



**SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E
BIOQUÍMICA**

**METABOLISMO DE LIPÍDIOS EM COMPLEXO CUMULUS-OVÓCITO
DURANTE A MATURAÇÃO *IN VITRO* EM SUÍNOS**

Aluna: Thainara Christie Ferreira Silva

Orientador: Prof. Dr. Maurício Machaim Franco

Uberlândia - MG

2023



**SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E
BIOQUÍMICA**

**METABOLISMO DE LIPÍDIOS EM COMPLEXO CUMULUS-OVÓCITO
DURANTE A MATURAÇÃO *IN VITRO* EM SUÍNOS**

Aluna: Thainara Christie Ferreira Silva

Orientador: Prof. Dr. Maurício Machaim Franco

Tese apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Doutora em Genética e Bioquímica (Área Genética).

Uberlândia - MG

2023

Dados Internacionais de Catalogação na Publicação (CIP)
Sistema de Bibliotecas da UFU, MG, Brasil.

S586m Silva, Thainara Christie Ferreira, 1991-
2023 Metabolismo de lipídios em complexo cumulus-ovócito durante a
maturação in vitro em suínos [recurso eletrônico] / Thainara Christie
Ferreira Silva. - 2023.

Orientador: Maurício Machaim Franco.
Tese (Doutorado) - Universidade Federal de Uberlândia, Programa
de Pós-Graduação em Genética e Bioquímica.
Modo de acesso: Internet.
Disponível em: <http://doi.org/10.14393/ufu.te.2023.7007>
Inclui bibliografia.

1. Genética. I. Franco, Maurício Machaim, 1965-, (Orient.). II.
Universidade Federal de Uberlândia. Programa de Pós-Graduação em
Genética e Bioquímica. III. Título.

CDU: 575

André Carlos Francisco
Bibliotecário – CRB-6/2047

**METABOLISMO DE LIPÍDIOS EM COMPLEXO CUMULUS-OVÓCITO DURANTE
A MATURAÇÃO *IN VITRO* EM SUÍNOS**

ALUNA: Thainara Christie Ferreira Silva

COMISSÃO EXAMINADORA

Presidente: Prof. Dr. Maurício Machaim Franco

Examinadores: Profa. Dra. Margot Alves Nunes Dode (EMBRAPA)

Dra. Mariana Groke Marques (EMBRAPA)

Prof. Dr. Robson Carlos Antunes (UFU)

Prof. Dr. Nilson Penha Silva (UFU)

Data da Defesa: 02 / 02 / 2023



UNIVERSIDADE FEDERAL DE UBERLÂNDIA
 Coordenação do Programa de Pós-Graduação em Genética e Bioquímica
 Av. Pará 1720, Bloco 2E, Sala 244 - Bairro Umuarama, Uberlândia-MG, CEP 38400-902
 Telefone: +55 (34) 3225-8438 - www.ppggb.ibtec.ufu.br - ppggb@ufu.br



ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Genética e Bioquímica				
Defesa de:	Tese de Doutorado Acadêmico/PPGGB				
Data:	Dois de fevereiro de dois mil e vinte e três	Hora de início:	14:50h	Hora de encerramento:	18:15h
Matrícula do Discente:	11823GBI013				
Nome do Discente:	Thainara Christie Ferreira Silva				
Título do Trabalho:	Metabolismo de lipídios em complexo cumulus-ovócito durante a maturação <i>in vitro</i> em suínos.				
Área de concentração:	Genética				
Linha de pesquisa:	Genética, Epigenética, Biologia e Melhoramento de Plantas e Animais.				
Projeto de Pesquisa de vinculação:	Desenvolvimento de um teste molecular multilocus para seleção e classificação de ovócitos bovinos utilizados na clonagem por transferência nuclear.				

Aos dois dias do mês de fevereiro de dois mil e vinte e três, às 14:50 horas, reuniu-se via web conferência pela Plataforma *Google Meet*, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, Resolução de nº 06/2020 e Resolução nº 19/2022 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Dr^a. Margot Alves Nunes Dode, Dr^a. Mariana Groke Marques, Dr. Robson Carlos Antunes, Dr. Nilson Penha Silva e Dr. Maurício Machaim Franco, orientador (a) do (a) candidato (a) e demais convidados presentes conforme lista de presença. Iniciando os trabalhos o (a) presidente da mesa, Dr. Maurício Machaim Franco apresentou a Comissão Examinadora e o (a) candidato (a), agradeceu a presença do público, e concedeu o (à) Discente a palavra para a exposição do seu trabalho. A duração da apresentação do (a) Discente e o tempo de arguição e resposta foram conforme as normas do Programa de Pós-graduação em Genética e Bioquímica. A seguir o (a) senhor (a) presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir o (a) candidato (a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais. Em face do resultado obtido, a Banca Examinadora considerou o candidato (a):

APROVADO (A).

Esta defesa de Tese de Doutorado é parte dos requisitos necessários à obtenção do título de Doutor. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram

encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.



Documento assinado eletronicamente por **Nilson Penha Silva, Professor(a) do Magistério Superior**, em 02/02/2023, às 18:18, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do [Decreto nº 8.539, de 8 de outubro de 2015](#).



Documento assinado eletronicamente por **Robson Carlos Antunes, Professor(a) do Magistério Superior**, em 02/02/2023, às 18:19, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do [Decreto nº 8.539, de 8 de outubro de 2015](#).



Documento assinado eletronicamente por **Mariana Groke Marques, Usuário Externo**, em 02/02/2023, às 18:20, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do [Decreto nº 8.539, de 8 de outubro de 2015](#).



Documento assinado eletronicamente por **Maurício Machaim Franco, Usuário Externo**, em 02/02/2023, às 18:20, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do [Decreto nº 8.539, de 8 de outubro de 2015](#).



Documento assinado eletronicamente por **Margot Alves Nunes Dode, Usuário Externo**, em 03/02/2023, às 11:00, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do [Decreto nº 8.539, de 8 de outubro de 2015](#).



A autenticidade deste documento pode ser conferida no site https://www.sei.ufu.br/sei/controlador_externo.php?acao=documento_conferir&id_orgao_acesso_externo=0, informando o código verificador **4084922** e o código CRC **C992DCC9**.

AGRADECIMENTOS

A Deus pelo amparo, pela saúde e por toda força necessária para que eu pudesse chegar até aqui. Agradeço Seu amor de Pai que me fez mais forte para alcançar meus objetivos.

Aos meus pais, Vicente e Zilma, que me apoiaram incessantemente, me guiaram e me proporcionaram as condições necessárias para todas as minhas conquistas. Obrigada por serem tão amorosos e dedicados.

Ao meu marido, Guilherme, por compreender a distância e principalmente por me apoiar e tornar mais fácil a nossa caminhada. Agradeço pelo incentivo de sempre, por seu amor e principalmente pelo exemplo que é para mim.

Agradeço aos meus irmãos Phillipe e Graciela, por sempre acreditarem em mim, me dando força e apoio nos momentos difíceis e tornando meus dias melhores.

A minha vó Emiliana, que sempre orou por mim e torceu pelo meu sucesso.

Ao meu orientador, Maurício, por confiar em mim, por me guiar sempre e por tornar possível essa conquista. Obrigada por todos os ensinamentos, por todos os conselhos e pela sua amizade.

Aos amigos que fiz na Embrapa Recursos Genéticos e Biotecnologia, Márcia, Luna, Paloma, Amanda, pelas horas de conversa, pelo companheirismo e parceria principalmente nos momentos em que a saudade da família apertava, pelas muitas piadas e risadas que só nós entendíamos. Agradeço a vocês e à Ligiane e Nayara pela amizade e momentos de aprendizado e diversão.

A todos os funcionários da Embrapa, pesquisadores, técnicos, funcionários da limpeza, Aninha e a Dacilene pelas conversas na hora do almoço.

À Embrapa Recursos Genéticos e Biotecnologia por fornecer toda a estrutura para a realização desse trabalho.

À Universidade Federal de Uberlândia e ao Programa de Pós-Graduação em Genética e Bioquímica pelo programa de doutorado oferecido. Agradeço à secretária do programa, Janaína de Souza Mota, pela dedicação de sempre.

À Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) pela bolsa de estudos oferecida.

SUMÁRIO

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS.....	viii
LISTA DE FIGURAS.....	xii
LISTA DE TABELAS.....	xiv
Apresentação.....	1
Capítulo I – Fundamentação teórica.....	2
Complexo cumulus-ovócito e maturação ovocitária.....	2
Maturação <i>in vitro</i> e o metabolismo de lipídios.....	4
Homeostase lipídica em ovócitos.....	6
Lipogênese e esterificação.....	7
Vias lipolíticas (lipólise e lipofagia).....	9
β -oxidação.....	11
Idade da fêmea e o metabolismo de lipídios.....	13
Referências.....	16
Capítulo II – Cumulus-oocyte complexes from cycling sows show differences in lipid metabolism compared to cumulus-oocyte complexes from prepubertal gilts during <i>in vitro</i> maturation.....	24
Abstract.....	25
Introduction.....	26
Materials and methods.....	29
Experimental design.....	29
Chemicals and reagents.....	29
In vitro maturation and collection of oocyte and cumulus cells.....	29
RNA isolation and cDNA synthesis.....	30
qPCR.....	31
Assessment of lipid droplet accumulation.....	35
DNA isolation and sodium bisulfite treatment.....	36
PCR amplification, cloning, and sequencing.....	36

Statistical analysis.....	38
Results.....	39
Gene expression analysis in cumulus cells.....	39
Lipid droplet accumulation in oocytes.....	39
DNA methylation of FABP3 and NR3C1 genes in cumulus cells and oocytes...	42
Discussion.....	44
Conclusions.....	51
Acknowledgments.....	51
Supplementary data.....	52
References.....	53
Capítulo III – Expression profile of genes related to lipophagy in oocytes from prepubertal gilts and cycling sows during in vitro maturation.....	61
Abstract.....	62
Acknowledgments.....	73
Conflict of Interest.....	73
Author Contributions.....	73
References.....	74
Considerações finais.....	76
ANEXOS.....	77
Extração de RNA de Células do Cumulus e Ovócitos.....	78
Síntese de cDNA.....	80
Extração de DNA de células do cumulus (Salting OUT).....	81
Extração de DNA de ovócito (choque térmico).....	82
Extração de DNA de sêmen (Salting OUT).....	83
Tratamento com bissulfito de sódio.....	85
Clonagem bacteriana.....	86
Mini preparação de plasmídeo – Miniprep.....	90
Coloração de ovócitos (Bodipy).....	91

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

ACACA - *acetyl-CoA carboxylase alpha*

ACACB - *acetyl-CoA carboxylase beta*

ACAD - *acyl-CoA dehydrogenase - short/medium/long-chain*

ACSL - *acyl-CoA synthetase*

ACSS2 - *acyl-CoA synthetase short chain family member 2*

ADRP - *adipose differentiation-related protein*

ATG - *Autophagy related*

ATGL - *adipose triacylglycerol lipase*

ATP – adenosina trifosfato

cAMP - AMP cíclico

CC – Células do cumulus

cDNA - Complementary DNA

CD36 - *cluster of differentiation 36*

CGI-58 - *comparative gene identification – 58*

COC – Complexo cumulus-ovócito

CO₂ – dióxido de carbono

CpG – Citosina-fosfato-Guanina

CPT1 - *Carnitine palmitoyltransferase 1*

CPT2 - *Carnitine palmitoyltransferase 2*

CS-GV - immature COCs of cycling sows

CS-IVM - COCs of cycling sows subjected to the IVM

DG - diacilglicerol

DNA – Ácido desoxirribonucleico

dNTP – Desoxirribonucleotídeos fosfatados

EDTA – Ácido etilenodiamino tetra-acético

FA – ácidos graxos

FABP3 - *fatty acid binding protein 3*

FAO – oxidação de ácidos graxos

FASN - *Fatty Acid Synthase*

FF - fluido folicular

FFA – ácidos graxos livres

GV – Vesícula germinativa

GVBD – Quebra da vesícula germinativa

HADHA - *hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha*

HSD11 β 1 - *Hydroxysteroid 11-beta dehydrogenase 1*

HSL - *hormone-sensitive lipase*

IVM - *in vitro* maturation

LC3 - MAP1LC3B - *microtubule-associated proteins 1A/1B light chain 3B*

LD – Gota lipídica

LH – Hormônio Luteinizante

LPL - *Lipoprotein lipase*

M – Molar

mA – Miliampere

MIV – Maturação *in vitro*

MG - monoacilglicerol

mg – Miligrama

MgCl₂ – Cloreto de magnésio

MGL - *monoacylglycerol lipase*

MII – Metáfase II

min – Minutos

mL – Mililitro

mm – Milímetro

mM – Milimolar

mRNA – RNA mensageiro

mTORC1 - *mechanistic target of rapamycin complex 1*

NR3C1 - *Nuclear receptor subfamily 3 group C member 1*

PAP - *Phosphatidic acid phosphatase*

PBS – Solução salina em tampão fosfato

PCR – Reação em cadeia da polimerase

PG-GV – immature COCs of prepubertal gilts

PG-IVM - COCs of prepubertal gilts subjected to the IVM

pH – Potencial hidrogeniônico

PIVE – Produção *in vitro* de embriões

PKA - proteína-cinase dependente de cAMP

PLIN – Perilipin

PNPLA2 - *patatin-like phospholipase domain-containing protein 2*

p62 - SQSTM1 - *Sequestosome-1*

RABs - *Ras-related protein*

RNA – Ácido ribonucleico

rpm – Rotação por minuto

s – Segundo

SLC27A - long-chain fatty acid transport protein

TBE – Tampão Tris-Borato-EDTA

TCM-199 – Meio de Cultura de Tecidos-199

TFEB - *Transcription Factor EB*

TFE3 - *Transcription factor E3*

TG – Triacilglicerol ou triacilglicerídeo

TIFOI - Transferência intrafolicular de ovócitos imaturos

TIP47 - *tail-interacting protein of 47 kDa*

TRA – Técnica de Reprodução Assistida

U – Unidades

UI – Unidades Internacionais

°C – Graus Celsius

µg – Micrograma

µL – Microlitro

µM – Micromolar

ng – Nanograma

µm – Micrômetro

LISTA DE FIGURAS

Capítulo I

Figura 1: Esquema do desenvolvimento folicular e maturação ovocitária em mamíferos.....	3
Figura 2: Esquema do metabolismo de lipídios em célula de mamíferos.....	13

Capítulo II

Figure 1: Scheme of genes related to lipid metabolism in cumulus cells based on known protein functions in other cell types.....	31
Figure 2: Relative abundance of mRNA encoding the lipid metabolism genes, as determined by RT-qPCR in immature and in vitro matured cumulus cells from prepubertal and cycling female pigs.....	39
Figure 3: Area of lipid droplets relative to the total area of oocytes matured in different systems.....	40
Figure 4: Representative images following confocal microscopy (LSM Leica sp8; 488-nm argon laser, original magnification _20) of lipid droplets (LDs) in Bodipy 493/503-stained pig oocytes.....	40
Figure 5: Methylation pattern of FABP3 (above) and NR3C1 (below) genes in cumulus cells (yellow), oocyte (pink), and sperm (control group).....	41
Figure 6: Analysis of comparative methylation by CpG of FABP3 (above) and NR3C1 (below) genes in cumulus cells (yellow) and oocytes (pink).....	42
Figure 7: Schematic representation of transcription factor (TFs- gray boxes) and its possible binding site in the FABP3 gene (represented by the black line)...	48
Figure S1. Representation of the <i>FABP3</i> and <i>NR3C1</i> genes structures, GC content, and CpG island prediction.....	51

Capítulo III

Figure 1: Scheme of genes related to lipophagy based on protein function known in many cell types.....	66
Figure 2: Relative abundance of mRNA encoding the lipophagy genes, as determined by RT-qPCR in immature and in vitro matured oocytes from cycling sows.....	69

Figure 3: Relative abundance of mRNA encoding the lipophagy genes, as determined by RT-qPCR in immature and in vitro matured oocytes from prepubertal gilts..... 69

LISTA DE TABELAS

Capítulo II

Table 1. Gene nomenclature, GenBank access number, gene function, primer sequences, melt temperature, amplicon sizes and primer efficiency for each gene used for gene expression in qPCR.....	33
Table 2. Gene identification, primer sequences, GenBank accession number, primer positions, CpG island position and amplicon size.....	36
Table 3. Nested PCR conditions for the FABP3 and NR3C1 genes.....	37
Table 4. Transcription factors and binding site sequence the FABP3 gene.....	49

Capítulo III

Table 1: Gene nomenclature, GenBank access number, primer sequences, melt temperature, amplicon sizes and primer efficiency for each gene used for gene expression in qPCR.....	67
--	----

Apresentação

A espécie suína apresenta semelhanças fisiológicas com os seres humanos e por isso é amplamente utilizada em estudos de edição genômica com foco em pesquisas relacionadas a doenças humanas e xenotransplantes. No entanto, a produção *in vitro* de embriões (PIVE) de suínos, necessária para os estudos de edição genômica, possui uma baixa eficiência devido, dentre outras coisas, à competência dos ovócitos, que afeta a maturação *in vitro* (MIV). Sabe-se que ovócitos de fêmeas pré-púberes possuem menor competência, no entanto, as razões permanecem não totalmente compreendidas. Muitos estudos têm relacionado o metabolismo de lipídios à competência do ovócito por ser uma importante fonte energética para a maturação do gameta. Portanto, a compreensão de como a idade da fêmea afeta o metabolismo de lipídios do complexo cumulus-ovócito durante a MIV se faz necessária para a adaptação e melhorias dos protocolos de MIV.

Esta tese está subdividida em três capítulos. No capítulo I é apresentada a fundamentação teórica. Nesse capítulo, é demonstrada a importância do metabolismo de lipídios para a maturação de ovócitos, como esse metabolismo pode ser afetado pela idade da fêmea e todo o conhecimento necessário para o entendimento dos assuntos abordados nos demais capítulos.

O capítulo II está em formato de artigo na língua inglesa. O artigo denominado “Cumulus-oocyte complexes from cycling sows show differences in lipid metabolism compared to cumulus-oocyte complexes from prepubertal gilts during *in vitro* maturation” foi submetido para publicação na revista *Molecular Reproduction and Development* e apresenta resultados da comparação de expressão de genes do metabolismo de lipídios em células do cumulus de marrãs pré-púberes e porcas cíclicas, além de dosagem de lipídios em ovócitos dessas fêmeas e padrão de metilação do DNA de dois genes diferencialmente expressos.

O capítulo III está em formato de artigo na língua inglesa. O artigo denominado “Expression profile of genes related to lipophagy in oocytes from prepubertal gilts and cycling sows during *in vitro* maturation” foi submetido para publicação na revista *Genetics and Molecular Biology* e apresenta as diferenças de expressão de genes relacionados à lipofagia em ovócitos de marrã e porca durante a MIV.

No final, são apresentados em “Anexos” os protocolos utilizados em todos os experimentos realizados nesta tese.

Capítulo I – Fundamentação Teórica

Complexo cumulus-ovócito e maturação ovocitária

Com o crescente avanço das técnicas de reprodução assistida torna-se necessária cada vez mais a compreensão dos mecanismos moleculares do complexo cumulus-ovócito (COC), que é essencial para a aquisição de competência e maturação ovocitária. No COC o gameta feminino é envolvido pelas células do cumulus (CC) que comunicam entre si e com o ovócito através das junções gap (Gilula et al., 1978; Eppig and Ward-Bailey, 1982; Furger et al., 1996), pelas quais são trocados íons, nutrientes e RNAs mensageiros (mRNA), essenciais para o metabolismo do ovócito e preparo do gameta para o desenvolvimento embrionário inicial.

O COC é formado durante a foliculogênese e ovogênese. Inicialmente, as ovogônias formadas a partir da diferenciação das células germinativas primordiais (CGPs), originam os ovócitos primários através do processo de divisão meiótica (Wear et al., 2016). A meiose é interrompida na fase de diplóteno da prófase I e o ovócito é então coberto por uma camada de células da granulosa, formando os folículos primordiais (figura 1). Os folículos primordiais sofrem alteração no formato das células que passam de pavimentoso para cúbico, formando o folículo primário (Fair, 2003). No folículo secundário há aumento das camadas de células somáticas e início da formação da cavidade antral (Andrade et al., 2019). No momento da formação do antro as células da granulosa se separam das CC formando, portanto, o COC (figura 1).

Nos ovócitos de fêmeas mamíferas, como descrito acima, durante a vida pré-natal ocorre o início da divisão meiótica e imediatamente a meiose para em estágio de diplóteno da prófase I (Borum, 1961; Yokoo and Sato, 2004). O ovócito nesse estágio possui um núcleo chamado de vesícula germinativa (GV – do inglês, *germinal vesicle*) e permanece assim durante toda a diferenciação folicular, desde o folículo primordial até o antral (figura 1). *In vivo*, a retomada meiótica se inicia após um pico de hormônio luteinizante (LH), durante a puberdade, que é caracterizado inicialmente pela quebra da vesícula germinativa (GVBD – do inglês, *GV breakdown*). Nesse momento, o gameta sai da fase de diplóteno da prófase I e progride até a metáfase II (MII) (Iwashita et al., 1998; Yokoo and Sato, 2004),

processo conhecido como maturação nuclear (figura 1), e permanece em MII até a fecundação. Durante o período pré-ovulatório, as CCs começam o processo de expansão, onde ficam embutidas em uma matriz mucificada, perdendo a comunicação com o ovócito e entre si (figura 1) (Gilula et al., 1978).

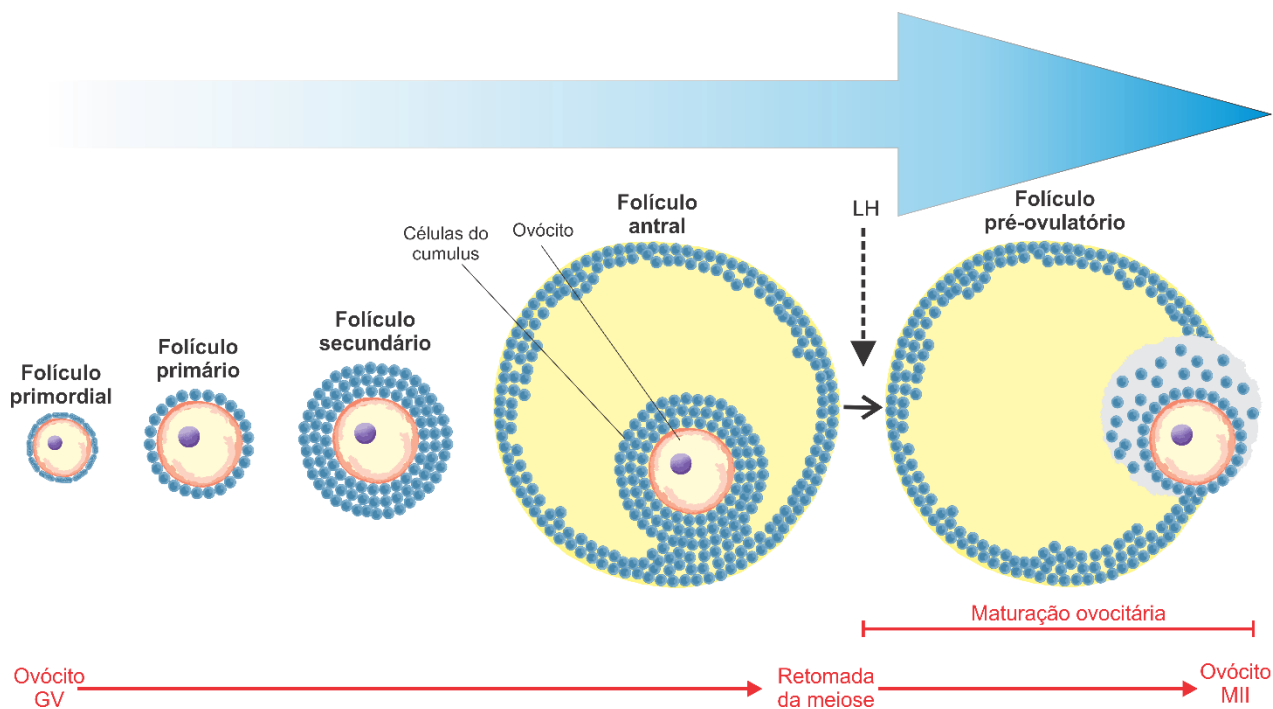


Figura 1. Esquema do desenvolvimento folicular e maturação ovocitária em mamíferos. Durante a vida pré-natal o gameta inicia a divisão meiótica e para no estágio de diplóteno da prófase I (ovócito GV). Os folículos se desenvolvem passando de primordial, primário e secundário para antral. No folículo antral observa-se o ovócito envolvido por células do cumulus, formando o complexo cumulus-ovócito. A retomada meiótica ocorre *in vivo* após o pico de LH durante a puberdade. Ocorre então a maturação ovocitária, onde o gameta finaliza a meiose I e inicia a meiose II até parar novamente em estágio de metáfase II (ovócito MII). No folículo pré-ovulatório ocorre a expansão das células do cumulus, onde essas perdem a conexão entre si e com ovócito e ficam embebidas em uma matriz mucificada.

Concomitante à maturação nuclear descrita acima, o ovócito passa ainda pela maturação citoplasmática e molecular (Fulka Jr et al., 1998; Dekel, 2005). A maturação citoplasmática se caracteriza pela reorganização de organelas mediada pelos microtúbulos e microfilamentos do citoesqueleto, deslocamento das mitocôndrias para áreas de maior gasto energético, deslocamento do complexo de Golgi para área perinuclear e deslocamento dos grânulos corticais para a periferia do ovócito (Roelen, 2020). A maturação molecular é caracterizada pelo acúmulo de

nutrientes, substratos e mRNA, os quais são necessários para o término da maturação, fecundação e para o futuro desenvolvimento embrionário inicial (Watson, 2007; Gottardi and Mingoti, 2009; Macaulay et al., 2016). A maioria desses mRNAs são sintetizados e acumulados no ovócito durante o período de crescimento ovocitário (De Sousa et al., 1998), ou seja, antes da retomada da meiose (figura 1). Após a retomada da meiose, ocorre a condensação dos cromossomos, diminuindo a síntese de mRNA no gameta (Wu et al., 1996; Mamo et al., 2011; Macaulay et al., 2014; Sprícigo et al., 2014). Assim, o desenvolvimento embrionário inicial é altamente dependente dos estoques de mRNAs e proteínas acumulados no ovócito (De Sousa et al., 1998; De La Fuente and Eppig, 2001) até que ocorra a ativação do genoma embrionário (transição materno-zigótica), que em suínos ocorre no estágio de embrião de 4 células (Telford et al., 1990). A sincronia entre a maturação nuclear, citoplasmática e molecular é essencial para a aquisição da competência ovocitária e consequentemente para o desenvolvimento embrionário.

A competência do ovócito é definida como a capacidade intrínseca do gameta de se tornar apto à fecundação, clivar e gerar um embrião viável (Zuccotti et al., 2011) e esta é progressivamente adquirida durante os estágios finais da foliculogênese e ovogênese. Ovócitos competentes são essenciais para a produção *in vitro* de embriões (PIVE), visto que a técnica possui limitações e, portanto, a maior competência ovocitária pode melhorar as taxas de produção de embriões.

Maturação *in vitro* e o metabolismo de lipídios

Um dos passos importantes da PIVE é a maturação *in vitro* (MIV), onde o ovócito passa pela maturação nuclear, citoplasmática e molecular em um ambiente adverso, necessitando de atenção especial. Em fêmeas mamíferas, fisiologicamente, apenas alguns ovócitos são ovulados e aptos à fecundação. A MIV permite explorar uma maior parte dos ovócitos presentes no ovário e, consequentemente, explorar ainda mais o potencial reprodutivo da fêmea. Nesse estágio, muitos eventos ocorrem para tornar o ovócito apto para a fecundação. Dentre esses eventos, o metabolismo de lipídios se destaca por ser uma importante

fonte energética para a maturação e o desenvolvimento embrionário inicial (Yokoo and Sato, 2004; Auclair et al., 2013; Dai et al., 2021).

No geral, a reserva lipídica deve suprir as necessidades energéticas requeridas durante os processos de maturação, fecundação e desenvolvimento embrionário inicial. Portanto, acredita-se que *in vivo* o ovócito e as células do cumulus trabalham juntos para o acúmulo de lipídios durante o período de crescimento ovocitário e maturação nuclear (Silva et al., 2011; Roelen, 2020). Porém, muitos trabalhos têm mostrado que esse acúmulo se intensifica durante a MIV em algumas espécies (Spate et al., 2014; del Collado et al., 2017; Faria et al., 2021). Em suínos, os efeitos adversos do sistema *in vitro* sobre a maturação de ovócitos já foi demonstrado. O perfil transcricional de ovócitos maturados *in vitro* apresentou diferenças comparado com o perfil de ovócitos maturados *in vivo* (Spate et al., 2014). Essas diferenças afetam a competência do ovócito e consequentemente tornam as taxas de maturação e produção de embriões mais baixas (Yuan et al., 2017). As razões pelas quais o ovócito maturado *in vitro* é menos competente que os maturados *in vivo* ainda não são totalmente claras, no entanto, sabe-se que o sistema *in vitro* causa alterações no metabolismo de lipídios do COC (Sanchez-Lazo et al., 2014; Faria et al., 2021).

O metabolismo de lipídios desempenha um papel importante no COC em várias espécies (Prates et al., 2014; Sanchez-Lazo et al., 2014; Braga et al., 2019; Ibayashi et al., 2021), especialmente em suínos, cujos ovócitos possuem um alto conteúdo lipídico (Paczkowski et al., 2013). No entanto, algumas diferenças no metabolismo de lipídios em suínos já foram relatadas em CC e ovócitos durante a MIV (Paczkowski et al., 2013; Braga et al., 2019) quando comparados com outras espécies ou fêmeas de diferentes idades. De acordo com Paczkowski et al., (2013), a oxidação de ácidos graxos (FA – do inglês *fatty acid*) é ainda mais importante na MIV de ovócitos da espécie suína, comparada com as espécies bovina e camundongos, que possuem teor lipídico médio e baixo, respectivamente.

Os lipídios são compostos químicos insolúveis em água com diversas funções biológicas, dentre as quais destaca-se o armazenamento de energia, ou seja, são compostos de suma importância para a vida celular. Os principais lipídios de armazenamento são os óleos e gorduras, que são derivados dos FA. Os FAs são armazenados em forma de triglicerídeos, ou triacilgliceróis (TG), que são as

gorduras mais abundantes nas células dos mamíferos e formam as gotas lipídicas (LD – do inglês *lipid droplet*) (Aardema et al., 2011). As LDs possuem estrutura única, sendo formadas por um núcleo hidrofóbico de lipídios neutros e uma monocamada de membrana fosfolipídica. Na superfície, elas são formadas por muitas proteínas, sendo predominante os membros da família PAT (PLIN – *perilipin*; ADRP - *adipose differentiation-related protein* e TIP47 - *tail-interacting protein of 47 kDa*) (Onal et al., 2017). As proteínas PLIN revestem a LD e a estabilizam e protegem da degradação pelas lipases (Kaushik and Cuervo, 2015). As LDs são abundantes em ovócitos e embriões, principalmente na espécie bovina (Paczkowski et al., 2013; Faria et al., 2021) e suína (McEvoy et al., 2000).

As CCs também contribuem para o acúmulo de lipídios em gametas. Del Collado et al. (2017) mostraram que, em COCs bovinos submetidos à MIV, a proteína FABP3 (*fatty acid binding protein 3*) se liga a ácidos graxos livres (FFA – *free fatty acid*) para transportá-los via *gap junction* das CCs para o ovócito. De acordo com os autores, esse transporte de FA pelo FABP3 ocorre apenas no sistema *in vitro* e resulta em um acúmulo de lipídios no ovócito. Além disso, o funcionamento inadequado de mitocôndrias também contribui para o estoque de lipídios. Faria et al (2021), comparando o sistema de maturação *in vivo* com a MIV e com a transferência intrafolicular de ovócitos imaturos (TIFOI), onde os ovócitos imaturos são transferidos para um folículo pré-ovulatório para serem maturados *in vivo* (Sprícigo and Dode, 2017), observaram que apenas ovócitos submetidos à MIV apresentaram acúmulo de lipídios. Os autores sugerem que esse acúmulo se dá pelo funcionamento deficiente das mitocôndria, resultando em menor gasto energético via β -oxidação (del Collado et al., 2017; Faria et al., 2021).

Homeostase lipídica em ovócitos

A quantidade de lipídios presente no citoplasma de ovócitos varia entre diferentes espécies (Paczkowski et al., 2013). De acordo com McEvoy et al. (2000), ovócitos suínos possuem maior teor de ésteres de ácidos graxos, sendo $161 \pm 18 \mu\text{g}$ a cada 1000 ovócitos. Destes, $74,25 \mu\text{g}$ são de triacilgliceróis e $40,9 \mu\text{g}$ de fosfolipídios. Acredita-se que a maior quantidade de lipídios dos ovócitos nessa espécie ocorra devido ao maior tempo entre ovulação e implantação embrionária,

o que exige maior gasto energético e, portanto, maior acúmulo lipídico (Sturmey et al., 2009).

A remoção de LD em ovócitos MII de camundongos resultam em uma rápida síntese de novas LDs, enquanto a adição de LD, provenientes de ovócitos MII, em embriões resulta em uma quebra rápida dessas novas LDs (Aizawa et al., 2019; Ibayashi et al., 2021). Isso sugere que há mecanismos que regulam a quantidade ideal de lipídios em ovócitos e embriões, ou seja, há um balanço entre síntese e degradação de lipídios para manutenção da homeostase lipídica (Ibayashi et al., 2021). Esses mecanismos, como lipogênese, lipólise e lipofagia, devem manter a quantidade de lipídios essencial para a maturação do ovócito e o desenvolvimento embrionário de cada espécie.

Lipogênese e esterificação

A biossíntese de FA ocorre no citosol e inicia-se pela ativação do acetato para a formação de acetil-CoA pelo ACSS2 (*Acyl-CoA synthetase short chain family member 2*) (Liu et al., 2018), conforme observado na figura 2. Posteriormente, o acetil-CoA é carboxilado pelo ACACA (Acetyl-CoA Carboxylase Alpha) formando malonil-CoA (Barber et al., 2005; Vysochan et al., 2017), em um processo irreversível de duas etapas (figura 2). A transformação de acetil-CoA para formar malonil-CoA por ACACA é uma etapa limitante para a síntese de FA, ou seja, um importante ponto de regulação da síntese de lipídios (Wakil and Abu-Elheiga, 2009). Além disso, o malonil-CoA também bloqueia a β -oxidação através da inibição da CPT1 (Carnitine palmitoyltransferase 1) (McGarry and Brown, 1997; Ruderman et al., 2003), que é um transportador de membrana da mitocôndria (figura 2). A inibição da β -oxidação diminui o gasto energético e consequentemente induz o acúmulo de lipídios.

A partir do acetil-CoA e malonil-CoA, o FASN (*Fatty Acid Synthase*) catalisa a síntese de FA (Wakil and Abu-Elheiga, 2009), conforme observado na figura 2. O FASN é um complexo multienzimático que promove alongamento da cadeia carbonada em quatro etapas. Esse alongamento ocorre inicialmente com dois carbonos provenientes do acetil-CoA, e os demais carbonos, adicionados dois a dois, são sempre provenientes do malonil-CoA até a formação de uma cadeia de 16 carbonos, formando o palmitato. Este é o principal precursor de outros ácidos

graxos de cadeia longa. A formação de demais ácidos graxos, por sua vez, ocorre no retículo endoplasmático liso através do sistema de alongamento de ácidos graxos, onde diferentes enzimas estão envolvidas (Ridgway and McLeod, 2008; Nelson and Cox, 2014).

O aumento de expressão dos genes *ACSS2*, *ACACA* e *FASN* já foi relatado em CCs (Sanchez-Lazo et al., 2014; Del Collado et al., 2017b; Pawlak et al., 2020) e ovócitos (Jin et al., 2017; Faria et al., 2021) durante a MIV. O aumento da lipogênese nessas células é associado ao maior acúmulo de lipídios através da formação de novas LD. A suplementação do fluido folicular (FF) com FA durante a MIV aumentou a expressão de *ACACA* nas CCs e formação de LDs (Pawlak et al., 2020). Em ovócitos, a utilização de etomoxir, um análogo do malonyl-coa, levou à menor taxa de maturação dos ovócitos ou apoptose das células, através da inibição da oxidação de ácidos graxos (FAO – do inglês fatty acid oxidation) (Paczkowski et al., 2013; Sanchez-Lazo et al., 2014; Valsangkar and Downs, 2015). Em camundongos o knockout de *ACACB* (Acetyl-CoA carboxylase beta), que transforma acetil-CoA em malonyl-CoA, resultou em maior FAO e retomada meiótica (Valsangkar and Downs, 2015). Os altos níveis de malonyl-coa suprimem a retomada da meiose e a FAO através do bloqueio de CPT1, que transporta FA para a mitocôndria para gerar ATP. Esses trabalhos mostram que lipogênese e FAO devem atuar de formas opostas. Se ambas ocorressem simultaneamente, esse ciclo constituiria um processo falho e de desperdício de energia (Nelson and Cox, 2014).

A maior parte dos FA sintetizados podem ser armazenados em forma de triacilgliceróis (TG), figura 2. Os TGs possuem dois precursores que são o acil-CoA graxo (figura 2) e o glicerol-3-fosfato. O primeiro é formado a partir de FA pela ação da enzima ACSL (acyl-CoA synthetase). O glicerol-3-fosfato sofre acilação por duas moléculas de acil-CoA graxo formando ácido fosfatídico. Este é então hidrolisado pela enzima PAP (Phosphatidic acid phosphatase) formando 1,2-diacilglicerol (DG). Os DGs são convertidos em TGs com um terceiro acil-CoA graxo por transesterificação misto (Nelson and Cox, 2014). Uma das vias de regulação da formação de TG é através de hormônios glicocorticoides. No fígado, o cortisol se liga ao receptor e induz a expressão de genes (Phosphoenolpyruvate carboxykinase) e consequentemente a formação de TG. No entanto, no tecido

adiposo o cortisol possui efeito oposto, ou seja, diminui a expressão de genes e síntese de TG. Em CCs de humanos, a ligação de cortisol ao seu receptor NR3C1 (do inglês *Nuclear receptor subfamily 3 group C member 1*) induziu a lipólise nessas células durante a MIV e promoveu maior energia para maturação dos ovócitos adjacente e maior qualidade embrionária (Simerman et al., 2015).

Vias lipolíticas (lipólise e lipofagia)

A lipólise e a β -oxidação são essenciais para a maturação ovocitária. Alguns trabalhos têm demonstrado a importância da energia gerada pela FAO em CCs e ovócitos para a MIV (Somfai et al., 2011; Paczkowski et al., 2013; Sanchez-Lazo et al., 2014; Simerman et al., 2015; Jin et al., 2017; Lowe et al., 2017; Carrillo-González et al., 2020; Pawlak et al., 2020). De acordo com Paczkowski et al. (2013), quanto maior o conteúdo lipídico no citoplasma do ovócito mais importante é a FAO para a célula. No entanto, altas taxas de lipídios no citoplasma do ovócito, principalmente provocado pela MIV, comprometem o sucesso, por exemplo, da criopreservação (Galeati et al., 2011; Mateo-Otero et al., 2021) e podem causar lipotoxicidade (Wu et al., 2010; Marei et al., 2019). A criopreservação permite conservar gametas e embriões por um maior período, facilitando o transporte e preservando o potencial reprodutivo de gametas (Yurchuk et al., 2018; Leme et al., 2020). Portanto, alguns estudos têm sido realizados a fim de reduzir o conteúdo lipídico citoplasmático (Takahashi et al., 2013; Carrillo-González et al., 2020; Dias et al., 2020; Xu et al., 2020), principalmente durante a MIV, promovendo maior FAO e maior fornecimento de energia. A lipólise promove liberação de FA, que é então oxidado nas mitocôndrias, processo conhecido como β -oxidação (figura 2). A partir da β -oxidação de FA há o fornecimento de energia (ATP) para manutenção celular, maturação ovocitária e desenvolvimento embrionário.

A lipólise é uma via catabólica que ocorre tanto no citoplasma quanto nas gotas lipídicas e depende da ativação direta de lipases. Nos adipócitos, após sinalização hormonal para a mobilização de TG, ocorre a produção de AMP cíclico (cAMP), que ativa a PKA (proteína-quinase dependente de cAMP). A PKA fosforila HSL (*hormone-sensitive lipase*) e as perilipinas (PLINs) na superfície da LD. Quando fosforiladas as proteínas PLIN sofrem dissociação da proteína CGI-58 (*comparative gene identification – 58*) (Yamaguchi et al., 2004). As PLINs

fosforiladas se associam à HSL fosforiladas, enquanto o CGI-58 se liga e ativa a proteína ATGL ou PNPLA2 (*adipose triacylglycerol lipase* ou *patatin-like phospholipase domain-containing protein 2*). A ATGL quebra o TG em diacilglicerol (DG) liberando um FA. A HSL associada a PLIN hidrolisa o DG formando monoacilglicerol (MG) liberando outro FA. Por fim, a lipase MGL (*monoacylglycerol lipase*) hidrolisa o MG para liberação do terceiro FA e o glicerol. Outra importante lipase é a LPL (*Lipoprotein lipase*), que desempenha um papel importante na quebra de TG em lipoproteínas (figura 2). Porém, Sanchez-Lazo et al. (2014) observaram aumento da expressão de *LPL* em células do cumulus de bovinos durante as primeiras horas de MIV, sugerindo também um papel dessa lipase na maturação de ovócitos.

Outra forma de liberação de FA a partir de TG para produção de energia via β -oxidação é chamada de lipofagia. As pesquisas sobre o metabolismo de lipídios em COCs têm focado principalmente em lipogênese e lipólise (Sanchez-Lazo et al., 2014; Jin et al., 2017; Braga et al., 2019; Faria et al., 2021); no entanto, nos últimos anos, a autofagia seletiva de LD tem sido alvo de novos estudos (Singh et al., 2009). A lipofagia atua juntamente com a lipólise para manutenção da homeostase lipídica (Zechner et al., 2017).

Os mecanismos moleculares envolvidos na lipofagia ainda são pouco compreendidos (Zechner et al., 2017; Shin, 2020). Dentre os mecanismos mais estudados de autofagia, a macroautofagia se destaca. Inicialmente ocorre a formação de uma membrana em torno da LD, conhecida como fagóforo (Van Limbergen et al., 2009), conforme observado na figura 2. O fagóforo é formado por proteínas RABs (*Ras-related protein*) e ATGs (*Autophagy related*). Através da proteína LC3 (MAP1LC3B - *microtubule-associated proteins 1A/1B light chain 3B*) o fagóforo se liga à LD que possui a proteína p62 (SQSTM1 - *Sequestosome-1*) em sua superfície (figura 2) (a presença de p62 na LD é uma marca para que ocorra a lipofagia). O fagóforo se expande com ajuda das proteínas PNPLAs presentes na LD até a fusão das extremidades, formando uma estrutura fechada conhecida como autofagossomo (Van Limbergen et al., 2009), observado na figura 2. Essa estrutura se funde com o lisossomo formando o autolisossomo, onde lipases lisossomais degradam o TG presente nas LDs liberando FFA para o citoplasma da célula (figura 2). As lipases lisossomais são sintetizadas a partir de fatores de transcrição TFEB

(*Transcription Factor EB*) e TFE3 (*Transcription factor E3*), que, por sua vez, são reguladas pelo mTORC1 (*mechanistic target of rapamycin complex 1*) (Shin, 2020; Haidar et al., 2021).

O estudo de lipofagia em ovócitos ainda é escasso, apesar de muitos estudos focarem na autofagia de modo geral (Tsukamoto et al., 2008; Peters et al., 2020), ou seja, não há estudos que mostrem a importância da autofagia de LD para a homeostase lipídica e consequentemente para a maturação ovocitária. Em embriões de camundongo, Tatsumi et al. (2018) induziram a lipofagia e observaram redução no número e tamanho das LD, o que resultou em retardamento do desenvolvimento embrionário. Segundo os autores, esses embriões em que a lipofagia era induzida eram menos propensos à lipotoxicidade. No entanto, a perda da homeostase lipídica prejudicou o desenvolvimento embrionário.

β-oxidação

Os FA liberados via lipólise ou lipofagia são transportados para a mitocôndria para serem oxidados. O processo de β-oxidação é o primeiro passo dessa oxidação e ocorre em quatro etapas para converter os FA de cadeia longa em acetil-CoA. Este entra então no ciclo do ácido cítrico para ser oxidado a CO₂. Para entrar na matriz mitocondrial os FA de cadeia longa (acima de 14 carbonos) precisam passar pelo ciclo da carnitina (Vanella et al., 2000), que possui três etapas. Na primeira, a acil-CoA sintetase, que está presente na membrana externa da mitocôndria, catalisa a ligação do FA à coenzima A formando um acil-CoA graxo (Nelson and Cox, 2014). Na segunda etapa, o acil-CoA graxo é ligado à carnitina pela CPT1 (*Carnitine palmitoyltransferase 1*) (figura 2) formando acil-graxo-carnitina, que é então transportada para o espaço intermembrana. O transportador de acil-carnitina na membrana interna permite a entrada do acil-graxo-carnitina na matriz mitocondrial por difusão facilitada. Na terceira etapa, a CPT2 (*Carnitine palmitoyltransferase 2*) (figura 2), presente na membrana interna, transfere o acil-graxo da carnitina para a coenzima A, regenerando o acil-CoA graxo (Dunning and Robker, 2012).

Na primeira reação da β-oxidação a ACAD (Acyl-CoA Dehydrogenase - short/medium/long-chain) (apresentado na figura 2 como ACADS) produz uma ligação dupla entre os átomos de carbono α e β. Na segunda, uma molécula de

água é adicionada à ligação dupla pela enoil-CoA hidratase. No terceiro passo, a β -hidroxiacil-CoA desidrogenase forma β -cetoacil-CoA. No último passo, chamado de tiólise, a enzima tiolase catalisa a reação de β -cetoacil-CoA com uma molécula livre de coenzima A. Dessa forma, o fragmento de dois carbonos da extremidade carboxílica do FA original é separado em forma de acetil-CoA. As quatro reações se repetem até a liberação total dos grupos acetil-CoA. Quando a cadeia acil-graxo possui 12 carbonos ou mais, as três últimas reações da β -oxidação são catalisadas por um complexo multienzimático associado à membrana interna da mitocôndria, chamado de HADHA (*hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha*) (figura 2), cuja subunidade α possui atividade da enoil-CoA hidratase e β -hidroxiacil-CoA desidrogenase, e a subunidade β possui atividade tiolase (Nelson and Cox, 2014).

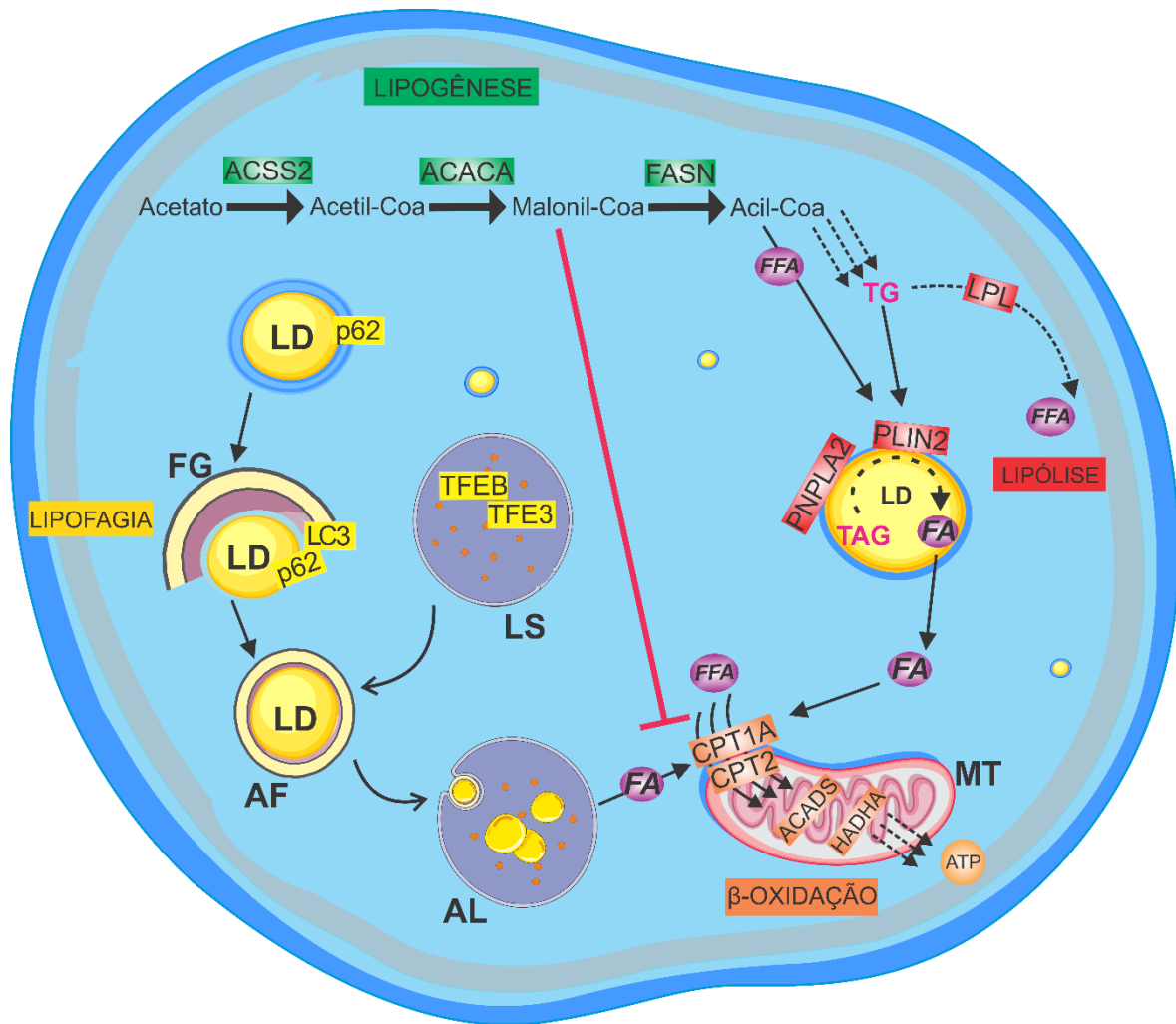


Figura 2. Esquema do metabolismo de lipídios em célula de mamíferos. Em verde observa-se as enzimas responsáveis pela síntese de FFA (em roxo), chamada de lipogênese. Em vermelho observa-se os genes envolvidos na quebra do triacilglicerol (lipólise), tanto no citoplasma quanto na LD, para liberação dos FA. Em amarelo destaca-se as enzimas responsáveis pela autofagia/lipofagia das LDs. A LD é englobada pelo fagóforo formando o autofagossomo. Este se une ao lisossomo formando o autolisossomo, onde o triacilglicerol da LD é quebrado também para liberar FA. Os FA são então transportados para as mitocôndrias e sofrem oxidação (β -oxidação, em laranja) para formação de energia. FFA = ácido graxo livre; FA = ácido graxo; TG ou TAG = triacilglicerol; ATP = adenosina trifosfato; LD = gota lipídica; MT = mitocôndria; FG = fagóforo; AF = autofagossomo; LS = lisossomo; AL = autolisossomo.

Idade da fêmea e o metabolismo de lipídios

A importância dos lipídios para o COC e para o desenvolvimento embrionário levanta a questão sobre os aspectos que afetam o metabolismo de lipídios. A idade da fêmea é um parâmetro que vem sendo amplamente estudado em várias espécies por ser um dos fatores que afeta o metabolismo de lipídios em ovócitos e células do cumulus. Na espécie humana, as pesquisas focam no envelhecimento

dos ovócitos e seus prejuízos para a reprodução (Ben-Meir et al., 2015; Liu et al., 2018b; Llonch et al., 2021). No entanto, nos animais os estudos focam em ovócitos de fêmeas mais jovens comparados com fêmeas adultas (Braga et al., 2019; Succu et al., 2021; Malyszka et al., 2023). No geral, ovócitos de fêmeas que não atingiram a puberdade possuem menor competência de desenvolvimento (Marchal et al., 2001; Gruben et al., 2003; Paczkowski and Krisher, 2010; Paczkowski et al., 2011; Pawlak et al., 2012; Braga et al., 2019).

Em suínos, ovócitos de marrãs pré-púberes são rotineiramente utilizadas para a PIVE (Grabowska et al., 2016; Moreira et al., 2016; Yuan et al., 2017). Portanto, identificar de que forma a idade afeta o complexo cumulus-ovócito e entender os aspectos moleculares que tornam o ovócito de fêmeas pré-púberes menos competente se faz cada vez mais necessário para o desenvolvimento de novos protocolos de PIVE. Nesse sentido, o metabolismo de lipídios é um excelente ponto de partida, pois alguns trabalhos já mostraram alterações metabólicas em gametas de marrãs pré-púberes durante a MIV. Braga et al. (2019) mostraram diferenças na composição lipídica e expressão de genes do metabolismo lipídico durante a MIV em ovócitos de marrãs pré-púberes comparados com os de porcas cíclicas. Além disso, Pawlak et al., (2012) observaram diferenças com relação a maturação citoplasmática nos ovócitos e na composição de ácidos graxos no fluido folicular (FF) também em fêmeas de diferentes idades na espécie suína. Essas diferenças encontradas em animais com diferentes maturidades sexuais mostram a importância do metabolismo de lipídios para a aquisição de competência e maturação do ovócito.

Ovócitos de marrãs pré-púberes apresentaram maior expressão de *CPT2*, gene relacionado à β -oxidação, e de *PLIN2*, cuja proteína está presente na superfície da LD, durante a MIV, sugerindo que durante esse período ocorra maior lipólise e oxidação de FA nessas células do que nos ovócitos de porca (Braga et al., 2019). Além disso, Braga et al. (2019) também encontraram aumento de expressão de *SLC27A1* (*Long-chain fatty acid transport protein 1*), cuja proteína, localizada na membrana celular, transporta FA do meio externo (FF) para o citoplasma, nos ovócitos de porcas cíclicas durante a MIV, enquanto ovócitos de marrã pré-púbere não apresentaram diferença de expressão. Isso sugere um maior acúmulo de lipídios nos ovócitos de porca e maior gasto de energia nos ovócitos

de marrã (Braga et al., 2019). De acordo com Faria et al (2021), o maior acúmulo de lipídios em ovócitos de bovinos se dá pela menor atividade mitocondrial. Segundo Pedersen et al. (2016), a localização de LD e morfologia e quantidade de mitocôndrias em ovócitos de marrãs pré-púberes sofrem alterações durante a MIV, o que não ocorre nos ovócitos de fêmeas púberes. Esses resultados reforçam a ideia de diferenças no metabolismo de lipídios geradas por maior atividade mitocondrial nos gametas das fêmeas jovens. A nutrição pode explicar a diferença de metabolismo lipídico nos COCs desses animais, uma vez que ela afeta a composição hormonal e de FA no sangue e consequentemente no FF (Warzych et al., 2011; Moreira et al., 2016; Naskar et al., 2016; Pawlak et al., 2018).

Diferentemente da MIV de outras espécies animais, a MIV de suínos requer suplementação com fluido folicular (FF) (Pawlak et al., 2020). Este é composto principalmente por FAs, no entanto, sua composição é inconstante, o que gera uma variação significativa na maturação dos ovócitos (Malyszka et al., 2023). A concentração de FA no FF depende de muitos fatores, como a idade da fêmea (Grupe et al., 2003; Pawlak et al., 2012; Naskar et al., 2016; Pawlak et al., 2018), tamanho do folículo (Bender et al., 2010) e estação do ano (Zeron et al., 2001).

O fluido folicular de fêmeas pré-púberes apresentam maior conteúdo de FA total, sendo composto principalmente por ácidos palmítico e oleico (Pawlak et al., 2018). De acordo com Itami et al. (2018), altas concentrações de ácido palmítico podem ser prejudiciais para o ovócito suíno. Já o FF de fêmeas suínas púberes possuem maior quantidade de ácidos graxos poliinsaturados, linoleico e aracdônico (Pawlak et al., 2018). Segundo Pawlak et al (2020), a suplementação do meio de maturação com ácidos graxos insaturados altera a expressão de genes do metabolismo lipídico em CC e aumenta a taxa de blastocistos.

A suplementação do meio de maturação com FF de fêmeas suínas com diferentes idades sexuais altera diferentemente a expressão de genes do metabolismo de lipídio (*PLIN2* e *ACACA*) em embriões (Pawlak et al., 2018). Além disso, a diferença de composição de FA no FF também afeta o metabolismo de lipídios em CCs e ovócitos (Malyszka et al., 2023). Fluido folicular com alto teor de FA provocou maior acúmulo de lipídios em CCs e ovócitos de marrã pré-púberes, enquanto baixo teor de FA reduziu a quantidade de lipídios nos gametas durante a MIV (Malyszka et al., 2023). Essa maior acúmulo de lipídios em CCs em meios com

alto teor de FA é justificado pelo papel das CCs em regular o acúmulo e metabolismo de lipídios no ovócito, protegendo-o contra excesso de FA e lipotoxicidade durante a maturação (Aardema et al., 2013; Lolicato et al., 2015). Alguns estudos com CC bovinas já demonstraram que a composição do FF afeta os níveis de lipídios (Aardema et al., 2013; Lolicato et al., 2015).

Ovócitos e CCs possuem proteínas de membrana que transportam FA do meio externo para o interior da célula, como por exemplo CD36 (*cluster of differentiation 36*, ou FAT - *fatty acid translocase*) e SLC27A1 (Sanchez-Lazo et al., 2014; Simerman et al., 2015; Braga et al., 2019). No entanto, as CCs são capazes de filtrar e diminuir o acesso de FA prejudiciais ao ovócito (Aardema et al., 2013; Lolicato et al., 2015; Aardema et al., 2017). Os FA provenientes do FF são então armazenados em LD ou utilizados como fonte de energia via β -oxidação (Simerman et al., 2015). Além disso, o FF é também composto por hormônios que afetam o metabolismo de lipídios em COCs. Naskar et al. (2016) mostraram que os níveis de cortisol no FF são afetados pela maturidade sexual da fêmea suína e pelos níveis do hormônio no sangue. O cortisol, que pode ser convertido a partir da cortisona pelo HSD11 β 1 (*Hydroxysteroid 11-beta dehydrogenase 1*), se liga ao seu receptor NR3C1 (*Nuclear receptor subfamily 3 group C member 1*) nas CC ou ovócito e promove a expressão de genes que induzem lipólise na célula (Simerman et al., 2015). Simerman et al., (2015) mostraram que a maior concentração de cortisol no FF humano diminuiu a quantidade de lipídios nas CC, sugerindo a atividade lipolítica desse hormônio. Juntos, esses trabalhos demonstram a importância do conteúdo do FF para o metabolismo de lipídios em COCs durante a MIV e consequentemente para o desenvolvimento embrionário. Portanto, o uso de um FF padronizado pode ajudar a melhorar a qualidade dos ovócitos suínos maturados *in vitro*.

Neste trabalho, objetivou-se identificar de que forma a idade da fêmea afeta o metabolismo de lipídios em COC de fêmeas suínas e entender os aspectos moleculares que tornam o ovócito de marrã pré-púbere menos competente.

Referências

- Aardema, H., et al., (2013). "Bovine cumulus cells protect maturing oocytes from increased fatty acid levels by massive intracellular lipid storage." Biology of reproduction **88**(6): 164, 161-115. <https://doi.org/10.1095/biolreprod.112.106062>
- Aardema, H., et al., (2017). "Stearoyl-CoA desaturase activity in bovine cumulus cells protects the oocyte against saturated fatty acid stress." Biology of reproduction **96**(5): 982-992. <https://doi.org/10.1095/biolreprod.116.146159>
- Aardema, H., et al., (2011). "Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence." Biology of reproduction **85**(1): 62-69. <https://doi.org/10.1095/biolreprod.110.088815>
- Aizawa, R., et al., (2019). "Synthesis and maintenance of lipid droplets are essential for mouse preimplantation embryonic development." Development **146**(22): dev181925. <https://doi.org/10.1242/dev.181925>
- Andrade, G. M., et al., (2019). "Intrafollicular barriers and cellular interactions during ovarian follicle development." Animal Reproduction **16**: 485-496. <https://doi.org/10.21451/1984-3143-AR2019-0051>
- Auclair, S., et al., (2013). "Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes." American Journal of Physiology-Endocrinology and Metabolism **304**(6): E599-E613. <https://doi.org/10.1152/ajpendo.00469.2012>
- Barber, M. C., et al., (2005). "Structure and regulation of acetyl-CoA carboxylase genes of metazoa." Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids **1733**(1): 1-28. <https://doi.org/10.1016/j.bbalip.2004.12.001>
- Ben-Meir, A., et al., (2015). "Coenzyme Q10 restores oocyte mitochondrial function and fertility during reproductive aging." Aging Cell **14**(5): 887-895. <https://doi.org/10.1111/accel.12368>
- Bender, K., et al., (2010). "Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows." Reproduction **139**(6): 1047-1055. <https://doi.org/10.1530/REP-10-0068>
- Borum, K. (1961). "Oogenesis in the mouse: a study of the meiotic prophase." Experimental cell research **24**(3): 495-507. [https://doi.org/10.1016/0014-4827\(61\)90449-9](https://doi.org/10.1016/0014-4827(61)90449-9)
- Braga, T. F., et al., (2019). "The dynamics of gene expression, lipid composition and DNA methylation reprogramming are different during in vitro maturation of pig oocytes obtained from prepubertal gilts and cycling sows." Reproduction in Domestic Animals **54**(9): 1217-1229. <https://doi.org/10.1111/rda.13501>
- Carrillo-González, D. F., et al., (2020). "l-Carnitine supplementation during in vitro maturation and in vitro culture does not affect the survival rates after vitrification and warming but alters Inf-T and ptgs2 gene expression." International Journal of Molecular Sciences **21**(16): 5601. <https://doi.org/10.3390/ijms21165601>
- Dai, J. G., et al., (2021). "Mechanisms of lipid metabolism promoted by berberine via peroxisome proliferator-activated receptor gamma during in vitro maturation of porcine oocytes." Animal Science Journal **92**(1): e13582. <https://doi.org/10.1111/asj.13582>
- De La Fuente, R. and J. J. Eppig (2001). "Transcriptional activity of the mouse oocyte genome: companion granulosa cells modulate transcription and chromatin remodeling." Developmental Biology **229**(1): 224-236. <https://doi.org/10.1006/dbio.2000.9947>

- De Sousa, P. A., et al., (1998). "Temporal patterns of embryonic gene expression and their dependence on oogenetic factors." Theriogenology **49**(1): 115-128. [https://doi.org/10.1016/S0093-691X\(97\)00406-8](https://doi.org/10.1016/S0093-691X(97)00406-8)
- Dekel, N. (2005). "Cellular, biochemical and molecular mechanisms regulating oocyte maturation." Molecular and cellular endocrinology **234**(1-2): 19-25. <https://doi.org/10.1016/j.mce.2004.09.010>
- Del Collado, M., et al., (2017b). "In vitro maturation impacts cumulus–oocyte complex metabolism and stress in cattle." Reproduction **154**(6): 881-893. <https://doi.org/10.1530/REP-17-0134>
- del Collado, M., et al., (2017). "Fatty acid binding protein 3 and transzonal projections are involved in lipid accumulation during in vitro maturation of bovine oocytes." Scientific reports **7**(1): 1-13. <https://doi.org/10.1038/s41598-017-02467-9>
- Dias, L. R. O., et al., (2020). "Effect of delipidant agents during in vitro culture on the development, lipid content, gene expression and cryotolerance of bovine embryos." Reproduction in domestic animals **55**(1): 11-20. <https://doi.org/10.1111/rda.13579>
- Dunning, K. R. and R. L. Robker (2012). "Promoting lipid utilization with L-carnitine to improve oocyte quality." Animal reproduction science **134**(1-2): 69-75. <https://doi.org/10.1016/j.anireprosci.2012.08.013>
- Eppig, J. J. and P. F. Ward-Bailey (1982). "The mechanism of cumulus cell-oocyte uncoupling: Evidence for the participation of both cumulus cells and oocytes." Gamete Research **6**(2): 145-154. <https://doi.org/10.1002/mrd.1120060208>
- Fair, T. (2003). "Follicular oocyte growth and acquisition of developmental competence." Animal reproduction science **78**(3-4): 203-216. [https://doi.org/10.1016/S0378-4320\(03\)00091-5](https://doi.org/10.1016/S0378-4320(03)00091-5)
- Faria, O., et al., (2021). "Maturation system affects lipid accumulation in bovine oocytes." Reproduction, Fertility and Development **33**(5): 372-380. <https://doi.org/10.1071/RD20254>
- Fulka Jr, J., et al., (1998). "Nuclear and cytoplasmic determinants involved in the regulation of mammalian oocyte maturation." Molecular human reproduction **4**(1): 41-49. <https://doi.org/10.1093/molehr/4.1.41>
- Furger, C., et al., (1996). "Human granulosa cells in culture exhibit functional cyclic AMP-regulated gap junctions." MHR: Basic science of reproductive medicine **2**(8): 541-548. <https://doi.org/10.1093/molehr/2.8.541>
- Galeati, G., et al., (2011). "Pig oocyte vitrification by cryotop method: effects on viability, spindle and chromosome configuration and in vitro fertilization." Animal reproduction science **127**(1-2): 43-49. <https://doi.org/10.1016/j.anireprosci.2011.07.010>
- Gilula, N. B., et al., (1978). "Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex." The Journal of cell biology **78**(1): 58-75. <https://doi.org/10.1083/jcb.78.1.58>
- Gottardi, F. and G. Mingoti (2009). "Maturação de oócitos bovinos e influência na aquisição da competência para o desenvolvimento do embrião." Revista Brasileira de Reprodução Animal **33**(2): 82 - 94.
- Grabowska, R., et al., (2016). "Quality of oocytes in prepubertal and pubertal swine." Turkish Journal of Veterinary and Animal Sciences **40**(1): 89-94. <https://doi.org/10.3906/vet-1502-16>
- Gruppen, C. G., et al., (2003). "Relationship between donor animal age, follicular fluid steroid content and oocyte developmental competence in the pig."

- Reproduction, Fertility and Development **15**(2): 81-87.
<https://doi.org/10.1071/RD02086>
- Haidar, M., et al., (2021). "Lipophagy: a new player in CNS disorders." Trends in Endocrinology & Metabolism **32**(11): 941-951.
<https://doi.org/10.1016/j.tem.2021.08.010>
- Ibayashi, M., et al., (2021). "Homeostatic regulation of lipid droplet content in mammalian oocytes and embryos." Reproduction **162**(6): R99-R109.
<https://doi.org/10.1530/REP-21-0238>
- Itami, N., et al., (2018). "Palmitic acid induces ceramide accumulation, mitochondrial protein hyperacetylation, and mitochondrial dysfunction in porcine oocytes." Biology of reproduction **98**(5): 644-653. <https://doi.org/10.1093/biolre/ioy023>
- Iwashita, J., et al., (1998). "Essential role of germinal vesicle material in the meiotic cell cycle of *Xenopus* oocytes." Proceedings of the National Academy of Sciences **95**(8): 4392-4397. <https://doi.org/10.1073/pnas.95.8.4392>
- Jin, J. X., et al., (2017). "Melatonin regulates lipid metabolism in porcine oocytes." Journal of pineal research **62**(2): e12388. <https://doi.org/10.1111/jpi.12388>
- Kaushik, S. and A. M. Cuervo (2015). "Degradation of lipid droplet-associated proteins by chaperone-mediated autophagy facilitates lipolysis." Nature cell biology **17**(6): 759-770. <https://doi.org/10.1038/ncb3166>
- Leme, L., et al., (2020). "Effect of sex on cryotolerance of bovine embryos produced in vitro." Theriogenology **141**: 219-227.
<https://doi.org/10.1016/j.theriogenology.2019.05.002>
- Liu, M.-J., et al., (2018b). "Resveratrol improves in vitro maturation of oocytes in aged mice and humans." Fertility and Sterility **109**(5): 900-907.
<https://doi.org/10.1016/j.fertnstert.2018.01.020>
- Liu, X., et al., (2018). "Acetate production from glucose and coupling to mitochondrial metabolism in mammals." Cell **175**(2): 502-513. e513.
<https://doi.org/10.1016/j.cell.2018.08.040>
- Llouch, S., et al., (2021). "Single human oocyte transcriptome analysis reveals distinct maturation stage-dependent pathways impacted by age." Aging Cell **20**(5): e13360. <https://doi.org/10.1111/accel.13360>
- Lolicato, F., et al., (2015). "The cumulus cell layer protects the bovine maturing oocyte against fatty acid-induced lipotoxicity." Biology of reproduction **92**(1): 16, 11-16. <https://doi.org/10.1095/biolreprod.114.120634>
- Lowe, J., et al., (2017). "Supplementation of culture medium with L-carnitine improves the development and cryotolerance of in vitro-produced porcine embryos." Reproduction, Fertility and Development **29**(12): 2357-2366.
<https://doi.org/10.1071/RD16442>
- Macaulay, A. D., et al., (2014). "The gametic synapse: RNA transfer to the bovine oocyte." Biology of Reproduction **91**(4): 90, 91-12.
<https://doi.org/10.1095/biolreprod.114.119867>
- Macaulay, A. D., et al., (2016). "Cumulus cell transcripts transit to the bovine oocyte in preparation for maturation." Biology of reproduction **94**(1): 16, 11-11.
<https://doi.org/10.1095/biolreprod.114.127571>
- Malyszka, N., et al., (2023). "Distinct dynamics of lipid accumulation by porcine cumulus cells during in vitro maturation with follicular fluid of low and high fatty acid contents." Theriogenology **195**: 93-102.
<https://doi.org/10.1016/j.theriogenology.2022.10.015>
- Mamo, S., et al., (2011). "Sequential analysis of global gene expression profiles in immature and in vitro matured bovine oocytes: potential molecular markers

- of oocyte maturation." BMC Genomics **12**(1): 151. <https://doi.org/10.1186/1471-2164-12-151>
- Marchal, R., et al., (2001). "Meiotic and developmental competence of prepubertal and adult swine oocytes." Theriogenology **56**(1): 17-29. [https://doi.org/10.1016/S0093-691X\(01\)00539-8](https://doi.org/10.1016/S0093-691X(01)00539-8)
- Marei, W. F., et al., (2019). "Proteomic changes in oocytes after in vitro maturation in lipotoxic conditions are different from those in cumulus cells." Scientific reports **9**(1): 1-12. <https://doi.org/10.1038/s41598-019-40122-7>
- Mateo-Otero, Y., et al., (2021). "Cryopreservation and oxidative stress in porcine oocytes." Research in Veterinary Science **135**: 20-26. <https://doi.org/10.1016/j.rvsc.2020.12.024>
- McEvoy, T., et al., (2000). "Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida." Journal of reproduction and fertility **118**(1): 163-170. <https://doi.org/10.1530/reprod/118.1.163>
- McGarry, J. D. and N. F. Brown (1997). "The mitochondrial carnitine palmitoyltransferase system—from concept to molecular analysis." European journal of biochemistry **244**(1): 1-14. <https://doi.org/10.1111/j.1432-1033.1997.00001.x>
- Moreira, F., et al., (2016). "Metabolic and reproductive parameters in prepubertal gilts after omega-3 supplementation in the diet." Animal reproduction science **170**: 178-183. <https://doi.org/10.1016/j.anireprosci.2016.05.008>
- Naskar, S., et al., (2016). "Steroid and metabolic hormonal profile of porcine serum vis-à-vis ovarian follicular fluid." Veterinary world **9**(11): 1320. <https://doi.org/10.14202/vetworld.2016.1320-1323>
- Nelson, D. L. and M. M. Cox (2014). "Princípios de bioquímica de Lehninger." Porto Alegre: Artmed. **6. ed.**: 1220p.
- Onal, G., et al., (2017). "Lipid droplets in health and disease." Lipids in health and disease **16**(1): 1-15. <https://doi.org/10.1186/s12944-017-0521-7>
- Paczkowski, M. and R. Krisher (2010). "Aberrant protein expression is associated with decreased developmental potential in porcine cumulus–oocyte complexes." Molecular reproduction and development **77**(1): 51-58. <https://doi.org/10.1002/mrd.21102>
- Paczkowski, M., et al., (2013). "Comparative importance of fatty acid beta-oxidation to nuclear maturation, gene expression, and glucose metabolism in mouse, bovine, and porcine cumulus oocyte complexes." Biology of reproduction **88**(5): 111, 111-111. <https://doi.org/10.1095/biolreprod.113.108548>
- Paczkowski, M., et al., (2011). "Alterations in the transcriptome of porcine oocytes derived from prepubertal and cyclic females is associated with developmental potential." Journal of animal science **89**(11): 3561-3571. <https://doi.org/10.2527/jas.2011-4193>
- Pawlak, P., et al., (2012). "No single way to explain cytoplasmic maturation of oocytes from prepubertal and cyclic gilts." Theriogenology **78**(9): 2020-2030. <https://doi.org/10.1016/j.theriogenology.2012.07.023>
- Pawlak, P., et al., (2020). "Fatty acid induced lipolysis influence embryo development, gene expression and lipid droplet formation in the porcine cumulus cells." Biology of reproduction. <https://doi.org/10.1093/biolre/iaaa045>
- Pawlak, P., et al., (2018). "The consequences of porcine IVM medium supplementation with follicular fluid become reflected in embryo quality, yield and gene expression patterns." Scientific reports **8**(1): 1-12. <https://doi.org/10.1038/s41598-018-33550-4>

- Pedersen, H. S., et al., (2016). "Ultrastructure and mitochondrial numbers in pre- and postpubertal pig oocytes." Reproduction, Fertility and Development **28**(5): 586-598. <https://doi.org/10.1071/RD14220>
- Peters, A. E., et al., (2020). "Autophagy in female fertility: a role in oxidative stress and aging." Antioxidants & redox signaling **32**(8): 550-568. <https://doi.org/10.1089/ars.2019.7986>
- Prates, E., et al., (2014). "A role of lipid metabolism during cumulus-oocyte complex maturation: impact of lipid modulators to improve embryo production." Mediators of inflammation **2014**. <https://doi.org/10.1155/2014/692067>
- Ridgway, N. and R. McLeod (2008). Biochemistry of lipids, lipoproteins and membranes, Elsevier.
- Roelen, B. A. (2020). "Bovine oocyte maturation: Acquisition of developmental competence." Reproduction, Fertility and Development **32**(2): 98-103. <https://doi.org/10.1071/RD19255>
- Ruderman, N. B., et al., (2003). "Minireview: malonyl CoA, AMP-activated protein kinase, and adiposity." Endocrinology **144**(12): 5166-5171. <https://doi.org/10.1210/en.2003-0849>
- Sanchez-Lazo, L., et al., (2014). "Fatty acid synthesis and oxidation in cumulus cells support oocyte maturation in bovine." Molecular endocrinology **28**(9): 1502-1521. <https://doi.org/10.1210/me.2014-1049>
- Shin, D. W. (2020). "Lipophagy: molecular mechanisms and implications in metabolic disorders." Molecules and Cells **43**(8): 686.
- Silva, R. C., et al., (2011). "Ultrastructural characterization of porcine oocytes and adjacent follicular cells during follicle development: lipid component evolution." Theriogenology **76**(9): 1647-1657. <https://doi.org/10.1016/j.theriogenology.2011.06.029>
- Simerman, A. A., et al., (2015). "Intrafollicular cortisol levels inversely correlate with cumulus cell lipid content as a possible energy source during oocyte meiotic resumption in women undergoing ovarian stimulation for in vitro fertilization." Fertility and sterility **103**(1): 249-257. <https://doi.org/10.1016/j.fertnstert.2014.09.034>
- Singh, R., et al., (2009). "Autophagy regulates lipid metabolism." Nature **458**(7242): 1131-1135. <https://doi.org/10.1038/nature07976>
- Somfai, T., et al., (2011). "Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes." Reproduction, Fertility and Development **23**(7): 912-920. <https://doi.org/10.1071/RD10339>
- Spate, L. D., et al., (2014). "Dickkopf-related protein 1 inhibits the WNT signaling pathway and improves pig oocyte maturation." PloS one **9**(4): e95114. <https://doi.org/10.1371/journal.pone.0095114>
- Sprícigo, J., et al., (2014). "Vitrification of bovine oocytes at different meiotic stages using the Cryotop method: assessment of morphological, molecular and functional patterns." Cryobiology **69**(2): 256-265. <https://doi.org/10.1016/j.cryobiol.2014.07.015>
- Sprícigo, J. F. W. and M. A. N. Dode (2017). "Transferência intrafolicular de ovócitos imaturos (TIFOI) em bovinos." Embrapa Recursos Genéticos e Biotecnologia-Artigo em periódico indexado (ALICE).
- Sturmey, R., et al., (2009). "Role of fatty acids in energy provision during oocyte maturation and early embryo development." Reproduction in domestic animals **44**: 50-58. <https://doi.org/10.1111/j.1439-0531.2009.01402.x>

- Succu, S., et al., (2021). "Vitrification of In Vitro Matured Oocytes Collected from Adult and Prepubertal Ovaries in Sheep." JoVE (Journal of Visualized Experiments)(173): e62272. <https://doi.org/10.3791/62272-v>
- Takahashi, T., et al., (2013). "Supplementation of culture medium with L-carnitine improves development and cryotolerance of bovine embryos produced in vitro." Reproduction, Fertility and Development **25**(4): 589-599. <https://doi.org/10.1071/RD11262>
- Tatsumi, T., et al., (2018). "Forced lipophagy reveals that lipid droplets are required for early embryonic development in mouse." Development **145**(4): dev161893. <https://doi.org/10.1242/dev.161893>
- Telford, N. A., et al., (1990). "Transition from maternal to embryonic control in early mammalian development: a comparison of several species." Molecular Reproduction and Development **26**(1): 90-100. <https://doi.org/10.1002/mrd.1080260113>
- Tsukamoto, S., et al., (2008). "The role of autophagy during the oocyte-to-embryo transition." Autophagy **4**(8): 1076-1078. <https://doi.org/10.4161/auto.7065>
- Valsangkar, D. S. and S. M. Downs (2015). "Acetyl CoA carboxylase inactivation and meiotic maturation in mouse oocytes." Molecular reproduction and development **82**(9): 679-693. <https://doi.org/10.1002/mrd.22505>
- Van Limbergen, J., et al., (2009). "Autophagy: from basic science to clinical application." Mucosal immunology **2**(4): 315-330. <https://doi.org/10.1038/mi.2009.20>
- Vanella, A., et al., (2000). "L-propionyl-carnitine as superoxide scavenger, antioxidant, and DNA cleavage protector." Cell Biology and Toxicology **16**(2): 99-104. <https://doi.org/10.1023/A:1007638025856>
- Vysochan, A., et al., (2017). "ACSS2-mediated acetyl-CoA synthesis from acetate is necessary for human cytomegalovirus infection." Proceedings of the National Academy of Sciences **114**(8): E1528-E1535. <https://doi.org/10.1073/pnas.1614268114>
- Wakil, S. J. and L. A. Abu-Elheiga (2009). "Fatty acid metabolism: target for metabolic syndrome." Journal of lipid research **50**(Supplement): S138-S143. <https://doi.org/10.1194/jlr.R800079-JLR200>
- Warzych, E., et al., (2011). "Maternal nutrition affects the composition of follicular fluid and transcript content in gilt oocytes." Vet Med **56**(4): 156-167. <https://doi.org/10.17221/1573-VETMED>
- Watson, A. J. (2007). "Oocyte cytoplasmic maturation: a key mediator of oocyte and embryo developmental competence." Journal of Animal Science **85**(13 Suppl): E1-3. <https://doi.org/10.2527/jas.2006-432>
- Wear, H. M., et al., (2016). "From primordial germ cells to primordial follicles: a review and visual representation of early ovarian development in mice." Journal of ovarian research **9**(1): 1-11. <https://doi.org/10.1186/s13048-016-0246-7>
- Wu, B., et al., (1996). "Temporal distinctions in the synthesis and accumulation of proteins by oocytes and cumulus cells during maturation in vitro of bovine oocytes." Molecular Reproduction and Development **45**(4): 560-565. [https://doi.org/10.1002/\(SICI\)1098-2795\(199612\)45:4<560::AID-MRD18>3.0.CO;2-#](https://doi.org/10.1002/(SICI)1098-2795(199612)45:4<560::AID-MRD18>3.0.CO;2-#)
- Wu, L. L.-Y., et al., (2010). "High-fat diet causes lipotoxicity responses in cumulus–oocyte complexes and decreased fertilization rates." Endocrinology **151**(11): 5438-5445. <https://doi.org/10.1210/en.2010-0551>

- Xu, H., et al., (2020). "The Effect of L-Carnitine Additive During In Vitro Maturation on the Vitrification of Pig Oocytes." Cellular Reprogramming **22**(4): 198-207.
<https://doi.org/10.1089/cell.2020.0014>
- Yamaguchi, T., et al., (2004). "CGI-58 interacts with perilipin and is localized to lipid droplets: possible involvement of CGI-58 mislocalization in Chanarin-Dorfman syndrome." Journal of Biological Chemistry **279**(29): 30490-30497.
<https://doi.org/10.1074/jbc.M403920200>
- Yokoo, M. and E. Sato (2004). "Cumulus-oocyte complex interactions during oocyte maturation." International review of cytology **235**: 251-282.
[https://doi.org/10.1016/S0074-7696\(04\)35006-0](https://doi.org/10.1016/S0074-7696(04)35006-0)
- Yuan, Y., et al., (2017). "Quadrupling efficiency in production of genetically modified pigs through improved oocyte maturation." Proceedings of the National Academy of Sciences **114**(29): E5796-E5804.
<https://doi.org/10.1073/pnas.1703998114>
- Yurchuk, T., et al., (2018). "Science of cryopreservation in reproductive medicine—Embryos and oocytes as exemplars." Early human development **126**: 6-9.
<https://doi.org/10.1016/j.earlhumdev.2018.08.016>
- Zechner, R., et al., (2017). "Cytosolic lipolysis and lipophagy: two sides of the same coin." Nature Reviews Molecular Cell Biology **18**(11): 671-684.
<https://doi.org/10.1038/nrm.2017.76>
- Zeron, Y., et al., (2001). "Seasonal changes in bovine fertility: relation to developmental competence of oocytes, membrane properties and fatty acid composition of follicles." Reproduction **121**(3): 447-454.
<https://doi.org/10.1530/rep.0.1210447>
- Zuccotti, M., et al., (2011). "What does it take to make a developmentally competent mammalian egg?" Human reproduction update **17**(4): 525-540.
<https://doi.org/10.1093/humupd/dmr009>

Capítulo II – Cumulus-oocyte complexes from cycling sows show differences in lipid metabolism compared to cumulus-oocyte complexes from prepubertal gilts during *in vitro* maturation

Artigo submetido à revista Molecular Reproduction and Development

Thainara Christie Ferreira Silva¹, Margot Alves Nunes Dode^{2,3}, Thiago Felipe Braga^{4,5}, Mariana Groke Marques⁶, Luna Nascimento Vargas¹, Otávio Augusto Costa de Faria³, Andressa Pereira de Souza⁷, Daniela Albring⁸, Alexandre Rodrigues Caetano² and Maurício Machaim Franco^{1,2,9,10*}

¹ Programa de Pós-Graduação em Genética e Bioquímica, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil;

² Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil;

³ Pós-graduação em Biologia Animal, Universidade de Brasília, Brasília, DF, Brazil;

⁴ Centro Universitário do Cerrado, Patrocínio, MG, Brazil;

⁵ Instituto Master de Ensino Presidente Antônio Carlos, Araguari, MG, Brazil;

⁶ Embrapa Suínos e Aves, Concórdia, SC, Brazil;

⁷ Universidade do Estado de Santa Catarina – UDESC, SC, Brazil;

⁸ Universidade do Contestado – UnC, SC, Brazil;

⁹ Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil.

¹⁰ Instituto de Biotecnologia, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil;

*Corresponding author:

E-mail: mauricio.franco@embrapa.br

Abstract

This study aimed to evaluate the effects of donor age on lipid metabolism during *in vitro* maturation (IVM) of pigs cumulus-oocyte complexes (COCs). We evaluated transcript levels of genes, measured total lipid content, and evaluated DNA methylation in COCs from prepubertal gilts and cycling sows. The expression of six genes (*ACACA*, *ACSS2*, *FASN*, *FABP3*, *SLC27A4*, *PLIN2*) increased after 44h of IVM in the sow group. In the gilt group, only *FASN* expression increased, while *NR3C1* expression decreased after IVM. The measurement of lipid droplets in oocytes showed an accumulation of lipids in sow oocytes during IVM, while gilt oocytes showed a decrease in lipid droplets. *FABP3* and *NR3C1* methylation patterns exhibited a demethylation pattern in cumulus cells (CCs) and oocytes from gilts and sows and showed statistical differences between groups. CCs from sows had a better capacity to change the transcription levels of the major genes involved in lipid metabolism during IVM than CCs from gilts. This difference may be involved in the accumulation of lipids, acquisition of competence, and maturation of enclosed oocytes. Our results contribute to a better understanding of the mechanisms involved in lipid metabolism and the acquisition of competence in porcine COCs.

Keywords: donor age, embryo production, fatty acid, gene expression, pig.

Introduction

In vitro maturation (IVM) is an essential step in *in vitro* embryo production because it determines embryonic development. It has been increasingly studied and used in humans (De Vos et al., 2021) and it is widely used in domestic animals. Immature pig (*Sus scrofa*) oocytes are also used for various *in vitro* techniques, such as somatic cell nuclei transfer, cloning, and transgenic animal production (Lunney, 2007). In addition, owing to their biological similarities, pigs are used as models for studying human diseases and xenotransplantation.

However, the success of *in vitro* maturation depends on the competence of the female gamete since only competent oocytes can generate a viable embryo. Competence is influenced by many factors including the age of the oocyte donor. In general, oocytes from younger females, such as prepubertal animals, have a decreased capacity to develop into embryos in many species (Braga et al., 2019; Grupen et al., 2003; Marchal et al., 2001; Pawlak et al., 2012). Nevertheless, oocytes from prepubertal gilts are routinely used in pigs for basic research and *in vitro* embryos (Grabowska et al., 2016; Moreira et al., 2016; Yuan et al., 2017), despite having lower developmental competence than those of cyclic sows (Braga et al., 2019; Sherrer et al., 2004). Although the reasons for the oocytes of younger females being less competent than those of cycling females have been extensively studied (Braga et al., 2019; Cocero et al., 2019; Fang et al., 2016; Grupen et al., 2003; Hendriks et al., 2015; Romar et al., 2011; Schwarzer et al., 2014), they remain unclear.

Recently, we found that genes involved in lipid metabolism showed different transcript levels in less competent oocytes during IVM (Braga et al., 2019), suggesting that lipid metabolism may play an important role in the acquisition of

competence. Lipids are important signaling molecules in oocytes (Dunning et al., 2014; Paczkowski et al., 2014; Prates et al., 2014). Lipid metabolism is essential in both cumulus cells (CCs) and oocytes. It is a crucial energy source for gametes (Auclair et al., 2013; Prates et al., 2014; Yokoo & Sato, 2004), especially in pigs, whose oocytes have a high lipid content (Paczkowski et al., 2013).

Cumulus cells play a fundamental role in gamete maturation and subsequent embryonic development as they provide nutrients to the oocyte and supply part of the oocyte's energy (Aardema et al., 2013; Auclair et al., 2013; Lolicato et al., 2015; Macaulay et al., 2016; Prates et al., 2014; Sanchez-Lazo et al., 2014; Tatemoto et al., 2000; Yokoo & Sato, 2004). CCs maintain a bidirectional connection between themselves and oocytes (gap junctions), which is essential for the synchrony between follicular growth and oocyte development and maturation *in vivo* and *in vitro* (Aardema et al., 2019; Yokoo & Sato, 2004; Zuccotti et al., 2011). They transfer paracrine factors, regulatory signals, and messenger RNA molecules (mRNA) (Macaulay et al., 2014; Macaulay et al., 2016) to the oocyte through these gap junctions. In addition, the lipid metabolism in CCs acts as a protection to the oocyte against the excess of fatty acids present in the follicular fluid (Pawlak et al., 2020). These are essential for oocytes to acquire competence for fertilization and embryo production (Barrett & Albertini, 2010; Dieci et al., 2013; Macaulay et al., 2016).

Based on our previous results, where oocytes showed differences in gene expression (Braga et al., 2019), and on the bidirectional communication between oocytes and CCs, we hypothesized that major genes involved in lipid metabolism in CCs from prepubertal gilts may have different expression patterns compared with CCs from cycling sows during IVM. Furthermore, we measured lipid droplets in the oocytes from both groups of animals. Finally, we investigated whether the

expression of some differentially expressed genes was controlled by DNA methylation in CCs and oocytes from both groups.

Materials and methods

Experiments were performed per the Brazilian Law for Animal Protection and approved by the Ethics Committee on Animal Use at EMBRAPA (Brazilian Agricultural Research Corporation) Genetic Resources and Biotechnology.

Experimental design

Immature and *in vitro* matured cumulus–oocyte complexes were collected from prepubertal gilts and cycling sows. In total, eight groups were analyzed: immature oocytes from prepubertal gilts and from cycling sows, *in vitro* matured oocytes from gilts and from sows, cumulus cells not submitted to IVM system from prepubertal gilts and from cycling sows, and cumulus cells submitted to IVM from gilts and sows. Cumulus cells submitted and non-submitted to IVM from gilts and sows were used for analysis of gene expression and DNA methylation. Oocytes were used to measure lipids and DNA methylation.

Chemicals and reagents

The chemicals and reagents used to prepare the IVM medium were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

***In vitro* maturation and collection of oocyte and cumulus cells**

The biological material was collected and handled as described by Braga et al. (2019). Prepubertal gilts and cycling sows were separated in the corrals and were identified phenotypically between sows and gilts (female that has had at least one litter and nulliparous, respectively). Furthermore, they were slaughtered separately. Only ovaries containing the corpus luteum or albicans were used for the sow group. Ovaries were collected and immediately transported to the laboratory in a saline solution at 30–35 °C. Cumulus–oocyte complexes (COCs) were aspirated from follicles having 2–6 mm diameter within a maximum period of one hour after

collection. COCs with homogenous cytoplasm and more than three compact layers of cumulus cells were used and divided into four experimental groups: (1) immature COCs of prepubertal gilts (PG-GV); (2) COCs of prepubertal gilts subjected to the *in vitro* maturation system (PG-IVM); (3) immature COCs of cycling sows (CS-GV); (4) COCs of cycling sows subjected to the *in vitro* maturation system (CS-IVM). Immature oocytes are identified as GV (germinal vesicle) and matured oocytes as IVM (*in vitro* matured).

In vitro maturation was performed following the protocol described by Marques et al. (2007) and Braga et al. (2019). COCs were subjected to *in vitro* maturation for 44 h in TCM199 supplemented with 3.05 mM glucose, 0.91 mM sodium pyruvate, 50 IU/mL gentamycin, 0.57 mM cysteine, 10% (v/v) porcine follicular fluid (from cycling sows), 10 ng/mL EGF, 10 UI/mL eCG and 10 UI/mL hCG for the first 22 h at 38 °C and 5% CO₂ (v/v) in air. Then, the COCs were washed and moved to IVM media without eCG and hCG for 22 h. Immature COCs and COCs subjected to IVM from prepubertal and cycling females were denuded by repeated pipetting. Cumulus cells were frozen at –80 °C in RNA*later* (Applied Biosystems Foster City, CA, USA) for gene expression analysis. Oocytes were fixed and stained for lipid droplet measurements. CCs and oocytes were frozen at –80 °C in phosphate buffered saline (PBS) for methylation analyses.

RNA isolation and cDNA synthesis

Four pools of CCs from an average of 50 COCs from each experimental group were used to isolate the total RNA. Total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen - Venlo, NL) according to the manufacturer's instructions and quantified using the QuantiFluor RNA System (Promega - Madison, WI, USA). Complementary DNA (cDNA) synthesis was performed using GoScript Reverse Transcriptase

(Promega), 72,45 ng of total RNA for all samples and 0.5 µg/µL of Oligo-dT primers (500 µg/mL; Promega), and 0.5 µg/µL of random primers (500 µg/mL; Promega) in a final volume of 30 µL. Reactions were performed at 25 °C for 5 min and 42 °C for 60 min, followed by enzyme inactivation at 70 °C for 15 min.

qPCR

Gene selection was based on the involvement of these genes in lipid metabolism. The functions of each gene are shown in figure 1. We evaluated the expression levels of acyl-CoA synthetase short-chain family member 2 (*ACSS2*), acetyl-CoA carboxylase alpha (*ACACA*), and fatty acid synthase (*FASN*) for lipogenesis. The long-chain fatty acid transport protein 4 (*SLC27A4*) and fatty acid binding protein 3 (*FABP3*) genes were selected for transport. We chose carnitine palmitoyltransferase I A (*CPT1*), carnitine palmitoyltransferase 2 (*CPT2*), acyl-CoA dehydrogenase short-chain (*ACADS*), and hydroxyacyl-coa dehydrogenase trifunctional multienzyme complex subunit alpha (*HADHA*) for the β-oxidation.

The genes related to lipolysis were perilipin 2 (*PLIN2*), patatin-like phospholipase domain containing 2 (*PNPLA2*), lipoprotein lipase (*LPL*), hydroxysteroid 11-beta dehydrogenase 1 (*HSD11β1*), and nuclear receptor subfamily 3 group C member 1 (*NR3C1*). The gene nomenclature, GenBank accession numbers, gene functions, and primer sequences are listed in Table 1.

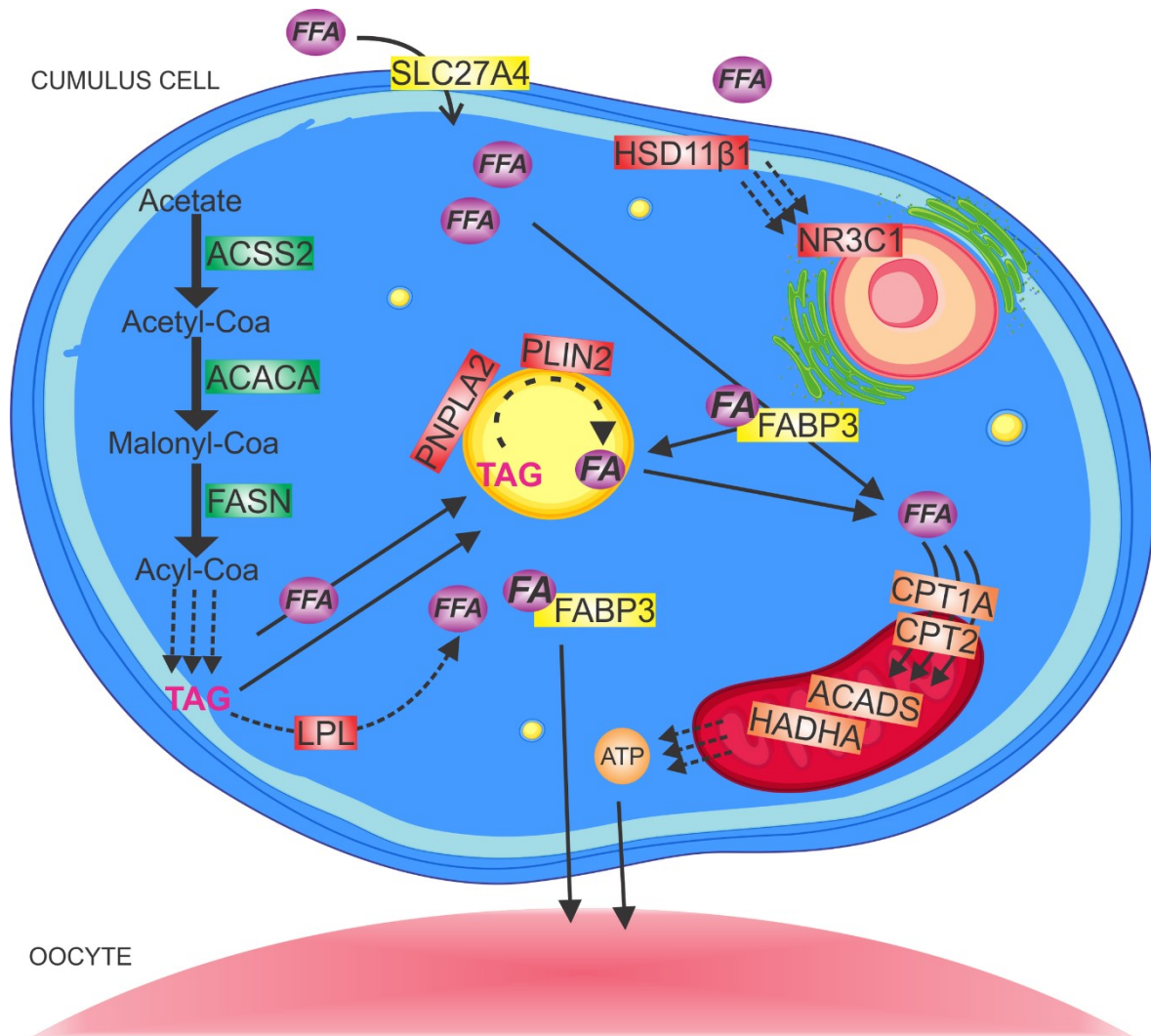


Figure 1. Scheme of genes related to lipid metabolism in cumulus cells based on known protein functions in other cell types. In this scheme, free fatty acids (FFA) are represented in purple. The synthesis of FA (green) is represented in the cytoplasm with the formation of FFA and triacylglycerol (TAG). The three dotted arrows represent the esterification process, which occurs in the smooth endoplasmic reticulum (not shown) resulting in the formation of TAG. TAG can be stored in lipid droplets (LD, yellow ball) or undergo lipolysis (red) to form free FA (dotted arrow) in the cytoplasm and LD. Lipolysis can also be stimulated by cortisol via nuclear receptors (the nucleus is light pink in the right corner of the image). The transformation of cortisone into cortisol and its binding to the nuclear receptor is represented by the three-dotted arrows next to the nucleus. FA carriers are depicted in yellow. The thin black arrows indicate the direction of the FA, which can be stored in the LD, transported to the oocyte through gap junctions or to the mitochondria (dark pink) to undergo β -oxidation (orange) and form *adenosine triphosphate* (ATP). In the mitochondria, the carnitine cycle and the formation of ATP by the citric acid cycle are represented by three dotted arrows.

Real-time PCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) using GoTaq qPCR Master Mix (Promega). Four biological and three technical replicates were used. All primer pairs were designed using exon-exon junctions. Therefore, genomic DNA was not amplified. Primer efficiency ranged from 86 to 111%. The specificity of each primer pair was determined by the melting curve and amplicon size visualized on agarose gel. Each PCR was run in a final volume of 25 μ L using 1 μ L of cDNA and 0.5 μ M of each primer (forward and reverse). The cycling conditions were as follows: 95 °C for 5 min, followed by 50 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Transcript levels were normalized using the geometric average of the endogenous genes β -actin and GAPDH and calculated using the $\Delta\Delta$ Ct method with efficiency correction (Pfaffl, 2001). The CS-GV group was utilized as the control group because COCs from cycling animals are desirable biological samples for *in vitro* production (IVP).

Table 1. Gene nomenclature, GenBank access number, gene function, primer sequences, melt temperature, amplicon sizes and primer efficiency for each gene used for gene expression in qPCR.

Gene	Name	Access Number	Function	Primer Sequence	MT (°C)	Amplicon size (bp)	Eff%
PLIN2	Perilipin 2	NM_214200.2	LL	(F) CGGTGCTCTCCCTATCATCC (R) ACAGCCCCCTTAGCATTGGC	83.19	147	106.444
PNPLA2	Patatin like phospholipase domain containing 2	XM_021076533.1	LL	(F) AACGTCTGCAGCACCTTCAT (R) TCGTAGAGAGGCAGGTTGTC	85.83	107	90.523
ACADS	Acyl-CoA dehydrogenase short chain	XM_005670708.3	β O	(F) GGACCTATCCTGAAGTTTGGCT (R) CCTGGTTCAGTGAGGGCAAA	82.78	101	101.62
HADHA	Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha	NM_213962.2	β O	(F) GTACGAAAGCAGACCAAAGGC (R) GGGTCTGACCACGGTAAAGTC	82.94	175	96.118
CPT1A	Carnitine palmitoyltransferase I A	NM_001129805.1	β O	(F) TAAACGGATGACGGCTCTGG (R) CTGTTCAACATCAGGGGTCC	84.51	159	111.577
CPT2	Carnitine palmitoyltransferase 2	NM_001246243.1	β O	(F) CCTCTGGATATGTCCCAGTAT (R) CTCACAATCTTCCCGTCTTG	84.68	164	96.968
SLC27A4	Long-chain fatty acid transport protein 4	XM_021069609.	FAT	(F)TCTCCCTTCTGTTCTCTAC (R)GGTGGCAGCGAATAAGAT	89.16	170	107.222
ACSS2	Acyl-CoA synthetase short-chain family member 2	XM_005672862.3	FAS	(F)GGATGAAAGGGGCAACTACCA (R)CTTGGAACAGAAAGCTCACGG	82.22	150	107.05
ACACA	Acetyl-CoA carboxylase alpha	XM_021066227.1	FAS	(F)GCAAGGTCGAGACCGAAAGAA (R)CCGGATTGCACGCTCATTTTC	83.12	199	107.026
FASN	Fatty acid synthase	NM_001099930.1	FAS	(F)GAGGGGGCTATTGCATCAAGGA (R)GCAGGAACTCGGACATAGCG	88.22	173	90.719
LPL	Lipoprotein lipase	NM_214286.1	LL	(F)AGAACC GTTGCAACAACTTGG (R)AGATCTCAAAGGCCTGGTTGG	82.36	174	102.906
HSD11β1	Hydroxysteroid 11-beta dehydrogenase 1	NM_001348961.2	LL	(F)CAACTTCCTCAGCTACGTGGT (R)TGCTTGCAGAATAGGGAGCA	86.82	134	86.022

NR3C1	Nuclear receptor subfamily 3 group C member 1	NM_001008481.1	LL	(F)AGTTCCCAAGGACGGTTTGAA (R)TGACTGGAGTTTCCTCCCTC	79.11	108	98.907
FABP3	Fatty acid binding protein 3	NM_001099931.1	FAT	(F)GTCTCTTTCTCAGCCTAGCCC (R)TGGTAGGCTTGGTCATGTTGG	85.88	151	100.809
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	AF017079.1	EC	(F)GTCTGGAGAAACCTGCCAAATA (R)CCCAGCATCAAAGGTAGAAGAG	85.46	152	95.378
β-actin	Beta-actin	U07786.1	EC	(F)GACCACCTTCAACTCGATCAT (R)GATCTCCTTCTGCATCCTGTC	85.85	124	93.65

(MT) melt temperature; (Eff%) primer efficiency; (F) forward; (R) reverse; (bp) base pair; (LL) lipolysis; (βO) β-oxidation; (FAT) fatty acid transport; (FAS) fatty acid synthesis; (EC) endogenous control.

Assessment of lipid droplet accumulation

Staining and lipid measurements in the oocytes were performed as previously described by Faria et al. (2021). Denuded oocytes were fixed in 4% paraformaldehyde for 1 h, washed with PBS, and stored at 4 °C in 1% paraformaldehyde for up to seven days. Oocytes were then washed in PBS supplemented with 0.3% polyvinylpyrrolidone (PBS/PVP), incubated for 30 min at room temperature in PBS supplemented with 0.2% Triton, washed in PBS/PVP, and stained with Bodipy 493/503 (Molecular Probes; 20 µg/mL) for 1 h at room temperature. After staining, oocytes were placed individually in 35 mm plates in 4 µL drops of anti-fade solution (SlowFade; Molecular Probes) and evaluated using a confocal microscope (LSM Leica Sp8 Laser Dissection Microscope, New Orleans, LA, USA). Four groups of oocytes were evaluated: (1) immature oocytes from prepubertal gilts (Oc PG-GV; n = 33); (2) *in vitro*-matured oocytes from prepubertal gilts (Oc PG-MII; n = 38); (3) immature oocytes from cycling sows (Oc CS-GV; n = 34); and (4) *in vitro*-matured oocytes from cycling sows (Oc CS-MII; n = 36), using the same microscope parameters. All samples were analyzed and photographed

using an X20 objective, a 488 nm argon laser for viewing lipid droplets, and a fluorescence spectrum between 488 and 552 nm. Oocytes were submitted one by one to 20 cross-sections with a 2.96 μm interval. Z-stacking was used to create an image of the overlapping sections. The images were adjusted on a grain (eight bits images) using the ImageJ program (National Institutes of Health). The background was corrected. Lipid quantification was based on the ratio of the area occupied by lipids in the cytoplasm to the total area of the oocyte.

DNA isolation and sodium bisulfite treatment

For methylation analyses, genomic DNA was isolated from a pool of 30 oocytes from each experimental group, as described by Mendonça et al. (2015). Genomic DNA from three pools of CCs and the sperm of three mature males (control) was isolated using a salting-out procedure described in Carvalho et al. (2012). DNA samples from oocytes, CCs, and sperm were treated with sodium bisulfite using the EZ DNA Methylation-Lightning Kit (Zymo Research) according to the manufacturer's protocol. After bisulfite treatment, the DNA samples were stored at -80°C until PCR amplification.

PCR amplification, cloning, and sequencing

Genomic regions of two genes that showed differences in expression, *FABP3*, and *NR3C1*, were chosen for methylation analysis. We evaluated CpG islands in exon 1 and 5' UTR of *FABP3* and *NR3C1*, respectively, as they are potential regulator regions of gene expression (figure S1). Primer sequences, GenBank accession numbers, CpG island positions, and amplicon sizes for both genes are shown in Table 2. Two rounds of amplification were performed for both genes. PCR components were as follows: 1 $\times$ Taq buffer, 1.0 mM or 1.5 mM MgCl_2 for the first and second rounds, respectively, 0.4 mM dNTPs, 1 U Platinum Taq polymerase

(Invitrogen), 0.5 μ M of each primer (forward and reverse) and 3 μ l of bisulfite-treated DNA for the first round and 0.5 μ L of amplicon for the second round, in a final volume of 20 μ l. The cycling conditions are presented in Table 3.

Table 2. Gene identification, primer sequences, *GenBank* accession number, primer positions, CpG island position and amplicon size.

Gene	Primer sequences (5'→3')	GenBank accession	Primer positions	CpG island position	Amplicon size (bp)	Number of CpG sites
FABP3 OUT	F:GAAAGGGGATTGGT TTTGTTAGTT R:AATCRTCCTCACCA ATTAAC TTCAT	NM_00109 9931	752-1127	exon 1	376	22
FABP3 INN	F:GTAGYGT TTGTATG GGGTTTTTTA R:AATCRTCCTCACCA ATTAAC TTCAT		877-1127		251	
NR3C1 OUT	F:GTTTTTATAYGT TAAGTTGTTTATT R:AAAAAAAACCA ACRCTATCA	NM_00100 8481	1292-1591	5'UTR	300	29
NR3C1 INN	F:GTTTTTATAYGT TAAGTTGTTTATT R:CRAC TAATAAAA ATTTACAAACTCC		1292-1509		218	

(F) forward; (R) reverse; (bp) base pair;

Amplicons were purified using the Wizard SV Gel and PCR Clean-Up System (Promega), according to the manufacturer's protocol. Subsequently, they were cloned into the TOPO TA Cloning vector (Invitrogen) and transferred into DH5 α cells using a heat shock protocol. Plasmid DNA was isolated using the miniprep protocol described by Russell and Sambrook (2001). Individual clones were sequenced and analyzed as described Silveira et al. (2018). The DNA sequences were compared with GenBank NM_001099931 for *FABP3* and NM_001008481 for *NR3C1*.

Table 3. Nested PCR conditions for the FABP3 and NR3C1 genes

Gene	Reaction	Initial denaturing	Cycles (35 cycles for the 1 ^a reaction and 28-40 cycles for the 2 ^a reaction)			Final extension
			Denaturing	Anneling	Extension	
FABP3	1 ^a	94°C; 4 min	94°C; 40 s	58°C; 1 min	72°C; 1 min	72°C; 15 min
	2 ^a	94°C; 4 min	94°C; 40 s	58°C; 1 min	72°C; 1 min	72°C; 15 min
NR3C 1	1 ^a	94°C; 4 min	94°C; 40 s	55°C; 1 min	72°C; 1 min	72°C; 15 min
	2 ^a	94°C; 4 min	94°C; 40 s	54°C; 1 min	72°C; 1 min	72°C; 15 min

Statistical analysis

Transcript levels and DNA methylation comparisons among groups (PG-GV, PG-IVM, CS-GV, and CS-IVM) were performed using analysis of variance (ANOVA) and Tukey's test or Kruskal–Wallis's and Mann–Whitney's U tests for normally and non-normally distributed data, respectively. Data related to the accumulation of lipid droplets in oocytes were analyzed by ANOVA using the generalized linear mixed model (GLIMMIX). All analyses were performed using the Action version 3.7 (Estatcamp, 2014) and GraphPad Prism software version 7.00 (GraphPad Software, La Jolla, California, USA; www.graphpad.com).

Results

Gene expression analysis in cumulus cells

Cumulus cells from the four experimental groups were subjected to gene expression analysis and the results are depicted in Figure 2. No differences ($P > 0.05$) were detected in the transcript levels for any of the genes between the groups at the same maturation stage. However, when the expression of the genes was compared before and after maturation, seven of the 14 genes showed changes in transcript levels. The transcript levels of *ACSS2*, *ACACA*, *FABP3*, *SLC27A4*, and *PLIN2* genes increased ($P < 0.05$) during IVM only in CCs from the cycling sows (figure 2). *FASN* expression increased ($P < 0.05$) during maturation in both groups, while *NR3C1* was the only gene that demonstrated a decrease in transcript levels ($P < 0.05$) during maturation (figure 2) in the prepubertal gilts group.

Lipid droplet accumulation in oocytes

Analyses showed that the lipid content in immature oocytes was similar ($P > 0.05$) between cycling sows and prepubertal gilts. After maturation, an increase in lipid content was observed in the cycling sow group (figures 3 and 4). In contrast, in the prepubertal gilt group, there was a decrease in lipid content ($P < 0.05$) during IVM. When mature oocytes from both groups were compared, higher amounts of lipids were observed in the sow group than in the prepubertal gilt group (figures 3 and 4).

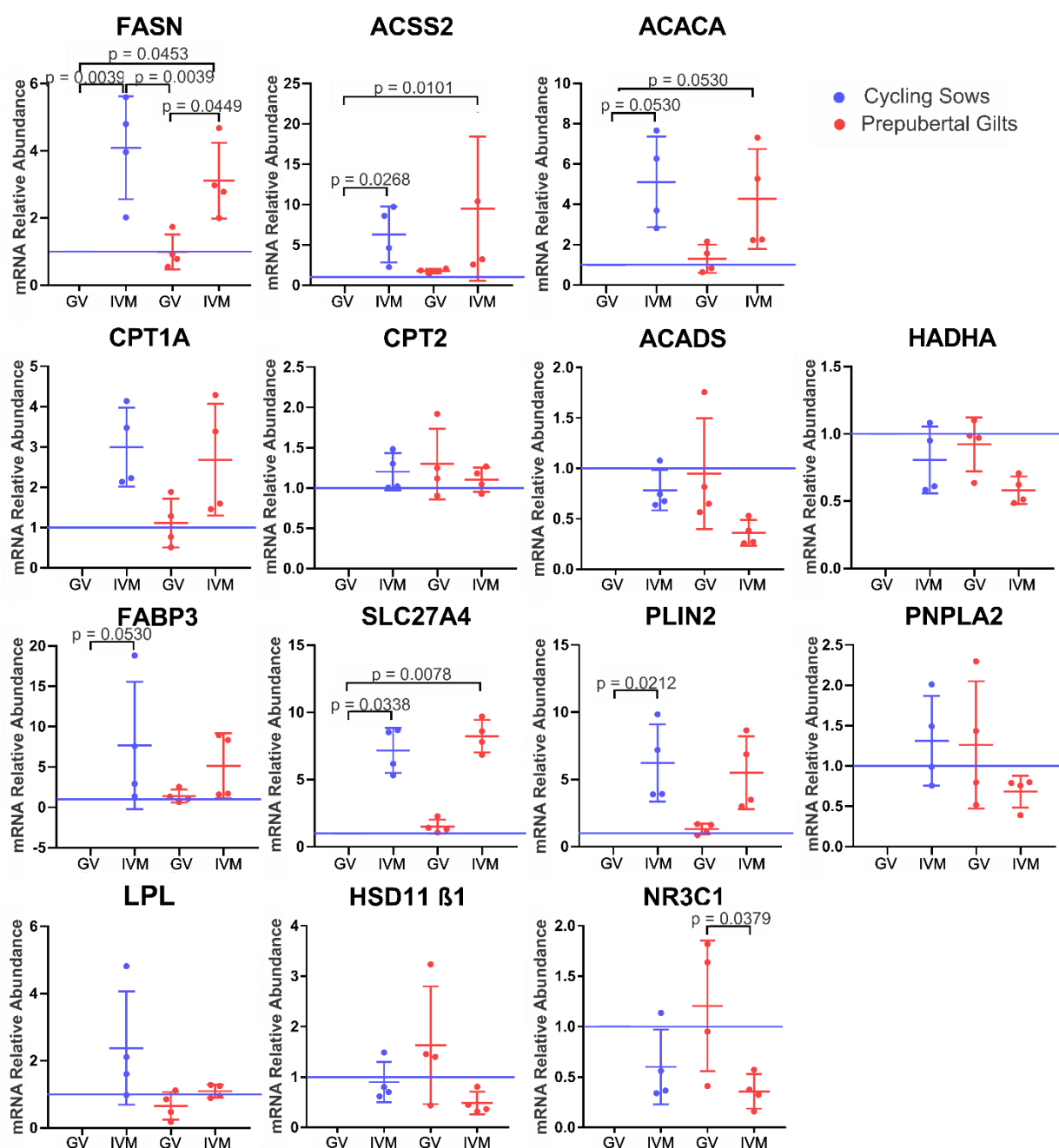


Figure 2. Relative abundance of mRNA encoding the lipid metabolism genes, as determined by RT-qPCR in immature and *in vitro* matured cumulus cells from prepubertal and cycling female pigs. Data are means $\pm$ standard deviation (SD) of the mean of four biological replicates. The data (mean $\pm$ SD) were normalized using the formula $\Delta\Delta C_t$ (Pfaffl, 2001), with the geometric mean of β -actin and *GAPDH* as the endogenous controls. The blue line represents the mean of the CS-GV group that was used as a control group.

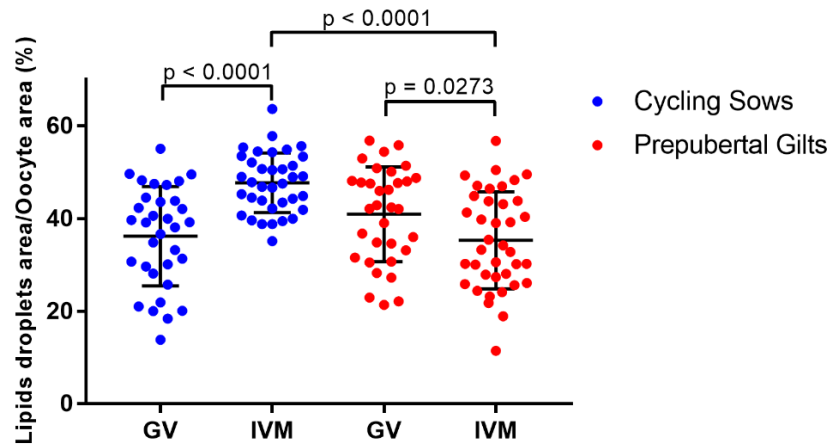


Figure 3. Area of lipid droplets relative to the total area of immature and *in vitro* matured oocytes from cycling sows and prepubertal gilts. Data are the mean $\pm$ standard deviation.

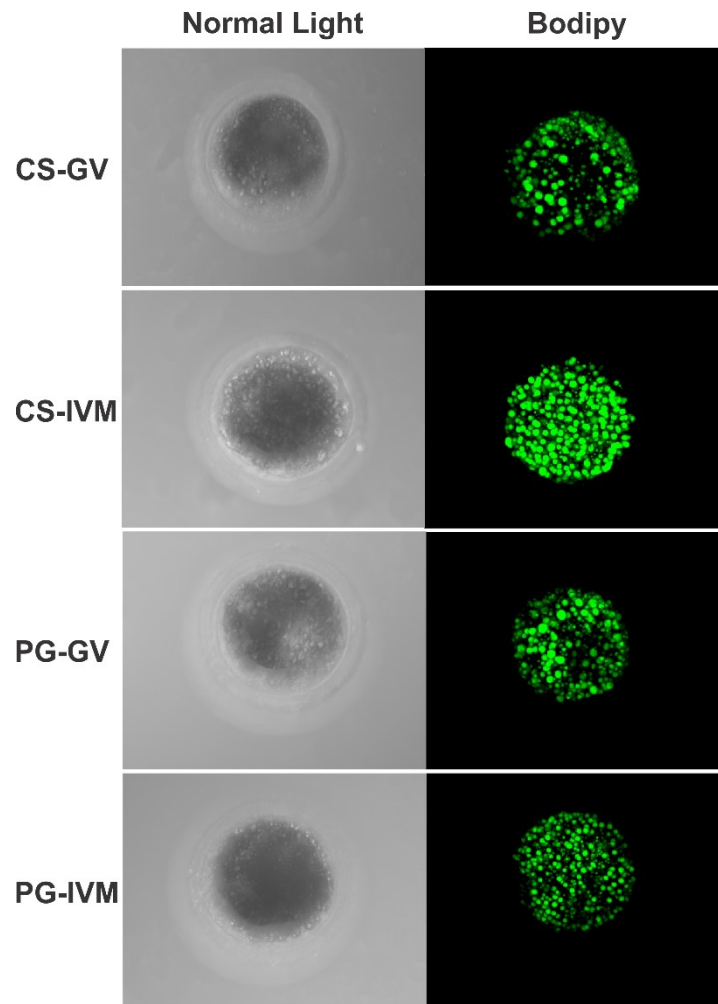


Figure 4. Representative images following confocal microscopy (LSM Leica sp8; 488-nm argon laser, original magnification _20) of lipid droplets (LDs) in Bodipy 493/503-stained pig oocytes: immature oocyte from cycling sow (CS-GV); *in vitro* matured oocyte from cycling sow (CS-IVM); immature oocyte from prepubertal gilt (PG-GV); *in vitro* matured oocyte from prepubertal gilt (PG-IVM).

DNA methylation of FABP3 and NR3C1 genes in cumulus cells and oocytes

We evaluated the DNA methylation patterns of two genes that showed differences in expression (*FABP3* and *NR3C1*) in oocytes and CCs from the four groups (PG-GV, PG-IVM, CS-GV, and CS-IVM). We used sperm as a control group. The methylation percentage of each of the eight experimental groups and the control group are described in figure 5. For both genes, we found demethylated or hypomethylated alleles in all the groups (figure 5). Despite this, there were statistically significant differences between groups, as shown in figure 5.

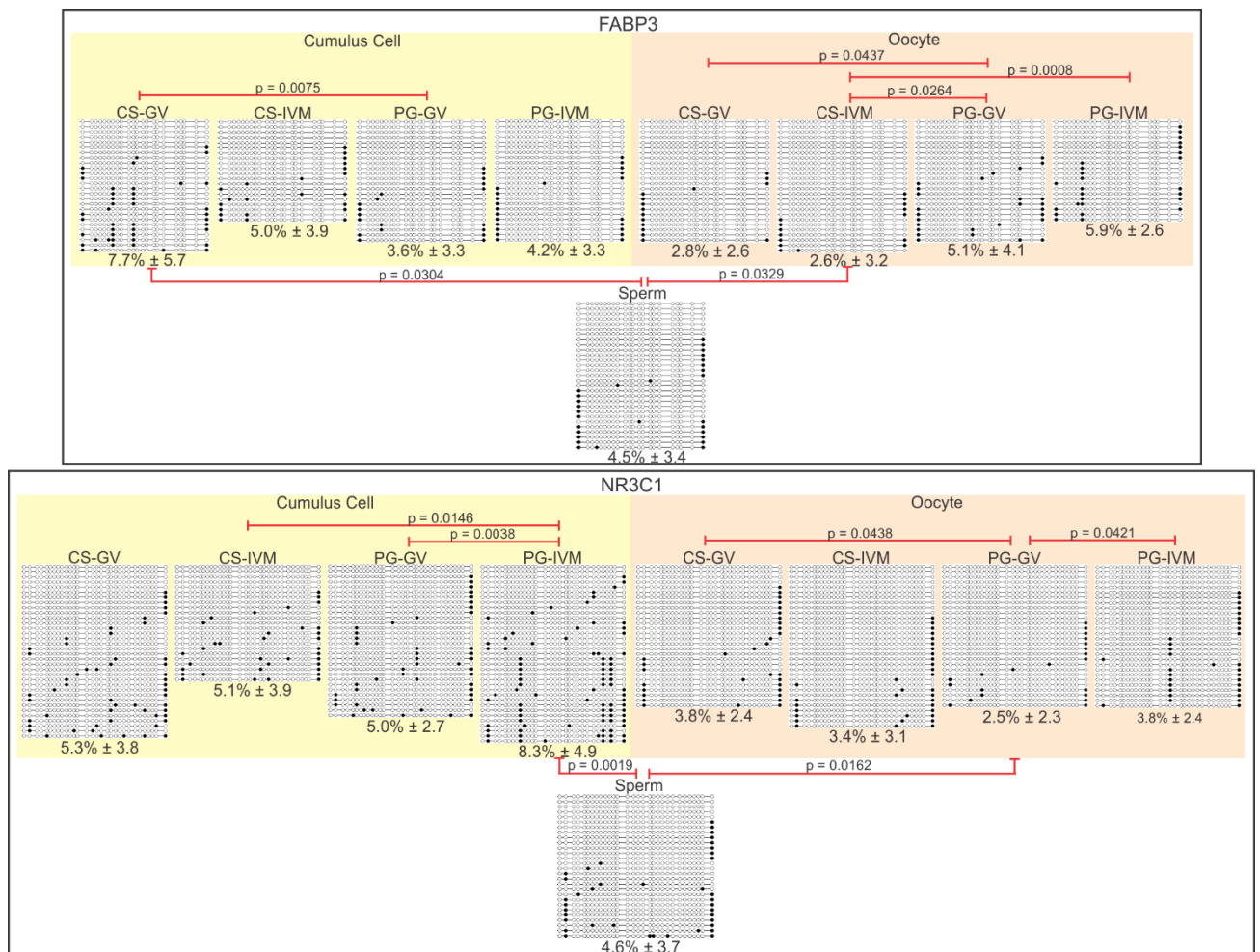


Figure 5. Methylation pattern of *FABP3* (above) and *NR3C1* (below) genes in cumulus cells (yellow), oocyte (pink), and sperm (control group). White circles represent unmethylated cytosines and black circles represent methylated cytosines. The p values are represented by red bars between the groups that indicated a statistical difference. CS = cycling sows; PG = prepubertal gilts; GV = germinal vesicle; IVM = *in vitro* matured.

In cumulus cells, *FABP3* showed higher methylation in CS-GV group compared with PG-GV and control. In oocytes, the gilt group showed higher methylation in both GV and IVM compared with sows. *NR3C1* showed higher methylation in cumulus cells from PG-IVM group. In oocytes, *NR3C1* showed less methylation compared to the other groups. We also evaluated the percentage of specific methylation for each CpG site and the results are shown in figure 6.

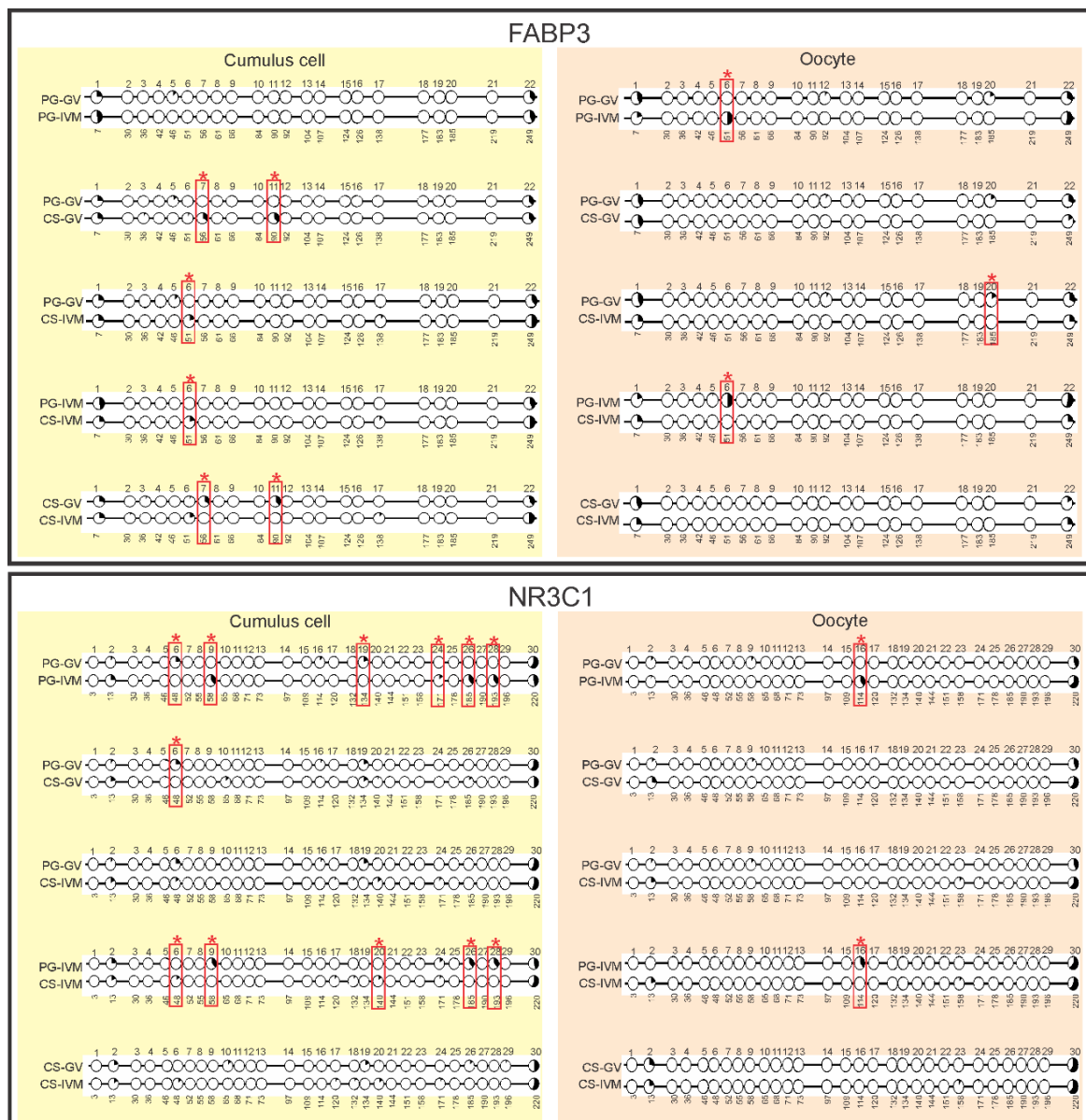


Figure 6. Analysis of comparative methylation by CpG of *FABP3* (above) and *NR3C1* (below) genes in cumulus cells (yellow) and oocytes (pink). The circles represent the percentage of methylation in each CpG. Red rectangles and asterisks indicate CpGs that revealed statistical differences ($p < 0.05$) between groups. CS = cycling sows; PG = prepubertal gilts; GV = germinal vesicle; IVM = *in vitro* matured.

Discussion

Our results showed a difference in the dynamics of expression in lipid metabolism genes between pig females with different sexual maturity, suggesting a greater accumulation of lipids in sow COCs during IVM. Indeed, we showed a greater accumulation of LD in cycling sow oocytes and a decrease in lipid content in prepubertal gilt oocytes during IVM. Finally, we determined and associated the methylation pattern to the expression of two differentially expressed genes (one with increased expression and the other with decreased expression during IVM).

In a recent study by the authors, oocytes from prepubertal and cycling animals showed differences in the transcript levels of genes related to lipid metabolism (Braga et al., 2019). These results suggest that the effect of donor age on oocyte competence may be associated with the presence and utilization of fatty acids (FA) (Braga et al., 2019). We hypothesized that the low developmental competence of oocytes from younger animals could be related to the ability of CCs to metabolize lipids, considering cumulus cells also play an important role in the processes of energy production for oocytes. We analyzed the transcript levels of genes involved in the metabolism of FA, such as lipogenesis, FA transport, β -oxidation, and lipolysis, in CCs from prepubertal and cycling females before and after IVM to test this hypothesis.

Our results showed that no differences were found when comparing gilts versus sow groups at the same maturation stage, GV or MII. However, seven genes showed different expression levels between GV and MII when compared within the same group. Therefore, they presented different expression dynamics during IVM in CCs from gilts compared with CCs from sows. Genes related to the synthesis and transport of FA and *PLIN2* were upregulated during IVM only in the sow group.

These results show that the dynamics of gene expression in pig CCs is also different in females of different sexual ages, as observed in oocytes (Braga et al., 2019).

Of the three genes related to FA synthesis, the transcript levels of two, *ACSS2* and *ACACA*, increased during IVM in the cyclic sow group, but not in the gilts. However, only *FASN* was increased in both groups (figure 2). In addition, there was an increase in the expression of genes related to the transport of FA (*SLC27A4* and *FABP3*) in the sow group. These results suggest that CCs from sows synthesize *de novo* FAs during oocyte maturation and capture more FA from the follicular fluid (FF), which can be stored in lipid droplets or transported to the oocyte. In bovine CCs, Sanchez-Lazo et al. (2014) evaluated the expression of these three genes (*ACSS2*, *ACACA*, and *FASN*). They found increased expression after IVM, highlighting the importance of lipogenesis in these cells, indicating a potential relationship with the formation of lipid droplets in oocytes. Recently, Del Collado et al. (2017) showed that FABP3 protein plays an essential role in transporting FA directly to the oocyte via gap junctions. Therefore, these results suggest that sow CCs accumulate FA from lipogenesis and FF and that these lipids are transported to the gamete by FABP3, most likely as an energy source for the gamete or embryonic development. Cycling sows also showed higher levels of *SLC27A4* expression in oocytes after IVM but there was no increase in the transcript levels in the gametes or CCs from prepubertal gilts (Braga et al., 2019), suggesting that both CCs and oocytes from sows accumulated FA from FF.

Regarding β -oxidation and lipolytic activity, none of the genes studied changed their transcript levels during IVM in either group (figure 2). Lipolytic activity is associated with lower lipid accumulation in lipid droplets and increased fatty acid β -oxidation (FAO) activity for energy production (Sanchez-Lazo et al., 2014). These results

suggest that CCs do not consume the lipid stock. Fatty acid β -oxidation is important in CCs and oocytes from mice, cattle, and pigs (Downs et al., 2009; Paczkowski et al., 2013; Brisard et al., 2014; Sanchez-Lazo et al., 2014; Uzbekova et al., 2015). However, the transcript levels of genes of this pathway in pig CCs before and after IVM remain undetermined. Based on other species, we expected an increase in the expression of these genes (Sanchez-Lazo et al., 2014), suggesting higher production of energy that could be transported to the oocyte to support nuclear maturation, acquisition of competence, and early embryonic development. However, we found no differences in the transcript levels of these genes in either gilts or sows, suggesting that this pathway does not increase ATP production in CCs. Paczkowski et al. (2013) showed that pig oocytes, which have a higher lipid content than other species, are more sensitive to FAO inhibition. This may be because gametes use only small amounts of lipids for the maintenance and nuclear maturation of the oocyte, with most of the lipid content being accumulated for embryonic development. The high lipid content in pig gametes is justified by the long embryonic pre-implantation period and the size of the litter, whose embryos require energy from the lipids until the development of the placenta in polytocous species (Prates et al., 2014). Therefore, CCs are important sources of FA for oocyte competence. In contrast, the results obtained for oocytes (Braga et al., 2019), suggest that the gilt gamete has changed the levels of *CPT2* expression, indicating that it may perform more β -oxidation, while the sow gamete is more likely to hold the stock of FA in lipid droplets (LD) for early embryonic development. This could explain the lower competence of these oocytes, as it may indicate a higher energy consumption.

Furthermore, *PLIN2* expression increased in the sow group during IVM but did not differ in the gilt group (figure 2). *PLIN2* is a protein that coats lipid droplets (LDs), stabilizes, and protects them from lipase degradation (Kaushik and Cuervo, 2015). This suggests that in the sow group, LD may suffer less degeneration during IVM and have a greater accumulation of FA than that in the gilt group. Unlike the results found for sows, the *NR3C1* gene showed fewer transcripts in PG-IVM than in PG-GV, which suggests less lipolytic activity in gilt CCs and may be related to the cortisol level from FF (Simerman et al., 2015).

Although there were no statistical differences in the transcript levels between gilts and sows, the difference in the transcript levels of the five genes (*ACSS2*, *ACACA*, *FABP3*, *SLC27A4*, and *PLIN2*), specifically in the sow group and one (*NR3C1*) in the gilt group (figure 2), suggested that FA metabolism in CCs is different between them. It further suggested an accumulation of lipids in the sow gamete, indicating the importance of lipids for the acquisition of competence by the oocytes. To evaluate whether the gene expression results reflect lipid accumulation, we measured the lipid content before and after maturation. During IVM, lipid accumulation was observed in oocytes from the cycling sow (figures 3 and 4). A decrease in lipid content was observed in the prepubertal gilt group. An increase in LDs in oocytes during maturation has been reported in cattle (Ordoñez-Leon et al., 2014; Faria et al., 2021; Melo-Sterza and Poehland, 2021). It was suggested to be associated with *in vitro* systems, lipotoxicity, and low cryotolerance (Melo-Sterza and Poehland, 2021). Oocytes of different species show differences in energy metabolism and lipid content, which may be related to the time of gamete maturation and the pre-implantation period of the embryo (Melo-Sterza and Poehland, 2021). Therefore, we hypothesize that the accumulation of lipids that are harmful to oocytes

of other species, such as bovine, murine, and human, can be expected in pig oocytes. Apparently, this increase in LDs in cycling sow oocytes is physiological and the difference in lipid metabolism of CCs and oocytes between sows and gilts may explain the lower competence of prepubertal gilt oocytes.

The reason for the CCs of prepubertal gilts having a different gene expression dynamic from the CCs of cyclic sows, for genes related to lipid metabolism, and sow oocytes accumulating lipids while gilt oocytes exhibit the opposite behavior remains unclear. However, it may be related to the FF content. Some studies in cattle have shown that FF composition can affect lipid levels in CCs (Aardema et al., 2013; Lolicato et al., 2015). The influence of female age on FF has been demonstrated in pigs (Gruppen et al., 2003; Pawlak et al., 2012; Naskar et al., 2016; Pawlak et al., 2018). Therefore, these results suggest that FF alters the metabolism of FA in swine CCs and oocytes and affects embryonic development. There is evidence that porcine embryos use lipids as an energy source during development until the blastocyst stage (Niu et al., 2015; Lowe et al., 2017).

Regarding DNA methylation, we evaluated whether the difference in gene expression of CC could be associated with epigenetic factors and determined the methylation pattern in oocytes. Cumulus cells from the CS-GV group showed more total methylation in *FABP3* than those from the PG-GV and sperm groups. Despite showing a demethylation pattern this difference may be related to the methylation of specific CpG sites, as shown in figure 6. When we examined specific CpGs, we observed that cytosines 7 and 11 were methylated only in the CS-GV group, while the PG-GV and CS-IVM groups did not show methylation at these CpG sites. Methylation of these specific cytosines may interfere with the binding of transcription factors (TF) that allow gene expression. When we analyzed the sequence in a

database of transcription factors (TRANSFAC) (Tsunoda & Takagi, 1999), we observed that the TFs SREBP1 and SP1 possibly bind to the promoter region of *FABP3* at cytosines 7 and 11, respectively (figure 7 and table 4). SREBP1 occupies the promoters of many target genes involved in several biological pathways, including genes involved in lipid metabolism, which may result in the repression or expression of target genes (Reed et al., 2008). Furthermore, Reeds et al. (2008) demonstrated that SREBP1 cooperates extensively with SP1 throughout the genome. Further research is required to determine whether the TFs SREBP1 and SP1 act on the promoter region of *FABP3*, resulting in its expression and whether these TFs bind to methylated cytosines.

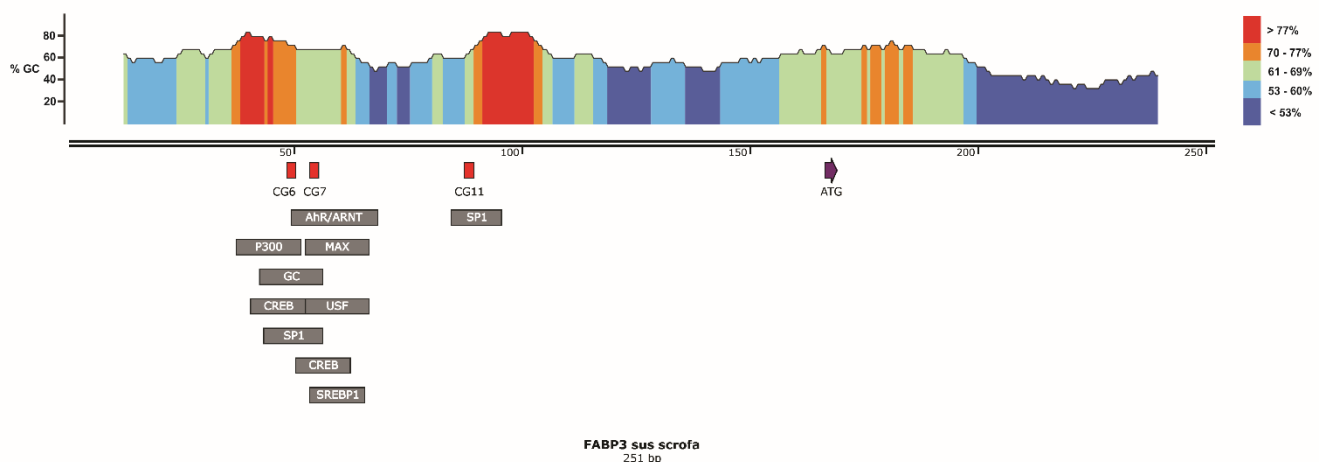


Figure 7. Schematic representation of transcription factor (TFs- gray boxes) and its possible binding site in the *FABP3* gene (represented by the black line). Red rectangles represent cytosines 6, 7, and 11 of the studied region. The image was generated using SnapGene Viewer 6.0.2 software (from Insightful Science; available at snapgene.com).

Regarding the *NR3C1* gene, CCs from the PG-IVM group showed more methylation compared with the PG-GV group, which may explain the decrease in gene expression that we found in these prepubertal gilt cells. Although DNA methylation is stable in somatic tissues and cells (Franco, 2017; Zeng and Chen, 2019), we

observed an increase in DNA methylation of the *NR3C1* gene in CCs during IVM. The same behavior was observed for the *NR3C1* gene in oocytes, that is, an increase in methylation during IVM in the prepubertal gilt group. Therefore, these results demonstrate that the study of epigenetic mechanisms in CCs has great potential for identifying epigenetic markers of oocyte quality.

Table 4. Transcription factors and binding site sequence the FABP3 gene

Target cytosine	Transcription Factor	Matrix ID	Similarity	Consensus sequence	Input sequence
CG6	P300	M00033	0.811520	NNNRGGAGTNNNS	GGCGTACGCCACGC
	CREB	M00113	0.812779	NNGNTGACGYNN	GTACGCCACGCC
CG6 and CG7	GC	M00255	0.919402	NRGGGGCGGGGCK	ACGCCACGCCTCGT
	SP1	M00196	0.860863	NGGGGGCGGGGYN	CGCCACGCCTCGT
	AhR/ARNT	M00237	0.804604	GRGKATYGCGTGMSWNSCC	GCCTCGTCACGTGACGCTA
	MAX	M00119	0.851842	NNANCACGTGNTNN	TCGTACGTGACGC
CG7	USF	M00187	0.986022	GYCACGTGNC	GTCACGTGAC
	USF	M00122	0.915786	NNRNCACGTGNYNN	TCGTACGTGACGC
	SREBP1	M00220	0.960955	NATCACGTGAY	CGTCACGTGAC
CG11	SP1	M00008	0.804926	GRGGCRGGGW	AGCGCGGGCT

S = C or G nucleotide; **W** = A or T nucleotide; **R** = A or G nucleotide; **Y** = C or T nucleotide; **K** = G or T nucleotide; **M** = A or C nucleotide; **N** = any base pair. (TRANSFAC R.3.4 - <https://tfbind.hgc.jp/>)

Conclusions

Cumulus cells from sows were found to have a better capacity to dynamically change the transcription levels of the major genes involved in FA metabolism during IVM. In addition, oocytes from cycling sows showed lipid accumulation, whereas oocytes from prepubertal gilts showed a decrease in the amount of lipids during IVM. Although we found differences in methylation patterns, all groups were highly demethylated. It is possible that these methylation differences had no biological effects. Our results may contribute to a better understanding of the mechanisms involved in lipid metabolism in porcine COCs of different sexual maturity and the acquisition of oocyte competence.

Acknowledgments

We thank Amanda Oliveira Moura, Nayara Ribeiro Kussano, and Ligiane Leme for their commitment to establishing the *in vitro* maturation protocol. We thank Leticia Prates Martins. We thank Embrapa Genetic Resources and Biotechnology and Embrapa Swine and Poultry for the financial support, and we thank slaughterhouses Frimelo (Luziânia - GO) and Bom Corte (Formosa – GO) and its employees for the supply of biological material.

Supplementary data

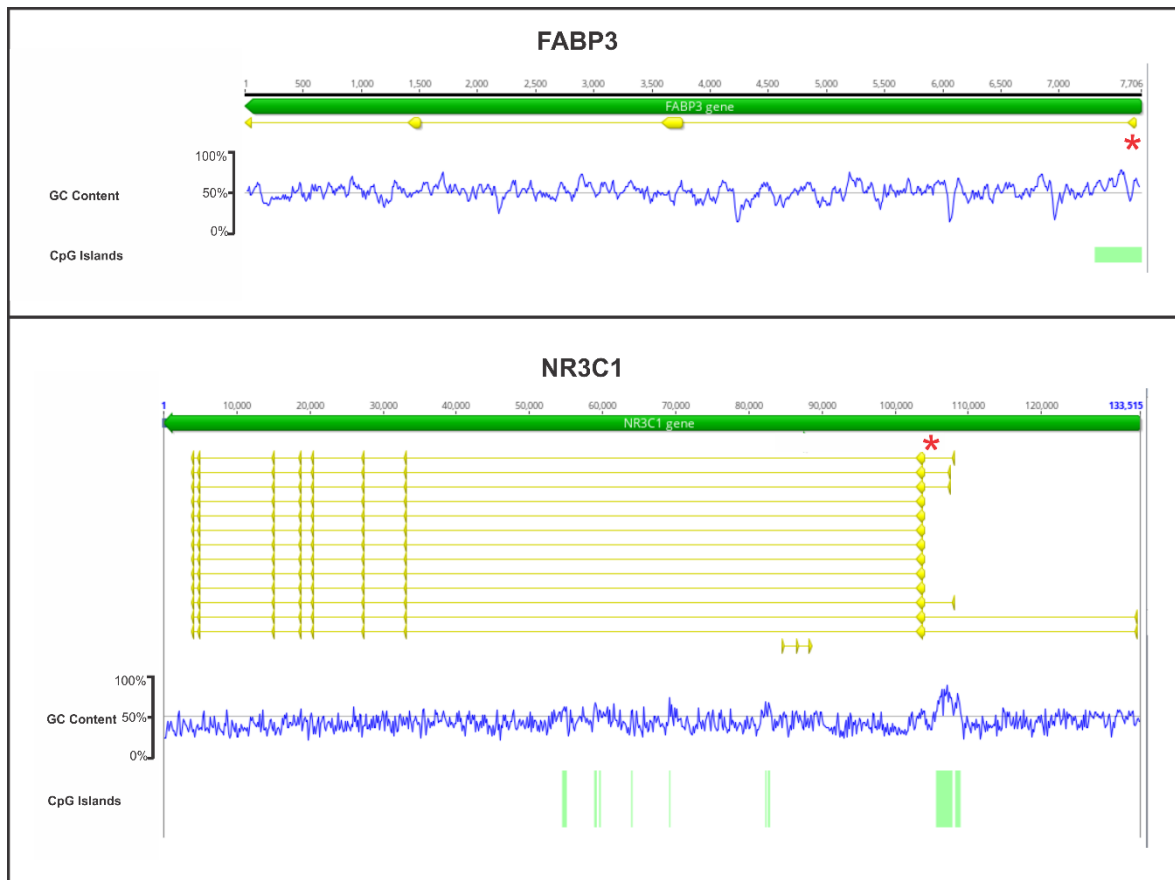


Figure S1. Representation of the *FABP3* and *NR3C1* genes structures, GC content, and CpG island prediction. Green bars represent the input sequence; below, a red asterisk represents the regions that were chosen for methylation analysis, and yellow lines with yellow arrows represent introns and exons, respectively. The GC content and CpG islands are predicted for each gene. The graphs were generated using Geneious v2020.0.5 (Biomatters, Auckland, New Zealand).

References

- Aardema, H., et al., (2013). "Bovine cumulus cells protect maturing oocytes from increased fatty acid levels by massive intracellular lipid storage." Biology of reproduction **88**(6): 164, 161-115.
- Aardema, H., et al., (2019). "An overview on how cumulus cells interact with the oocyte in a condition with elevated NEFA levels in dairy cows." Animal reproduction science.
- Aardema, H., et al., (2017). "Stearoyl-CoA desaturase activity in bovine cumulus cells protects the oocyte against saturated fatty acid stress." Biology of reproduction **96**(5): 982-992.
- Aardema, H., et al., (2011). "Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence." Biology of reproduction **85**(1): 62-69.
- Aizawa, R., et al., (2019). "Synthesis and maintenance of lipid droplets are essential for mouse preimplantation embryonic development." Development **146**(22): dev181925.
- Auclair, S., et al., (2013). "Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes." American Journal of Physiology-Endocrinology and Metabolism **304**(6): E599-E613.
- Barber, M. C., et al., (2005). "Structure and regulation of acetyl-CoA carboxylase genes of metazoa." Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids **1733**(1): 1-28.
- Barrett, S. L. and D. F. Albertini (2010). "Cumulus cell contact during oocyte maturation in mice regulates meiotic spindle positioning and enhances developmental competence." Journal of assisted reproduction and genetics **27**(1): 29-39.
- Ben-Meir, A., et al., (2015). "Coenzyme Q10 restores oocyte mitochondrial function and fertility during reproductive aging." Aging Cell **14**(5): 887-895.
- Bender, K., et al., (2010). "Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows." Reproduction **139**(6): 1047-1055.
- Borum, K. (1961). "Oogenesis in the mouse: a study of the meiotic prophase." Experimental cell research **24**(3): 495-507.
- Braga, T. F., et al., (2019). "The dynamics of gene expression, lipid composition and DNA methylation reprogramming are different during in vitro maturation of pig oocytes obtained from prepubertal gilts and cycling sows." Reproduction in Domestic Animals **54**(9): 1217-1229.
- Brisard, D., et al., (2014). "Tribbles expression in cumulus cells is related to oocyte maturation and fatty acid metabolism." Journal of ovarian research **7**(1): 1-13.
- Canovas, S., et al., (2017). "DNA methylation and gene expression changes derived from assisted reproductive technologies can be decreased by reproductive fluids." Elife **6**: e23670.
- Carrillo-González, D. F., et al., (2020). "l-Carnitine supplementation during in vitro maturation and in vitro culture does not affect the survival rates after vitrification and warming but alters Inf-T and ptgs2 gene expression." International Journal of Molecular Sciences **21**(16): 5601.

- Carvalho, J. O., et al., (2012). "The methylation patterns of the IGF2 and IGF2R genes in bovine spermatozoa are not affected by flow-cytometric sex sorting." Molecular reproduction and development **79**(2): 77-84.
- Cocero, M. J., et al., (2019). "Ovine oocytes display a similar germinal vesicle configuration and global DNA methylation at prepubertal and adult ages." Theriogenology **138**: 154-163.
- Dai, J. G., et al., (2021). "Mechanisms of lipid metabolism promoted by berberine via peroxisome proliferator-activated receptor gamma during in vitro maturation of porcine oocytes." Animal Science Journal **92**(1): e13582.
- De Vos, M., et al., (2021). "Perspectives on the development and future of oocyte IVM in clinical practice." Journal of assisted reproduction and genetics **38**(6): 1265-1280.
- Dekel, N. (2005). "Cellular, biochemical and molecular mechanisms regulating oocyte maturation." Molecular and cellular endocrinology **234**(1-2): 19-25.
- Del Collado, M., et al., (2017b). "In vitro maturation impacts cumulus-oocyte complex metabolism and stress in cattle." Reproduction **154**(6): 881-893.
- del Collado, M., et al., (2017). "Fatty acid binding protein 3 and transzonal projections are involved in lipid accumulation during in vitro maturation of bovine oocytes." Scientific reports **7**(1): 1-13.
- Desmet, K., et al., (2016). "Exposure of bovine oocytes and embryos to elevated non-esterified fatty acid concentrations: integration of epigenetic and transcriptomic signatures in resultant blastocysts." BMC genomics **17**(1): 1-18.
- Dias, L. R. O., et al., (2020). "Effect of delipidant agents during in vitro culture on the development, lipid content, gene expression and cryotolerance of bovine embryos." Reproduction in domestic animals **55**(1): 11-20.
- Dieci, C., et al., (2013). "The effect of cilostamide on gap junction communication dynamics, chromatin remodeling, and competence acquisition in pig oocytes following parthenogenetic activation and nuclear transfer." Biology of reproduction **89**(3): 68, 61-11.
- Downs, S. M., et al., (2009). "Fatty acid oxidation and meiotic resumption in mouse oocytes." Molecular reproduction and development **76**(9): 844-853.
- Dunning, K. R. and R. L. Robker (2012). "Promoting lipid utilization with L-carnitine to improve oocyte quality." Animal reproduction science **134**(1-2): 69-75.
- Dunning, K. R., et al., (2014). "Lipids and oocyte developmental competence: the role of fatty acids and β -oxidation." Reproduction **148**(1): R15-R27.
- Eppig, J. J. and P. F. Ward-Bailey (1982). "The mechanism of cumulus cell-oocyte uncoupling: Evidence for the participation of both cumulus cells and oocytes." Gamete Research **6**(2): 145-154.
- Estatcamp, E. (2014). "Software Action." São Carlos: Estatcamp-Consultoria em estatística e qualidade.
- Fang, Y., et al., (2016). "Global DNA methylation and related mRNA profiles in sheep oocytes and early embryos derived from pre-pubertal and adult donors." Animal reproduction science **164**: 144-151.
- Faria, O., et al., (2021). "Maturation system affects lipid accumulation in bovine oocytes." Reproduction, Fertility and Development **33**(5): 372-380.
- Franco, M. M. (2017). "Epigenética no melhoramento genético e reprodução animal."

- Franco, M. M. (2017). "Epigenetics in genetic improvement and animal reproduction." Latin American Archives of Animal Production **25**(1-2).
- Fulka Jr, J., et al., (1998). "Nuclear and cytoplasmic determinants involved in the regulation of mammalian oocyte maturation." Molecular human reproduction **4**(1): 41-49.
- Furger, C., et al., (1996). "Human granulosa cells in culture exhibit functional cyclic AMP-regulated gap junctions." MHR: Basic science of reproductive medicine **2**(8): 541-548.
- Garcia-Macia, M., et al., (2021). "A Mammalian Target of Rapamycin-Perilipin 3 (mTORC1-Plin3) Pathway Is Essential to Activate Lipophagy and Protects against Hepatosteatosis." Hepatology **74**(6): 3441-3459.
- Gilula, N. B., et al., (1978). "Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex." The Journal of cell biology **78**(1): 58-75.
- Grabowska, R., et al., (2016). "Quality of oocytes in prepubertal and pubertal swine." Turkish Journal of Veterinary and Animal Sciences **40**(1): 89-94.
- Grupen, C. G., et al., (2003). "Relationship between donor animal age, follicular fluid steroid content and oocyte developmental competence in the pig." Reproduction, Fertility and Development **15**(2): 81-87.
- Haidar, M., et al., (2021). "Lipophagy: a new player in CNS disorders." Trends in Endocrinology & Metabolism **32**(11): 941-951.
- Han, J., et al., (2016). "Deoxynivalenol exposure induces autophagy/apoptosis and epigenetic modification changes during porcine oocyte maturation." Toxicology and applied pharmacology **300**: 70-76.
- Hendriks, W. K., et al., (2015). "Maternal age and in vitro culture affect mitochondrial number and function in equine oocytes and embryos." Reproduction, Fertility and Development **27**(6): 957-968.
- Ibayashi, M., et al., (2021). "Homeostatic regulation of lipid droplet content in mammalian oocytes and embryos." Reproduction **162**(6): R99-R109.
- Itami, N., et al., (2018). "Palmitic acid induces ceramide accumulation, mitochondrial protein hyperacetylation, and mitochondrial dysfunction in porcine oocytes." Biology of reproduction **98**(5): 644-653.
- Iwashita, J., et al., (1998). "Essential role of germinal vesicle material in the meiotic cell cycle of *Xenopus* oocytes." Proceedings of the National Academy of Sciences **95**(8): 4392-4397.
- Jia, L., et al., (2019). "Homocysteine impairs porcine oocyte quality via deregulation of one-carbon metabolism and hypermethylation of mitochondrial DNA." Biology of reproduction **100**(4): 907-916.
- Jin, J. X., et al., (2017). "Melatonin regulates lipid metabolism in porcine oocytes." Journal of pineal research **62**(2): e12388.
- Kanninen, T. T., et al., (2013). "The role of autophagy in reproduction from gametogenesis to parturition." European Journal of Obstetrics & Gynecology and Reproductive Biology **171**(1): 3-8.
- Kaushik, S. and A. M. Cuervo (2015). "Degradation of lipid droplet-associated proteins by chaperone-mediated autophagy facilitates lipolysis." Nature cell biology **17**(6): 759-770.
- Kloska, A., et al., (2020). "Lipophagy and lipolysis status in lipid storage and lipid metabolism diseases." International Journal of Molecular Sciences **21**(17): 6113.

- Li, L.-Y., et al., (2021). "Interplay and cooperation between SREBF1 and master transcription factors regulate lipid metabolism and tumor-promoting pathways in squamous cancer." Nature communications **12**(1): 1-17.
- Li, L., et al., (2020). "Characterization of metabolic patterns in mouse oocytes during meiotic maturation." Molecular cell **80**(3): 525-540. e529.
- Lim, S. A., et al., (2022). "Lipid metabolism in T cell signaling and function." Nature Chemical Biology **18**(5): 470-481.
- Lin, T., et al., (2018). "α-Solanine impairs oocyte maturation and quality by inducing autophagy and apoptosis and changing histone modifications in a pig model." Reproductive Toxicology **75**: 96-109.
- Liu, M.-J., et al., (2018b). "Resveratrol improves in vitro maturation of oocytes in aged mice and humans." Fertility and Sterility **109**(5): 900-907.
- Liu, X., et al., (2018). "Acetate production from glucose and coupling to mitochondrial metabolism in mammals." Cell **175**(2): 502-513. e513.
- Llouch, S., et al., (2021). "Single human oocyte transcriptome analysis reveals distinct maturation stage-dependent pathways impacted by age." Aging Cell **20**(5): e13360.
- Lolicato, F., et al., (2015). "The cumulus cell layer protects the bovine maturing oocyte against fatty acid-induced lipotoxicity." Biology of reproduction **92**(1): 16, 11-16.
- Lowe, J., et al., (2017). "Supplementation of culture medium with L-carnitine improves the development and cryotolerance of in vitro-produced porcine embryos." Reproduction, Fertility and Development **29**(12): 2357-2366.
- Lunney, J. K. (2007). "Advances in swine biomedical model genomics." International journal of biological sciences **3**(3): 179.
- Luo, B., et al., (2015). "Effects of histone acetylation status on the early development of in vitro porcine transgenic cloned embryos." Cellular Reprogramming (Formerly Cloning and Stem Cells) **17**(1): 41-48.
- Macaulay, A. D., et al., (2014). "The gametic synapse: RNA transfer to the bovine oocyte." Biology of reproduction **91**(4): 90, 91-12.
- Macaulay, A. D., et al., (2016). "Cumulus cell transcripts transit to the bovine oocyte in preparation for maturation." Biology of reproduction **94**(1): 16, 11-11.
- Malyszka, N., et al., (2023). "Distinct dynamics of lipid accumulation by porcine cumulus cells during in vitro maturation with follicular fluid of low and high fatty acid contents." Theriogenology **195**: 93-102.
- Marchal, R., et al., (2001). "Meiotic and developmental competence of prepubertal and adult swine oocytes." Theriogenology **56**(1): 17-29.
- Marques, M. G., et al., (2007). "In vitro maturation of pig oocytes with different media, hormone and meiosis inhibitors." Animal reproduction science **97**(3-4): 375-381.
- McEvoy, T., et al., (2000). "Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida." Journal of reproduction and fertility **118**(1): 163-170.
- McGarry, J. D. and N. F. Brown (1997). "The mitochondrial carnitine palmitoyltransferase system—from concept to molecular analysis." European journal of biochemistry **244**(1): 1-14.
- Melo-Sterza, F. d. A. and R. Poehland (2021). "Lipid Metabolism in Bovine Oocytes and Early Embryos under In Vivo, In Vitro, and Stress Conditions." International Journal of Molecular Sciences (Web) **22**(7): 3421.

- Mendonça, A. d. S., et al., (2015). "Characterization of the IGF2 imprinted gene methylation status in bovine oocytes during folliculogenesis." PloS one **10**(10): e0142072.
- Mensah, L. B., et al., (2017). "mTORC1 signalling mediates PI3K-dependent large lipid droplet accumulation in Drosophila ovarian nurse cells." Biology Open **6**(5): 563-570.
- Moreira, F., et al., (2016). "Metabolic and reproductive parameters in prepubertal gilts after omega-3 supplementation in the diet." Animal reproduction science **170**: 178-183.
- Nakashima, A., et al., (2017). "Role of autophagy in oocytogenesis, embryogenesis, implantation, and pathophysiology of pre-eclampsia." Journal of Obstetrics and Gynaecology Research **43**(4): 633-643.
- Naskar, S., et al., (2016). "Steroid and metabolic hormonal profile of porcine serum vis-à-vis ovarian follicular fluid." Veterinary world **9**(11): 1320.
- Nelson, D. L. and M. M. Cox (2014). "Princípios de bioquímica de Lehninger." Porto Alegre: Artmed. **6. ed.**: 1220p.
- Niu, Y., et al., (2015). "Distribution and content of lipid droplets and mitochondria in pig parthenogenetically activated embryos after delipation." Theriogenology **83**(1): 131-138.
- Onal, G., et al., (2017). "Lipid droplets in health and disease." Lipids in health and disease **16**(1): 1-15.
- Ordoñez-Leon, E., et al., (2014). "Lipid droplet analysis using in vitro bovine oocytes and embryos." Reproduction in domestic animals **49**(2): 306-314.
- Paczkowski, M. and R. Krisher (2010). "Aberrant protein expression is associated with decreased developmental potential in porcine cumulus-oocyte complexes." Molecular reproduction and development **77**(1): 51-58.
- Paczkowski, M., et al., (2014). "Fatty acid metabolism during maturation affects glucose uptake and is essential to oocyte competence." Reproduction **148**(4): 429-439.
- Paczkowski, M., et al., (2013). "Comparative importance of fatty acid beta-oxidation to nuclear maturation, gene expression, and glucose metabolism in mouse, bovine, and porcine cumulus oocyte complexes." Biology of reproduction **88**(5): 111, 111-111.
- Paczkowski, M., et al., (2011). "Alterations in the transcriptome of porcine oocytes derived from prepubertal and cyclic females is associated with developmental potential." Journal of animal science **89**(11): 3561-3571.
- Pawlak, P., et al., (2012). "No single way to explain cytoplasmic maturation of oocytes from prepubertal and cyclic gilts." Theriogenology **78**(9): 2020-2030.
- Pawlak, P., et al., (2020). "Fatty acid induced lipolysis influence embryo development, gene expression and lipid droplet formation in the porcine cumulus cells." Biology of reproduction.
- Pawlak, P., et al., (2018). "The consequences of porcine IVM medium supplementation with follicular fluid become reflected in embryo quality, yield and gene expression patterns." Scientific reports **8**(1): 1-12.
- Pedersen, H. S., et al., (2016). "Ultrastructure and mitochondrial numbers in pre- and postpubertal pig oocytes." Reproduction, Fertility and Development **28**(5): 586-598.
- Peters, A. E., et al., (2020). "Autophagy in female fertility: a role in oxidative stress and aging." Antioxidants & redox signaling **32**(8): 550-568.

- Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic acids research **29**(9): e45-e45.
- Prates, E., et al., (2014). "A role of lipid metabolism during cumulus-oocyte complex maturation: impact of lipid modulators to improve embryo production." Mediators of inflammation **2014**.
- Reed, B. D., et al., (2008). "Genome-wide occupancy of SREBP1 and its partners NFY and SP1 reveals novel functional roles and combinatorial regulation of distinct classes of genes." PLoS genetics **4**(7): e1000133.
- Ridgway, N. and R. McLeod (2008). Biochemistry of lipids, lipoproteins and membranes, Elsevier.
- Roelen, B. A. (2020). "Bovine oocyte maturation: Acquisition of developmental competence." Reproduction, Fertility and Development **32**(2): 98-103.
- Romar, R., et al., (2011). "Expression of maternal transcripts during bovine oocyte in vitro maturation is affected by donor age." Reproduction in Domestic Animals **46**(1): e23-e30.
- Ruderman, N. B., et al., (2003). "Minireview: malonyl CoA, AMP-activated protein kinase, and adiposity." Endocrinology **144**(12): 5166-5171.
- Russell, D. W. and J. Sambrook (2001). Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Cold Spring Harbor, NY.
- Sanchez-Lazo, L., et al., (2014). "Fatty acid synthesis and oxidation in cumulus cells support oocyte maturation in bovine." Molecular endocrinology **28**(9): 1502-1521.
- Schübeler, D. (2015). "Function and information content of DNA methylation." Nature **517**(7534): 321-326.
- Schwarzer, C., et al., (2014). "Maternal age effect on mouse oocytes: new biological insight from proteomic analysis." Reproduction **148**(1): 55-72.
- Sherrer, E., et al., (2004). "Fertilization and blastocyst development in oocytes obtained from prepubertal and adult pigs." Journal of animal science **82**(1): 102-108.
- Shin, D. W. (2020). "Lipophagy: molecular mechanisms and implications in metabolic disorders." Molecules and Cells **43**(8): 686.
- Silva, R. C., et al., (2011). "Ultrastructural characterization of porcine oocytes and adjacent follicular cells during follicle development: lipid component evolution." Theriogenology **76**(9): 1647-1657.
- Silveira, M. M., et al., (2018). "DNA methylation profile at a satellite region is associated with aberrant placentation in cloned calves." Placenta **70**: 25-33.
- Simerman, A. A., et al., (2015). "Intrafollicular cortisol levels inversely correlate with cumulus cell lipid content as a possible energy source during oocyte meiotic resumption in women undergoing ovarian stimulation for in vitro fertilization." Fertility and sterility **103**(1): 249-257.
- Singh, R., et al., (2009). "Autophagy regulates lipid metabolism." Nature **458**(7242): 1131-1135.
- Somfai, T., et al., (2011). "Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes." Reproduction, Fertility and Development **23**(7): 912-920.
- Spate, L. D., et al., (2014). "Dickkopf-related protein 1 inhibits the WNT signaling pathway and improves pig oocyte maturation." PloS one **9**(4): e95114.

- Sprícigo, J. F. W. and M. A. N. Dode (2017). "Transferência intrafolicular de ovócitos imaturos (TIFOI) em bovinos." Embrapa Recursos Genéticos e Biotecnologia-Artigo em periódico indexado (ALICE).
- Srirattana, K., et al., (2022). "Strategies to Improve the Efficiency of Somatic Cell Nuclear Transfer." International Journal of Molecular Sciences **23**(4): 1969.
- Sturmey, R., et al., (2009). "Role of fatty acids in energy provision during oocyte maturation and early embryo development." Reproduction in domestic animals **44**: 50-58.
- Succu, S., et al., (2021). "Vitrification of In Vitro Matured Oocytes Collected from Adult and Prepubertal Ovaries in Sheep." JoVE (Journal of Visualized Experiments)(173): e62272.
- Sun, Y.-C., et al., (2018). "The role of autophagy during murine primordial follicle assembly." Aging (Albany NY) **10**(2): 197.
- Takahashi, T., et al., (2013). "Supplementation of culture medium with L-carnitine improves development and cryotolerance of bovine embryos produced in vitro." Reproduction, Fertility and Development **25**(4): 589-599.
- Tatemoto, H., et al., (2000). "Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during in vitro maturation: role of cumulus cells." Biology of reproduction **63**(3): 805-810.
- Tatsumi, T., et al., (2018). "Forced lipophagy reveals that lipid droplets are required for early embryonic development in mouse." Development **145**(4): dev161893.
- Tsukamoto, S., et al., (2008). "The role of autophagy during the oocyte-to-embryo transition." Autophagy **4**(8): 1076-1078.
- Tsunoda, T. and T. Takagi (1999). "Estimating transcription factor bindability on DNA." Bioinformatics (Oxford, England) **15**(7): 622-630.
- Uzbekova, S., et al., (2015). "MALDI mass spectrometry imaging of lipids and gene expression reveals differences in fatty acid metabolism between follicular compartments in porcine ovaries." Biology **4**(1): 216-236.
- Valsangkar, D. S. and S. M. Downs (2015). "Acetyl CoA carboxylase inactivation and meiotic maturation in mouse oocytes." Molecular reproduction and development **82**(9): 679-693.
- Van Limbergen, J., et al., (2009). "Autophagy: from basic science to clinical application." Mucosal immunology **2**(4): 315-330.
- Vanella, A., et al., (2000). "L-propionyl-carnitine as superoxide scavenger, antioxidant, and DNA cleavage protector." Cell Biology and Toxicology **16**(2): 99-104.
- Vysochan, A., et al., (2017). "ACSS2-mediated acetyl-CoA synthesis from acetate is necessary for human cytomegalovirus infection." Proceedings of the National Academy of Sciences **114**(8): E1528-E1535.
- Wakil, S. J. and L. A. Abu-Elheiga (2009). "Fatty acid metabolism: target for metabolic syndrome." Journal of lipid research **50**(Supplement): S138-S143.
- Warzych, E., et al., (2011). "Maternal nutrition affects the composition of follicular fluid and transcript content in gilt oocytes." Vet Med **56**(4): 156-167.
- Xu, H., et al., (2020). "The Effect of L-Carnitine Additive During In Vitro Maturation on the Vitrification of Pig Oocytes." Cellular Reprogramming **22**(4): 198-207.
- Yamaguchi, T., et al., (2004). "CGI-58 interacts with perilipin and is localized to lipid droplets: possible involvement of CGI-58 mislocalization in Chanarin-Dorfman syndrome." Journal of Biological Chemistry **279**(29): 30490-30497.

- Yang, G., et al., (2021). "Dux-mediated corrections of aberrant H3K9ac during 2-cell genome activation optimize efficiency of somatic cell nuclear transfer." Cell stem cell **28**(1): 150-163. e155.
- Yokoo, M. and E. Sato (2004). "Cumulus-oocyte complex interactions during oocyte maturation." International review of cytology **235**: 251-282.
- Yu, X.-X., et al., (2018). "Ascorbic acid induces global epigenetic reprogramming to promote meiotic maturation and developmental competence of porcine oocytes." Scientific reports **8**(1): 1-12.
- Yuan, Y., et al., (2017). "Quadrupling efficiency in production of genetically modified pigs through improved oocyte maturation." Proceedings of the National Academy of Sciences **114**(29): E5796-E5804.
- Zechner, R., et al., (2017). "Cytosolic lipolysis and lipophagy: two sides of the same coin." Nature Reviews Molecular Cell Biology **18**(11): 671-684.
- Zeng, Y. and T. Chen (2019). "DNA methylation reprogramming during mammalian development." Genes **10**(4): 257.
- Zeron, Y., et al., (2001). "Seasonal changes in bovine fertility: relation to developmental competence of oocytes, membrane properties and fatty acid composition of follicles." Reproduction **121**(3): 447-454.
- Zuccotti, M., et al., (2011). "What does it take to make a developmentally competent mammalian egg?" Human reproduction update **17**(4): 525-540.

Capítulo III – Expression profile of genes related to lipophagy in oocytes from prepubertal gilts and cycling sows during *in vitro* maturation

Artigo submetido a revista Genetics and Molecular Biology

Thainara Christie Ferreira Silva¹, Margot Alves Nunes Dode^{2,3}, Luna Nascimento Vargas¹, Amanda Oliveira Moura⁴, Nayara Ribeiro Kussano³, Ligiane Oliveira Leme⁵ and Maurício Machaim Franco^{1,2,4,6*}

¹ Programa de Pós-Graduação em Genética e Bioquímica, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil;

² Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil;

³ Pós-graduação em Biologia Animal, Universidade de Brasília, Brasília, DF, Brazil;

⁴ Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil;

⁵ Universidade Federal do Espírito Santo, Vitória, ES, Brazil;

⁶ Instituto de Biotecnologia, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil;

*Corresponding author:

E-mail: mauricio.franco@embrapa.br

Abstract

Lipid metabolism plays an important role in cumulus-oocyte complex, acting as an important energy source during oocyte maturation and early embryonic development. Several studies have evaluated lipid metabolism in oocytes, however, there is no information on whether oocyte use lipophagy as a source of fatty acids and energy or if lipophagy is affected by oocyte competence and female age. Here, we evaluated the expression levels of genes involved in lipophagy in oocytes from cycling sows and prepubertal gilts during *in vitro* maturation (IVM) using quantitative real-time PCR. Ten genes were selected for the study: *ATG5*, *ATG7*, *RAB7*, *RAB10*, *LC3*, *PNPLA2*, *p62*, *TFEB*, *TFE3*, and *mTORC1*. During IVM, the expression of all genes increased in sow group ($P < 0.05$), whereas *TFEB* was the only gene whose expression increased in gilt group. These results suggest that lipophagy may be active in oocytes of sows or that the gametes accumulate mRNA of lipophagy-related genes to prepare the lipid droplet for use as an energy source during early embryonic development. Our results contribute to a better understanding of the mechanisms involved in lipid metabolism in oocytes with different competences and may also contribute to new discoveries regarding the importance of lipophagy in embryology.

Keywords: embryo production, fatty acid, lipid metabolism, lipophagy, pig.

Oocyte competence is an important requirement for *in vitro* embryo production, because only competent oocytes are capable of complete maturation and develop as an embryo after fertilization. In general, oocytes from younger females have lower competence and, consequently, a decreased capacity to develop into embryos in many species (Marchal et al., 2001; Grupen et al., 2003; Pawlak et al., 2012; Braga et al., 2019). In pigs, oocytes from prepubertal gilts are routinely used for basic research and *in vitro* embryo production (Grabowska et al., 2016; Moreira et al., 2016; Yuan et al., 2017).

Recently, we showed that there are different dynamics in the expression of genes related to lipid metabolism in cumulus cells (data submitted for publication) and oocytes (Braga et al., 2019) from sows compared to gilts. Furthermore, we showed that during *in vitro* maturation (IVM), oocytes from sows accumulate lipid droplets (LDs), whereas lipid expenditure is observed in oocytes from gilts. These differences in metabolism among the cumulus-oocyte complexes (COCs) from animals of different sexual maturities show the importance of lipid metabolism for the acquisition of competence and oocyte maturation in pigs.

Lipid metabolism plays an important role in the COC in several species (Prates et al., 2014; Sanchez-Lazo et al., 2014; Braga et al., 2019; Ibayashi et al., 2021) especially in pigs, where they have a high lipid content (Paczkowski et al., 2013). Lipids are an important energy source for sustaining oocyte maturation and early embryonic development (Paczkowski et al., 2013). However, lipid metabolism during IVM of cycling sow oocytes seems to differ from the oocytes from other species and those from prepubertal gilts (Paczkowski et al., 2013; Braga et al., 2019).

Several studies have investigated lipid metabolism in oocytes, with a focus on well-described pathways such as lipolysis and lipogenesis (Sanchez-Lazo et al., 2014; Braga et al., 2019; Faria et al., 2021). Studies on lipophagy have been conducted in recent years but only in the context of human diseases, associating the process with metabolic disorders caused by an imbalance in the amount of lipids in different cells (Haidar et al., 2021). Therefore, little is known about the contribution of this mechanism to oocyte maturation and, consequently, its importance in gametogenesis and embryogenesis.

Lipophagy is a pathway that, along with lipolysis, maintains LD homeostasis. The molecular mechanisms governing this pathway are not well understood (Shin, 2020). The interaction between lipophagy and lipolysis, the triacylglycerol degrading pathways, to regulate lipid metabolism and energy homeostasis is not fully understood (Zechner et al., 2017). Indeed, the contribution of each pathway to LD homeostasis is unclear and may vary among different cell types (Zechner et al., 2017). Therefore, the study of lipophagy in oocytes from cycling sows and prepubertal gilts, especially during oocyte maturation, is important for determining the extent to which female gametes use lipophagy as a source of fatty acids and energy. This study contributes to the understanding of lipophagy and its importance in oocyte physiology.

Based on our previous results, where oocytes from cycling sows accumulate lipids during IVM and considering that both lipolysis and lipophagy are part of the lipolytic pathway, we hypothesized that lipophagy is silenced or reduced in these oocytes; conversely, we hypothesized that lipophagy would be augmented in oocytes from prepubertal gilts because they consume lipids during IVM. To test this hypothesis,

we analyze the transcript levels of genes involved in lipophagy in these gametes before and after IVM.

Experiments were performed in accordance with the Brazilian Law for Animal Protection and approved by the Ethics Committee on Animal Use at EMBRAPA (Brazilian Agricultural Research Corporation) Genetic Resources and Biotechnology. The chemicals and reagents used to prepare the IVM medium were purchased from Sigma (St. Louis, MO, USA), unless otherwise indicated.

The biological material was collected and handled as described by Braga *et al*, (2019). Briefly, ovaries from cycling sows, containing corpus luteum or albicans, and prepubertal gilt ovaries were collected and transported to the laboratory in saline solution at 30–35 °C. Cumulus–oocyte complexes were aspirated from the follicles that were 2–6 mm in diameter. Only the COCs with a homogenous cytoplasm and more than three compact layers of cumulus cells were used. Some COCs were denuded via repeated pipetting and immediately stored in a stabilization solution (RNA/later™, Applied Biosystems· Foster City, CA, USA). The remaining COCs were subjected to IVM, which was performed according to the protocol described by Marques *et al*. (2007) and Braga *et al*. (2019). Briefly, COCs were subjected to *in vitro* maturation for 44 h in TCM199 supplemented with 3.05 mM glucose, 0.91 mM sodium pyruvate, 50 IU/mL gentamycin, 0.57 mM cysteine, 10% (v/v) porcine follicular fluid from cycling sows, 10 ng/mL EGF, and hormones for the first 22 h (10 IU/mL eCG and 10 IU/mL hCG) at 38 °C and 5% CO₂ (v/v) in air. After IVM, COCs were denuded via repeated pipetting, and only the oocytes that presented a polar body were stored in RNA/later™ and frozen at –80 °C for gene expression analysis. Oocytes were then classified into four experimental groups: immature (GV) and *in*

vitro matured (MII) oocytes from cycling sows and GV and MII oocytes from prepubertal gilts.

For the prepubertal gilts (n = 3) and cycling sows (n = 4), pools of 25 oocytes from each experimental group were used to isolate total RNA using the RNeasy Plus Micro Kit (Qiagen®, Venlo, NL), according to the manufacturer's instructions. Total RNA was used to synthesize complementary DNA (cDNA) using GoScript Reverse Transcriptase (Promega®, Madison, WI, USA) and 0.5 µg/µL of random primers (500 µg/mL; Promega) in a final volume of 40 µL. Reactions were performed at 25 °C for 5 min and 42 °C for 60 min, followed by enzyme inactivation at 70 °C for 15 min.

Genes were selected based on their function and their involvement in lipophagy and are schematically represented in Figure 1. Some genes participate in phagophore formation, such as *autophagy-related (ATG) 5*, *ATG7*, *microtubule-associated proteins 1A/1B light chain 3 (MAP1LC3A or LC3)*, and *ras-related proteins Rab-10* and *Rab-7*. Others code proteins that are present on the surface of LDs include patatin-like phospholipase domain-containing enzyme 2 (PNPLA2, also known as adipose triglyceride lipase or ATGL) and sequestosome 1 (SQSTM1 or p62). We also evaluated genes that regulate the expression of lysosomal lipases and lysosomal biogenesis, namely, *transcription factor EB (TFEB)* and *transcription factor binding to IGHM enhancer 3 (TFE3)*. Finally, the expression of the mammalian target of rapamycin complex 1 (*mTORC1*) gene, a negative regulator of autophagy, was evaluated. All gene nomenclature, GenBank accession numbers, primer sequences, and primer efficiencies are listed in Table 1.

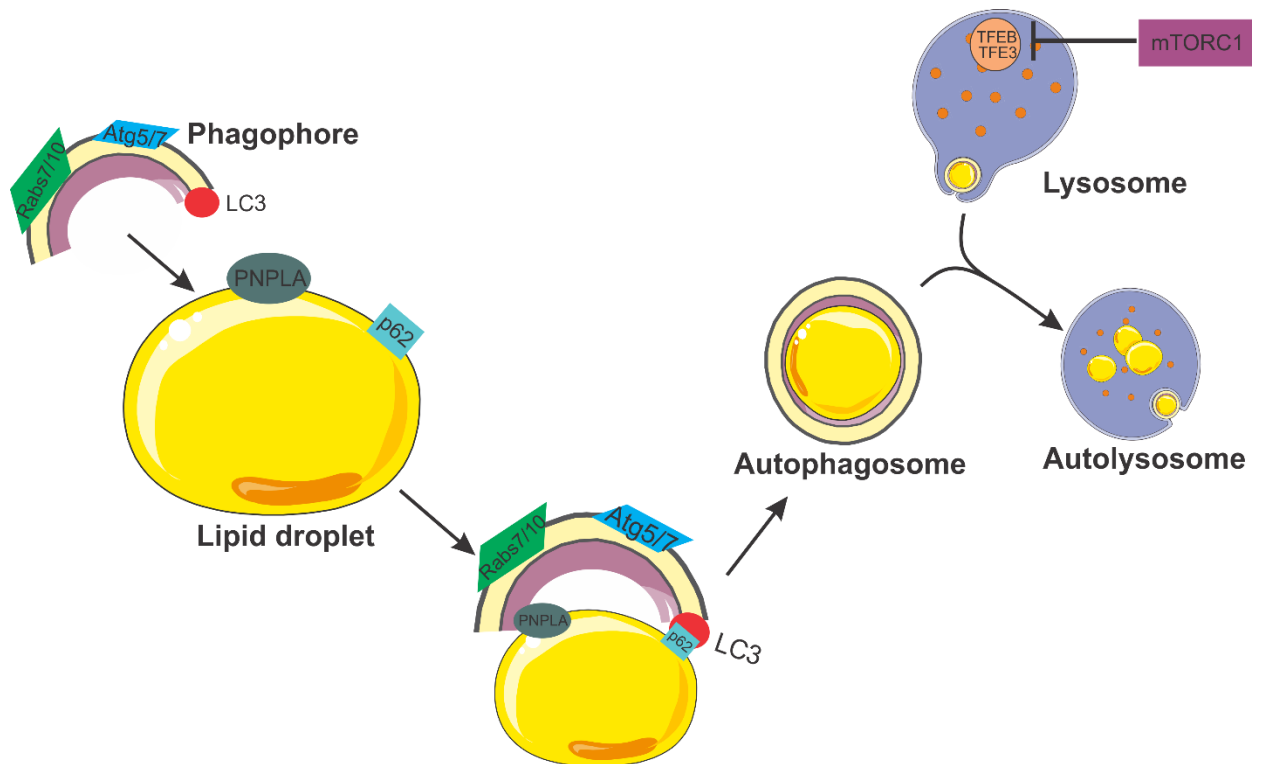


Figure 1. Genes related to lipophagy based on protein function in many cell types. In this scheme, the phagophore, formed by the proteins Rabs and Atgs, binds LC3 to the p62 protein on the surface of the LD. The phagophore expands with the help of PNPLA proteins present on the surface of the LD and engulfs it totally or partially. The union of the phagophore with the LD forms the autophagosome, which is engulfed by the lysosome to form an autolysosome. The transcription factors TFEB and TFE3 are responsible for the synthesis of lysosomal lipases, which degrade LD in the autolysosome. mTORC1 is an inhibitor of these transcription factors. The degradation of triacylglycerol present in LDs allows the release of free fatty acids that undergo oxidation in the mitochondria for energy production.

Real-time PCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) using GoTaq qPCR Master Mix (Promega). Three technical replicates were used for this study. All primer pairs were designed using exon-exon junctions; thus, no amplification of genomic DNA was observed. The specificity of each primer pair was determined by observing the melting curve and amplicon size visualized on agarose gel. Each PCR was run in a final volume of 25 μ L using 1 μ L of cDNA and 0.5 μ M of each primer (forward and reverse).

The cycling conditions were as follows: 95 °C for 5 min, followed by 50 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min.

Table 1. Gene nomenclature, GenBank access number, primer sequences, melt temperature, amplicon sizes and primer efficiency for each gene used for gene expression in qPCR.

Gene	Name	Access Number	Primer Sequence	MT (°C)	Amplicon size (bp)	Eff%
ATG5	Autophagy related 5	NM_001037152.2	(F) CTGCACTGTCCATCAAAGGAT (R) TTCCGATTGATGGCCCCAA	80.08	176	102.805
ATG7	Autophagy related 7	NM_001190285.1	(F) TCTCTGCCTTCCAGAGAGTAT (R) CGGGACTCCTTCTGTTTGAC	80.94	127	100.039
LC3	Microtubule-associated proteins 1A/1B light chain 3B	NM_001170827.1	(F) CCTGGACAAGACCAAGTTCC (R) GAAGCCATCCTCATCCTTCTC	87.28	187	90.389
RAB10	ras-related protein Rab-10	NM_001243060.1	(F) TGAGCATGCCAACGAAGATG (R) GCTCTCTTGCAATCTGTTCTCC	77.33	107	103.158
RAB7	ras-related protein Rab-7	XM_021069431.1	(F) GTTCTCATCCAGGCCAGTC (R) GGAAGGCTTGCTCCACATT	85.91	185	100.932
PNPLA2	Patatin like phospholipase domain containing 2	XM_021076533.1	(F) AACGTCTGCAGCACCTTCAT (R) TCGTAGAGAGGCAGGTTGTC	85.83	107	90.523
p62	Sequestosome 1	XM_003123639.4	(F) GAATAAGATCGCCCTGGAGTC (R) TAGACGGGTCCACCTCTTT	82.99	116	97.732
TFEB	Lysosomal biogenesis transcription factor EB	XM_021098671.1	(F) CCAAGGAGCGACAGAAGAAG (R) GATGGTGGCCTTGTTCCA	84.02	140	85.229
TFE3	Transcription factor binding to IGHM enhancer 3	XM_003135074.6	(F) CAGGCAGTGCTCCCAAC (R) TGGACTCCAGGCTAATGATCT	82.65	102	94.45
mTORC1	Mammalian target of rapamycin complex 1	XM_013983042.2	(F) GATTCCAGCAACGGCTTTC (R) ATGGCTGGCAGTGTCTC	86.94	119	83.286
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	AF017079.1	(F) GTCTGGAGAAACCTGCCAAATA (R) CCCAGCATCAAAGGTAGAAGAG	85.46	152	95.378
β-actin	Beta-actin	U07786.1	(F) GACCACCTTCAACTCGATCAT (R) GATCTCCTTCTGCATCCTGTC	85.85	124	93.65

(MT) melt temperature; (Eff%) primer efficiency; (F) forward; (R) reverse; (bp) base pair;

Transcript levels were normalized using the geometric averaging of the endogenous genes β -actin and GAPDH and calculated using the $\Delta\Delta\text{Ct}$ method with efficiency correction (Pfaffl, 2001). The GV group was used as the control group. We compared the levels of gene expression in prepubertal gilts and cycling sows separately but compared before and after IVM in each group of animals. Transcript level comparisons between groups were performed using Student's t-test or the Mann-Whitney test for normally and non-normally distributed data, respectively. All analyses were performed using Action version 3.7 (Estatcamp, 2014) and GraphPad Prism version 7.00 (GraphPad Prism Software, San Diego, CA, USA). The gene expression results are shown in Figures 2 and 3. For cycling sows all 10 genes (*ATG5*, *ATG7*, *RAB7*, *RAB10*, *LC3*, *PNPLA2*, *p62*, *TFEB*, *TFE3*, and *mTORC1*) were detected in immature and mature oocytes and showed an increase in transcript levels during IVM ($P < 0.05$). For prepubertal gilts, all genes were also detected in immature and mature oocytes; however, only *TFEB* increased in expression during IVM ($P < 0.05$).

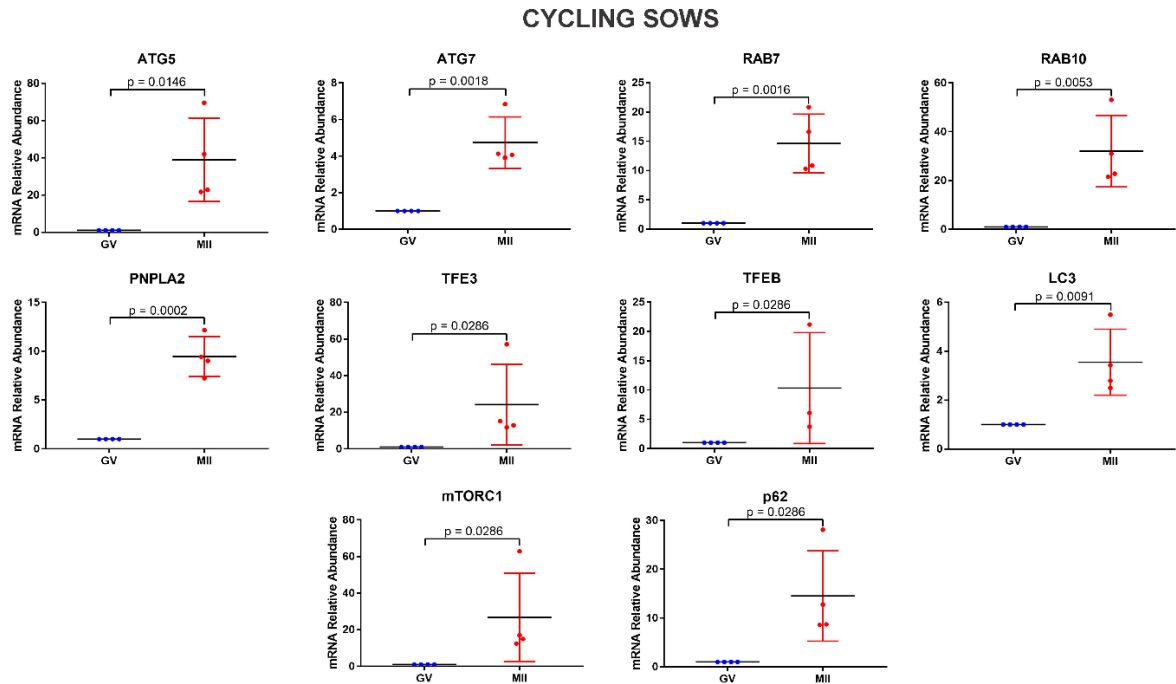


Figure 2. Relative abundance of mRNA encoding the lipophagy genes, as determined using RT-qPCR in immature and *in vitro* matured oocytes from cycling sows. Data are presented as mean $\pm$ standard deviation of the mean of four biological replicates. Data were normalized using the $\Delta\Delta C_t$ method (Pfaffl 2001), with the geometric mean of β -actin and *GAPDH* as the endogenous controls.

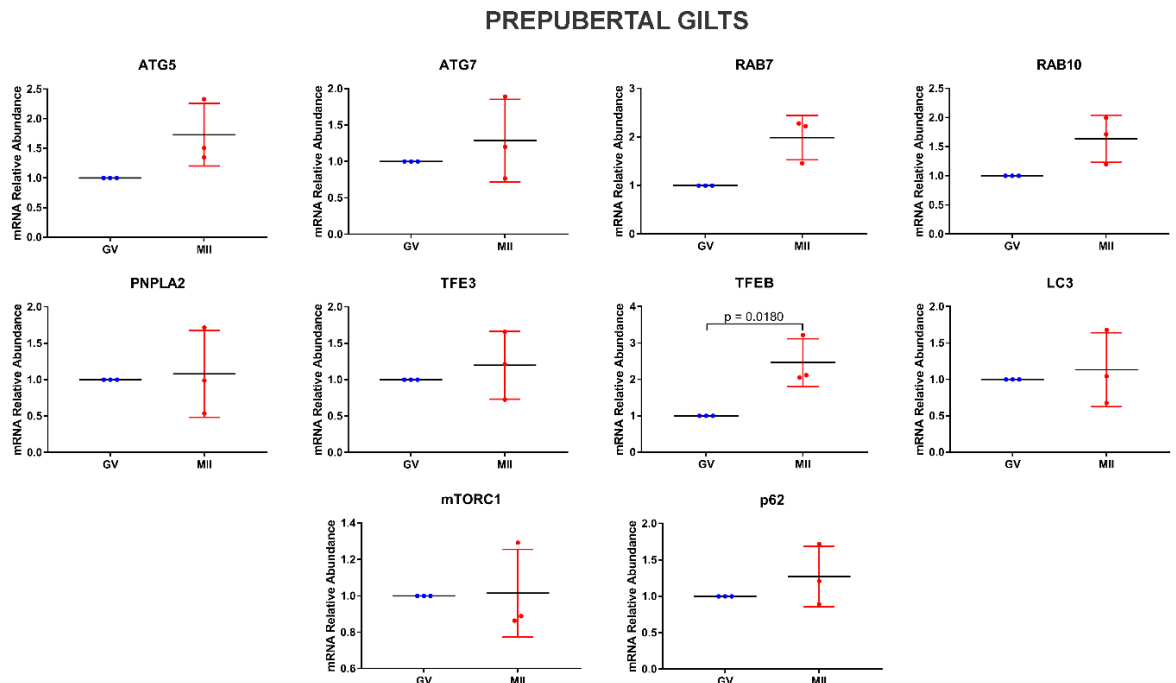


Figure 3. Relative abundance of mRNA encoding the lipophagy genes, as determined using RT-qPCR in immature and *in vitro* matured oocytes from prepubertal gilts. Data are presented as mean $\pm$ standard deviation of the mean of four biological replicates. Data were normalized using the $\Delta\Delta C_t$ method (Pfaffl 2001), with the geometric mean of β -actin and *GAPDH* as the endogenous controls.

In recent studies from our group, oocytes (Braga et al., 2019) and cumulus cells (data submitted to publication) from cycling animals exhibited differences in lipid metabolism during *in vitro* maturation compared to those from the prepubertal gilts. We have extended these observations to lipophagy, a process that had previously not been studied in oocytes of any species during IVM. We hypothesized that the dynamics of this pathway would differ between oocytes from cycling sows and those from prepubertal gilts. To test this hypothesis, we used oocytes from prepubertal gilts and from cycling sows and characterized, for the first time, the expression of genes related to lipophagy in immature and *in vitro* matured oocytes with different competences.

Our results showed that all genes were increased in expression in the cycling sow group after IVM. This result was unexpected because the lipolytic pathway is reduced in oocytes from cycling sows (Braga et al., 2019). It is possible that, in this group, the lipophagic pathway is active, providing fatty acids for cell maintenance; alternatively, the oocyte may accumulate lipophagic-related mRNAs with the effect of readying the cell for using LDs as an energy source during early embryonic development (Tsukamoto et al., 2008; Nakashima et al., 2017). In the gilt group, only one gene (*TFEB*) showed an increase in its transcript levels during IVM, suggesting that the lipophagic pathway may not be required for fatty acid supply in prepubertal gilt oocytes and that demand is met by the classical lipolytic pathway.

The importance of autophagy in processes such as the development of primordial follicles, embryogenesis, and placental development has already been demonstrated (Tsukamoto et al., 2008; Kanninen et al., 2013; Sun et al., 2018). Autophagy contributes to organelle turnover, bioenergetic management, and immune regulation. It regulates cell death and survival during pregnancy and

maintains cellular homeostasis (Kanninen et al., 2013). However, studies on lipophagy in reproduction are scarce. Tatsumi et al. (2018) demonstrated that lysosomal degradation of LDs is necessary for early embryonic development. The authors induced lipophagy in mouse embryos and observed that this pathway was not involved in protecting the cell from lipotoxicity. In addition, forced consumption of LDs at early stages impairs embryonic development at later stages; therefore, an appropriate level of lipid storage is required for preimplantation embryo development (Tatsumi et al., 2018). Here, we show that the expression of genes related to lipophagy increased during IVM in oocytes from cycling sows. This finding suggested that lipophagy may be a protective mechanism against lipotoxicity in these oocytes, given their high lipid content, and that during maturation the oocyte prepares the molecular machinery of the lipophagic pathway for embryonic development.

As the mTORC1 protein is a negative regulator of autophagy (Kloska et al., 2020), it was expected that the expression of this gene would have a different dynamic compared to other genes, whose proteins actively participate in autophagy. However, we observed similar expression dynamics among all the genes. According to Zechner et al. (2017), mTOR signaling has an antilipolytic role that leads to a decrease in lipolysis. According to Mensah et al. (2017), mTORC1 participates in the formation of LDs. Garcia-Macia et al. (2021) suggested that mTORC1 is essential in the liver to activate lipophagy. Taken together, these results suggest that mTORC1 participates in the accumulation of lipids in the oocytes of cycling sows through the inhibition of lipolysis and formation of LDs. Furthermore, mTORC1 may have different functions in different cell types and may be actively participating in lipophagy in oocytes.

In conclusion, the expression of genes related to lipophagy during IVM showed different dynamics between cycling sows and prepubertal gilts. Oocytes from cycling sows showed increased expression during IVM, whereas oocytes from prepubertal gilts showed no difference in the expression of most genes. Our results contribute to a better understanding of the mechanisms involved in lipid metabolism in porcine COCs during maturation and may contribute to new discoveries about the importance of lipophagy in gametogenesis and embryogenesis.

Acknowledgments

We thank Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Embrapa Genetic Resources and Biotechnology for their financial support. We also thank the slaughterhouses at Frimelo (Luziânia-GO) and Bom Corte (Formosa-GO) and their employees for supplying biological material.

Conflict of Interest

The authors have no conflicts of interest to declare.

Author Contributions

TCFS, MAND and MMF conceived and designed the study; TCFS, LNV and AOM collected the samples; TCFS, LNV, AOM, NRK and LOL conducted experiments. TCFS performed molecular analysis and wrote the manuscript; MAND and MMF made a critical review, adding improvements through comments and rewriting sentences. All authors read and approved the final version.

References

- Braga, T. F., et al. (2019). "The dynamics of gene expression, lipid composition and DNA methylation reprogramming are different during in vitro maturation of pig oocytes obtained from prepubertal gilts and cycling sows." Reproduction in Domestic Animals **54**(9): 1217-1229.
- Estatcamp, E. (2014). "Software Action." São Carlos: Estatcamp-Consultoria em estatística e qualidade.
- Faria, O., et al. (2021). "Maturation system affects lipid accumulation in bovine oocytes." Reproduction, Fertility and Development **33**(5): 372-380.
- Garcia-Macia, M., et al. (2021). "A Mammalian Target of Rapamycin-Perilipin 3 (mTORC1-Plin3) Pathway Is Essential to Activate Lipophagy and Protects against Hepatosteatosis." Hepatology **74**(6): 3441-3459.
- Haidar, M., et al. (2021). "Lipophagy: a new player in CNS disorders." Trends in Endocrinology & Metabolism **32**(11): 941-951.
- Ibayashi, M., et al. (2021). "Homeostatic regulation of lipid droplet content in mammalian oocytes and embryos." Reproduction **162**(6): R99-R109.
- Kanninen, T. T., et al. (2013). "The role of autophagy in reproduction from gametogenesis to parturition." European Journal of Obstetrics & Gynecology and Reproductive Biology **171**(1): 3-8.
- Kloska, A., et al. (2020). "Lipophagy and lipolysis status in lipid storage and lipid metabolism diseases." International Journal of Molecular Sciences **21**(17): 6113.
- Marques, M. G., et al. (2007). "In vitro maturation of pig oocytes with different media, hormone and meiosis inhibitors." Animal reproduction science **97**(3-4): 375-381.
- Mensah, L. B., et al. (2017). "mTORC1 signalling mediates PI3K-dependent large lipid droplet accumulation in Drosophila ovarian nurse cells." Biology Open **6**(5): 563-570.
- Nakashima, A., et al. (2017). "Role of autophagy in oocytogenesis, embryogenesis, implantation, and pathophysiology of pre-eclampsia." Journal of Obstetrics and Gynaecology Research **43**(4): 633-643.
- Paczkowski, M., et al. (2013). "Comparative importance of fatty acid beta-oxidation to nuclear maturation, gene expression, and glucose metabolism in mouse, bovine, and porcine cumulus oocyte complexes." Biology of reproduction **88**(5): 111, 111-111.
- Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic acids research **29**(9): e45-e45.
- Prates, E., et al. (2014). "A role of lipid metabolism during cumulus-oocyte complex maturation: impact of lipid modulators to improve embryo production." Mediators of inflammation **2014**.
- Sanchez-Lazo, L., et al. (2014). "Fatty acid synthesis and oxidation in cumulus cells support oocyte maturation in bovine." Molecular endocrinology **28**(9): 1502-1521.
- Shin, D. W. (2020). "Lipophagy: molecular mechanisms and implications in metabolic disorders." Molecules and Cells **43**(8): 686.
- Singh, R., et al. (2009). "Autophagy regulates lipid metabolism." Nature **458**(7242): 1131-1135.

- Sun, Y.-C., et al. (2018). "The role of autophagy during murine primordial follicle assembly." Aging (Albany NY) **10**(2): 197.
- Tatsumi, T., et al. (2018). "Forced lipophagy reveals that lipid droplets are required for early embryonic development in mouse." Development **145**(4): dev161893.
- Tsukamoto, S., et al. (2008). "The role of autophagy during the oocyte-to-embryo transition." Autophagy **4**(8): 1076-1078.
- Zechner, R., et al. (2017). "Cytosolic lipolysis and lipophagy: two sides of the same coin." Nature Reviews Molecular Cell Biology **18**(11): 671-684.

Considerações finais

O complexo cumulus-ovócito (COC) de fêmeas suínas com diferentes idades sexuais apresenta mecanismos distintos em relação ao metabolismo de lipídios. Nas fêmeas púberes, que possuem ovócitos mais competentes, o gameta apresentou um acúmulo de lipídios durante a MIV, o que, aparentemente, é resultado da contribuição das células do cumulus e composição do fluido folicular. Além disso, a via de lipofagia nesses gametas parece estar ativa para manutenção da homeostase lipídica, a fim de evitar excesso de lipídios durante a MIV e lipotoxicidade durante o desenvolvimento embrionário inicial.

Por outro lado, os ovócitos menos competentes, pertencente às marrãs pré-púberes, apresentaram mecanismos opostos àqueles apresentados pelas porcas cíclicas em relação ao metabolismo de lipídio. Nesses gametas, houve diminuição da quantidade de lipídios durante a MIV, o que indica uma atividade lipolítica ativa. No entanto, a via da lipofagia está aparentemente silenciada ou diminuída, indicando que, em ovócitos suínos, lipólise e lipofagia podem ser vias opostas, mas ambas contribuem para manutenção da quantidade de lipídios.

Com relação à lipofagia, os resultados de expressão gênica não são suficientes para determinar se a via está de fato ativa ou não. Portanto, mais estudos são necessários para a compreensão dessa via em gametas e a relevância dela na homeostase lipídica.

Os resultados apresentados mostram que de fato os ovócitos de marrãs pré-púberes ainda não estão prontos para serem maturados, por não terem adquirido a competência durante os estágios finais de foliculogênese. Portanto, esses gametas necessitam de novos protocolos de maturação *in vitro* que garantam um comportamento mais parecido aos gametas competentes.

ANEXOS

ANEXO 1

EXTRAÇÃO DE RNA DE CÉLULAS DO CUMULUS E OVÓCITOS

- Kit: RNeasy® Plus Micro Kit (QIAGEN)
- As células devem ser armazenadas em RNAlater a -80° C

Antes de iniciar a extração de ovócitos:

- Carrier RNA 310 ng/ µL (Preparar solução a 4 ng/ µL)

$$C_i \times V_i = C_f \times V_f$$

$$310 \times V_i = 4 \times 80 \mu\text{L}$$

$$V_i = 1,036 \mu\text{L} + 78,96 \mu\text{L de RLT plus}$$

- Preparar a solução na hora de usar

- 1) Centrifugar os microtubos com as amostras – 6.250 rpm / 2 minutos
Retirar o sobrenadante deixando o menor volume possível
- 2) Adicionar 75 µL de Buffer RLT plus
 - Fazer o mais rápido possível para evitar degradação
- 3) Transferir o lisado para tubo de 1,5 ml, adicionar 5 µL do Carrier RNA e ajustar o volume final para 350 µL com Buffer RLT plus
 - Vórtex por 1 minuto
- 4) Transferir o lisado para coluna gDNA eliminator (borda roxa)
 - Centrifugar por 30 s / 10000 rpm
 - Descartar a coluna e guardar o filtrado
- 5) Adicionar 350 µL de etanol 70% ao filtrado e homogeneizar
- 6) Transferir o lisado para coluna RNeasy MinElute (rosa)
 - Centrifugar por 15 s / 10000 rpm
 - Descartar o filtrado
- 7) Adicionar 700 µL de Buffer RW1 na coluna RNeasy MinElute
 - Centrifugar por 15 s / 10000 rpm
 - Descartar o filtrado
- 8) Adicionar 500 µL de Buffer RPE na coluna RNeasy MinElute
 - Centrifugar por 15 s / 10000 rpm
 - Descartar o filtrado
- 9) Adicionar 500 µL de etanol 80% na coluna RNeasy MinElute
 - Centrifugar por 2 min / 10000 rpm

- Descartar o filtrado e o tubo coletor
- 10) Colocar a coluna RNeasy MinElute em novo tubo coletor
 - Centrifugar por 5 min / 13500 rpm com a tampa da coluna aberta
 - Descartar o filtrado e o tubo coletor
- 11) Colocar a coluna RNeasy MinElute em tubo de 1,5 ml
 - Adicionar 14 µL de água RNase free diretamente na membrana da coluna
 - Centrifugar por 1 min / 13500 rpm
- 12) Armazenar o RNA eluído em freezer -80 °C

ANEXO 2

SÍNTESE DE cDNA

- Kit: GoScript™ Reverse Transcription System Protocol (Promega)

Volume final do cDNA = 30 µL

Reagente	Concentração inicial	Concentração final	Volume final (µl)
RNA	----	72,45 ng	10,5
Oligo(dT)	500 µg/ml	0,5 µg/µl	1,0
Hexâmero	500 µg/ml	0,5 µg/µl	1,0
Incubar em termociclador a 70°C por 5 minutos; depois incubar em gelo por 5 minutos			
Água	----	----	2,5
GoScript Buffer	5x	1x	6,0
MgCl ₂	25 mM	5 mM	6,0
Nucleotide Mix	10 mM	0,5 mM	1,5
RNasin	40 U/µL	20 U	0,5
Enzima RT			1,0

Termociclador: $\left\{ \begin{array}{l} 25^{\circ}\text{C por 5 min} \\ 42^{\circ}\text{C por 1 hora} \\ 70^{\circ}\text{C por 15 min} \end{array} \right.$

- Armazenar em freezer -20 °C

ANEXO 3

EXTRAÇÃO DE DNA DE CÉLULAS DO CUMULUS (Salting OUT)

- Amostras de CC armazenadas em PBS a -80°C
- Usar PBS estéril (autoclavado)
- Ligar banho-maria 55°C
- Resfriar a centrífuga a 4°C
 - 1) Centrifugar as células por 1 min / 13500 rpm e descartar o sobrenadante
 - 2) Adicionar 1ml de PBS, centrifugar por 1 min / 13500 rpm e descartar o sobrenadante, deixando um pouco de PBS para ressuspender o pellet vortexando
 - 3) Adicionar 600 µL de solução de lise nuclear e 4 µL de RNase A (10ng/ µL) e incubar a 55°C por 2 horas
 - Solução de lise nuclear:

[

50 mM de Tris ph 7,8
25 mM de EDTA
400 mM de NaCl
1% de SDS
 - 4) Adicionar 200 µL de solução de precipitação de proteína (NaCl 6M) e vortexar por 30 segundos
 - Incubar em gelo por 5 minutos
 - Centrifugar (16,220 rpm) por 20 minutos a 4°C
 - Transferir o sobrenadante para um novo tubo de 2 ml e descartar o pellet
 - 5) Adicionar 600 µL de isopropanol gelado e misturar por inversão até ver o DNA precipitado e incubar no freezer -20°C overnight
 - 6) No dia seguinte, centrifugar (16,220 rpm) por 30 minutos a 4°C
 - 7) Descartar o sobrenadante e lavar o pellet com 1,2 ml de etanol 70%
 - Centrifugar por 10 minutos e descartar o sobrenadante
 - Deixar o pellet secar por 10 minutos
 - 8) Ressuspender em 50 µL de TE e deixar overnight na geladeira.
 - TE = tampão de eluição:

[

10 mM de Tris pH 7,5
1 mM de EDTA

ANEXO 4

EXTRAÇÃO DE DNA DE OVÓCITO (choque térmico)

- Preparando a enzima Pronase E (50 mg/ml)
 - Protease from *Streptomyces griseus* (Sigma/Merck) - Referência: P8811
 - Preparar 200 µL (0,2 ml)

50 mg ----- 1 ml

X ----- 0,2 ml

X = 10 mg = pesar 0,01 g de pronase e adicionar 200 uL de PBS

- Ovócitos estavam acondicionados em PBS a -80°C

- 1) Retirar as amostras do freezer e descongelar em gelo
- 2) Centrifugar os tubos 10000 rpm por 4 minutos para decantar o material
- 3) Juntar todos em um tubo só, de 0,2 ml
- 4) Retirar o excesso de PBS para ficar apenas 16 uL
- 5) Lavar todos os tubos com 4 uL de pronase (Concentração final de 10mg/ml)
 - Pronase: $C_1 \times V_1 = C_2 \times V_2$
 $50 \times V_1 = 10 \times 20$
 $V_1 = 4 \text{ uL}$
- 6) Incubar em termociclador a 37°C por 30 minutos seguido de 85°C por 15 minutos
- 7) Choque térmico: colocar o tubo contendo os ovócitos em nitrogênio líquido e imediatamente em termociclador a 95°C por 1 minuto
 - Repetir por 5 vezes
 - Armazenar em freezer -20 °C

ANEXO 4

EXTRAÇÃO DE DNA DE SÊMEN (Salting out)

- Solução de lise ($V_f = 350 \mu\text{L}$)

- Utiliza-se $300 \mu\text{L}$ de solução de lise por amostra de sêmen, portanto para preparar solução de lise para uma amostra, calcula-se para o volume final de $350 \mu\text{L}$ da seguinte forma:

{	50 mM de Tris pH 7,8
	5mM de EDTA
	100 mM de NaCl
	2% de SDS

- Preparar 10 mL de solução com os reagentes acima

- <u>Tris 1M</u> $C_i \times V_i = C_f \times V_f$ $1000\text{mM} \times V_i = 50\text{mM} \times 10 \text{ mL}$ $V_i = 0,5 \text{ mL} = 500 \mu\text{L}$	- <u>EDTA 0,5M</u> $C_i \times V_i = C_f \times V_f$ $500\text{mM} \times V_i = 5\text{mM} \times 10$ $V_i = 0,1 \text{ mL} = 100 \mu\text{L}$
- <u>NaCl 6M</u> $C_i \times V_i = C_f \times V_f$ $6000\text{mM} \times V_i = 100\text{mM} \times 10$ $V_i = 0,166 \text{ mL} = 166 \mu\text{L}$	- <u>SDS 10%</u> $C_i \times V_i = C_f \times V_f$ $10\% \times V_i = 2\% \times 10$ $V_i = 2 \text{ mL} = 2000 \mu\text{L}$

- Completar o volume que faltar com água (7,234 mL de água)
- No momento de uso da solução de lise, adiciona-se os demais reagentes:

- <u>β-mercaptoetanol</u> $C_i \times V_i = C_f \times V_f$ $98\% \times V_i = 0,3\% \times 350$ $V_i = 1,07 \mu\text{L}$	- <u>DTT</u> $C_i \times V_i = C_f \times V_f$ $1000\text{mM} \times V_i = 5\text{mM} \times 350$ $V_i = 1,75 \mu\text{L}$	- <u>Proteinase K</u> $C_i \times V_i = C_f \times V_f$ $20 \times V_i = 0,5 \times 350$ $V_i = 8,75 \mu\text{L}$
---	---	--

- 338,43 μL da solução de lise (Tris+EDTA+NaCl+SDS) + 1,07 μL de β -mercaptoetanol + 1,75 μL de DTT + 8,75 μL de Proteinase K = 350 μL (usar 300 μL para extração de 1 amostra de sêmen).

- 1) Lavar o sêmen 2 vezes com 1 mL de PBS
 - Centrifugar a 13.000 rpm por 30 seg.
- 2) Ressuspender os espermatozoides em 300 μL de solução de lise
 - Incubar *overnight* a 55°C
- 3) Adicionar 100 μL de solução de precipitação de proteína
 - Vórtex por 30 s
 - Incubar em gelo por 5 min
 - Centrifugar o tubo por 8 min / 13.000 rpm a 4 °C
- 4) Transferir o sobrenadante para um tubo novo
- 5) Adicionar 300 μL de isopropanol gelado e inverter o tubo mais que 10 vezes
- 6) Incubar no freezer (-20°C) *overnight*
- 7) Centrifugar os tubos por 30 min / 13.000 rpm, 4°C
- 8) Descartar o sobrenadante e acrescentar 600 μL etanol 70%
 - Centrifugar por 10 min / 13.000 rpm e descartar o etanol invertendo o tubo
- 9) Centrifugar por 30 s para retirar o que restou do etanol com o auxílio de uma pipeta e deixar o tubo com o pellet secar por aproximadamente 10 minutos
- 10) Reidratar o pellet com 50 μL TE
 - Deixá-lo no banho-maria a 55°C por 1 hora, homogeneizando o tubo nos primeiros 15 minutos. Retirar quando o pellet estiver dissolvido completamente
 - TE: 10 mM de Tris pH 7,5
1 mM de EDTA
- 11) Estocar no freezer a -20°C

ANEXO 5

TRATAMENTO COM BISSULFITO DE SÓDIO

- Kit: EZ DNA methylation-lightning™ (Zymo)
- Amostras tratadas com bissulfito de sódio devem ser armazenadas no freezer -80°C por no máximo 30 dias.

- 1) Tratar 500 ng de DNA no volume de 20 µL
- 2) Adicionar 130 µL de *lightning conversion reagent* em 20 µL da amostra (500ng)
- 3) Termociclador
 - 98° C / 8 min
 - 54 °C / 1 hora
- 4) Adicionar 600 µL de M-binding buffer na coluna e inserir a coluna no tubo coletor
- 5) Adicionar a amostra do passo 2 na coluna do passo 4
 - Fechar a tampa e inverter 10 vezes
 - Centrifugar 30 s / 10000 g e descartar o filtrado
- 6) Adicionar 100 µL de M-wash buffer na coluna
 - Centrifugar 30 s / 13000 rpm e descartar o filtrado
- 7) Adicionar 200 µL de L-desulphonation buffer na coluna
 - Deixar em temperatura ambiente por 15 – 20 min
 - Centrifugar 30 s / 13000 rpm e descartar o filtrado
- 8) Adicionar 200 µL de M-wash buffer na coluna
 - Centrifugar 30 s / 13000 rpm e descartar o filtrado
- 9) Repetir a etapa 8 e colocar a coluna em novo tubo coletor
- 10) Adicionar 10 µL de M-elution buffer diretamente no centro da coluna
 - Centrifugar 30 s / 13000 rpm
 - Armazenar no freezer -80 °C

ANEXO 6

CLONAGEM BACTERIANA

Meio SOC:

{ 2% de Triptona 0.5% de extrato de levedura 10 mM de NaCl 2,5 mM de KCl 10 mM de MgCl₂ 10 mM de MgSO₄ 20 mM de glicose	

- Preparar 10 mL de solução com os reagentes acima

- <u>Triptona 2%</u> Pesar 2 g de triptona	- <u>Extrato de levedura</u> Pesar 0,05 g
- <u>NaCl 500mM</u> MM = 58,44 g/mol 29,22g-----1000ml X -----15 ml X = 0,4383g Pesar 0,4383g de NaCl e adicionar 15ml de água destilada. Preparar o NaCl 10mM a partir dessa solução de 500mM	- <u>KCl 250mM</u> MM = 74,5513 g/mol 18,63g-----1000ml X -----15 ml X = 0,2794g Pesar 0,2794g de KCl e adicionar 15ml de água destilada. Preparar o KCl 2,5 mM a partir dessa solução de 250 mM
- <u>NaCl 10mM</u> $C_i \times V_i = C_f \times V_f$ $500\text{mM} \times V_i = 10\text{mM} \times 10$ $V_i = 0,2 \text{ mL} = 200 \mu\text{L}$	- <u>KCl 2,5mM</u> $C_i \times V_i = C_f \times V_f$ $250\text{mM} \times V_i = 2,5\text{mM} \times 10$ $V_i = 0,1 \text{ mL} = 100 \mu\text{L}$

- Colocar as quantidades de reagentes indicadas na tabela em 9ml de água destilada e ajustar o pH para 7.

- Autoclavar

- Depois que esfriar, adicionar os demais reagentes:

- <u>MgCl₂ 10mM</u> $C_i \times V_i = C_f \times V_f$ $1000\text{mM} \times V_i = 10\text{mM} \times 10\text{M}$ $V_i = 0,1 \text{ ml} = 100 \mu\text{L}$	- <u>MgSO₄ 100mM</u> $C_i \times V_i = C_f \times V_f$ $1000\text{mM} \times V_i = 10\text{mM} \times 10\text{M}$ $V_i = 0,1 \text{ ml} = 100 \mu\text{L}$	- Glicose <u>500mM</u> MM = 180,156 g/mol 90,078g-----1000ml X -----15 ml X = 1,35117g Pesar 1,35117g de glicose e adicionar 15ml de água destilada. Preparar a glicose 20mM a partir dessa solução de 500mM - Glicose <u>20mM</u> $C_i \times V_i = C_f \times V_f$ $500\text{mM} \times V_i = 20\text{mM} \times 10$ $V_i = 0,4 \text{ mL} = 400 \mu\text{L}$
---	--	--

- Ajustar o volume do meio para 10 ml com água destilada
- Filtrar o meio SOC com filtro para seringa 0,22 μM

Meio LB e LB-ágar:

{ 10 g de Triptona
 { 5g de extrato de levedura
 { 10g de NaCl

- Pesar reagentes e adicionar 800ml de água destilada
- Homogeneizar em um agitador magnético
- Completar o volume de água para 1000 ml
- Adicionar 7,5g em um dos frascos (LB-ágar)
- Separar o meio em 2 frascos (500 ml)
- Autoclavar
- No meio LB-ágar adicionar ampicilina (100 $\mu\text{g/ml}$):

$$100 \mu\text{g} = 0,1 \text{ mg}$$

$$C_i \times V_i = C_f \times V_f$$

$$200\text{mg} \times V_i = 0,1\text{mg} \times 500\text{ml}$$

$$V_i = 0,25 \text{ mL} = 250 \mu\text{L de ampicilina}$$

- O meio LB-ágar deve ser colocado em placas de Petri estéril e armazenadas em geladeira
- O meio LB pode ser armazenado em geladeira ou temperatura ambiente

- Ligação fragmento-vetor

Kit: TOPO™ TA Cloning pCR 2.1 (Invitrogen)

- 1) Usar 15 ng de DNA no volume máximo de 4 μL
- 2) Adicionar 1 μL de sal e 1 μL de TOPO
- 3) Colocar água milliQ na quantidade suficiente para 6 μL
- 4) Deixar em temperatura ambiente por 4 horas
 - Guardar no freezer -20°C ou seguir para a Transformação

- Transformação bacteriana

- Ligar banho-maria a 42°C e fluxo UV de 15 a 20 minutos
- 1) Descongelar as bactérias competentes DH5 α em gelo e colocar 75 μL em dois tubos eppendorff previamente gelados
 - 2) Adicionar 3 μL da ligação na solução de bactérias, lentamente e homogeneizar gentilmente com a mão
 - Incubar em gelo por 30 minutos
 - 3) Dar choque térmico: 42°C por 30 segundos seguido de 2 minutos no gelo
 - 4) No fluxo, adicionar 150 μL de SOC lentamente pela parede do tubo
 - 5) Agitar os tubos no shaker a 200 rpm, 37°C por 1 hora

- Cultivo bacteriano em placa

- Placas de LB-ágar com ampicilina, conforme protocolo acima
- Verificar se tem X-Gal (40 mg/ml) e IPTG (100mM)
- Ligar estufa 37°C

- Mix X-Gal e IPTG:

20 µL de X-Gal + 4 µL de IPTG por placa

- Pipetar 24 µL do mix em cada placa e espalhar bem com alça de vidro flambada

- Inverter e incubar em estufa 37°C até o momento de usar (deixar no mínimo 15 minutos)

- 1) Pipetar 70 µL de células transformadas em cada placa e espalhar bem com a alça de vidro

- 2) Inverter as placas e incubar em estufa 37°C por 12 a 16 horas

- Guardar na geladeira por no mínimo 2 horas antes de fazer o cultivo em tubo

- Cultivo bacteriano em tubo

- Verificar se tem meio LB e ampicilina (100 µg/ml)

- Identificar tubo Falcon de 15 ml

- Ligar UV 15 – 20 min

- 1) Adicionar 3 ml de meio LB com ampicilina nos tubos

- 2) Com palito, pegar as colônias brancas e colocar individualmente nos tubos

- 3) Colocar no shaker 250 rpm, 37°C por até 16 horas

- Guardar na geladeira

ANEXO 7

MINI PREPARAÇÃO DE PLASMÍDIO - MINIPREP

- Kit: PureYield™ Plasmid Miniprep System (Promega)

- 1) Colocar aproximadamente 1,5 ml da cultura de bactéria em tubos eppendorff de 1,5 e centrifugar a 13000 rpm por 3 min
- 2) Descartar o sobrenadante e vortexar o pellet por 30 s para ressuspender
- 3) Adicionar 100 µL Cell Lysis Buffer e misturar invertendo 10 vezes
- 4) Adicionar 350µl de Neutralization Solution gelado e misturar invertendo 10 vezes
 - Centrifugar a 13000 rpm por 10 min
- 5) Transferir o sobrenadante para a coluna PureYield™ Minicolumn
 - Centrifugar por 30 s / 13000 rpm
- 6) Adicionar 200 µl de Endotoxin Removal Wash (ERB) na minicoluna
 - Centrifugar por 30 s / 13000 rpm
 - Adicionar 400 µl de Column Wash Solution (CWC) na minicoluna
 - Centrifugar por 30 s / 13000 rpm
- 7) Transferir a minicoluna para um novo tubo de 1,5 ml e adicionar 30 µl de água nuclease-free e deixar por 1 min em temperatura ambiente
 - Centrifugar por 1 min / 13000 rpm
- 8) Armazenar em freezer -20 °C

ANEXO 8

COLORAÇÃO DE OVÓCITOS (Bodipy)

- Bodipy 493/ 503 (20 µg/mL – Invitrogen™; número de catálogo D3922)
- O corante deve ser preparado apenas no momento de uso e sempre protegido da luz
- Na preparação do triton, homogeneizar gentilmente com a mão sem deixar fazer bolha
- Ao homogeneizar todos os reagentes, observar se tudo foi diluído perfeitamente (pode demorar um pouco para homogeneizar o PBS/PVP e o triton)

<u>- Paraformaldeído 20%</u> Pesar 2g de paraformol e adicionar 10ml de PBS e diluir a 60°C	<u>- Paraformaldeído 4%</u> $C_i \times V_i = C_f \times V_f$ $20\% \times V_i = 4\% \times 1000 \mu\text{L}$ $V_i = 200 \mu\text{L} + 800 \mu\text{L de PBS}$	<u>- Paraformaldeído 1%</u> $C_i \times V_i = C_f \times V_f$ $20\% \times V_i = 1\% \times 1000 \mu\text{L}$ $V_i = 50 \mu\text{L} + 950 \mu\text{L de PBS}$
<u>- PBS/PVP (0,3%)</u> Pesar 0,03g de PVP e adicionar 10ml de PBS	<u>- Triton 0,2%</u> $C_i \times V_i = C_f \times V_f$ $100\% \times V_i = 0,2\% \times 2000 \mu\text{L}$ $V_i = 4\mu\text{L} + 1996 \mu\text{L de PBS/PVP}$	<u>- Bodipy</u> Diluir 0,02 g do corante com 50 µL de etanol absoluto (homogeneizar bem) e imediatamente adicionar 950 µL de PBS/PVP

Fixação:

- 1) Ovócitos GV ou MII devem ser inicialmente desnudados
- 2) Colocar as estruturas em paraformaldeído 4% por 1 hora
- 3) Colocar as estruturas em paraformaldeído 1% para guardar na geladeira

Coloração:

- 1) Em uma placa com 4 poços colocar 2 gotas de $\pm 400 \mu\text{L}$ de PBS/PVP e uma gota com Triton
- 2) Dar dois banhos nos ovócitos no PBS/PVP
- 3) Colocar na gota de Triton e deixar protegido da luz por 30 min

- 4) Em outra placa de 4 poços colocar 2 gotas de PBS/PVP e uma gota do corante Bodipy
- 5) Dar dois banhos nas estruturas e depois colocar no corante por 1 hora (luz apagada e protegido)
- 6) Dar 3 banhos vigorosos nas estruturas antes de colocar na placa de leitura para evitar background

Leitura:

- 1) Em uma placa para microscopia confocal (35x10 mm) colocar 4 gotas de 4 μ L de SlowFade™ (ThermoFisher)
- 2) Preencher a placa com óleo mineral (marca Corning)
- 3) Colocar 1 ovócito por gota e avaliar o mais rápido possível no Confocal Leica