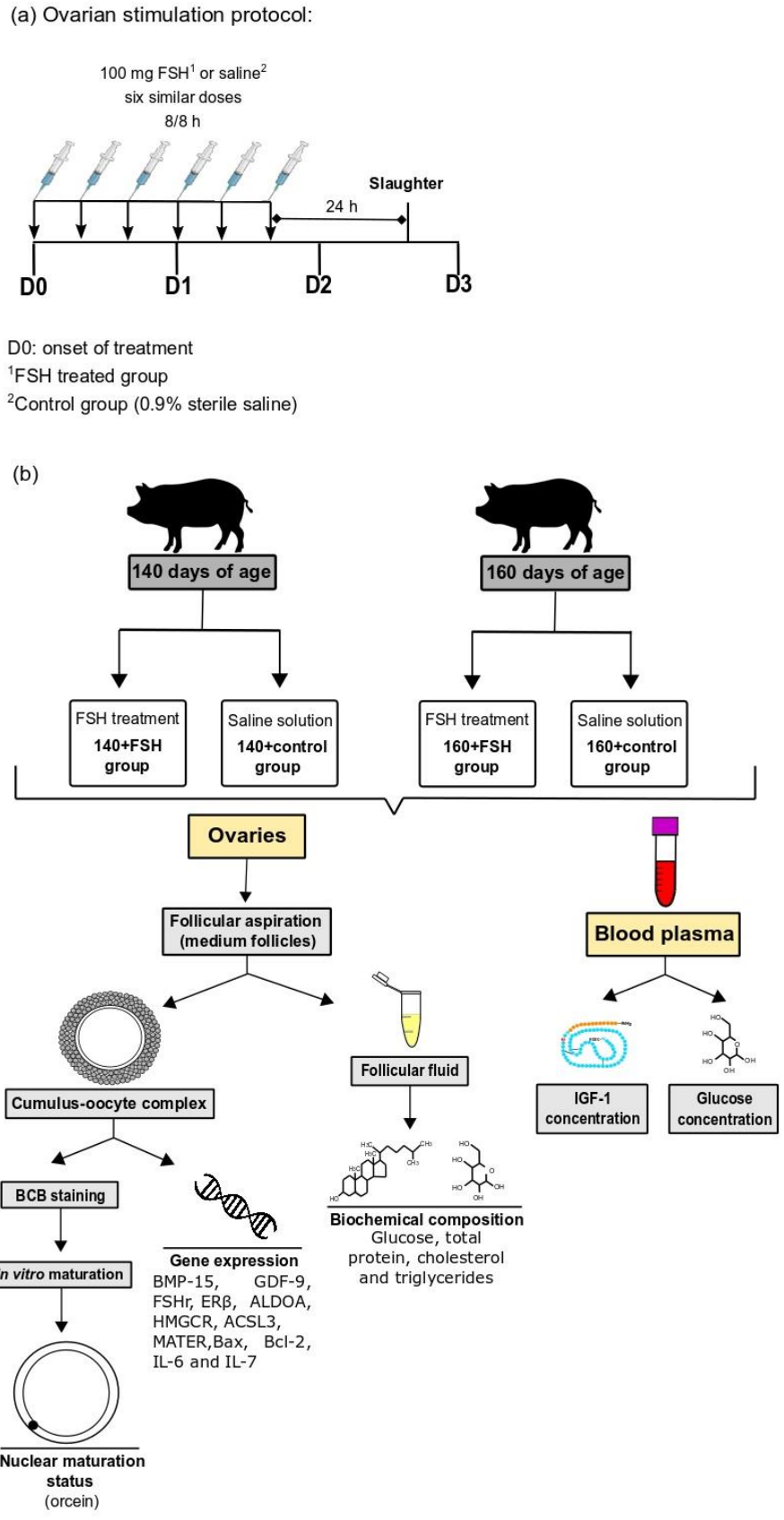

Fig. 1 Ovarian stimulation protocol (a) and (b) experimental design of the study. Effect of FSH stimulation and age (140 or 160 days) on ovarian follicular response, oocyte maturation and gene expression of cumulus-oocyte complexes obtained from prepubertal gilts.

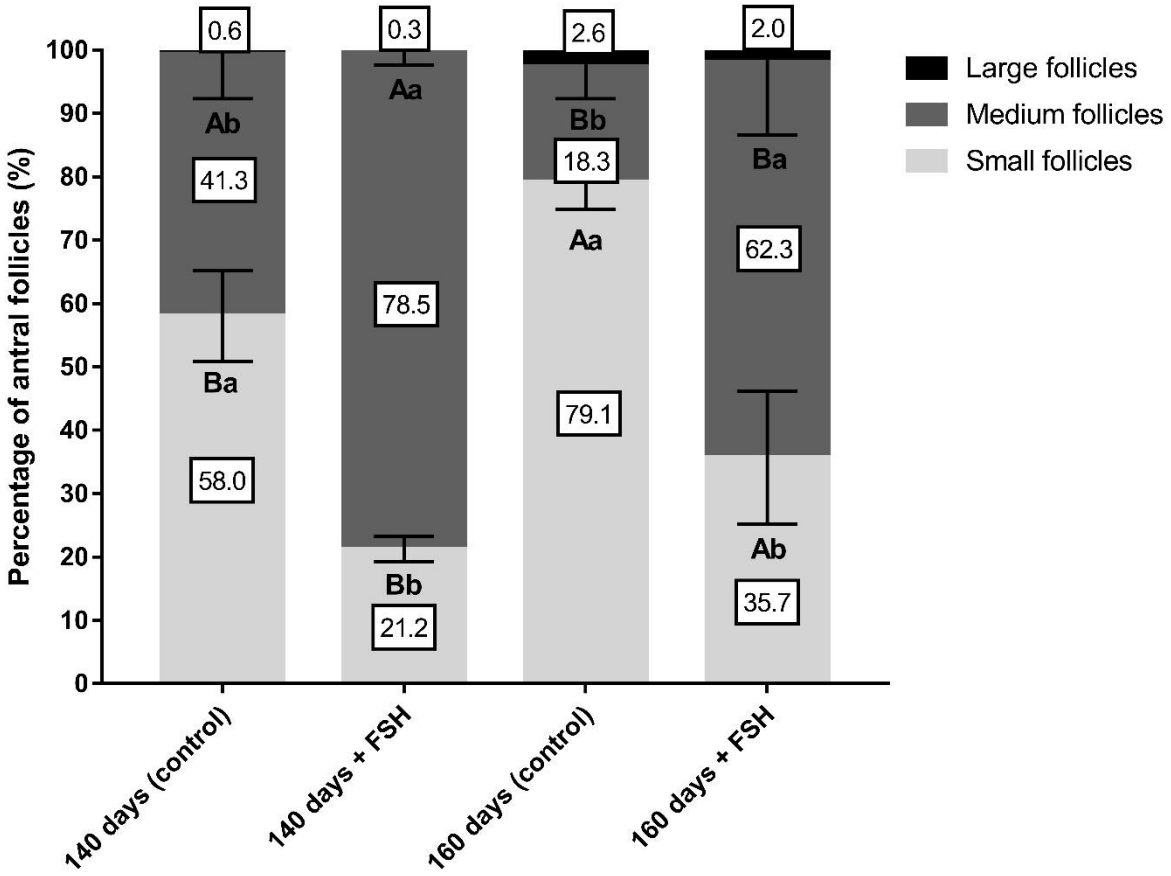

Fig. 2 Percentage of different antral follicles categories [small (1-3 mm), medium (3-6.49 mm) and large (≥6.5 mm) follicles] observed in ovaries obtained from prepubertal gilts at 140 or 160 days of age treated or not with FSH. Data are expressed as mean ± SEM (n = 7-10 per group). Different lower-case letters indicate differences ($P < 0.05$) between FSH and control (saline solution) groups according to Tukey-Kramer test. Different uppercase letters indicate differences ($P < 0.05$) between 140 days and 160 days groups according to Tukey-Kramer test.

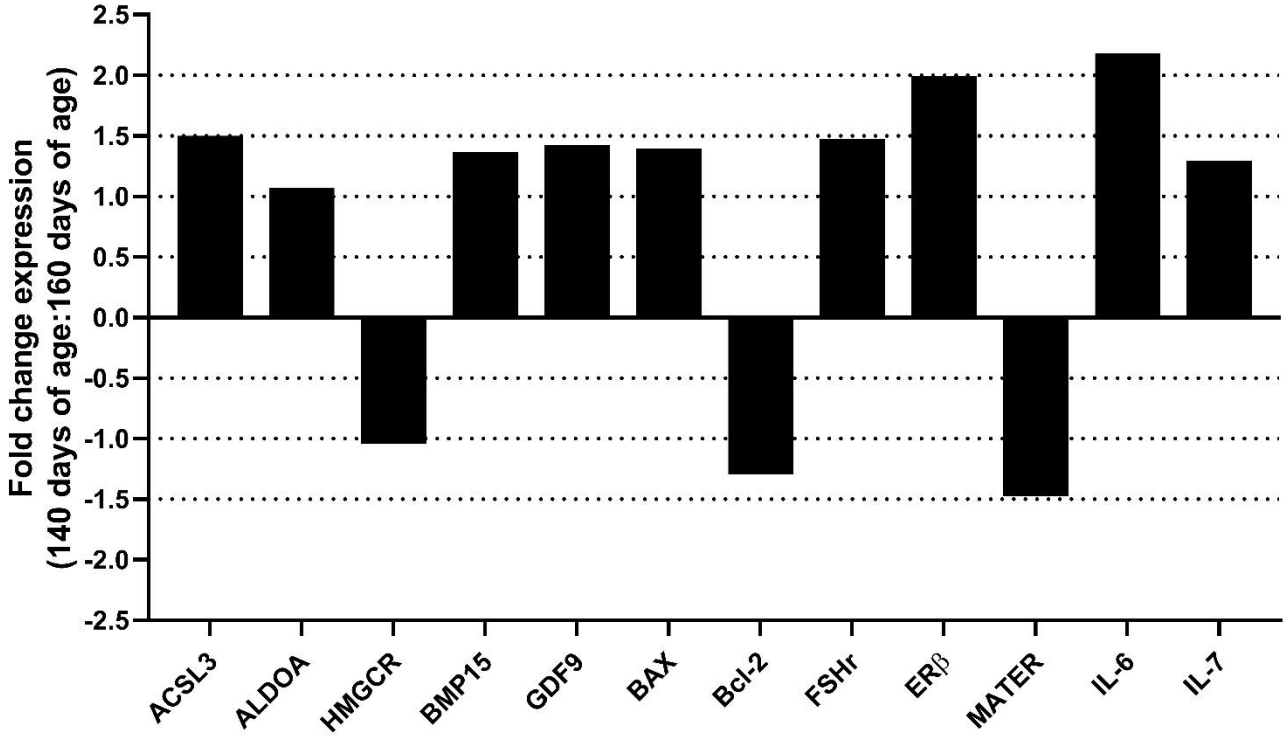

Fig. 3 Fold-change expression of *ACSL3, ALDOA, HMGCR, BMP15, GDF9, BAX, Bcl-2, FSHr, ERβ, MATER, IL-6 and IL-7* genes in immature cumulus-oocytes complexes (COCs) recovered from prepubertal gilts according to age (140 or 160 days) regardless of treatment (FSH or saline) received. Bars above the x-axis represents genes that were upregulated in COCs recovered from prepubertal gilts at 140 days of age. Bars below x-axis represent genes that were upregulated in COCs recovered from prepubertal gilts at 160 days of age. $P > 0.05$.

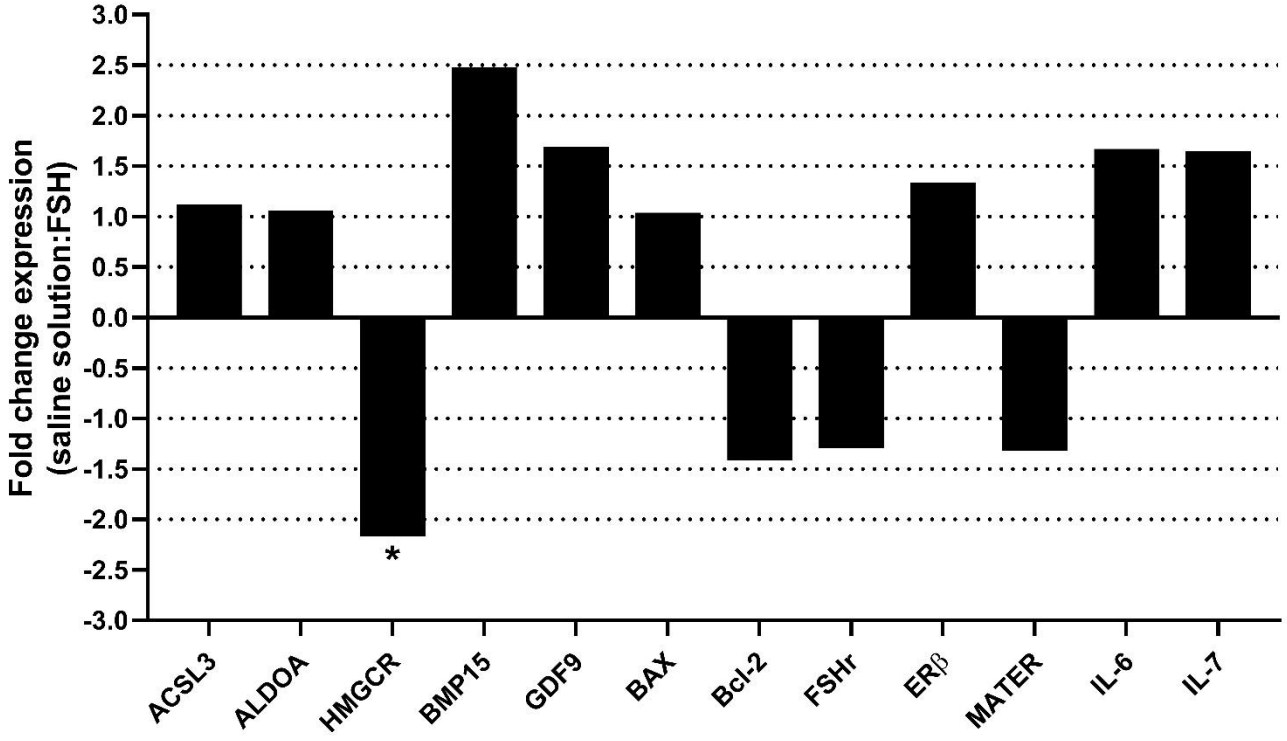

Fig. 4 Fold-change expression of *ACSL3, ALDOA, HMGCR, BMP15, GDF9, BAX, Bcl-2, FSHr, ERβ, MATER, IL-6 and IL-7* genes in immature cumulus-oocytes complexes (COCs) recovered from prepubertal gilts according to treatment (FSH or saline solution) regardless of age (140 or 160 days of age). Bars above the x-axis represents genes that were upregulated in COCs recovered from unstimulated prepubertal gilts (saline solution). Bars below x-axis represent genes that were upregulated in COCs recovered from FSH-stimulated prepubertal gilts. Asterisk indicates a significant difference ($P < 0.05$) in gene expression between COCs recovered from unstimulated and FSH-stimulated prepubertal gilts.

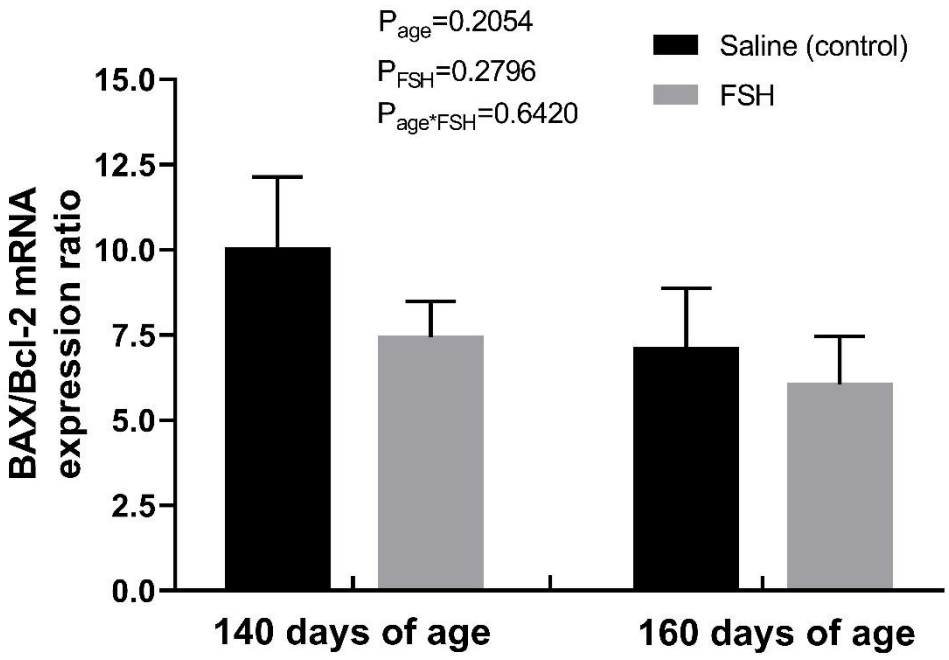

Fig. 5 BAX/Bcl-2 mRNA ratio in immature cumulus-oocytes complexes recovered from unstimulated or FSH-stimulated prepubertal gilts at 140 or 160 days of age. Data are expressed in arbitrary units as ls mean ± SEM (n = 7-10 per group). $P > 0.05$.

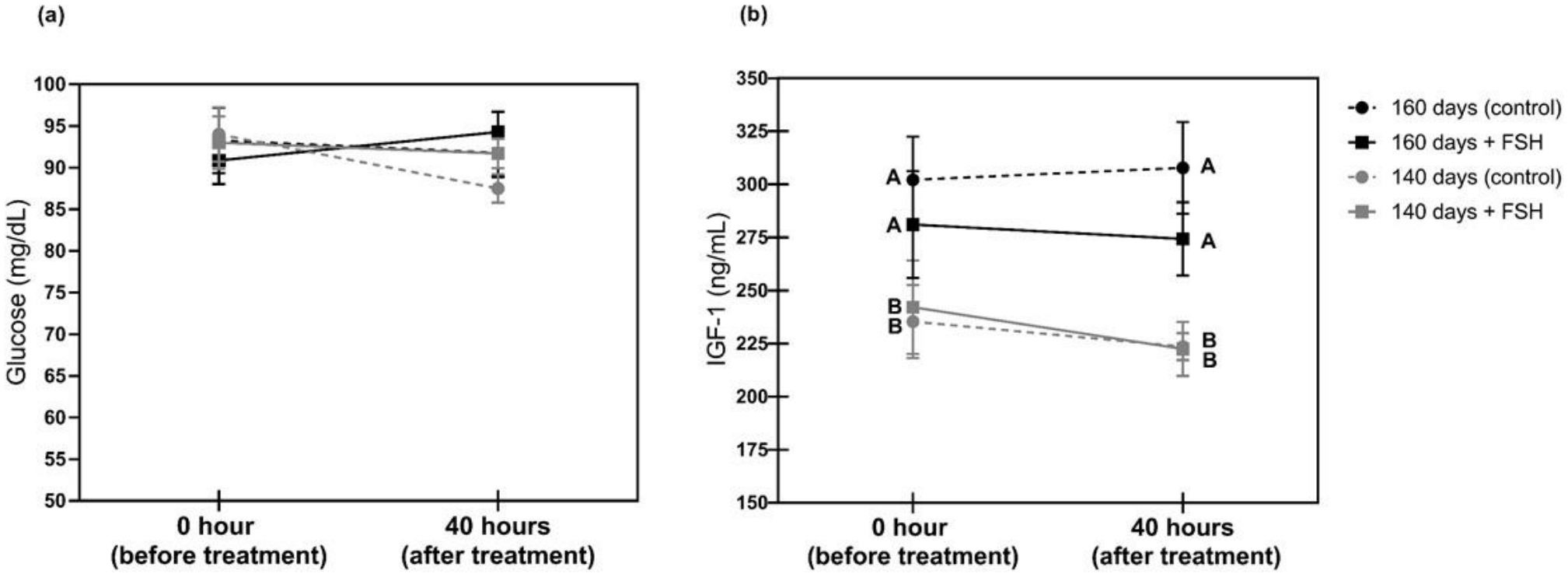

Fig. 6 Plasma glucose (a) and IGF-1 (b) concentrations in prepubertal gilts at 140 or 160 days of age before and after FSH or saline solution treatment. Data are expressed as mean ± SEM (n = 7-10 per group). Different uppercase letters indicate differences ($P < 0.05$) between 140 days and 160 days groups according to Tukey-Kramer test.

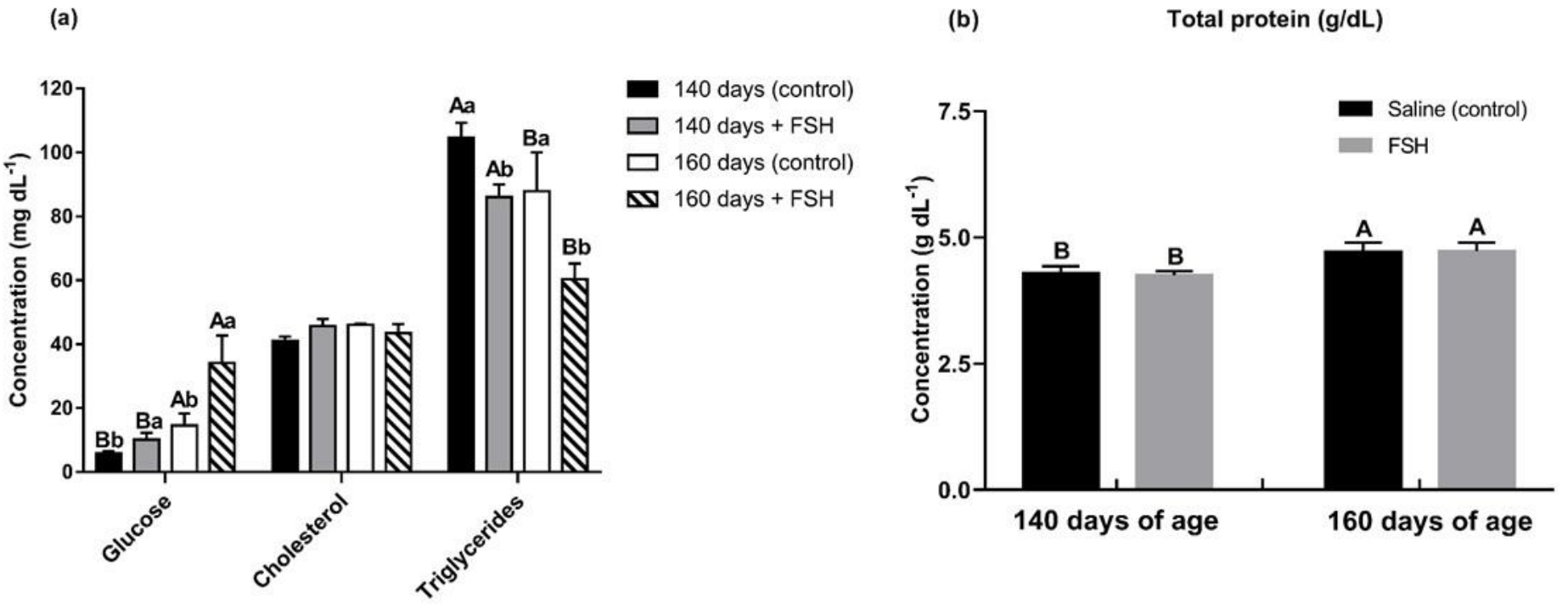

Fig. 7 Concentrations of glucose, cholesterol, triglycerides (a) and total protein (b) in follicular fluid obtained from medium follicles of prepubertal gilts at 140 or 160 days of age treated or not with FSH. Data are expressed as mean ± SEM (n = 7-10 per group). Different lower-case letters indicate differences ($P < 0.05$) between FSH and control (saline solution) groups according to Tukey-Kramer test. Different uppercase letters indicate differences ($P < 0.05$) between 140 and 160 days of age groups according to Tukey-Kramer test.

Supplementary Material

Table S1. Primers used for amplification in real time PCR reactions

Gene	GenBank accession number	Primer	Sequence
ACSL3	NM_001143698.1	Forward	TGTGAGACCAGAGCAGAA
		Reverse	GGACCTCCTAGAGTAGCATATAA
ALDOA	XM_021087995.1	Forward	GCCCTTTCCCAACACTTT
		Reverse	TGGCTGTTTATTGGCAGTAG
BAX	XM_003127290.5	Forward	CCTCCTCTCCTACTTTGGG
		Reverse	TCAGCCCATCTTCTTCCA
BCL-2	XM_021099593.1	Forward	ACCTGAGAAACCCTGGAA
		Reverse	GGTACGTCCCTCACAATAAAC
BMP15	NM_001005155.2	Forward	GTCCACGATGGTAGAACTTTAG
		Reverse	GTTGCTTTCTTCACTCCAAATC
ERβ	AF267736.1	Forward	GCTACAAACCAGTGTACGATAG
		Reverse	CTTCACCATTCCGACTTCATAG
FSHr	NM_214386.3	Forward	CTTAGAAGTGATCGAGGCAAAT
		Reverse	GCATCAGGGTCGATGTAAAG
GDF9	NM_001001909.1	Forward	GATCGTGTTACTGCTGTTGA
		Reverse	CAGGCTGCACTCACATTTA
HMGCR	NM_001122988.1	Forward	CCTTCTGGCTGTCAAGTATATC
		Reverse	ACTACCGGAGATGTGATAGG
IL-6	NM_214399.1	Forward	CCTGGAAGAAGATGCCAAAG
		Reverse	TTTGCCGAGGATGTACTTAATG
IL-7	NM_214135.2	Forward	CCTTGTTCTGTTGCCAGTAG
		Reverse	GTCATCGATGCTGACCATTAG
MATER	XM_005664875.3	Forward	GCCCTCCCTACCTACATATAC
		Reverse	TGACCTCCTCATGACCTTAC
GAPDH	NM_001206359.1	Forward	CTGGTGCTACGTATGTTGTG
		Reverse	AGAGATGATGACCCTCTTGG

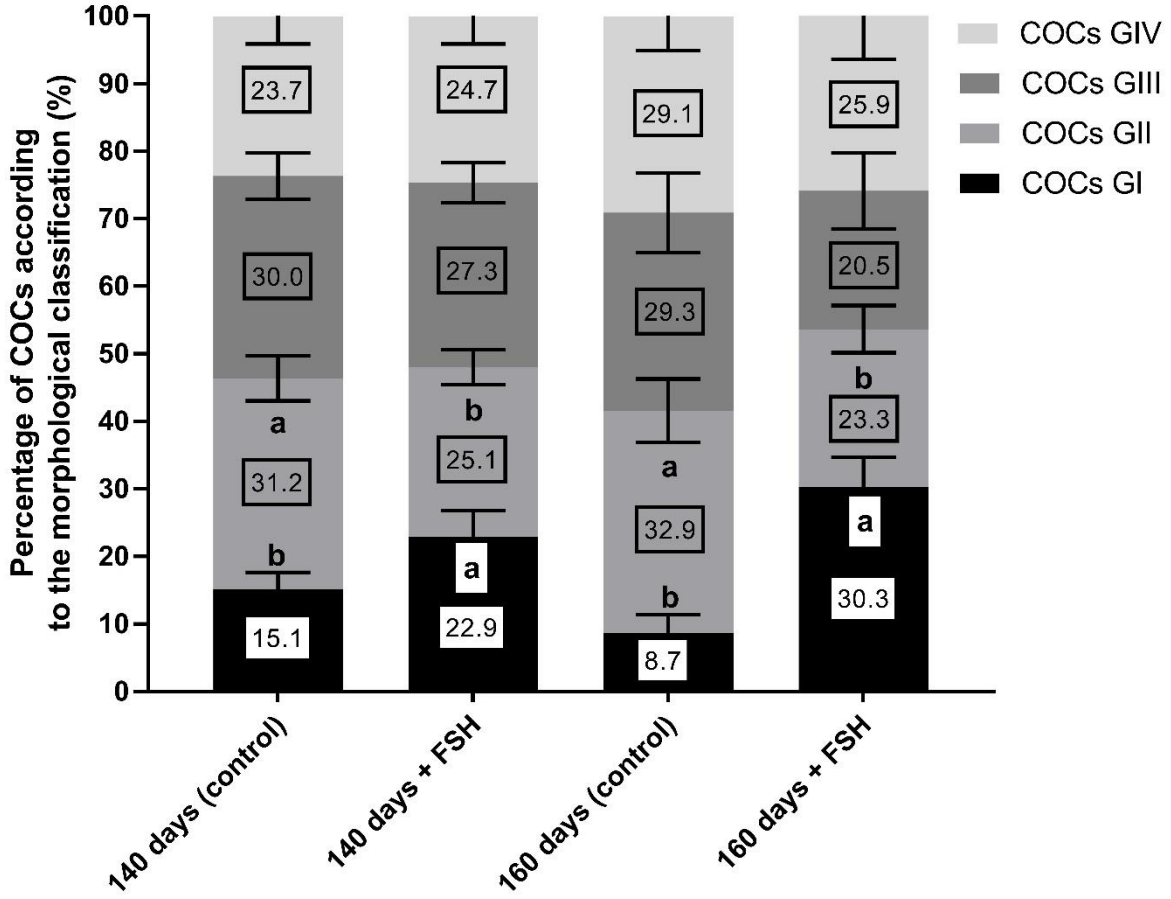

Fig. S1. Percentage of cumulus-oocyte complexes (COCs) according to morphological classification obtained from prepubertal gilts at 140 and 160 days of age treated or not with FSH. Data are expressed as mean ± SEM. Different lower-case letters indicate differences ($P < 0.05$) between FSH and control (saline solution) groups according to Tukey-Kramer test. Different uppercase letters indicate differences ($P < 0.05$) between 140 days and 160 days groups according to Tukey-Kramer test.

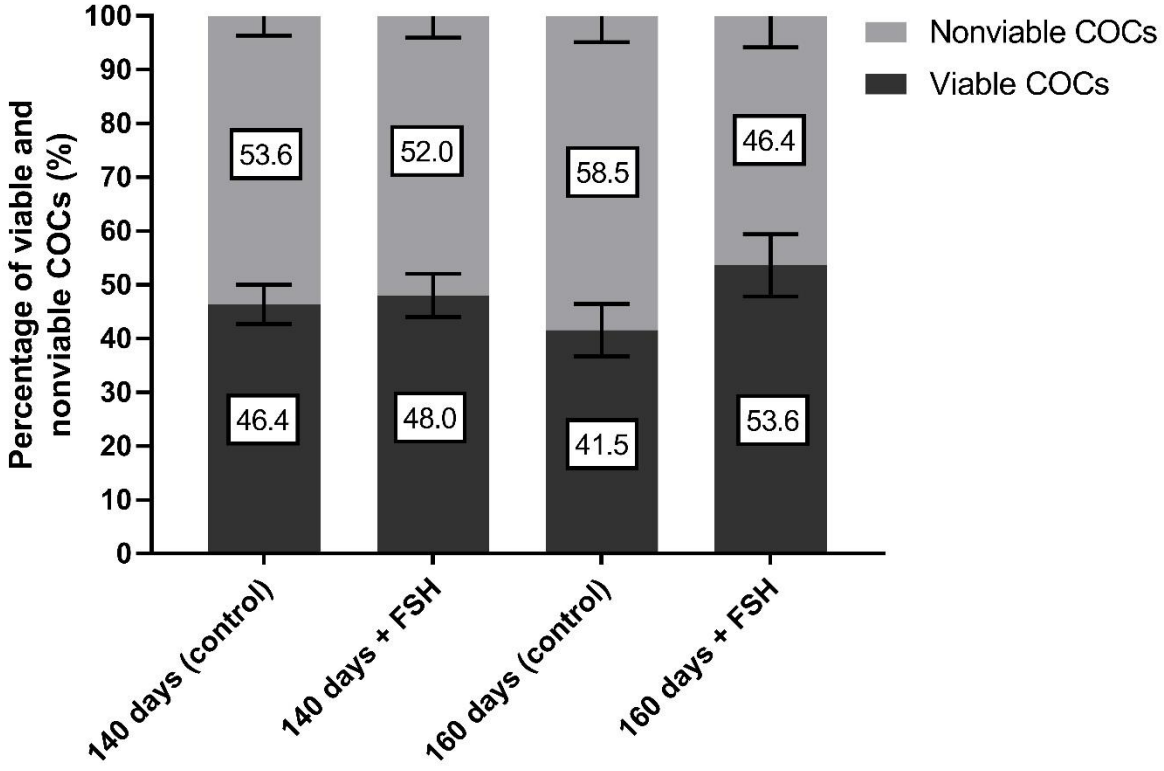

Fig. S2. Percentage of viable and nonviable cumulus-oocyte complexes (COCs) according to morphological classification obtained from prepubertal gilts at 140 and 160 days of age treated or not with FSH. Data are expressed as mean ± SEM. Viable COCs were considered those graded as I-II. Nonviable COCs were considered those graded as III-IV. $P > 0.05$.

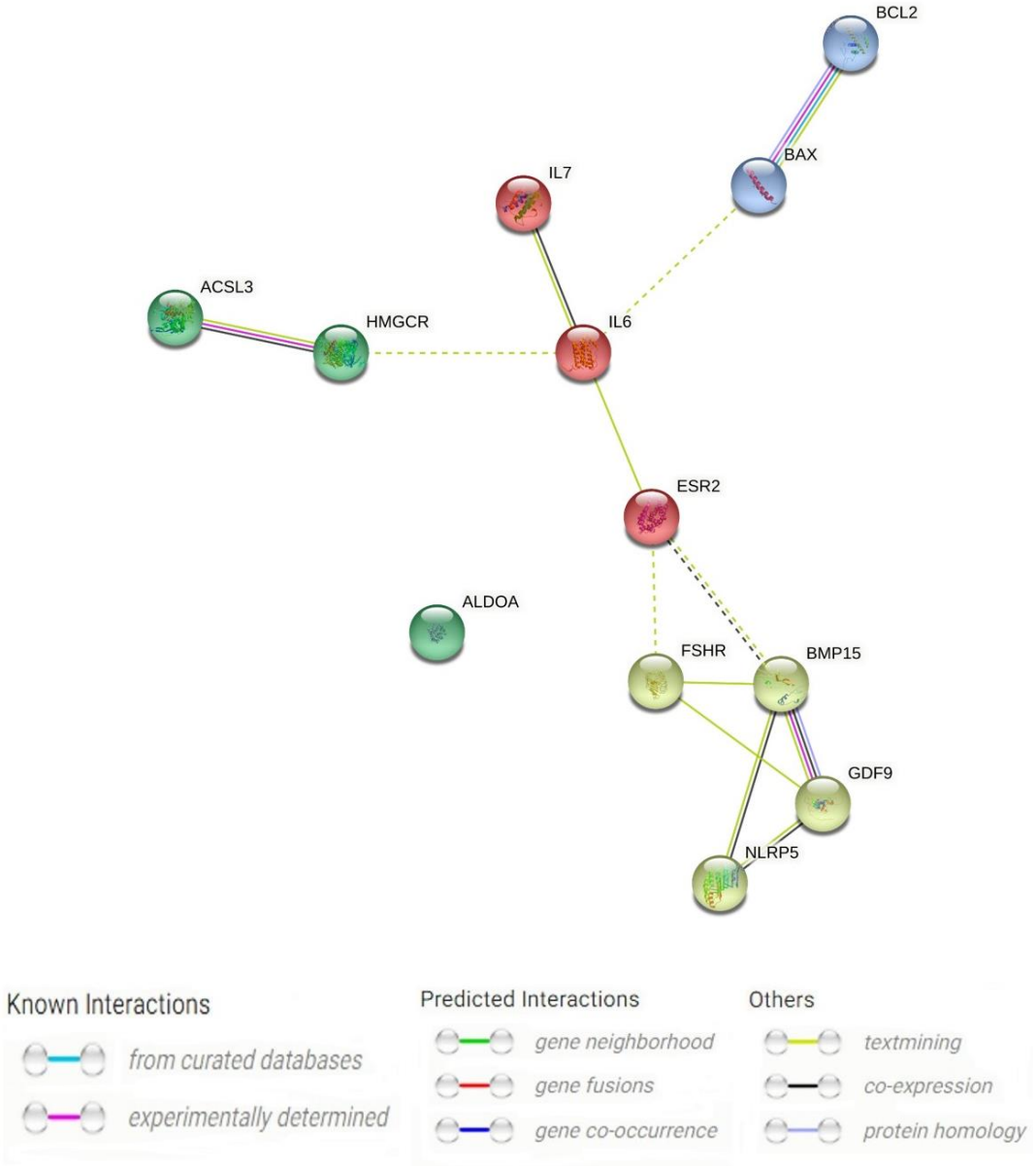

Fig. S3. STRING interaction network between genes related to steroidogenic (*FSHr* and *ESR2*/*ERβ*), maternal-effect required for early embryonic development (*NLRP5*/*MATER*), apoptosis (*BAX* and *Bcl-2*), energy metabolism (*ACSL3*, *ALDOA* and *HMGCR*), inflammatory response (*IL-6* and *IL-7*) and oocyte development (*BMP15* and *GDF9*) pathways evaluated in this study. The intensity of the edges reflects the strength of interaction score. The colored nodes represent query proteins and first shell of interactions. The filled nodes represent the known or predicted 3D protein structure.

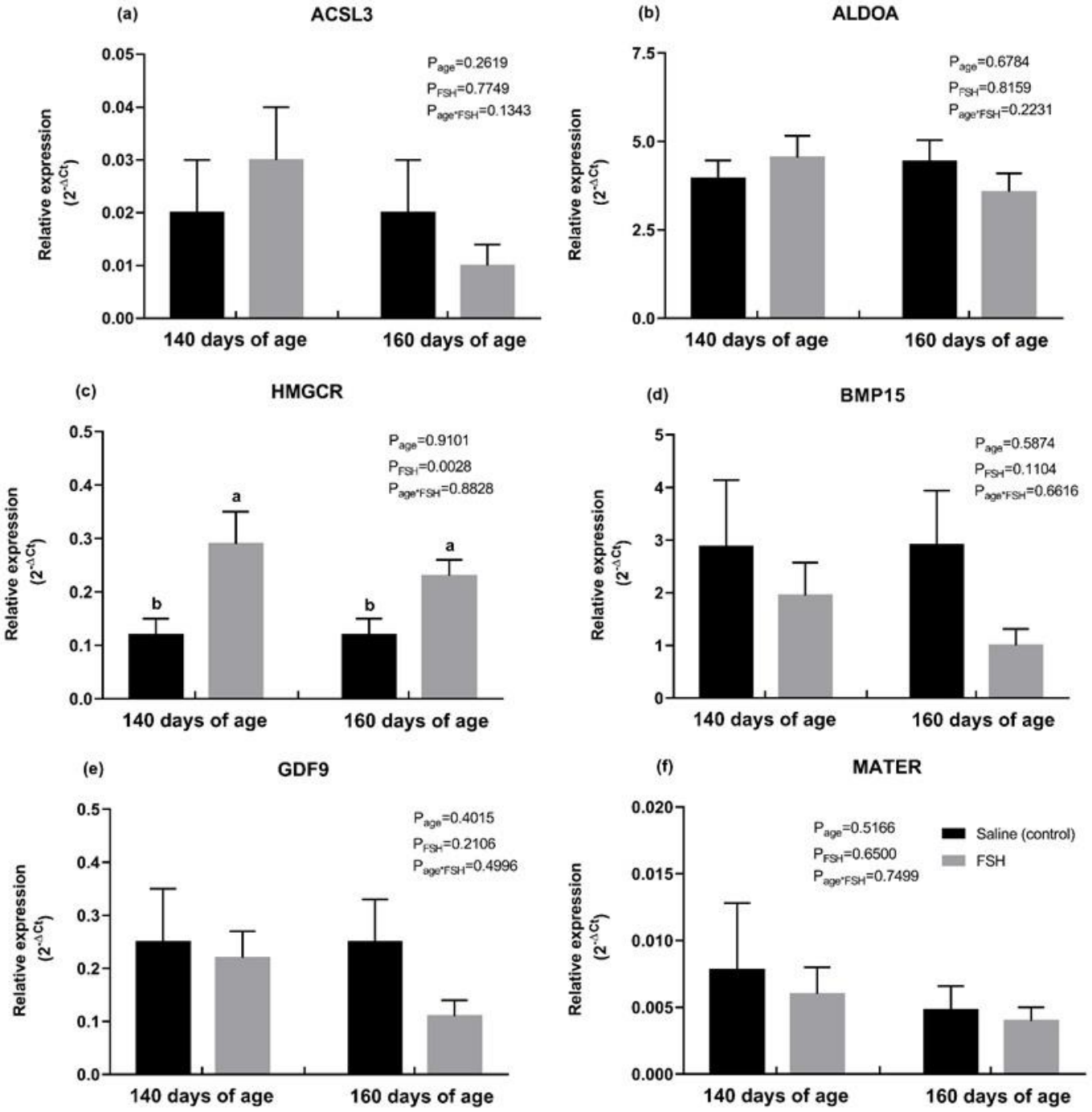

Fig. S4. Relative expression of *ACSL3*, *ALDOA*, *HMGCR*, *BMP15*, *GDF9* and *MATER* transcripts in immature COCs derived from prepubertal gilts at 140 or 160 days of age stimulated or not with FSH. Target genes levels were normalized by *GAPDH* (endogenous gene) expression using the $2^{-\Delta Ct}$ method. Data are presented in arbitrary unit as mean ± SEM (n = 7-10 per group). Different letters indicate differences (lower-case – saline *vs* FSH; uppercase – 140 *vs* 160 days of age; $P < 0.05$) between groups according to Tukey-Kramer test.

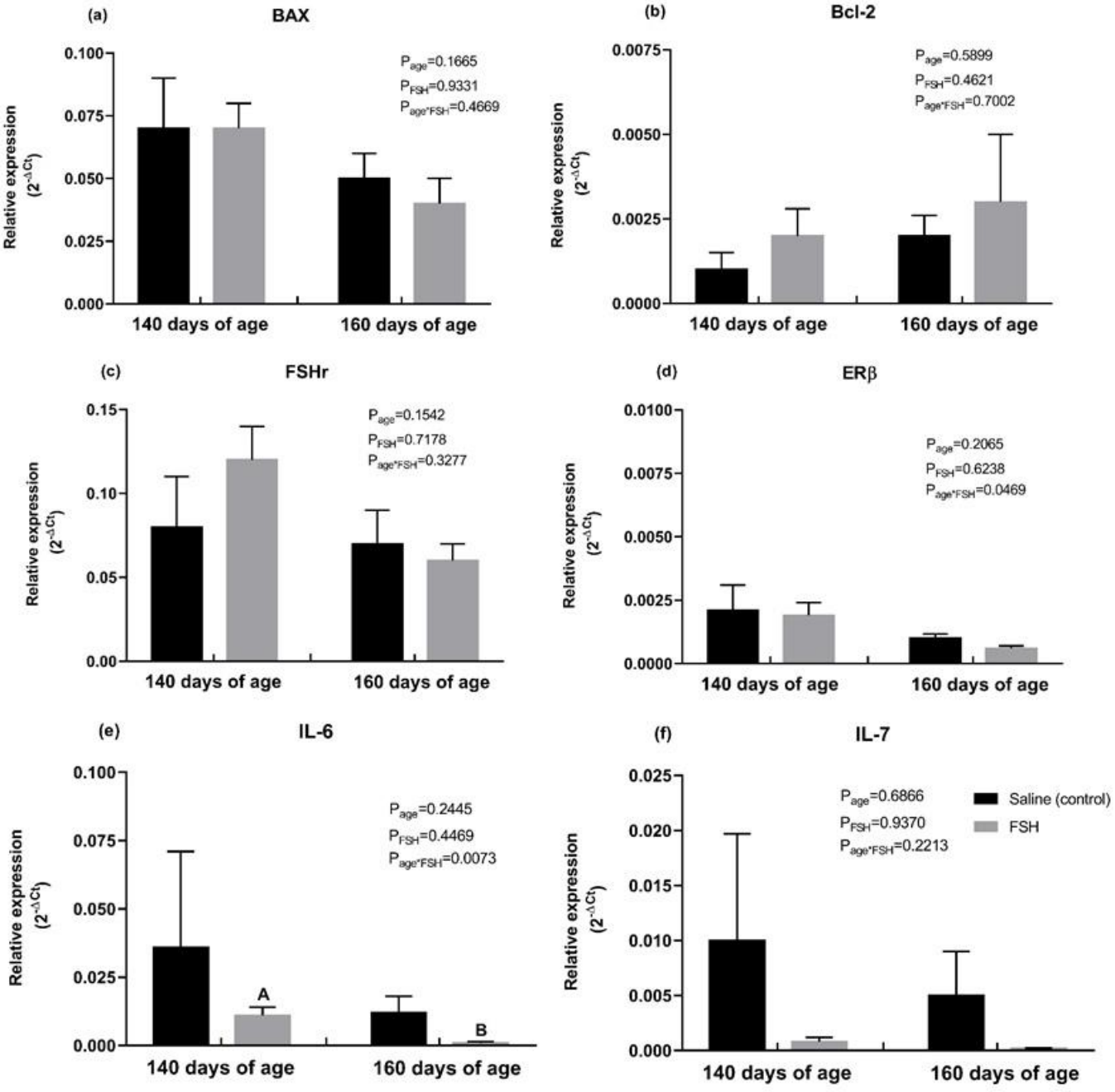

Fig. S5. Relative expression of *BAX*, *Bcl-2*, *FSHr*, *ERβ*, *IL-6* and *IL-7* transcripts in immature COCs derived from prepubertal gilts at 140 or 160 days of age stimulated or not with FSH. Target genes levels were normalized by *GAPDH* (endogenous gene) expression using the $2^{-\Delta Ct}$ method. Data are presented in arbitrary unit as mean ± SEM (n = 7-10 per group). Different letters indicate differences (lower-case – saline *vs* FSH; uppercase – 140 *vs* 160 days of age; $P < 0.05$) between groups according to Tukey-Kramer test.

3. MANUSCRIPT 2

Impact of FSH stimulation and age on ovarian and uterine traits and histomorphometry of prepubertal gilts*

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ABSTRACT

This study investigated the effect of age and FSH treatment on plasma concentration of estradiol (E2), ovarian follicle development, endometrial histomorphometry and ultrasonographic parameters of the ovaries and uterus in prepubertal gilts. Thirty-five prepubertal gilts were grouped according to age (140 or 160 days), and within each age, gilts were allotted to receive 100 mg of FSH [treated; G140+FSH (n = 10) and G160+FSH (n = 7)] or saline solution [control; G140+control (n = 10) and G160+control (n = 8)]. Before and after FSH treatment, blood was collected and transabdominal scanning of the ovaries and uterus were performed. After 24 h of the last FSH injection, gilts were slaughtered and ovaries and uterus were processed for histological and histomorphometric analysis. Biometric and histomorphometric parameters of uterus differed (P<0.05) between prepubertal gilts at 160 days and 140 days of age. Moreover, changes (P<0.05) in uterine and ovarian ultrasound images occurs between 140 and 160 days of age. Plasma E2 concentration increased (P<0.05) with age after FSH or saline treatment. FSH treatment did not affect (P>0.05) early stage of folliculogenesis in prepubertal gilts; however, number of early atretic follicles decreased (P<0.05) and luminal/glandular epithelium height and glandular diameter increased (P<0.05) after FSH treatment. FSH administration also increased (P<0.05) number of medium follicles in 140 days old gilts and decreased (P<0.05) number of small follicles in 160 days old gilts. Thus, injections of 100 mg of FSH stimulates endometrium epithelium and induce follicular growth to medium follicle size without affecting preantral stages in prepubertal gilts, also, macro- and microscopic morphometric changes occurs in the uterus as gilts approach the onset of puberty (160 days old).

Keywords: morphology, endometrium, pig, biometry, folliculogenesis, B-mode ultrasonography

3.1 Introduction

The modern swine production is a highly competitive market in which the highest productive animals presenting greater breeding values are selected to reach the demand for pork, that represents 40% of global meat consumption [1,2]. Consequently, reproducing animals at youngest ages is desirable for two reasons: i) to accelerate genetic progress by reducing the generation interval, and ii) reduces production costs since gilts represent a costly non-productive animal until obtaining descendants [3]. It has been shown that reproductive technologies, including in vivo and in vitro embryo production associated with the embryo transfer, allows breeding of gilts at prepubertal ages [4–6]. However, early age at mating in porcine specie might result in failure to maintain pregnancy due to the insufficient development of the uterus that has not yet reached maturity [7]. Thus, considering that porcine reproductive tract undergoes a series of growth changes from birth to attain puberty [8], it is relevant to determine if the development of reproductive system ceases during peripuberal period (140-160 days), and before the selection of gilts for breeding.

Hormonal ovarian stimulation is used in prepubertal females to increase follicle size and oocyte competence. For this purpose, follicle stimulating hormone (FSH), is a hormone widely used in animal and human reproductive medicine to induce multiple antral follicular selection and growth [9,10]. FSH is a glycoproteic hormone secreted by pituitary gland, which has a key role in reproductive function during different phases of reproductive development (fetal, neonatal, puberty and adult life) [10,11]. However, the presence of a functional FSH receptor (FSHR) is a prerequisite to mediate the action of FSH. FSHR is a transmembrane FSH-specific G protein-coupled receptor that transduce the FSH-induced signal in granulosa cells [12]. FSHR is present in porcine granulosa cells from primary follicles and persisted up to the preovulatory stage, indicating that FSH action mediated by functional FSHR is involved from the recruitment of primordial follicles into the growing pool until the follicular development through the preantral stage [13,14]. Innumerous studies evaluated the effect of FSH exogenous administration on ovarian antral follicular response of prepubertal females [15–17]. However, there is a lack of information on its impact on ovarian follicular development at preantral stages.

FSH acts mainly on ovaries regulating folliculogenesis and oogenesis, and stimulating ovarian estradiol production, that stimulates cell proliferation and angiogenesis in the endometrium [11,18–20]. Expression of the FSHR is detected in gonadal tissues (specifically, granulosa and Sertoli cells); however, FSHR can also be found in extra-gonadal tissues of reproductive tract [21–23]. In this context, it has been demonstrated that in ruminant and human

species, FSH affects uterine functions by changes in endometrium transcriptome, vascular density and endometrial morphology [24–28]. In pigs, the presence of FSH receptor in endometrium of prepubertal gilts was recently described [22,29], evidencing an extra-gonadal action of FSH in this specie. However, no data is available regarding the functional role of FSH in the uterus of prepubertal gilts and the effect of FSH treatment on uterine growth during peripuberal period.

Therefore, the aim of the current study was to investigate the effect of FSH treatment and age (140 and 160 d) on plasma concentrations of estradiol, and ovarian and uterine morphology of prepubertal gilts, using ultrasonographic and histomorphometrical approaches.

3.2 Material and methods

3.2.1 Ethics, location and animal conditions

The Ethics Committee in the use of farm animals at the Universidade Federal de Viçosa approved the present experimental design (CEUAP/UFV: 0118/2018). This experiment was conducted during December 2020 to May 2021 in the Pig Breeding Farm from Department of Animal Science at Universidade Federal de Viçosa, Viçosa, MG, Brazil.

Commercial line TN70 (Topigs Norsvin, Netherlands) gilts were housed in collective pens (2 animals/pen) and fed with soy/corn based diet and *ad libitum* water. Gilts were select based on the followed selection criteria (mean ± SD): birth weight (≥1.4 kg; 1.5 ± 0.2), teat number (≥14 teats; 16.0 ± 0.9), weaning weight (≥5.4 kg; 7.1 ± 1.1), 60 days weight (≥24 kg; 25.0 ± 2.8), 100 days weight (≥42 kg; 49.2 ± 4.1), adequate body and vulva conformation, and absence of physical defects such as hernias or lameness. To assess the effect of age, gilts used in this study were at 140 or 160 days of age and had a mean body weight of 100.3 ± 8.7 or 75.0 ± 4.4 kg (mean ± SD), respectively.

3.2.2 Experimental design and hormonal treatment

Thirty-five prepubertal gilts were separated according to the age (140 ± 4 days and 160 ± 4 days) and within each age, gilts were allotted to received FSH [treated; 100 mg of FSH; G140+FSH (n = 10) or G160+FSH (n = 10)] or saline [control; 0.9% sterile saline; G140+control (n = 10) or G160+control (n = 10)]. Hormonal stimulation consisted of a total dose of 100 mg FSH (Folltropin-V; Vetoquinol Saúde Animal, São Paulo, Brazil) divided into six similar doses, administered every 8 hours by intramuscular injection (Days 0-2) [30]. After 24 h of the last FSH injection (coasting period; Day 3), gilts were slaughtered and reproductive

tracts were collected (Fig. 1). Pubertal gilts that presented corpora haemorrhagica or corpora lutea in ovaries at post-mortem examination were excluded from the study.

3.2.3 Ultrasonographic examination

Gilts had their ovaries and uterus examined by transabdominal ultrasonography (Oxyson Kaixin DCU12 Vet Doppler, Jiangsu – China) using a 7.5-MHz multi-frequency microconvex transducer (model 6.5C20H1A). The transmitting zone of the transducer was coated with contact gel during ultrasonographic evaluations. For each prepubertal gilt, reproductive ultrasound evaluations were performed before (0 h) and after FSH/saline solution treatment (40 h after the first FSH injection). Gilts were moved into a purpose-designed crate to facilitate restraining during the evaluation, and the ultrasound scanning of reproductive tract was performed by applying the transducer ventrally on the abdominal wall in front of hind leg and lateral to the nipple line [31]. Ovaries were classified according to Bolamba et al. [32] into two morphological types (Fig. S1): 1) "grape" type (GT), characterized by the presence of many large follicles (> 6 mm), and 2) "honeycomb" type (HT), characterized by the presence of numerous small follicles (1-3 mm).

The uterine echotexture was subjectively classified according to Martinat-Botté et al. [33] as homogeneous (dark and homogenous uterine images) or heterogeneous (well-defined sections of the uterus; Fig. S1). The diameter of the uterine horns were measured in cross-sections images. For this, two measures of the diameter (maximum and minimum dimension) were taken in each cross-section image. In addition, two cross-sections were evaluated in each animal to reduce possible errors from variation in size and shape of cross-sections of the uterine horns. Considering an elliptical shape for uterine sectional images, the sectional area was calculated by the following formula: ½ x maximum diameter x ½ minimum diameter x π. Then, the sectional area of the cross-sectioned uterine horns of each animal (SAsono) was defined as the mean sectional area for each animal [34].

3.2.4 Post-mortem biometric measurements of ovaries and uterus

Firstly, the broad ligament of the uterus was removed and uterine body and horns were identified and dissected. Then, uterus and ovaries from each animal were weighed using an electronic digital analytical balance (Bioprecisa, F2104N, Curitiba - Brazil). The gonadosomatic index (GSI), which indicates the proportion of body tissue related to gamete production, was calculated as the ratio between gonadal weight and body weight [(ovarian weight/body weight) x 100]. For biometric measurements, the width and length of each uterine

horn and uterine body were recorded. In addition, the length, width and thickness of each ovary were measured using a caliper [35,36]. The number of small (< 3 mm), medium (3-6.49 mm) and large (≥ 6.5 mm) follicles on each ovary was assessed visually.

3.2.5 Histological analysis

3.2.5.1 Follicle classification and follicular density

Left ovaries were sliced in half, fixed in 10% neutral buffered formalin for 48 h, and stored in 70% alcohol. Then, half of each half-ovary (1/4 ovary) was dehydrated in increasing concentrations of alcohol, clarified in xylene, and embedded in paraffin [37]. One-quarter of the ovary embedded in paraffin was serially sectioned at 5-µm thickness with a minimum interval of 20 sections between each collected section to ensure that the same follicles were not counted in consecutive sections [38]. Five histological sections were stained with hematoxylin and eosin and evaluated using an optic microscope at 100 and 400x magnification. Follicles were counted only when they presented a visible and intact oocyte and classified as primordial, primary, secondary, pre-antral and antral according to the criteria established by [36,39]. Further, follicles presenting detachment of granulosa cells or collagen fibres and degenerative changes were identified as early or late atretic, respectively [38,40]. The area of each histological section were measured using Image J software (version 1.53; National Institute of Health, USA). Then, the number of follicles of each category per area (mm^2) was calculated by the following equation described by [38]:

$FD = NF / \Sigma A$

FD = follicle density (number of follicles/mm^2)

NF = number of follicles of a specific stage of development

Σ = sum of the area (mm^2) of the 5 histological sections

3.2.5.2 Granulosa layer, antrum and follicle areas of mature follicle

Granulosa layer area was determined by the difference between follicle area and antrum area [38]. For this, follicle and antrum areas were calculated by the ellipse area formula (π x radius of major axis x radius of minor axis) based on the ellipse shape of the ovarian follicle. Furthermore, follicle and antrum diameter were determinate by the mean of two perpendicular measurement in the same mature follicles, and the follicle diameter was measured considering up to the outer layer of the granulosa cells. Images of mature follicles (15-20 follicles/animal) were captured and the diameter of each follicle was measured using Image J software (version

1.53; National Institute of Health, USA). After the measurements, follicle diameter was classified into: F1 (≤0.5 mm), F2 (0.51-0.75 mm), F3 (0.76-1.0 mm) or F4 (>1.0 mm).

3.2.5.3 Endometrial histomorphometry

Fragments of medial portion of the right uterine horn (approximately 2 cm^2) were fixed in 10% neutral buffered formalin for 48 h and stored in 70% alcohol. Then, fragments were processed in a similar way as described above. Five different sections per animal were used and 20 sections between one and its next were discarded [25]. The images of tissue sections were obtained by an Olympus BX60 microscope at 4X and 10X magnification equipped with a digital camera (Olympus Q-Color 3™) using QCapture™ software. For the endometrial morphometry analysis, the Image J software (version 1.53; National Institute of Health, USA) was used to determine glandular diameter (GD), glandular epithelium height (GEH), luminal epithelium height (LEH), endometrial thickness (ET), and glandular density in each captured image. LEH was consider as the distance from basal membrane to the apical border of the epithelial cell, and ten measurements in each 5 randomly fields (n = 50/animal) were recorded using a 10X magnification. For GD and GEH, 50 randomly glands transversally cut were measured using a 10X magnification in each animal (n = 100 glandular measurements/animal). In each gland, the mean of two perpendicular diameters and two measurements from the basal lamina to the apical membrane of the epithelium cells were obtained for GD and GEH analysis, respectively. ET was consider as the distance from the apical region of the luminal epithelium to the end of endometrium, and three measurements in each 5 random fields (n = 15/animal) were recorded using a 4X objective magnification. Glandular density was determined on 10 histological images (10X objective magnification) using a 90-point Weibel grid with 2-point length 58.2 µm [41]. Only points overlaying glandular tissue were counted and results were expressed as total number of points over the glandular tissue [42,43].

3.2.6 Blood samples

Blood collection was performed at the first (0 h) and last (40 h after the first FSH injection) FSH/saline injection for the determination of plasma 17β-oestradiol (E2) concentration. Blood was collected into EDTA tube from the orbital venous sinus by insertion of 16 G needle (1,6x40 mm; BD, Juiz de Fora, Brazil) in medial eye canthus. Then, plasma was immediately separated by centrifugation at 1500 × *g* for 10 min and stored in microtubes at -20ºC for subsequent analysis.

3.2.7 E2 assay

Plasma E2 concentration was analyzed in an automatic gamma counter (model: 2470 Wizard; PerkinElmer, Waltham, MA, USA) using a solid-phase radioimmunoassay kit (17β-Oestradiol – ImmuChem Double Antibody; MP Biomedicals), according to the manufacturer's instructions. The intra-assay coefficient was 12%.

3.2.8 Statistical analysis

Statistical analysis was performed using Statistical Analysis System (SAS OnDemand; SAS Inst., Cary, NC, USA) software. The experiment was conducted in a completely randomized design in a 2 (treatment; FSH or saline) × 2 (age; 140 or 160 days) factorial scheme.

Quantitative variables were analyzed according to the following model:

$$Y_{ijk} = \mu + T_i + A_j + (TA)_{ij} + e_{ijk} \quad (1)$$

Where: Y_{ijk}, observed response; μ, constant; T_i, effect of the treatment; A_j, effect of the age; $(TA)_{ij}$, interaction; and $e_{ijk} \sim N(0, \sigma_e^2)$, error.

All quantitative variables were subjected to normality (Kolmogorov-Smirnov test) and homoscedasticity test (Bartlett test). Whenever necessary, data were submitted to arcsine transformation prior to a general linear model (GLM) factorial design analysis. If the main effects (FSH treatment and age) or their interaction were significant, mean values were compared using Tukey-Kramer's *post-hoc* test. Non-normal variables were submitted to Kruskal-Wallis test. Categorical variables (frequency of heterogeneous uterine echotexture and frequency of grape ovarian type) were arranged in contingency tables and analyzed by Fisher's exact test. Significance level was settle at $\alpha = 0.05$. Results are presented as mean and standard error of the mean (SEM).

3.3 Result

Puberal gilts at 160 days of age were excluded from the study. Thus, two gilts were excluded from control group [G160+control; final sample size (n) = 8] and three gilts were excluded from treated group [G160+FSH; final sample size (n) = 7].

3.3.1 Ovarian and uterine biometry from FSH-stimulated or unstimulated prepubertal gilts at 140 or 160 days of age

Tables 1 and 2 summarizes ovarian and uterine morphometry of prepubertal gilts at 140 or 160 days of age. Older prepubertal gilts were heavier ($P < 0.0001$) and presented higher

($P < 0.05$) ovarian weight than younger animals. FSH treatment increased ($P < 0.05$) ovarian weight, gonadosomatic index and ovarian volume in both ages (Table 1).

There was interaction ($P < 0.05$) between age and FSH treatment for number of small follicles on both ovaries. A lower number of small follicles was observed ($P < 0.05$) in FSH treated gilts compared with unstimulated gilts at 160 days of age. In contrast, the number of medium follicles was greater ($P < 0.05$) in FSH treated gilts compared with unstimulated gilts at 140 days of age. Regarding the effect of age, 160 days old prepubertal gilts presented higher ($P < 0.05$) number of medium follicles compared with 140 days old prepubertal gilts. The number of large follicles was not affected ($P > 0.05$) by age and FSH treatment (Table 1).

The biometrical parameters of the uterus were affected ($P < 0.05$) as 140 days gilts got older (Table 2). However, FSH treatment did not alter ($P > 0.05$) neither of these parameters.

3.3.2 Plasma E2 concentrations in prepubertal gilts at 140 or 160 days of age before and after FSH treatment

After FSH or saline treatment (40 h after the first FSH injection), prepubertal gilts at 160 days of age had higher ($P < 0.05$) plasma E2 concentrations compared with prepubertal gilts at 140 days of age. FSH stimulation did not affect ($P > 0.05$) plasma estradiol concentrations at such ages (Fig. 2).

3.3.3 Ultrasonographic evaluations of ovaries and uterus of prepubertal gilts at 140 or 160 days of age before and after FSH treatment

Data of ovarian and uterine ultrasonographic evaluations are shown in Table 3. After FSH or saline treatment, prepubertal gilts at 160 days of age presented greater ($P < 0.05$) SAsono values, frequency of uterus with heterogeneous echotexture and frequency of GT ovaries compared with prepubertal gilts at 140 days of age. Nonetheless, no differences ($P > 0.05$) between FSH and saline solution treatments were observed in the ultrasonographic parameters evaluated in this study.

3.3.4 Histomorphology and density of ovarian follicles population of FSH-stimulated or unstimulated prepubertal gilts at 140 or 160 days of age

Follicular density of each ovarian follicles category is shown in Fig. 3. The follicular density of ovarian follicles (primordial, primary, secondary, pre-antral, antral and late atretic follicles) were similar ($P > 0.05$) between FSH-stimulated and unstimulated prepubertal gilts.

On the other hand, FSH treatment decreased ($P < 0.05$) the number of early atretic follicles in 140 and 160 days old prepubertal gilts.

Regarding the effect of age, 160 days old prepubertal gilts presented lower ($P < 0.05$) number of secondary and early atretic follicles than 140 days old prepubertal gilts. However, the others developmental stages of ovarian follicles (primordial, primary, pre-antral, antral and late atretic) were not affected ($P > 0.05$) by age.

3.3.5 Granulosa layer measurement of mature follicles obtained from prepubertal gilts at 140 or 160 days of age treated with FSH or saline solution

Area data of granulosa layer, antrum and follicle from mature follicles are shown in Table S1. No differences ($P > 0.05$) between animal age and FSH treatment were observed in the area of granulosa layer, antrum and follicle of mature follicles. When considering larger follicles categories (F3 and F4), exogenous FSH administration in prepubertal gilts either at 140 or 160 days of age increased ($P < 0.05$) the area of granulosa layer in F4 follicles compared with their respective unstimulated controls. In addition, prepubertal gilts at 140 days of age showed larger ($P < 0.05$) areas of granulosa layer in F3 follicles.

3.3.6 Endometrial histomorphometry of FSH-stimulated or unstimulated prepubertal gilts at 140 or 160 days of age

The results of endometrial histomorphometry are shown in Fig. 4. Prepubertal gilts at 160 days of age presented greater ($P < 0.0001$) values in all parameters compared with 140 days gilts. Moreover, prepubertal gilts treated with FSH presented higher values ($P < 0.05$) of GD, GEH and LEH than unstimulated gilts at both ages. However, FSH stimulation did not affect ($P > 0.05$) ET and glandular density of prepubertal gilts at both ages.

3.4 Discussion

The present study evaluated the effect of age and FSH stimulation on plasma concentrations of estradiol, biometric data, ultrasonography and histomorphometry of ovaries and uterus of prepubertal gilts. The main findings that can be drawn from our study are: (i) FSH stimulation changes ovarian biometrical data and decrease early follicular atresia in both 140- and 160-day-old prepubertal gilts; (ii) macro-and microscopic characteristics and uterine ultrasound images changes from 140 to 160 days of age in prepubertal gilts; (iii) exogenous FSH do not impact uterine macroscopic features but alters uterine histomorphometric in prepubertal gilts.

In the present study, the age of prepubertal gilts did not affect ovarian biometrical measurements, with the exception of weight. In gilts, the ovarian tissue growth peak occurs at 16 weeks of age, and from 140 days of age to puberty ovaries does not undergoes major changes [8,44], which is in agreement with our findings. The increase in ovarian weight observed in prepubertal gilts from 140 to 160 days of age, is probably related with the increase in the number of small follicles as reported by [8]. In addition, in our study we observed an increase in plasma levels of estradiol from 140 to 160 days of age that can be associated with the emergence of preovulatory follicles, resulting in a change of ovarian status for GT accompanied by the increase of ovarian weight. Studies reported that FSH treatment stimulates small follicles to grow, which promotes changes on ovarian biometric characteristics improving ovarian development [45,46]. This observation is supported by our findings, which reported the increase in the number of medium follicles and decrease in small follicles in prepubertal gilts treated with FSH.

FSH has an important role in the regulation of ovarian follicular development, through granulosa cell proliferation, growth and maturation of antral follicles, and suppression of follicular atresia [11,47]. In our study, FSH treatment had no impact on the density of follicles at early stage of folliculogenesis. This can be due to the fact that FSH acts increasing the size of follicle rather than the number of follicles [48]. In addition, follicles that reached the antral stage are responsive to gonadotropins indicating that only follicles at late stages of folliculogenesis are responsive to FSH treatment [49]. Interestingly, [50] showed that treatment of host mice with FSH for 7 days or more increased the size of antral follicles of porcine ovarian xenografts. Indeed, our study showed that FSH treatment had a positive effect on the area of granulosa layer only in antral follicles with diameter > 1 mm (see Table S1). Increasing age and stimulation with FSH in prepubertal gilts decreased the occurrence of early follicular atresia, indicating a positive effect on follicular survival and on the development of a healthy follicle. FSH is an important survival factor in follicle since it inhibits granulosa cells apoptosis, and in porcine specie FSH seems to attenuate apoptosis of porcine granulosa cells through reduction of FasL mRNA abundance [51]. Since apoptosis of granulosa/theca cells is more pronounce in non-cyclic than cyclic gilts [52], we hypothesized that as gilts approach the age (160 days) near the onset of puberty a decrease of atretic follicles occurs to increase the number of oocytes capable of fertilization and embryonic development.

Changes occur on uterine traits (weight, length, and diameter) in prepubertal gilts from 140 to 160 days of age. Ovarian steroids (mainly E2) are required for normal uterine growth

during prepubertal period in gilts; also, growth of the uterine tissue closely follows the pattern of plasma E2 concentrations [7]. In gilts, blood E2 concentration is low throughout the prepubertal period but increases prior the achievement of puberty [53], probably associated with increase of uterine size to assure a proper uterine capacity during pregnancy. We hypothesized that as prepubertal gilts get older (160 days) and approach the age of puberty, uterine growth occurs in response to the increase of plasma E2 levels. Interestingly, transabdominal ultrasound (b-mode), in our study, was an adequate approach to detect phenotypic alterations in uterine dimensions of prepubertal gilts, similarly to that detected by post-mortem biometrical analysis. We observed that an increase in SAsono values occurs, as well as the frequency of prepubertal gilts presenting heterogeneous uterine echotexture from 140 to 160 days of age. This agrees with previous studies showing that cyclic gilts presented a greater value of SAsono and more heterogeneous echotexture than prepubertal gilts [34,54]. These observations indicate that uterine echographic images changes according to the age of prepubertal gilts, and transabdominal ultrasound can be a useful approach to detect differences in uterine size of live prepubertal gilt.

Endometrial histomorphometry is an objective and precise technique capable to identify alterations in the endometrium components caused by reproductive hormones on microscopic scale [55]. Although exogenous FSH treatment had no impact on uterine macroscopic characteristics, it led to increase in GD, GEH and LEH. Two possible mechanisms may be involved in FSH action on the porcine uterus. First, FSH might have a role in the regulation of uterine activity mediated by a direct binding to its receptor in endometrium of prepubertal gilts. To support this hypothesis, [22] showed the existence of FSH receptor in uterine glandular epithelium and endometrial luminal epithelium of prepubertal gilts. Second, FSH may indirectly regulate uterine function by acting on endometrial aromatase, inducing a local E2 synthesis in the endometrium. Previous studies reported that E2 induces proliferation of the luminal and glandular epithelium increasing the LEH and GEH [19,42,56], similarly our study observed this same effect on endometrium promoted by FSH stimulation. Moreover, [57] showed that FSH stimulation (10 or 100 ng mL^{-1}) increases *in vitro* E2 secretion in endometrial explants from cyclic gilts. This might indicate that FSH acts indirectly in endometrium through E2 secretion, supporting the proper functioning of the uterus.

The effect of age on endometrial histomorphometry in prepubertal gilts, indicates that as animals get older there is an increase in uterine histomorphometric parameters, consistent with the changes on biometric parameters observed in this study. Histologically, uterus is not

fully developed at birth and endometrial adenogenesis is completed during postnatal period in pigs [58]. Although porcine uterine wall histoarchitecture becomes functionally mature around 120 days after birth [59], this continues to develop prior the occurrence of puberty in response to hormonal stimulation. In this context, it was showed that ET and glandular density strongly increased over the first three months of age followed by less marked change until 180 days of age, an arrest in the growth and development of porcine endometrium is observed [60]. Some factors were identified as being responsible for the regulation of uterine development, such as growth factors (e.g., IGF1, IGF2 and FGFs) and hormones (e.g., ovarian steroids and prolactin) [58]. In pigs, uterine growth occurs independently of the ovary from birth to 60 days of age; however, ovarian steroids (mainly E2) regulate uterine histogenesis and development after this period [61]. Therefore, proliferation of uterine glands and the increase of luminal and glandular epithelia heights might be a reflection of the uterotrophic effects promoted by E2 elevation during peripuberal period.

In summary, uterine growth and development continues occurring from 140 to 160 days of age in prepubertal gilts, and possibly, it is regulated by the increased ovarian steroids that occurs during peripuberal period. FSH exogenous administration does not affect the early stage of folliculogenesis. Otherwise, it stimulates follicular growth at antral stages in prepubertal gilts. Changes on endometrial histomorphometry under FSH stimulation indicate that endometrial function is mediated by FSH action. However, further studies are necessary to elucidate the functional role of FSH in growth and development of uterus and endometrial function in prepubertal gilts.

DECLARATION OF INTEREST

The authors have no conflict of interest to declare.

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Table 1
Ovarian follicular population and ovarian biometric parameters of gilts at 140 or 160 days of age submitted or not (control) to FSH stimulation (mean ± SEM)

Parameter	Orientation	Treatment				*P value*		
		140 days of age		160 days of age				
		Saline (control)	FSH	Saline (control)	FSH	Age	FSH	Age*FSH
GSI (%; nºx10^{-3})*	-	9.5 ± 0.0	13.8 ± 0.0	9.0 ± 0.0	14.3 ± 0.0	0.9925	0.0016	0.7350
Weight (g)*	Right ovary	3.5 ± 0.0	5.1 ± 0.5	4.7 ± 0.3	6.5 ± 0.9	0.0262	0.0029	0.8394
	Left ovary	3.5 ± 0.3	5.2 ± 0.6	4.5 ± 0.2	7.2 ± 0.8	0.0093	0.0002	0.3130
Volume (cm^3)*	Right ovary	4.1 ± 0.3	6.1 ± 0.6	5.0 ± 0.7	5.2 ± 0.4	0.9504	0.0479	0.1103
	Left ovary	3.8 ± 0.4	6.8 ± 0.6	4.6 ± 0.3	7.0 ± 0.6	0.3079	<0.0001	0.5395
Nº small follicles (1-3 mm)*	Right ovary	37.7 ± 11.1	15.0 ± 2.5	87.7 ± 13.1	22.0 ± 5.0	0.0023	<0.0001	0.0389
	Left ovary	35.6 ± 8.2	16.2 ± 2.1	93.7 ± 10.4	28.0 ± 6.5	<0.0001	<0.0001	0.0149
Nº medium follicles (3.1-6.49 mm)	Right ovary	18.3 ± 2.5[b]	48.4 ± 3.8[a]	22.4 ± 6.1	49.4 ± 10.8	-	-	-
	Left ovary	20.8 ± 3.8[b]	60.1 ± 4.4[a]	19.4 ± 6.0	60.7 ± 14.9	-	-	-
Nº large follicles (6.5-12.9 mm)	Right ovary	0.3 ± 0.1	0.2 ± 0.2	2.6 ± 1.6	0.9 ± 0.6	-	-	-
	Left ovary	0.2 ± 0.1	0.3 ± 0.1	2.4 ± 1.4	2.0 ± 1.5	-	-	-

Within a row, mean values followed by lower-case (FSH *vs* control) letters differed between them by Kruskal-Wallis test.
*Tukey-Kramer test.

Table 2
Uterine biometric parameters of gilts at 140 or 160 days of age submitted or not (control) to FSH stimulation (mean ± SEM)

Parameter*	Orientation	Treatment				*P value*		
		140 days of age		160 days of age				
		Saline (control)	FSH	Saline (control)	FSH	Age	FSH	Age*FSH
Weight (g)	-	97.5 ± 9.7	126.5 ± 15.5	222.7 ± 39.3	223.9 ± 32.5	<0.0001	0.2872	0.5482
Uterine body diameter (cm)	-	1.8 ± 0.1	1.9 ± 0.1	2.3 ± 0.2	2.3 ± 0.1	0.0002	0.6767	0.9600
Uterine body length (cm)	-	1.8 ± 0.1	1.4 ± 0.1	2.4 ± 0.2	2.3 ± 0.2	<0.0001	0.0955	0.4067
Horn diameter (cm)	Right	1.4 ± 0.1	1.5 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	0.0001	0.3134	0.8802
	Left	1.4 ± 0.1	1.5 ± 0.1	1.9 ± 0.2	1.9 ± 0.1	0.0010	0.6039	0.7728
Horn length (cm)	Right	53.2 ± 3.7	54.5 ± 2.9	62.3 ± 2.7	56.1 ± 3.4	0.1201	0.4664	0.2656
	Left	53.1 ± 3.1	54.9 ± 2.8	64.0 ± 3.8	59.0 ± 3.4	0.0305	0.6288	0.3108

*Tukey-Kramer test.

Table 3

Mean sectional area of cross-section of uterine horns (SAsono) and frequency of ovaries classified as grape type according to the morphology and frequency of heterogeneous uterine echotexture in gilts at 140 or 160 days of age submitted or not (control) to FSH stimulation (mean ± SEM)

Parameter	Time	Treatment				*P value*		
		140 days of age		160 days of age				
		Saline (control)	FSH	Saline (control)	FSH	Age	FSH	Age*FSH
SAsono (cm^2)*[1]	Before	0.8 ± 0.1	0.7 ± 0.1	1.0 ± 0.3	0.8 ± 0.1	0.3096	0.3419	0.7553
	After	0.9 ± 0.1	1.1 ± 0.1	1.7 ± 0.4	2.0 ± 0.2	0.0001	0.2141	0.8597
Frequency of heterogeneous uterine echotexture % (n)[#2]	Before	0.0 (0/10)	0.0 (0/10)	12.5 (1/8)	0.0 (0/7)	0.4286	0.5143	-
	After	0.0 (0/10)	10.0 (1/10)	25.0 (2/8)	42.9 (3/7)	0.0370	0.2243	-
Frequency of grape ovarian type % (n)[#3]	Before	0.0 (0/10)	0.0 (0/10)	0.0 (0/8)	0.0 (0/7)	-	-	-
	After	0.0 (0/10)	0.0 (0/10)	25.0 (2/8)	28.6 (2/7)	0.0261	0.3974	-

*Tukey-Kramer. [#]Fisher's exact test.

[1]SAsono: sectional area of the cross-sectioned uterine horns of each animal. [2]Heterogeneous uterine echotexture: well-defined sections of the uterus.

[3]Grape type: presence of many large follicles (>6 mm).

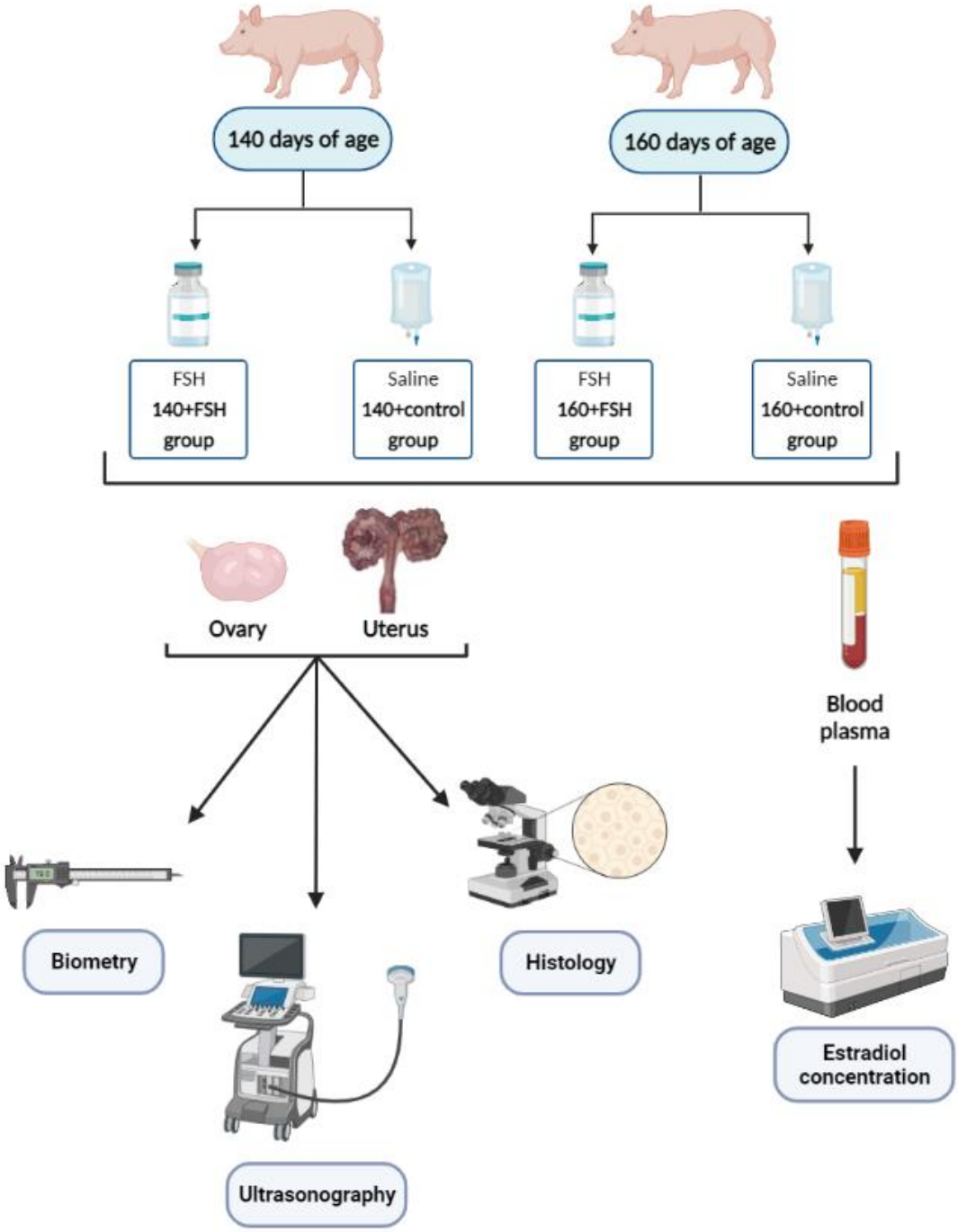

Fig. 1 Experimental design of the study. Effect of FSH stimulation on biometry, histomorphometry and ultrasonographic parameters of ovaries and uterus from prepubertal gilts at 140 or 160 days of age. FSH-treated groups were submitted to an ovarian stimulation protocol with multiple doses of FSH (total dose of 100 mg was divided into six equal doses given every 8 h). Control groups receive sterile 0.9% saline in a similar regime as described above. Figure created with BioRender.com.

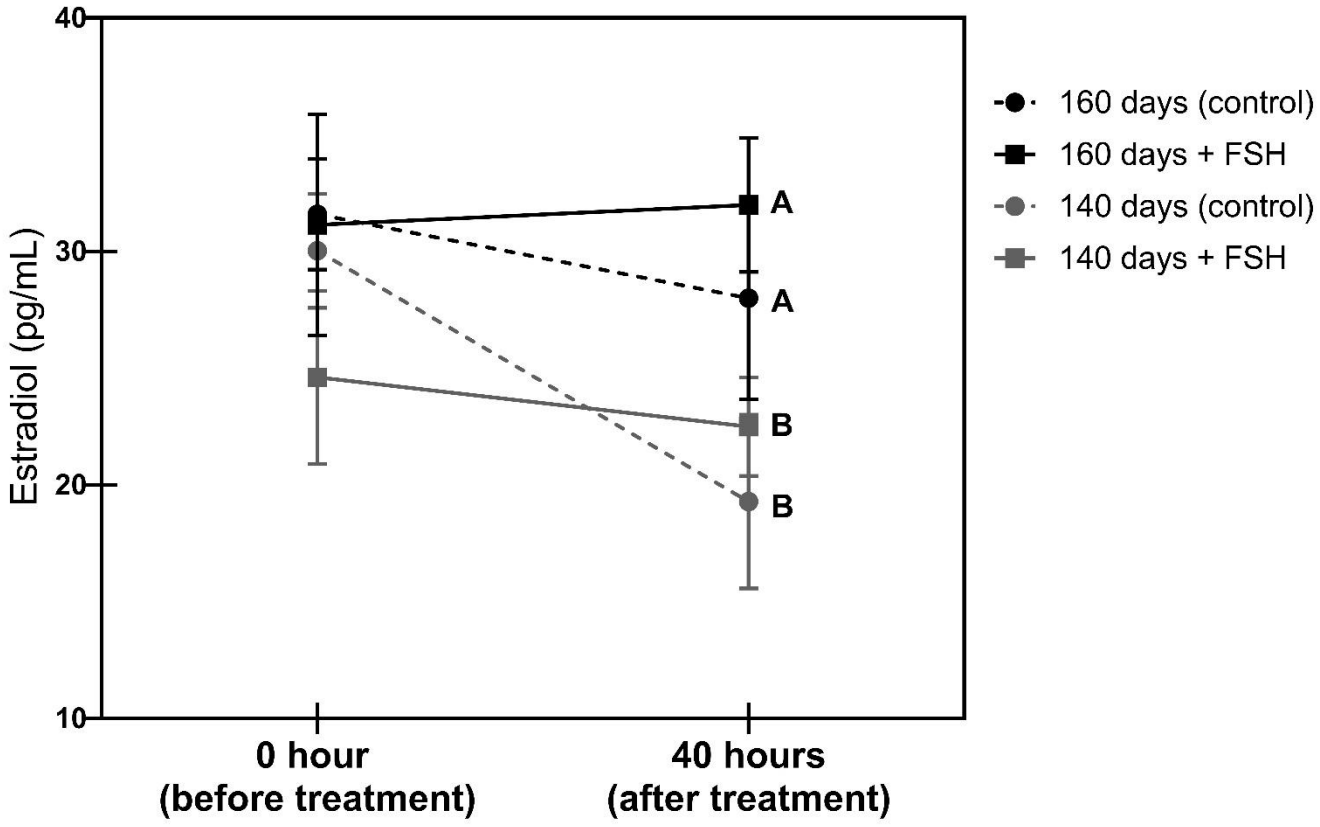

Fig. 2 Plasma estradiol concentrations in prepubertal gilts at 140 or 160 days of age before and after FSH or saline treatment. Data are expressed as mean ± SEM. Different uppercase letters indicate differences ($P < 0.05$) between 140 and 160 days of age groups according to Tukey-Kramer test.

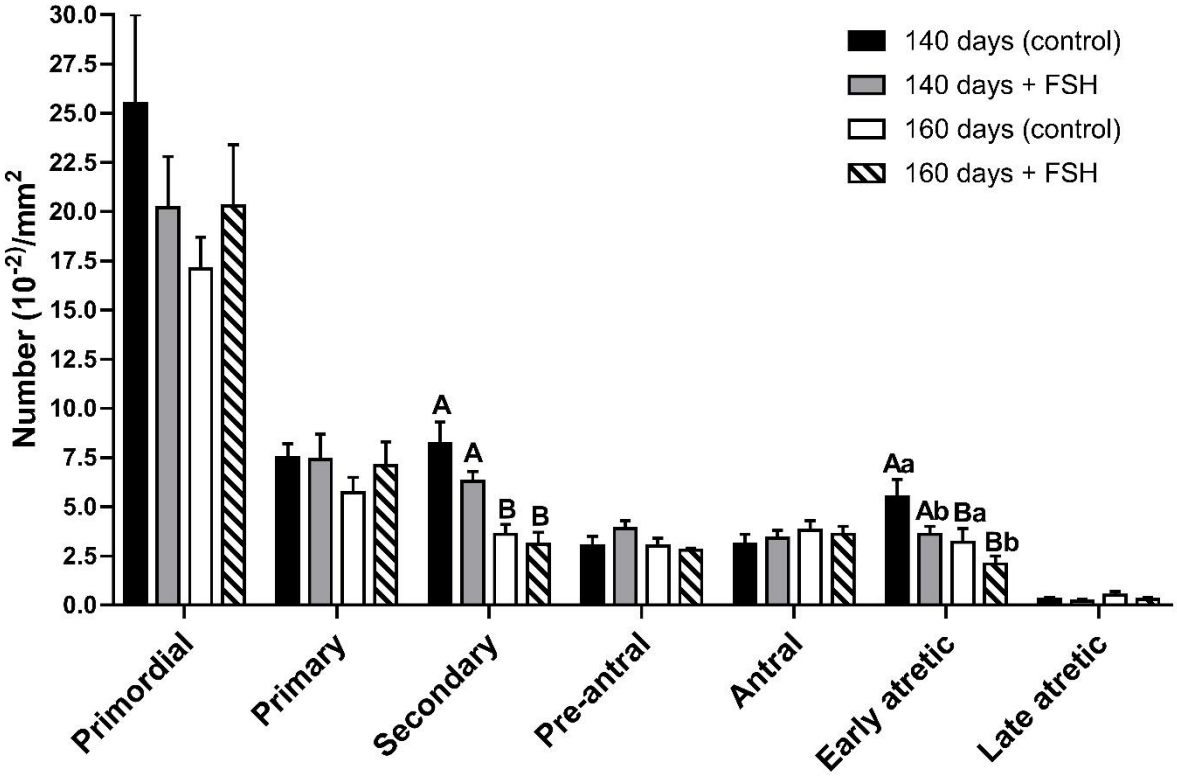

Fig. 3. Number of ovarian follicles (primordial, primary, secondary, pre-antral and antral) and atretic follicles (late and early) per mm^2 of ovarian tissue in gilts at 140 or 160 days of age submitted or not (control) to FSH stimulation (mean ± SEM). Different lower-case letters indicate differences ($P < 0.05$) between FSH and saline (control) groups according to Tukey-Kramer test. Different uppercase letters indicate differences ($P < 0.05$) between 140 days and 160 days groups according to Tukey-Kramer test.

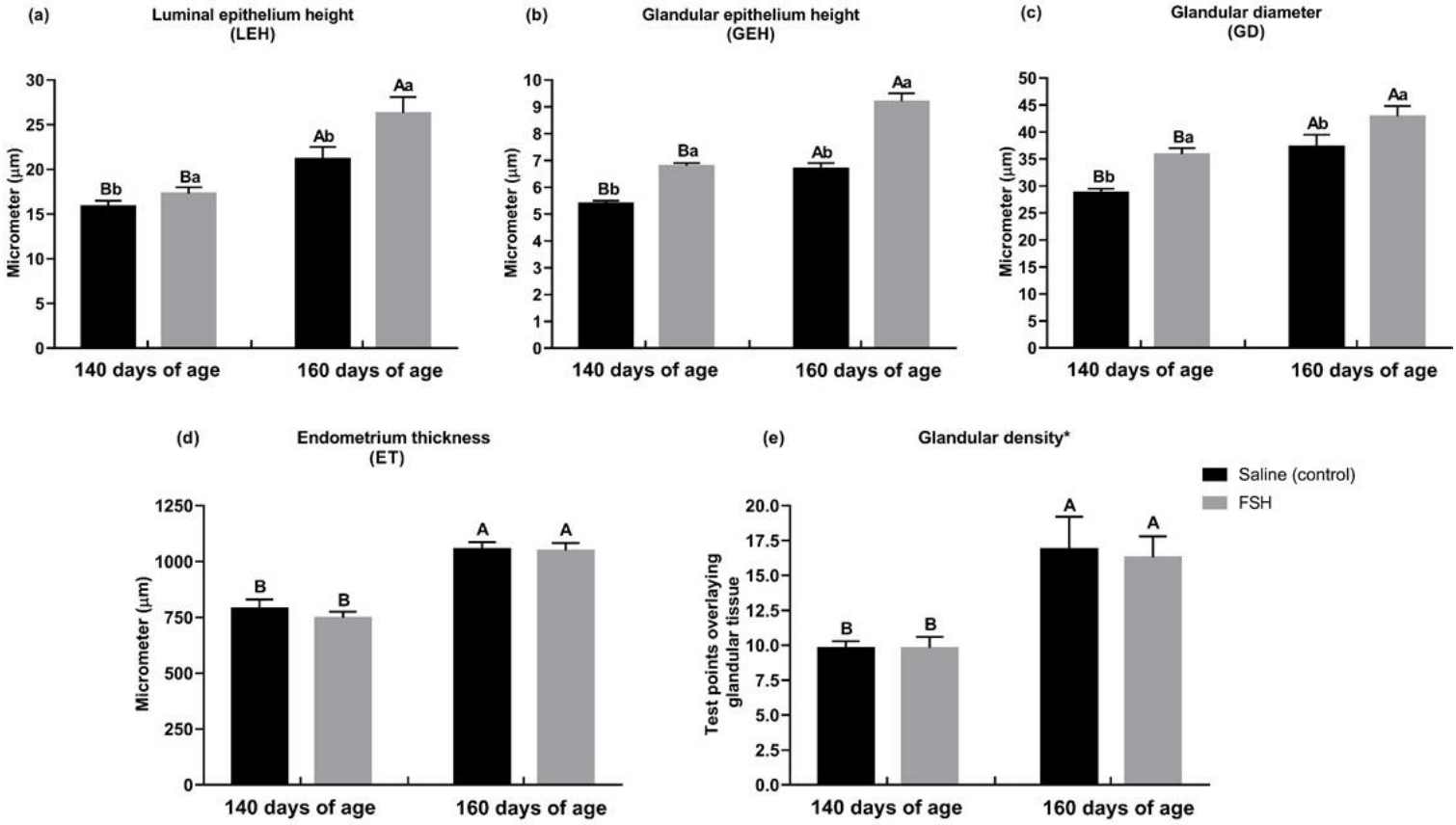

Fig. 4. Endometrium histomorphometry of prepubertal gilts at 140 or 160 days of age submitted or not (control) to FSH stimulation (mean ± SEM). Different lower-case letters indicate differences ($P < 0.05$) between FSH and saline (control) groups according to Tukey-Kramer test. Different uppercase letters indicate differences ($P < 0.05$) between 140 days and 160 days groups according to Tukey-Kramer test.*Expressed as points overlaying glandular tissue using the 90-point Weibel grid.

Supplementary Material

Table S1

Area (mm^2) of antrum, follicle and granulosa layer of mature follicles[1] from gilts at 140 or 160 days of age submitted or not (control) to FSH stimulation (mean ± SEM)

Parameter*	Type of mature follicle[1]	Treatment				*P value*		
		140 days of age		160 days of age				
		Saline (control)	FSH	Saline (control)	FSH	Age	FSH	Age*FSH
Area of follicular antrum (mm^2)	-	0.50 ± 0.06	0.57 ± 0.04	0.50 ± 0.04	0.53 ± 0.03	0.9170	0.4398	0.5387
Area of follicle (mm^2)	-	0.33 ± 0.05	0.39 ± 0.03	0.35 ± 0.04	0.36 ± 0.02	0.6541	0.3039	0.6239
Area of granulosa layer (mm^2)	F1-F4	0.17 ± 0.01	0.18 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.1535	0.0827	0.9700
	F1	0.08 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.6634	0.3966	0.6039
	F2	0.14 ± 0.01	0.13 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.2044	0.7971	0.1020
	F3	0.20 ± 0.01	0.19 ± 0.01	0.17 ± 0.01	0.17 ±0.01	0.0159	0.5240	0.7017
	F4	0.24± 0.04	0.30 ± 0.01	0.24 ± 0.01	0.32 ± 0.04	0.6696	0.0288	0.8361

*Tukey-Kramer test.

[1] Mature follicle were classified according to their diameters into F1 (≤ 0.5 mm), F2 (0.51-0.75 mm), F3 (0.76-1.0 mm) or F4 (>1.0 mm).

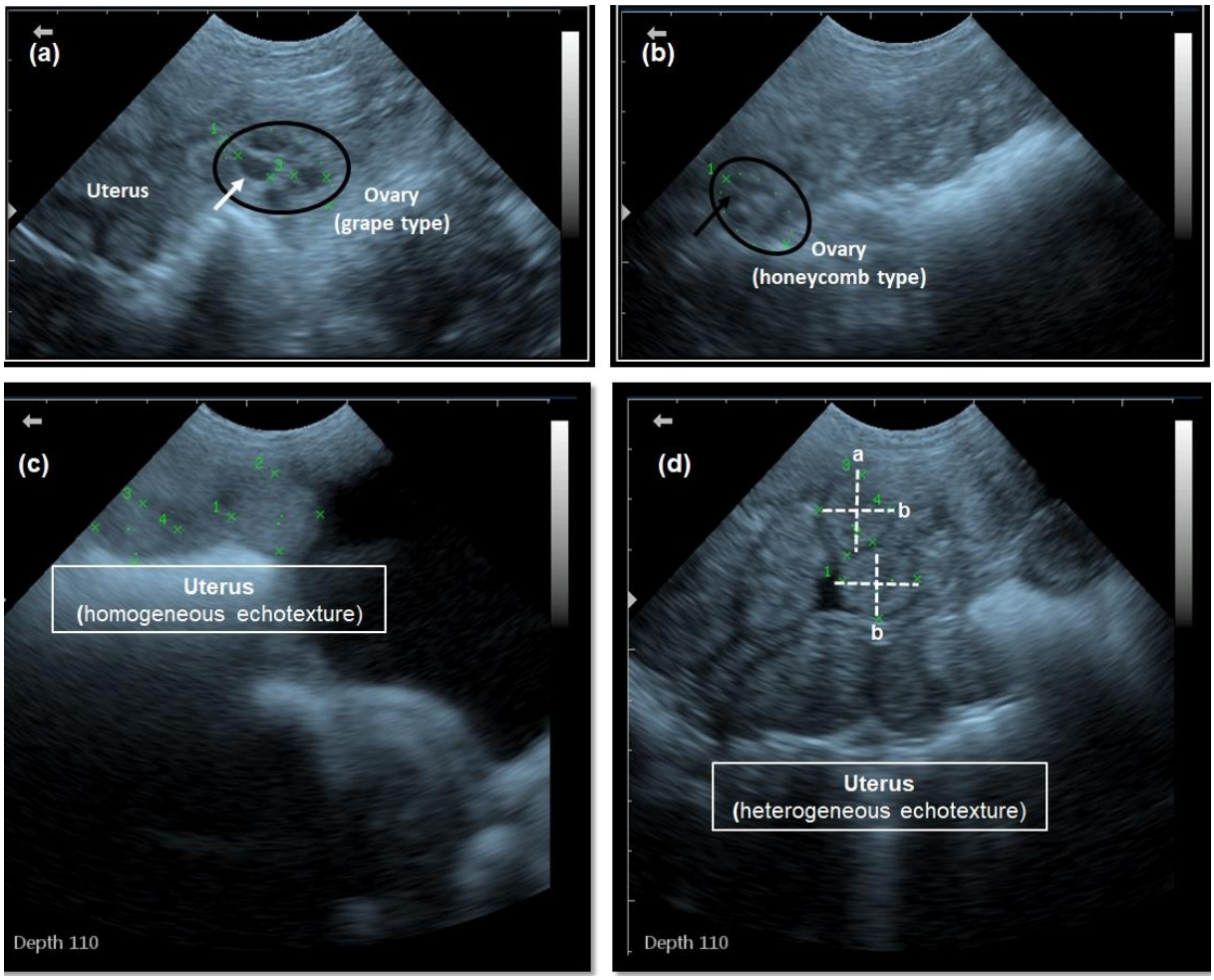

Fig. S1 Ultrasonographic images of ovary (a; b) and uterus (c; d) observed in prepubertal gilts at 160 or 140 days of age stimulated or not with FSH. (a) Grape type: presence of many large follicles (>6 mm). (b) Honeycomb type: presence of numerous small follicles (1-3 mm). (c) Homogeneous uterine echotexture. (d) Heterogeneous uterine echotexture. For uterine evaluation, two measurements (maximum – a; minimum – b) of two cross-sections of uterine horns were obtained to calculate the sectional area for each cross-section. The uterine sectional area for individual gilt (SAsono) was expressed as mean sectional areas calculated for each female. White arrow shows a large follicle; black arrow shows a small follicle.

4. GENERAL CONCLUSIONS

Os resultados obtidos nesta tese indicam que a idade e o estímulo com FSH provocam alterações nos ovários e no útero de marrãs pré-púberes. Em relação ao efeito da idade avaliada neste estudo, o crescimento e o desenvolvimento uterino continuam acontecendo durante os 140 e 160 dias de idade, sendo que alterações biométricas dos ovários não são observadas. No entanto, marrãs pré-púberes com 160 dias de idade apresentam aumento na frequência de ovários morfologicamente semelhantes ao cacho de uva (diversos folículos grandes > 6 mm) e nas concentrações plasmáticas de 17β-estradiol e IGF-1, que podem estimular o crescimento uterino e levar a alterações morfológicas nos componentes do endométrio (epitélio luminal/glandular). Estas alterações podem estar associadas a maturação fisiológica que o trato reprodutivo alcança durante o período pré-puberal para garantir o potencial embriotrófico e a capacidade funcional do útero para manter a prenhez após atingir a puberdade. Além disso, o avanço da idade de marrãs pré-púberes resulta em redução no número de folículos atrésicos iniciais e no aumento da disponibilidade de glicose no fluido folicular, que culminam no incremento do potencial do oócito para atingir a maturação oocitária.

O tratamento com FSH em marrãs pré-púberes com 140 ou 160 dias de idade promove melhora na resposta folicular ovariana associada a efeitos diretos no ambiente folicular, por meio do incremento da morfologia oocitária e no número de complexos cumulus-oócitos viáveis, e favorecimento do metabolismo da glicose em relação ao metabolismo dos triglicerídeos no fluido folicular. Estes efeitos culminam com aumento da capacidade do oócito em alcançar a maturação oocitária, potencialmente associado à melhoria da competência oocitária. Ainda, a administração de 100 mg de FSH dividida em subdoses múltiplas, causa redução no número de folículos atrésicos iniciais e modificação na morfologia ovariana, sem afetar os estágios pré-antrais da foliculogênese. O uso de FSH exógeno não promove alterações na biometria uterina, contudo, estimula a atividade do epitélio endometrial, incluindo o epitélio luminal e glandular. Isto pode estar envolvido na modulação da secreção histotrófica, fornecendo assim um melhor ambiente uterino para o embrião.

Em associação, os resultados apresentados nesta tese indicam que o avanço da idade e o tratamento prévio à aspiração folicular com 100 mg de FSH influenciam positivamente a maturação oocitária em marrãs pré-púberes, por meio da alteração do ambiente folicular. Estas estratégias otimizam e viabilizam o uso de marrãs pré-púberes como doadoras de oócitos em programas de produção *in vitro* de embriões, contribuindo na aceleração do progresso genético ao reduzir o intervalo entre gerações. No entanto, mais estudos são necessários incluindo as

análises da produção *in vitro* e da transferência de embriões, para suportar a validação do uso dessas estratégias na melhoria da eficiência da competência oocitária de oócitos provenientes de marrãs pré-púberes. Além disso, evidenciou-se efeito positivo do aumento da idade e do uso de FSH exógeno nos componentes do endométrio de marrãs pré-púberes. Embora outros estudos demostraram a presença do receptor de FSH de forma funcional no endométrio em diversas espécies, os resultados desta tese é um dos poucos estudos existentes que contribui no entendimento do papel do FSH, proveniente de fonte exógena, na regulação da atividade do endométrio em suínos. Esta ação no endométrio provocada pelo tratamento com FSH fornece perspectiva para utilizar o FSH com a finalidade de aumentar a receptividade endometrial. No entanto, estudos prospectivos são necessários para esclarecer essa ação endometrial promovida pelo tratamento exógeno com FSH e confirmar tais informações.

APPENDIX – CERTIFICATE OF ETHICS COMISSION ON THE USE OF FARM ANIMALS OF UNIVERSIDADE FEDERAL DE VIÇOSA (CEUAP-UFV)

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Viçosa, 03 de Abr. de 2019

CERTIFICADO

Certificamos que o projeto intitulado "**Estratégias para otimizar a produção in vivo e in vitro de embriões suínos**", protocolo **nº 0118/2018**, sob a responsabilidade de **Simone Eliza Facioni Guimarães** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo chordata, subfilo vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 8 de outubro de 2008, do decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo conselho nacional de controle da experimentação animal (concea), e foi apreciado pela comissão de ética no uso de animais de produção da universidade federal de viçosa (ceuap-ufv) em reunião de **13 de Dez. de 2018.**

Finalidade: **(X)Pesquisa ( )Ensino**

Vigência do Projeto: de **03 de Abr. de 2019 a 31 de Jul. de 2022**

Espécie/linhagem: **Suíno (*Sus scrofa*)** Nº de animais: **90**

Peso: **25 a 260 Kg** Idade: **60 a 120 dias** Sexo: **Fêmea** Origem: **Setor de Granja Melhoramento de Suínos DZO/UFV - CPF/CNPJ: 25.944.455/0001-96**

CERTIFICATE

We certify that the project entitled "**Strategies to improve the in vivo and in vitro production of porcine embryo**", protocol **nº 0118/2018**, under the responsibility of **Simone Eliza Facioni Guimarães** - which involves the production, maintenance and/or use of animals belonging to the phylum chordata, subphylum vertebrata (except man), for scientific research purposes (or education) - is in accordance with the law nº. 11.794, of October 8, 2008, Decree nº. 6899 of July 15, 2009, and the rules issued by the Brazilian National Council for Animal Experimentation Control (CONCEA), and was approved by the Ethics Commission on the use of farm animals of Universidade Federal de Viçosa (CEUAP-UFV) in its meeting on **Dec, 13th, 2018**.

Finality: **(X)Research ( )Education**

Duration of the Project: from **Apr, 03th, 2019** to **Jul, 31th, 2022**.

Species / strain: **Swine (*Sus scrofa*)** Nº of animals: **90**

Weight: **25 a 260 Kg** Age: **60 to 120 days** Sex: **Female** Source: **Setor de Granja Melhoramento de Suínos DZO/UFV - CPF/CNPJ: 25.944.455/0001-96**

Luciana Navajas Rennó

Luciana Navajas Rennó

Coordenadora da CEUAP/UFV