Fatty acids in goat follicular fluid: Effect on oocyte competence

Montserrat Roura Llerda

Universitat Autònoma de Barcelona

DOCTORAL THESIS

Fatty acids in goat follicular fluid: Effect on oocyte competence

Montserrat Roura Llerda

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dirigida per: Maria Teresa Paramio Nieto i María Gracia Catalá

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Universitat Autònoma de Barcelona

La Dra Maria Teresa Paramio Nieto, Catedràtica del Departament de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona,

I la Dra María Gracia Catalá, investigadora post-doctoral del Departament de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona,

CERTIFIQUEN

Que la tesi titulada "**Fatty acids in goat follicular fluid: Effect on oocyte competence**" presentada per Montserrat Roura Llerda per optar al grau de Doctora per la Universitat Autònoma de Barcelona, es va realitzar sota la nostra direcció i amb un finançament del Ministerio de Ciencia e Innovación (AGL2014-52408-R).

I per a que així consti, signem la present

Dra. Maria Teresa Paramio Nieto

Dra. María Gracia Catalá

Bellaterra, Setembre del 2016

Al meu pu,

Al Josep,

A la meva família,

Som Roures

això vol dir que creixem,

ens enfortim,

evolucionem.

Necessitem aigua

i donem ombra a qui la necessita.

Tenim història per crear boscos

i amb les nostres arrels,

fermem el terra que tenim als nostres peus.

Som nobles i bells,

renaixem de nosaltres mateixos

amb fulles més verdes i més fortes.

Sempre estarem aquí

Som Roures

Toni Roura.

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Fatty acids (FA) are an important source of energy for the growing and developing oocytes. Thus, the study of the follicular fluid, where these oocytes grow and mature could be of special interest as it could help to determine their developmental capacity. Additionally, it is widely accepted that oocytes from adult animals have better competence than those from its prepubertal counterparts. In previous studies in our lab, it was demonstrated that oocytes from prepubertal goats recovered from large follicles (> 3 mm) have the same competence to develop up to blastocyst as oocytes from adult females (Romaguera *et al*. 2011). Moreover, Catalá *et al*. 2015 showed a differential embryo production in goats among the seasons of the year, being significantly lower in autumn and higher in winter.

Our hypothesis was that the FA profile of FF from adult and prepubertal goats, as well as from different follicular sizes and seasons of the year could give us information to compare with oocyte developmental competence. Therefore, our objective was to determine if we could use the FA profile as a molecular marker for oocyte quality. Moreover, we wanted to give an explanation on how the follicular content could influence the lower developmental capacity of prepubertal oocytes.

The present thesis was divided in three experiments: in experiment 1, we analysed the FA profile of follicular fluid of adult and prepubertal goats, from large and small follicles and during autumn, winter, spring and summer, to try to identify potential markers of oocyte developmental competence, and compare this profile with the profile of other animals described in the literature. Among the 13 FA analysed, 9 FA and combinations of them were different according to the age of the donor. We observed higher percentages of total PUFA and omega 6 polyunsaturated fatty acids (n6 PUFA) and lower omega 3 polyunsaturated fatty acids (n3 PUFA) percentages on FF from prepubertal goats in comparison with adult goats, resulting in a higher n6:n3 ratio in prepubertal females. When comparing adult and prepubertal animals, the size of the follicle and the season of the year had no effect. However, when comparing FF FA concentrations in prepubertal animals, some differences were found among the follicle size and the season of the year mainly for n3 and n6 PUFA, being n6:n3 ratio higher in small follicles and on autumn compared with large follicles and the rest of the seasons of the year, respectively.

In experiment 2, we assessed oocyte competence of prepubertal goat oocytes adding different concentration ratios of linoleic (LA: n6 PUFA) and α-linolenic (ALA: n3 PUFA) acids in IVM media. We found that maturing oocytes with 200:50 μM LA:ALA ratio had a detrimental effect on oocyte developmental competence compared to the control groups (BSA and FCS) and the rest of the

treatments (100:50 and 50:50 µM) in IVF oocytes but not in parthenogenetically activated oocytes. Thus, in the 200:50 µM group there was a decrease in blastocyst production (2.63 %) compared to the other groups (≈ 13 %) after IVF.

According to the results of the experiment 2, the aim of the experiment 3 was to study the effect of LA:ALA ratios on prepubertal goat oocyte quality by assessing mitochondrial distribution and activity, ATP concentration and relative gene expression. Assessing mitochondrial activity, active mitochondria distribution and ATP concentration in the oocyte, we found that there was a change in this parameters when they were analysed on immature oocytes (collection point) compared to IVM oocytes (after 24 h of maturation). Moreover, the addition of 200:50 µM at IVM modified the mitochondrial activity of these oocytes, being higher compared with the other treatment groups, but no changes were observed in the active mitochondria distribution or ATP concentration. Concerning mRNA relative expression, we analysed 9 genes that are shown to be altered if the cell is under stress, and which development could be compromised: ATF4, DNMT1, GAPDH, GCLC, GPX1/GSH-Px, HSPA5/GPR78, RPL19, SLC2A1/GLUT1, SOD1/CuZnSOD. Among these genes, GPX1, RPL19 and SOD1 showed significant differences when comparing immature and IVM oocytes, but not among groups of treatment.

In conclusion, we found that FA profile of goat FF is similar to the follicular fluid found in cows, sheep and woman. The main differences that we found in goats were mainly due to the age of the female. However, we found a direct relationship between n6:n3 PUFA composition in follicular fluid regarding follicular size and season of the year, with previous results in our lab suggesting that this ratio could be a biomarker of oocyte competence. Moreover, we found that adding 200:50 µM LA:ALA had a detrimental effect on blastocyst production of prepubertal goat oocytes produced by IVF but not by parthenogenetic activation. Contrarily to what was previously concluded in another studies found in the literature, the negative effect of the highest LA:ALA ratio on oocyte competence was not related to impaired mitochondrial function, ROS production or ER stress according to the relative expression of the studied genes. Thus, we hypothesized that the effect of the addition of high concentrations of LA:ALA was related to an alteration on the structure of the plasma membrane caused for an incorporation of these fatty acids in the membrane phospholipids accompanied with ATP consumption.

Els àcids grassos (AAGG) són una important font d'energia pels oòcits que estan creixent o desenvolupant-se. Per tant, l'estudi del fluid fol·licular (FF), on aquests oòcits creixen i maduren pot ser d'especial interès ja que pot ajudar a determinar la capacitat que tenen per desenvolupar-se. A més, està àmpliament acceptat que els oòcits que provenen d'animals adults tenen millor competència que els que venen d'animals prepúbers. En estudis previs en el nostre laboratori, es va demostrar que els oòcits que provenen de fol·licles grans (> 3mm) de cabres prepúbers tenen la mateixa competència pel desenvolupament embrionari que els oòcits de femelles adultes (Romaguera i col. 2011). A més, Catalá i col. (2015) van demostrar que hi havia una producció diferencial d'embrions entre les estacions de l'any, sent significativament més baixes a la tardor que a l'hivern.

La nostra hipòtesi era que el perfil d'àcids grassos de cabres adultes i prepúbers, així com de diferents mides de fol·licle i de diferents estacions de l'any, ens podria donar informació per poder-ho relacionar amb la competència oocitària. Per tant, el nostre objectiu va ser determinar si es podria fer servir aquest perfil com a marcador molecular per la qualitat oocitària. A més, volíem donar una explicació sobre com el contingut del fol·licle podria influenciar la baixa capacitat de desenvolupament dels oòcits provinents de femelles prepúbers.

La present tesi es va dividir en tres experiments: en l'experiment 1, vàrem analitzar el perfil d'AAGG en FF de cabres adultes i prepúbers, de fol·licles grans i petits i durant les diferents estacions de l'any, per tal d'identificar potencials marcadors de la competència de l'oòcit per al desenvolupament, i comparar aquest perfil amb el d'altres espècies d'animals descrites en la bibliografia. D'entre els 13 AAGG analitzats, 9 AAGG i combinacions d'ells eren diferents segons l'edat de la donant. Vàrem observar un augment del percentatge del total d'àcids grassos poliinsaturats (PUFA) i dels PUFA omega 6 (n6) i una disminució dels PUFA omega 3 (n3) en cabres prepúbers comparat amb cabres adultes, resultant en una ràtio n6:n3 superior en animals prepúbers. La mida del fol·licle i l'estació de l'any no tenien efecte quan es comparaven els animals de les dues edats. Per contra, quan es comparaven els AAGG del FF en femelles prepúbers, es varen trobar diferències principalment en els PUFA n3 i n6, fent que la ràtio n6:n3 fos major en fol·licles petits i en la tardor que quan es comparaven amb fol·licles grans i amb la resta d'estacions respectivament.

En l'experiment 2, vàrem avaluar la competència de l'oòcit provinent de femelles prepúbers afegint diferents ràtios dels àcids linoleic (LA: n6 PUFA) i α-linolenic (ALA: n3 PUFA) en el medi de maduració *in vitro*. Vàrem observar la ràtio LA:ALA 200:50 μM tenia un efecte perjudicial sobre el desenvolupament embrionari dels oòcits quan es comparava amb els grups control i la

resta dels tractaments (100:50 i 50:50 µM), quan els oòcits eren fecundats *in vitro* (FIV) però no quan eren activats partenogenèticament. Així, en el grup de 200:50 µM hi va haver una disminució de la producció de blastocists (2.63 %) comparat amb els altres grups (≈ 13 %) després de la FIV.

Com a conseqüència dels resultats de l'experiment 2, l'objectiu de l'experiment 3 va ser l'estudi de l'efecte de les diferents ràtios de LA:ALA en l'oòcit de cabra prepúber avaluant l'activitat i distribució mitocondrial, la concentració d'ATP i l'expressió gènica relativa. Vàrem observar que hi havia un canvi si es comparava els oòcits en el moment de recol·lecció i després de 24h de MIV en l'activitat mitocondrial, en la distribució d'aquests orgànuls i en la producció d'ATP. A més, en el grup de 200:50 µM l'activitat mitocondrial era més alta que en la resta de grups. Pel que fa a l'expressió relativa de l'ARNm, es van analitzar 9 gens relacionats que es troben alterats si la cèl·lula pateix estrès, comprometent la seva viabilitat: ATF4, DNMT1, GAPDH, GCLC, GPX1/GSH-Px, HSPA5/GPR78, RPL19, SLC2A1/GLUT1, SOD1/CuZnSOD. D'entre aquests gens, es varen trobar diferències d'expressió entre les 0 i 24h de maduració pels gens: GPX1, RPL19 i SOD1, però no entre els diferents tractaments.

En conclusió, es va trobar que el perfil d'AAGG de FF de cabra és similar a la del fluid fol·licular de vaques, ovelles i dona. Les principals diferències trobades en les cabres es devien principalment a l'edat de la femella. No obstant això, es va trobar una relació directa entre la ràtio n6:n3 present en el FF segons mida del fol·licle i estació de l'any amb resultats previs en el nostre laboratori en la producció in vitro d'embrions, suggerint que aquesta ràtio podria ser un bon biomarcador de la competència dels oòcits. A més, vàrem veure que l'addició de 200: 50 µM de LA:ALA va tenir un efecte perjudicial sobre la producció de blastocist provinents d'ovòcits de cabra prepúbers produïts per FIV, però no per l'activació partenogenètica. L'efecte negatiu de la més alta ràtio de LA:ALA en la competència dels oòcits no estava relacionada, tal com s'havia observat en altres estudis, amb una funció mitocondrial alterada, ni amb un augment de la producció d'espècies reactives d'oxigen o d'estrès del reticle endoplasmàtic com a conseqüència dels resultats obtinguts en l'expressió relativa dels gens estudiats. Per tant, hem hipotetitzat que l'efecte de l'addició d'altes concentracions de LA: ALA es pot relacionar amb una alteració en l'estructura de la membrana plasmàtica causada per una incorporació d'aquests àcids grassos en els fosfolípids de la membrana acompanyats amb el consum d'ATP.

LIST OF ABBREVIATIONS

A	Adrenic acid
AA	Arachidonoc acid
ACS1	Acyl.CoA synthetase
ALA	α-Linolenic acid
ART	Assisted reproductive technology
ATP	Adenosine triphosphate
BCB	Brilliant Cresyl Blue
BSA	Soluble protein serum albumin
CLA	Conjugated linoleic acid
CoA	Coenzyme A
COC	Cumulus-oocyte complex
CPTI/II	Carnitine palmitoyl-transferase I and II
CYT c	Cytochrome C
DGLA	Dihomo-gammalinolenic acid
DHA	Docosaexaenoic acid
DPA	docosapentaenoic acid
EPA	Eicosapentanoic acid
ER	Endoplasmic reticulum
ESS	Oestrous sheep serum
ET	Embryo transfer
FA	Fatty acids
FAB_{pm}	Plasma membrane-associated fatty acid binding protein
$FAD^{+}/FADH_2$	flavin adenine dinucleotide
FAO	Fatty acid oxidation
FAT	Fatty acid translocase
FATP	Fatty acid transport proteins
FCS	Foetal calf serum
FF	Follicular fluid
FFA	Free fatty acids
GLA	Gamma linoleic acid
GSH	Glutathione
GV	Germinal vesicle
GVBD	Germinal vesicle break down
IVEP	*In vitro* embryo production
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
JIVET	Juvenile *in vitro* embryo transfer
LA	Linoleic acid
LOPU	Laparoscopic ovum pick-up
LTs	Leukotrienes
MOMP	Mitochondrial outer membrane permeabilization
MUFA	Monounsaturated fatty acids
NAD^{+}/NADH	Nicotinamide adenine dinucleotide

n3 PUFA	Omega 3 Polyunsaturated fatty acids
n6 PUFA	Omega 6 Polyunsaturated fatty acids
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NTC	Non template control
OPU	Ovum pickup
PGs	Prostaglandins
PUFA	Polyunsaturated fatty acids
PTP	Permeability transition pore
RVs	Resolvins
ROS	Reactive oxygen species
SCNT	Somatic cell nuclear transfer
SFA	Saturated fatty acids
SOF	Synthetic oviductal fluid
TCA	Tricarboxylic acid cycle/Citric acid cycle
TXs	Tromboxanes
UPR	Unfolded protein response

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Chapter 1

Introduction

In vitro embryo production (IVEP) has been an indispensable technology to increase the knowledge on developmental biology and physiology, as well as for emerging technologies such as transgenesis and stem cell reprogramming. Moreover, it provides a tool for better genetic diffusion of animals with high production values, preservation of endangered species, and even for the recovery of dead or extinct animals.

Juvenile *in vitro* Embryo Transfer (JIVET) is an assisted reproductive technology (ART) aimed to allow the reproduction of prepubertal (juvenile) females in order to reduce the generation interval and consequently improving genetic gain in breeding programmes. Increases of 5% of genetic gain can be reached using a combination of JIVET and sexed sperm (Morton, 2008). Offspring from prepubertal females can be achieved by the transfer of *in vitro* produced embryos using oocytes obtained from these females. However, is widely accepted that oocytes from prepubertal animals display a reduced developmental competence compared with oocytes derived from their adult counterparts (Gandolfi *et al*. 1998, Armstrong 2001, Palmerini *et al*. 2014, Leoni *et al*. 2015). In addition, the great majority of IVEP studies are performed with ovaries recovered from slaughterhouses, as is a cheap source of a vast number of oocytes. However, this implies that researchers are unaware of animal conditions and as a consequence, there is a high variability of the obtained results among biological repetitions and laboratories.

Oocyte competence or oocyte quality plays a pivotal role in IVEP and JIVET programmes. It is defined as its ability to resume meiosis (or mature), to cleave following fertilization, to develop to the blastocyst stage and to give rise to normal and fertile offspring after gestation. Oocyte competence is acquired gradually during the course of folliculogenesis as the oocyte grows and its somatic cell cohort differentiates (Eppig *et al*. 1994). It has been demonstrated that several factors affect oocyte competence such as: follicular diameter (Lonergan *et al*. 1994, Romaguera *et al*. 2010) , oocyte diameter (Anguita *et al*. 2007), grade of follicular (Vassena *et al.* 2003) and oocyte atresia (Anguita *et al*. 2009), phase of follicular wave (Machatkova *et al.* 2004), hormonal stimulation (reviewed by Sirard *et al*. 2006), oocyte maturation conditions (reviewed by Sutton *et al*. 2003), season (Sartori *et al*. 2002), nutrition (Fouladi-Nashta *et al*. 2007, Leroy *et al*. 2014), and donor's age (Gandolfi *et al*. 1998, Armstrong 2001, Rizos *et al*. 2005, Palmerini *et al*. 2014, Leoni *et al*. 2015). Several analysis on oocyte competence through embryo development have been tested out in our laboratory in goat (otherwise stated): cumulus-oocyte morphology (Martino *et al.* 1995), oocyte diameter by IVF (Anguita *et al*. 2007) and by ICSI (Jimenez-Macedo *et al*. 2006), and follicle diameter (Romaguera *et al*. 2010 and 2011). We found the same blastocyst outcome from prepubertal goat oocytes coming from large follicles (> 3mm diameter) than from adult goat oocytes (Romaguera *et al*. 2011), and concluded that the low embryo

development of prepubertal females was related to the small number of large follicules in these prepubertal ovaries. This was also observed in pigs by Bagg *et al*. (2007) using activated oocytes. In our lab, oocyte competence was also tested assessing oocyte growth selecting oocytes by Brilliant Cresyl Blue (BCB), a vital dye that differentiates fully grown oocytes (or BCB^+; coloured blue) from growing oocytes (BCB^-: non coloured): Rodriguez-Gonzalez *et al*. (2002) in prepubertal goats, Pujol *et al*. (2004) in heifers, and Catalá *et al*. (2011) in lamb oocytes showed that BCB+ oocytes had better competence and higher blastocyst yield than BCB- oocytes. Several authors have also used this test in cattle (Bhojwani *et al*. 2007, Torner *et al*. 2008) and pigs (Roca *et al*. 1998, Wongsrikeao *et al*. 2006), as it selects the competent oocytes.

Prepubertal female oocytes have been considered as study model for low quality oocytes. Besides, poor oocyte quality from high-yielding cows is considered one of the causes of the declined fertility of these females. Recent studies have highlighted the potential of metabolomic strategies in the assessment of embryo and oocyte quality (Singh *et al*. 2007, Sinclair *et al*. 2008, Revelli *et al*. 2009). Metabolomics is defined as the characterisation of the small-molecule metabolites found in an organism or biological sample. Low-molecular weight metabolites represent the intermediates or end products of the cell's regulatory processes, and their individual profile is also referred to as a 'metabolic fingerprint' (Kell *et al*. 2005). Several studies have taken a novel approach towards the assessment of oocyte quality by characterising specific classes of metabolites in the follicular fluid (FF), such as fatty acids (Zeron *et al*. 2001, Leroy *et al*. 2005), amino acids (Booth, Humpherson, Watson, & Leese, 2005) carbohydrates (Preis *et al*. 2005) and proteins (Zachut *et al*. 2016).

In small follicles, the oocytes are kept at meiotic arrest. However, in growing and preovulatory follicles the oocyte reinitiates the meiosis up to the Metaphase II in ruminants. This means that FF is changing according to follicle diameter and through oocyte competence achievement. Larger and healthier follicles are normally selected to be aspirated when recovering oocytes for IVEP programmes. The FF is also used as a supplement in the IVM media in goats and sheep (Cognie *et al.* 2004). FF is considered as non-defined substance because of its unknown composition, and differences in this affects IVEP. Several studies have shown a relationship between nutrition, plasma composition, FF and oocyte competence to develop up to blastocyst stage (Fouladi-Nashta *et al*. 2009, Leroy *et al*. 2012). Furthermore, it is known that negative energy balance (NEB) period in high producing dairy cows affects their fertility in a negative way (Leroy *et al*., 2012). Polyunsaturated fatty acids (PUFAs) constitute the major portion of the fatty acid content of the FF in small and large follicles (Homa *et al*. 1992), with linoleic acid (LA)

being the one with the highest concentration. Linolenic (ALA), oleic, palmitic and stearic acids are also present in substantial amounts.

Further examination of the PUFA content in the follicle revealed that the omega-6 (n-6) PUFAs were dominant in terms of both the absolute concentration and their percentage contribution in high-yielding cows of low fertility, whereas the omega-3 (n-3) PUFAs had a higher percentage contribution in the FF from heifers (good fertility) (Bender *et al.* 2010). Fouladi-Nashta *et al*. (2007) had shown that increased levels of dietary rumen-inert fatty acid mixture improved both the developmental potential of oocytes to the blastocyst stage and blastocyst quality. Zachut *et al*. (2010), feeding cows with flaxseed (a composition rich in n-3) or sunflower seed (rich in n-6) concluded that oocytes recovered from flaxseed feeding cows yielded more blastocysts after IVF. Thus, PUFA n-3 and n-6 (Omega 3 and 6 families) are believed to have significant health and reproductive benefits in animals. These compounds could improve fertility in high yielding milk cows by improving oocyte quality. Moreover, there are several studies on how different types of fatty acids affect oocyte quality (reviewed by Leroy *et al*. 2014).

In addition to the importance of FA as an energy source (Dunning *et al*. 2010 and 2012), PUFAs act as direct precursors for the synthesis of prostaglandins (PGE2α, PGF2α), progesterone (P4) and estradiol (E2). Wathes *et al*. (2007), in a review about the effect of PUFAs in male and female fertility, concluded that both n-6 and n-3 PUFAs can influence reproductive processes through a variety of mechanisms. They provide the precursors for prostaglandin synthesis and can modulate the expression patterns of many key enzymes involved in both prostaglandin and steroid metabolism. Moreover, some studies have shown the effect of the addition of FA in the *in vitro* maturation medium (IVM) on oocytes and subsequent embryonic development. Thus, Marei *et al*. (2009), testing the effect of different physiological concentrations (10, 50,100 and 200 µM) of ALA in IVM media, concluded that 50 µM resulted in a significantly higher blastocyst rate compared to control and higher ALA concentrations. In a later study, Marei *et al*. (2010), tested the same physiological concentrations of LA as the ones used with ALA (50, 100 and 200 µM), concluding that treatment of COCs with LA had a negative effect in a dose-dependent manner resulting in a significantly lower percentage of cleaved embryos and blastocyst yield. Moreover, Van Hoeck *et al*. (2011) demonstrated that exposing bovine oocytes during IVM to elevated levels of palmitic, oleic and stearic acids caused significant detrimental effects on embryo production, but this negative effect was not observed when the fatty acids were supplemented individually.

Taking all of these previous investigations into account, the aim of this thesis was to increase the knowledge on the relation between fatty acid composition on follicular fluid and goat oocyte quality, and the research of a plausible marker of oocyte competence to produce embryos *in vitro.* Additionally, we wanted to study the effect of the addition of a combination of LA and ALA (omega-6 and omega-3) to *in vitro* maturation media on prepubertal goat oocyte competence to develop up to blastocyst.

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Chapter 2

Literature review

2.1. Fatty acids: characteristics and chemical properties

Fatty acids are simply long-chain hydrocarbon (C-H) organic acids formed by an even number of carbon atoms, from 4 to 36, and a terminal carboxyl/acid group (COOH). In biological systems, they normally have between 14 and 24 carbon atoms, being the C16 and C18 the most abundant. They are stored in the body as triglycerides, and transported in plasma associated with albumin. When they are bound to albumin, they are in their free form or non-esterified (NEFAs: Non-esterified fatty acids).

Fatty acids are classified in three types or families, depending on the grade of saturation: saturated, monounsaturated and polyunsaturated. Saturated fatty acids (SFA) do not have double bounds in their structure, while unsaturated fatty acids have one (monounsaturated: MUFAs) or more than one (polyunsaturated: PUFAs) double bound. These double bounds are found naturally occurring in cis conformation, which produces a bend in the hydrocarbon tail, and are separated by at least a methylene (CH) group in PUFAs (figure 1).

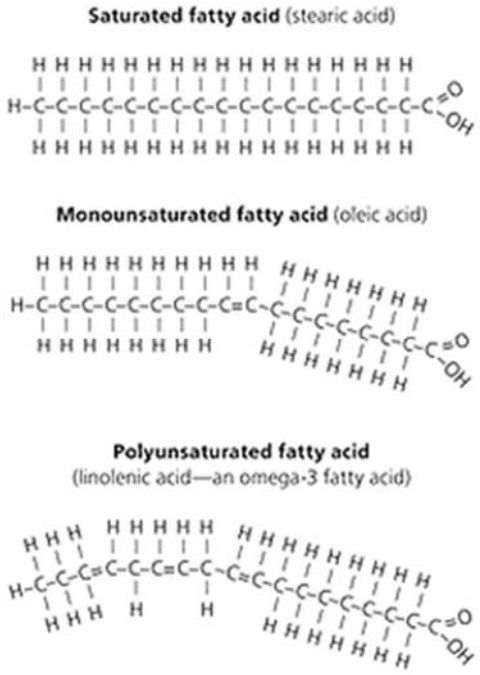

Figure 1 Representative structures of a saturated (Stearic), monounsaturated (oleic) and polyunsaturated (linoleic) fatty acids *(from White 2009)*

The physical properties and the melting points of the fatty acids, and of compounds that contain them, are largely determined by the length and degree of unsaturation of the hydrocarbon chain. Thus, the longer the fatty acyl chain and the fewer the double bonds, the lower the

solubility in water. Moreover, at room temperature (25ºC), the saturated fatty acids from 12:0 to 24:0 have a waxy consistency, whereas unsaturated FA of these lengths are oily liquids (Nelson *et al*. 2008) (Guyton *et al*. 2006).

Fatty acids have common names and systemic names. Systemic (or IUPAC) name comes from the name of the hydrocarbon from which is derived, and depends on the number of carbon atoms and the number of double bounds. For example, stearic acid C18:0 is the octadecanoic acid (derived from the octadecane hydrocarbon): octadec- (18 C atoms) and –ane (simple bounds). However, with the same number of carbon atoms, there are the unsaturated fatty acids: octadec**e**noic (oleic acid C18:1; one double bound), octadeca**di**enoic (linoleic acid C18:2; two double bounds) and octadeca**tri**enoic (alpha-linolenic acid C18:3; three double bounds). In the abbreviated name (table 1), the double bounds are represented with the delta (Δ) symbol and its position (number) from the carboxyl radical. Thus, oleic acid, 18:1cΔ9 has its first double bound between the ninth and the tenth carbon atoms. At a physiological pH, FAs are in the ionized form (-COO^-), and are called as a carboxylate form. For example, palmitate instead of palmitic acid.

2.2. Fatty acids of biological importance

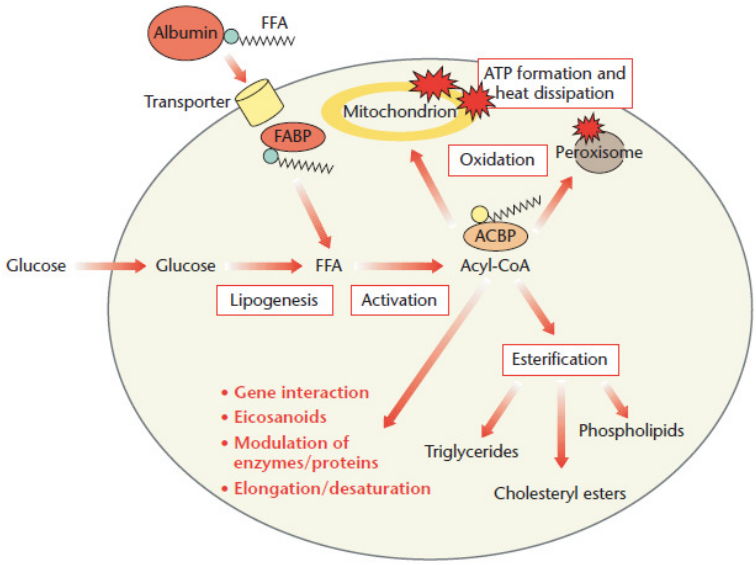

Figure 2. Free fatty acid uptake, metabolism and action in mammal cells *(from Rustan et al. 2009).*

Fatty acids, both free and part of complex molecules, have different important purposes in the body: metabolic energy generation and storage (important to compensate fasting periods), precursors of other molecules such as sterols, hormones, eicosanoids (prostaglandins, prostacyclins, leukotrienes, tromboxanes…), signalling molecules, gene regulators, but their most critical function is in the formation of cell membranes (figure 2) (Doege *et al*. 2006) (Rustan *et al*. 2009).

Among the totality of fatty acids present in the mammal body, there are some of them of biological importance due to its function (table 1).

Table 1. Some fatty acids of biological importance.

Common name	Systematic name	Carbon skeleton	Melting point (ºC)
Saturated fatty acids			
Capric	n-Decanoic acid	10:0	31.6
Lauric	n-Dodecanoic acid	12:0	44.2
Myristic	n-Tetradecanoic acid	14:0	53.9
Palmitic	n-Hexadecanoic acid	16:0	63.1
Stearic	n-Octadecanoic acid	18:0	69.6
Unsaturated fatty acids			
Palmitoleic	9-cis-Hexadecenoic acid	16:1cΔ9	-0.5
Oleic (ω9)	9-cis-octadecenoic acid	18:1cΔ9	13.4
Linoleic (ω6)	all-cis-9,12-octadecadienoic acid	18:2cΔ9,12	-5
Alpha-Linolenic (ω3)	all-cis-9,12,15-octadecatrienoic acid	18:3cΔ9,12,15	-11
Arachidonic (ω6)	all-cis-5,8,11,14-eicosatetraenoic acid	20:4cΔ5,8,11,14	-49.5
EPA (ω3)	all-cis-5,8,11,14,17-eicosatrienoic acid	20:5cΔ5,8,11,14,17	-54
DHA (ω3)	all-cis-4,7,10,13,16,19-docosahexaenoic acid	20:6cΔ4,7,10,13,16,19	-44

Capric, myristic, palmitic, stearic and oleic acids are found in significant quantities in milk in goat, sheep and cow (Castro-Gómez *et al*. 2014). High doses of palmitic acid causes apoptosis in cells like myocites (de Vries *et al*. 1997). Palmitoleic acid is also frequent in animal lipids.

Linoleic (LA:n6) and linolenic (ALA:n3) acids are essential fatty acids, which means that they cannot be synthesized in the body and must be provided by diet. Linoleic acid (LA) is the precursor of arachidonic acid, and Linolenic acid (ALA) is the precursor of eicosapentanoic aid (EPA) wich in turn are precursors of 2-series (more inflammatory) and 3-series (less inflammatory) prostaglandins respectively, tromboxanes and leukotrienes (all of them eicosanoids or signal molecules) (Egan *et al*. 1980) (figure 3).

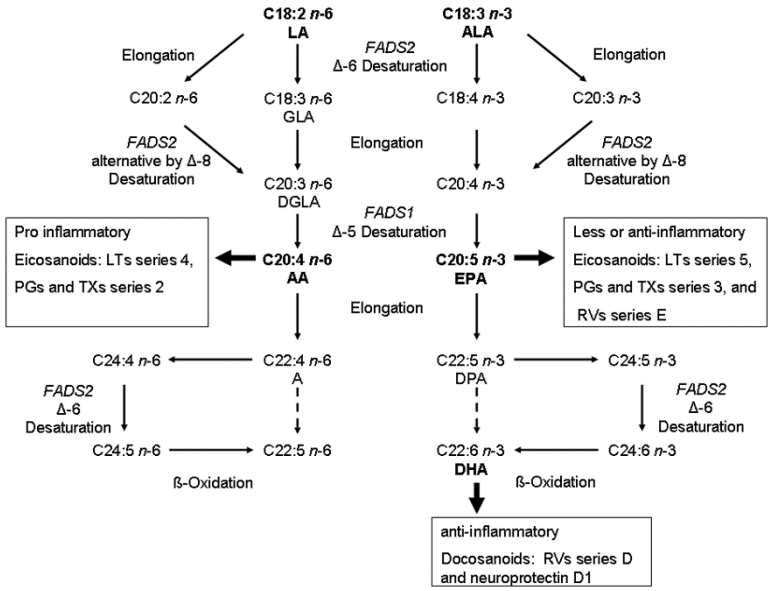

Figure 3. Metabolic pathways of n-6 and n-3 fatty acids. *LA = linoleic acid, GLA = gamma linolenic acid, DGLA = Dihomo-gammalinolenic acid, AA = arachidonoc acid, A = adrenic acid, ALA = alpha-limolenic acid, EPA, = eicospentaenoic acid, DPA = docosapentaenoic acid, DHA = docosahexaenoic acid; LTs = leukotrienes, PGs = prostaglandins, TXs = tromboxanes, RVs = Resolvins (from Rzehak et al. 2010).*

2.3. Metabolism of fatty acids

2.3.1. Fatty acid transport and cellular uptake

The three main sources from which cells can obtain fatty acids to produce energy are: fats from the diet, fats stored in the cell as lipid droplets and fats synthetized in one organ and exported to other cells. Insoluble free fatty acids (FFA) are transported through the blood associated with soluble protein serum albumin (BSA), which has about seven binding sites for FA with moderate to high affinity (van der Vusse, 2009) (McArthur *et al*. 1999). Bound to albumin, FA can be transported to different tissues.

There are several studies that suggest different models of FA transport across the cell membrane, FA transport protein function and its cytoplasmic regulation (McArthur *et al*. 1999, Vogel Hertzel *et al*. 2000, Luiken *et al*. 2002, Doege *et al*. 2006, Milger *et al*. 2006). Therefore, as reviewed by Schwenk *et al*. (2010), once albumin-FA complex reach the target cell or tissue, FA can be transported across the membrane through (figure 4):

1. **Simple diffusion.** As hydrophobic molecules, FA could cross the membrane without the need of a transporter. However, the FA molecule needs to be protonated (-COOH) to

diffuse. As mentioned before, at a physiological pH, the majority of the molecules are in the ionized form (-CCO^-), thuspm transport proteins are required.

2. **FAT/CD36** (fatty acid translocase) **(model 1)**. Alone or together with the peripheral $FABP_{pm}$ (plasma membrane-associated fatty acid binding protein) binds FA molecules to the cell surface to increase its concentration and increase the number of fatty acid diffusion across the membrane.
3. **FAT/CD36** (fatty acid translocase) **(model 2)**. Actively transport FA across the membrane.
4. **FATP** (Fatty acid transport proteins) **(model 1)**. A minority of FA are thought to be transported by FATP and rapidly activated by plasma membrane acyl-CoA synthetase (ACS1) to form acyl-CoA esters.
5. **FATP** (Fatty acid transport proteins) **(model 2)**. Very long chain fatty acids (> C22) are preferentially transported by FATPs, that directly convert the FA to acyl-CoA esters by its synthetase activity.

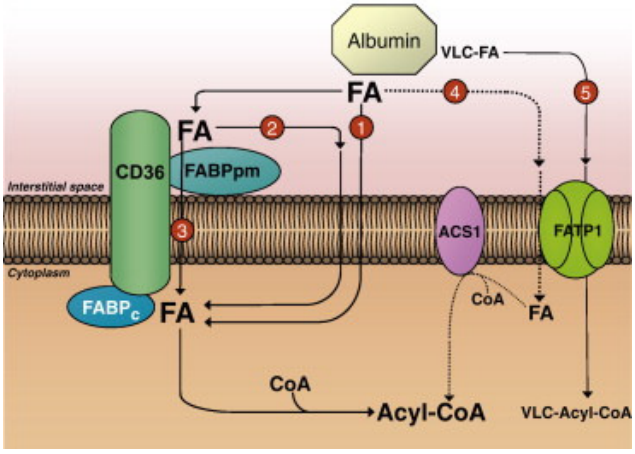

Figure 4. Representation of different models of fatty acid transport across the cell membrane *(from* Schwenk *et al*. 2010)

Once the fatty acids cross the plasma membrane, they are rapidly activated to their CoA thioesters (fatty acyl CoA) for subsequent metabolism. Afterwards, fatty acyl CoA molecules can be transported into the mitochondria to produce ATP (figure 5), modulate the transcription as transcription factors, synthetize several molecules for the cell function, or they can be used to synthesize membrane lipids.

2.3.2. Fatty acid oxidation

FA destined to oxidation enter mitochondria (figure 5) and CoA is enzymatically replaced by carnitine, which is catalysed by carnitine palmitoyl transferase I (CPTI). Then, fatty acyl-carnitine is translocated to mitochondrial matrix via carnitine acylcarnitine translocase, where carnitine palmitoyl transferase II (CPTII) replaces carnitine with intramitochondrial CoA. Finally, carnitine is recycled to intermembrane space and fatty acyl-CoA enters the fatty acid oxidation (FAO) process.

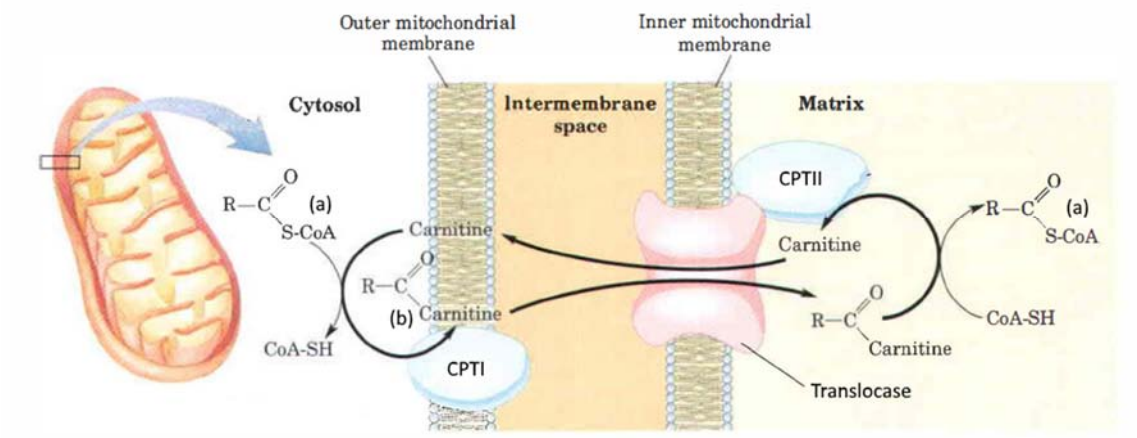

***Figure 5. Long-chain fatty acid entry into mitochondria**. Modified from Nelson et al. (2008). (a) Fatty acyl-CoA; (b) Fatty acyl-carnitine; CPTI-II: carnitine palmitoyl transferase I and II.*

Total oxidation of FA that lead up to the formation of ATP molecules has three main stages (figure 6):

- **Stage 1.** β-oxidation: long-chain FA undergo oxidative removal of successive two-carbon units in the form of acetyl-CoA starting from the carboxyl terminal, at the C-3 or β position, hence the name β-oxidation. Therefore, from a molecule of LA or ALA (C-18), 9 acetyl-CoA are obtained.
- **Stage 2.** Citric acid cycle (or tricarboxylic acid cycle,TCA): acetyl-CoA groups are oxidized to CO_2 molecules.
- **Stage 3.** Mitochondrial respiratory chain: electrons derived from stages 1 and 2 produce the reduced electron carriers NADH and $FADH_2$, which provide the electrons for ATP synthesis by oxidative phosphorylation.

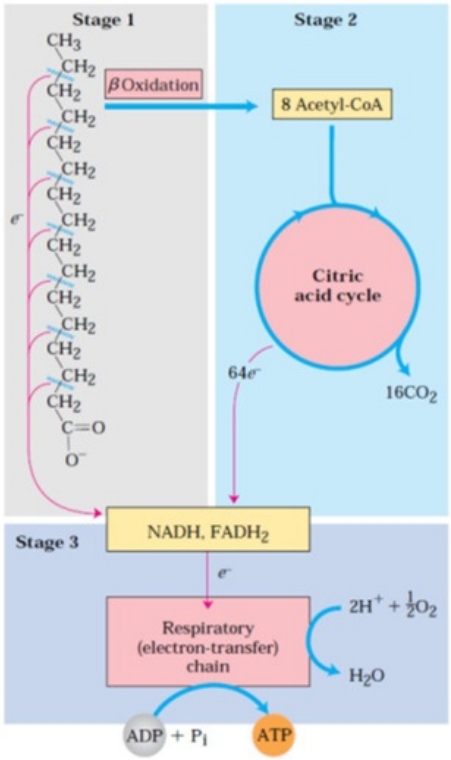

Figure 6. Stages of total oxidation of fatty acids for the formation of ATP molecules. *Example of the total oxidation of C-16 palmitic acid. Adapted from Nelson et al. (2008).*

2.3.3. Mitochondrial activity and ROS production

FA are an important source of energy for the cell, as mitochondrial FA metabolism generates high number of ATP molecules (108 ATP from a total oxidation of a single C16 palmitoyl-CoA molecule), compared with glucose metabolism (36 ATP per molecule). However, a part from the generation of energy in the form of ATP, needed for cell function, mitochondria produce reactive oxygen species (ROS), such as superoxide ion (O_2-) or non-reactive H_2O_2 (Turrens, 2003) as a consequence of FA modulation (reviewed by Schönfeld *et al*. 2008), or via FA catabolites (Seifert *et al*. 2010).

At normal physiological concentrations, oxygen and nitrogen free radical species play key roles in intracellular signalling, regulating many homeostatic mechanisms and mediating stress responses (Maiese *et al*. 2016). However, if ROS production is severely increased by mitochondria, and their detoxifying function is altered, it can lead to oxidative damage to mitochondrial proteins, membranes and DNA (Figure 7) (Murphy, 2009). As a consequence, the ability of the mitochondria to synthesize ATP and their wide range of metabolic functions (β-

oxidation, TCA, amino acid metabolism…) are impaired. Cell function may be perturbed and in the most severe cases apoptosis may result (Burton *et al*. 2002).

Several studies show the importance of mitochondria function for the reproductive outcome. For extensive reviews on the mitochondria importance in early mammalian development: Van Blerkom (2009 and 2011), Schatten *et al*. (2014), Jansen *et al*. (2004).

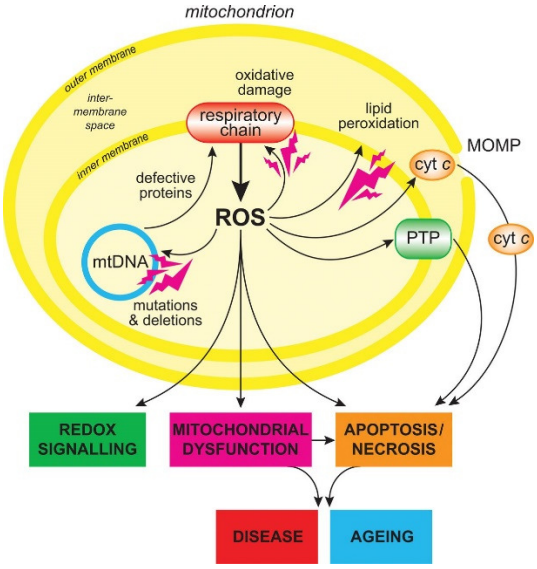

Figure 7. Effects of pathological increasing of mitochondrial ROS production*. Adapted from* Murphy (2009)*. A part from the impairing of normal mitochondrial functions, ROS can increase proteins such as cytochrome C (cyt C) in the cytosol via mitochondrial outer membrane permeabilization (MOMP) and thereby activate the cell's apoptotic machinery* (Gottlieb, Armour, Harris, & Thompson, 2003)*. In addition, mitochondrial ROS induce the mitochondrial permeability transition pore (PTP), which makes the membrane permeable to small molecules. Consequently, mitochondrial oxidative damage contributes to a wide range of pathologies.*

2.4. Fatty acids and reproduction

Polyunsaturated fatty acids have an important role on the regulation of male (Lin *et al*., 2016) and female reproductive function, as extensively reviewed by Wathes *et al*. (2007) probably owing to their effect on prostaglandin synthesis and steroidogenesis.

Simopoulos *et al*. (2003), described that human beings have evolved on a diet with a n-6:n-3 PUFA consumption ratio in the range of 1:1 to 2:1. In a typical modern westernized diet, this ratio ranges from 10:1 to 25:1, a situation thought to promote the pathogenesis of many diseases (Simopoulos 2008). This change in the diet is also affecting livestock due to intensively farming and little or no access to fresh pasture (rich in n3 PUFAs), and moreover, diets are

supplemented with fat derived from oilseeds rich in LA (n6 PUFAs). That could explain the decrease of the fertility in both human and farm animals.

Several authors have studied the alteration in reproduction outcomes in obese animals, due to a variation in energy metabolism and lipotoxicity (reviewed by Alves *et al*. 2015). Thus, it has been observed that an increase of the availability of fatty acids could imply an increase of mitochondrial dysfunction (mice: reviewed by Louden *et al*. 2015; human: reviewed by Schatten *et al*. 2014).

2.4.1. Fatty acids in follicular fluid and their effects on reproduction

Follicular Fluid (FF) composition can provide useful information about the stage of the follicle. Owing the close and physical relation between oocyte and FF, the composition of this fluid can be determinant in oocyte quality (Fair 2010, Leroy *et al*. 2012, Dumesic *et al*. 2015). It is a well-known fact that mammalian oocytes can resume meiosis spontaneously when removed from the follicular environment, which means that the components of immature or small follicles and oocyte surrounding somatic cells are responsible for regulating oocyte meiotic arrest. On the contrary, we could then think that in the growing or maturing antraal follicle there are the components needed for oocyte nuclear and cytoplasmic maturation. The surrounding environment and the somatic cells that accompany the oocyte (follicle and cumulus cells) are critical for oocyte health (Dumesic *et al*. 2015). There is an increase of knowledge of some single molecular markers for oocyte quality in FF (reviewed by Revelli *et al*. 2009), and wider number of molecular markers due to the appearance and establishment of new "omic" technologies such as transcriptomics, metabolomics (Gérard *et al*. 2015; cow, pig and horse) and proteomics (Zamah *et al*. 2015: Human; Zachut *et al*. 2016: cow; Fu *et al*. 2016: Buffalo). Such information is helpful when an accurate and non-invasive selection of oocytes with highest developmental potential is required.

Fatty Acids (FA) are an important component of the FF because they are an considerable source of energy for the growing and developing oocytes (McKeegan *et al*. 2011). An effort involving different groups of investigation has been done to study the effect on the oocyte of different FA in the FF. For example, Sinclair *et al*. (2008) studied the amino acid and FA composition of follicular fluid from follicles (6-8 mm) in a bovine model of IVM-IVF-IVC to try to use them as predictors of *in vitro* embryo development. The most abundant FA detected in FF was LA (≈30 %), followed by oleic acid (≈20%), palmitic (≈15%), stearic (≈11%) and ALA (≈10 %). Whilst they suggested that increasing levels of palmitic and stearic acids were associated with

morphologically poor or good cumulus-oocyte complex (COC), respectively, they concluded that FA composition of FF is not related to post-fertilization development *in vitro*, but rather it is related to oocyte quality. On the contrary, Matoba *et al*. (2014), on a similar study in a bovine model and analysing follicles <8 mm, showed that FF from oocytes that develop up to blastocyst stage compared with the ones that cleaved but subsequently degenerated was significantly lower in palmitic acid (72 µM vs 103.75 µM) and total saturated fatty acids (SFA) (148.54 µM vs 212.15 µM), and significantly higher in linolenic acid (25.11 µM vs 14.15 µM). These results are in accordance with previous studies in the same group (Bender *et al*.. 2010) where they analysed the FA in preovulatory dominant follicles comparing cows and heifers, which have well-characterised differences in fertility, being the heifers the ones with better oocyte development. Among the 25 FA analysed, there were significantly differences in 15 FA and total SFA, MUFA, PUFAS AND n-6 n-3 PUFA relation (table 2). They have also demonstrated that follicular fluid FA concentrations did not directly reflect serum concentrations, which means that the follicles have their own metabolism. They finally concluded that FF from cows contained higher levels of SFAs, which could be detrimental for oocyte maturation and embryo development. In a similar study, Leroy *et al*. 2005 compared FA profile of blood and dominant FF in high-yielding dairy cows. They found that total NEFA concentrations during negative energy balance (NEB) post-partum was ±40% lower compared with serum concentrations.

Moreover, in a study in FF in women who were following IVF treatment, O'Gorman *et al*. (2013) concluded that FA in FF could serve as potential biomarkers for oocyte developmental competence, as they found differences in the FA composition in FF from oocytes that cleaved compared to the ones that didn't cleave after fertilization. Palmitic, total SFA and n6:n3 ratio were significantly higher in the non-cleaved group, whilst stearic and arachidonic acids, DHA, total PUFA and total n-3 PUFA were increased in the cleaved group.

Besides, in all the studies mentioned, they coincide that the most abundant FA found in FF were (table 2):

Table 2. Concentration of some FA of biological importance in preovulatory follicles from cows and heifers. *Adapted from Bender et al. (2010).*

FATTY ACID	COW	HEIFER	P value
Myristic acid (C14:0)	7.65	3.92	0.001
Palmitoleic acid (C16:1)	35.35	10.98	<0.001
Palmitic acid (C16:0)	177.42	102.53	0.001
Linoleic acid (C18:2n6)	782.31	170.38	<0.001
Linolenic acid (C18:3n3)	107.16	53.54	0.002
Oleic acid (C18:1n9c)	741.60	307.41	<0.001
Stearic acid (C18:0)	190.83	110.18	0.005
Arachidonic acid (C20:4n6)	26.21	29.25	NS
EPA (C20:5n3)	34.22	36.31	NS
DHA (C22:6n3)	1.31	3.12	0.022
Total SFA	375.9	216.63	0.001
Total MUFA	776.95	318.39	<0.001
Total PUFA	951.21	293.00	<0.001
(n-3) PUFA	142.69	92.67	0.016
(n-6) PUFA	808.52	199.63	<0.001
(n-6)(n-3) PUFA ratio	5.66	2.15	<0.001
LA:ALA ratio	7.30	3.18	-

Values are expressed as means of concentration (µM). NS: non-significant differences.

Additionally, some authors have studied how metabolic status affects fertility, either by supplementing animal diets with FA (Mattos 2000: ruminants; Thangavelu *et al*. 2007: cow; Zeron *et al*. 2002: sheep), or due to metabolic disorders (Leroy *et al*. 2012; NEB in cows) (Sessions-Bresnahan *et al*. 2016; Wu *et al*. 2010, Louden *et al*. 2015; obesity; Fontana *et al*. 2016; over/under-nutrition).

As reviewed by Leroy *et al*. (2014), several studies have reported that changes in serum fatty acids in relation to diet are reflected in the FA composition of the FF (example: Adamiak 2005 PUFAs; Zeron *et al*. 2002). Additionally, several authors suggest that that the ovary could regulate the metabolism or the intake of FA (Fouladi-Nashta *et al*. 2009, Wonnacott *et al*. 2010, Zachut *et al*. 2010, Jungheim *et al*. 2011). Furthermore, it seems that the effect that supplementing FA have on reproduction outcomes depends on the fatty acid supplied (table 3).

Table 3.Summary of recent studies focusing on the effect of different types of fatty acids on oocyte and embryo quality in mammal models. *Adapted from Leroy et al. (2014).*

AUTHOR	FINDINGS
In vitro studies	
Leroy *et al*. (2005)	High NEFA during oocyte maturation hampers embryo development
Pereira *et al*. (2007)	Positive effect of trans-10, cis-12 CLA during *in vitro* embryo culture on cryotolerance
Marei *et al*. (2009)	Positive effect of ALA on oocyte maturation
Marei *et al*. (2010)	Negative effect of LA on oocyte maturation and development
Hughes *et al*. (2011)	EPA and DHA may increase oxidative damage in ovine oocytes
Haggarty *et al*. (2006)	Human embryos with higher unsaturated/saturated fatty acid ratios are more likely to develop
Lapa *et al*. (2011)	Improved development and embryo quality after trans-10, cis-12 CLA supplementation during bovine oocyte maturation
Jungheim *et al*. (2011)	Predominant human follicular fluid and serum NEFA were oleic, palmitic, linoleic and stearic acid. Elevated NEFA correlated with poor COC morphology.
Wonnacott *et al*. (2010)	Omega-6 fatty acids reduce embryo quality
Aardema *et al*. (2011)	Oleic acid buffers negative effects of palmitic and stearic acid and promotes maturation
Yang *et al*. (2012)	Lipid-rich human follicular fluid decreases murine oocyte maturation rate
Van Hoeck *et al*. (2011) (2013)	High NEFA during oocyte maturation affects D7 embryo physiology
Oba *et al*. (2013)	Serum high in NEFA adversely affects embryo development *in vitro*
Valckx *et al*. (2014)	High NEFA during *in vitro* murine follicle growth impairs oocyte competence
Sutton-mcdowall *et al*. (2016)	High NEFA during oocyte maturation induce ER stress in cattle COC affecting its metabolism and competence
Oseikria *et al*. (2016)	Low doses of DHA on oocyte IVM has beneficial effects on oocyte competence
In vivo studies	
Zeron *et al*. (2002)	Positive effects of fish oil-supplemented diets on oocyte quality and chilling
Adamiak *et al*. (2006)	Altered lipid intake is reflected in changed fatty acid composition in follicular fluid and cumulus oocyte complex
Bilby *et al*. (2006a,b)	Negative effects of n-6-rich diets on oocyte quality
Fouladi-Nashta *et al*. (2007)	Positive effects of 800 g Megalac (long-chain mainly saturated fatty acids) for 14 d on oocyte quality
Thangavelu *et al*. (2007)	Reduced embryo quality after OPU of cows supplemented with SFA compared with ALA or LA supplemented cows
Petit *et al*. (2008)	Negative effect of ALA on embryo quality at ET
Fouladi-Nashta *et al*. (2009)	Holstein cows fed palmitic and oleic, linoleic or linolenic acids had altered plasma fatty acid profile, but no effect on embryo development
Cerri *et al*. (2009)	Positive effect of LA on embryo quality at ET
Awasthi *et al*. (2010)	More intracellular lipid droplets in oocytes from overfed repeat breeder cows
Zachut *et al*. (2010)	Positive effect of flaxseed oil on oocyte quality in dairy cows
Wu *et al*. (2010)	High fat diet causes lipotoxicity in murine and human COCs decreasing fertility
Jungheim *et al*. (2011)	Elevated FF NEFA correlated with poor COC morphology
Ponter *et al*. (2012)	Diet high in LNA increases prostoglandin E2 synthase-1 in COCs
Guardieiro *et al*. (2013)	Negative effect of LA on embryo cryotolerance in Nellore heifers
Sessions *et al*. (2016)	Maternal obesity causes alterations in the follicle and the oocyte

COC: cumulus oocyte complex; NEFA: Non esterified fatty acids; CLA: conjugated linoleic acid; ALA: linolenic acid; LA: linoleic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; OPU: ovum pickup; SFA: saturated fatty acids; ET: embryo transfer.

2.4.2. Linoleic acid (LA) and alpha-linolenic acid (ALA) in follicular fluid

If the attention is focused on LA and ALA, and regardless the rest of FA, Childs *et al*. (2008) reported that in accordance with the diet, the concentration of LA in FF of cattle vary from 71 to 710 µM and ALA from 36 to 72 µM. Linoleic acid is the major fatty acid in bovine follicular fluid (Bender *et al*. 2010, Matoba *et al*. 2014) and it has an inhibitory effect on oocyte maturation *in vitro* if it is supplied in supraphysiological concentrations (Homa *et al*. 1992, Marei *et al*. 2010). More recently, Matoba *et al*. (2014) have not found differences in LA concentration in FF associated with oocytes developing to blastocysts or degenerated ones (121.2 and 114.1 µM, respectively) but these differences were significant in ALA concentrations (25.1 and 14.1 mM, respectively). Moreover, McKeegan *et al*. (2011) concluded that not only the concentration but also the ratio of LA and ALA is critical in mammalian reproductive success.

2.4.3. Importance of fatty acid metabolism on oocyte competence

Oocytes accumulate lipid droplets in their cytoplasm during follicular development (McEvoy *et al*. 2000). The colour of the cytoplasm reflects the amount of FA stored in the oocyte. In cows, oocytes with dark cytoplasm have more lipids (Leroy *et al*. 2005) and more mitochondria Jeong *et al*. (2009) than oocytes with pale cytoplasm, which resulted in better developmental competence (Jeong *et al*. 2009). The content of these FA is reduced during maturation and blastocyst development (Sturmey *et al*. 2003), highlighting the importance of the use FA for energy provision and oocyte quality (Ferguson *et al*. 2006, Sturmey *et al*. 2009, McKeegan *et al*. 2011, Collado-Fernandez *et al*. 2012, Dunning *et al*. 2014).

FAO and thus β-oxidation is necessary for oocyte development (reviewed by Dunning *et al*. 2014), either in species with low (Downs *et al*. 2009) or high (Sanchez-Lazo *et al*. 2014) accumulation of lipids in the cytoplasm (Paczkowski *et al.* 2013). Moreover, an inhibition of β-oxidation during oocyte maturation compromises its developmental capacity in mice (Downs *et al*. 2009, Dunning *et al*. 2010), pig (Sturmey *et al*. 2006) and cow (Ferguson and Leese 2006, Sanchez-Lazo *et al*. 2014). On the contrary, if β-oxidation is enhanced by adding fatty acids or substances like L-carnitine, oocyte developmental capacity is increased in mice (Dunning *et al*. 2010), pig (Somfai *et al*. 2011), lamb (Reader *et al.* 2015), cow (Sutton-McDowall *et al*. 2012, Ghanem *et al*. 2014). This effect is also observed when L-carnitine is added in *in vitro* follicle culture (Dunning *et al*. 2011). Moreover, You *et al*. (2012), treating oocytes with L-carnitine showed that it increased glutathione synthesis and thus, reduced ROS and enhanced embryo development in PA oocytes and after somatic cell nuclear transfer (SCNT).

However, some studies showed that elevated concentrations of fatty acids could be toxic and impair oocyte development. Lipotoxicity could affect oocyte competence by inducing endoplasmic reticulum (ER) stress and altering cell metabolism (reviewed by Alves *et al*. 2015 and Latham 2016). Such effect has been observed in cattle (Sutton-mcdowall *et al*. 2016) and mice (Wu *et al*. 2010, Yang *et al*. 2012).

2.4.4. Linoleic acid (LA) and alpha-linolenic acid (ALA) and oocyte quality and development

Studies *in vitro* have also shown a negative effect on oocyte nuclear maturation and embryo development at physiological concentrations of 50, 100 and 200 µM (Marei *et al*. 2010). Similarly, Khalil *et al*. (2013) reported the negative effects of 100 µM LA concentration on IVM media, but these effects were alleviated by the addition of antioxidants such as Vitamin E and glutathione peroxidase. Homa and Brown (1992), reported that follicular LA concentration is higher in small follicles than in large and developing cow follicles. *In vitro*, Marei *et al*. (2009) reported positive effects on oocyte maturation, embryo development and embryo quality with the addition of 50 µM of ALA, but when 200 µM concentration of ALA was added (supraphysiological concentration) it had a detrimental effect on oocyte nuclear maturation, characterised by the extrusion of an abnormal second polar body, which may indicate hypermaturation/ageing of the oocytes.

2.5. *In vitro* embryo production in small ruminants

In vitro embryo production (IVEP) is a multistep methodology comprising the following procedures: (1) IVM of oocytes recovered directly from the follicles, (2) IVF or coincubation of capacitated spermatozoa with *in vitro*-matured oocytes, and (3) IVC of zygotes to the blastocyst stage. At this stage, the embryo could be directly transferred to a recipient female or cryopreserved for future use.

In small ruminants, a critical factor affecting the overall efficiency of IVEP is the large variation in embryo response to superovulation treatments, the early regression of corpora lutea in goats, and the traumatic surgical procedure of embryo recovery. *In vitro* embryo production can overcome some of these limitations because superovulation is not needed as oocytes are recovered directly from the follicle in hormonally stimulated or non-stimulated females by laparoscopic ovum pick-up (LOPU). Furthermore, IVEP allows the production of progeny from non-fertile females, prepubertal, pregnant, lactating, and even dead or slaughtered females.

Since the birth of the first lamb and kid using IVF in the 80s, several studies have been carried out; however, results still are inconsistent and unpredictable. Moreover, significantly fewer research groups are working on embryo production in small ruminants than in cattle and pigs.

IVEP processes are intended to mimic the *in vivo* processes of embryo development in the female reproductive tract. During follicle growth, mammalian oocytes arrested at the germinal vesicle (GV) stage, acquire the capacity to resume meiosis. When removed from the young follicle, these COCs could be induced to IVM.

2.5.1. *In vitro* maturation (IVM)

A correct maturation of the cumulus-oocyte complex (COC), both nuclear and cytoplasmic, is one of the most important factors which determine the entry of the oocyte into metaphase II (MII), subsequent successful fertilization, as well as the ability of an embryo to undergo an appropriate growth and development (Watson, 2007). Performing the COC maturation under *in vitro* conditions provides an excellent opportunity to have cheap and abundant oocytes for carrying out basic research and for the application of emerging biotechnologies like cloning and transgenesis.

Several aspects of the IVM of small ruminants have been studied (Wani *et al*. 2000, Teotia *et al*. 2001, Rao *et al*. 2002). Although several culture media have been used for IVM, the most commonly used for goats and sheep is Tissue Culture Medium (TCM199) bicarbonate-buffered with Earle's salts. Some supplements or the IVM are known to stimulate small ruminant oocyte nuclear and cytoplasmic maturation: epidermal growth factor (EGF) (Guler *et al*. 2000, Zhou *et al*. 2005, van der Valk *et al*. 2010), mare serum (Motlagh *et al.* 2010), fetal calf serum (FCS), estrous sheep (ESS) or goat serum (Pawshe *et al*. 1996, Ghasemzadeh *et al*. 2000, Bebbere *et al*. 2010), insulin–like growth factor (IGF-I) (Guler *et al*. 2000, Magalhaes-Padilha *et al*. 2012), and cysteamine (de Matos *et al*. 2002, Urdaneta *et al*. 2004).

After the improvements in the IVM media, actually the most commonly media used to *in vitro* mature small ruminant oocytes is the TCM199 supplemented with 2-mM glutamine, 100-mM cysteamine, 0.3-mM sodium pyruvate, 10-ng/mL EGF, 10% if FCS or ESS, 5 to 10 mg/mL FSH, 5 to 10 mg/mL LH, and 1 mg/mL 17b-estradiol. Oocytes are usually cultured in groups (1 oocyte per 2–10 mL of medium) in microdrops at 38 ºC to 39 ºC in a humidified atmosphere of 5% CO_2 in air for 24 to 27 hours (Loi *et al*. 2008, Catala *et al*. 2011, Hammami *et al*. 2014). Nevertheless, there are some research teams using synthetic oviductal fluid (SOF) medium instead of TCM199

in sheep (Shabankareh *et al*. 2012) and goat (Ongeri *et al*. 2001, Bormann *et al*. 2003, Herrick *et al*. 2004) oocytes.

2.5.2. *In vitro* fertilization (IVF) and sperm capacitation.

Fertilization is a complex procedure whose success requires appropriate sperm selection, sperm capacitation, and IVF media. So, before fertilization, ejaculates need to be prepared to inseminate the oocytes. In goat, freshly ejaculated semen is usually used for IVF (Cox *et al*. 1994, Keskintepe *et al*. 1994, Anguita *et al*. 2007, Romaguera *et al*. 2011, Hammami *et al*. 2013). Some trials have been described where IVF was carried out using frozen–thawed sperm (Keskintepe *et al*. 1998, Rho *et al*. 2001, Bormann *et al*. 2003). The principal techniques used to select the most motile and viable sperm from the whole fresh ejaculate or from frozen-thawed sperm are swim-up and the centrifugation with discontinuous density gradients (Percoll or Ficoll), or commercial gradients. Once the spermatozoa are selected, capacitation is carried out *in vitro*. Several agents have been used to capacitate spermatozoa, but most laboratories use heparin or ESS for fresh and frozen buck and ram semen, respectively. Currently, buck sperm is incubated with 50 mg/mL heparin for 45 minutes previous to the IVF (Romaguera *et al*. 2011, Hammami *et al*. 2013), whereas 2% to 20% ESS is added to the IVF microdrops in sheep (Huneau *et al*. 1994, Walker *et al*. 1994, Catala *et al*. 2011). With respect to the medium and conditions used, IVF is usually carried out in SOF medium in sheep (M. G. Catala *et al*. 2011) and in Tyrode's albumin lactate pyruvate medium supplemented with hypotaurine in goats (Hammami *et al*., 2013). The final sperm concentration most used in the IVF drop is 1 to 4 x 10^6 spermatozoa/mL, and sperm and oocytes are co-incubated for 17 to 24 hours at 38ºC to 39ºC. Regarding the atmosphere used during the IVF period, although it has been suggested that low oxygen tension improves sheep blastocyst quality Leoni *et al*. 2007), most laboratories still use a humidified atmosphere of 5% CO2 in air.

2.5.3. Parthenogenetic activation

After the entry of the sperm, mammalian oocytes exhibit a series of multiple intracellular calcium ion transient increase induced by the sperm. These transient calcium peaks are propagated throughout the fertilized oocyte in the form of a wave and initiate both the cortical granule exocytosis and escape from MII arrest to become a zygote (reviewed by Loi *et al*. 1998, Nakada *et al*. 1998).

Oocyte activation protocols have been developed to induce artificially the intracellular calcium levels in the oocyte cytoplasm. This is achieved by exposing the oocyte to a calcium ionomicyn

or ionophore and subsequently culturing it with a persistent kinase inhibitor such as 6-DMAP (6-dimethyl amino purine). The treatment with ionomycin alone caused resumption of meiosis but not pronuclear formation and the 6-DMAP alone did not cause resumption of meiosis or pronuclear formation. So, it is important the combination of the two compounds to reach the pronuclear stage (Susko-Parrish *et al*. 1994). In sheep, Alexander *et al*. (2006) using the combination of this two compounds produce 21 % of blastocysts; they had also showed that using cycloheximide in place of the 6-DMAP could also produce blastocyst but in a lower percentage (15%). Loi *et al*. (1998), using the combination of ionomicyn and 6-DMAP to activate sheep nuclear transfer oocytes reached an efficiency of 83% of blastocyst compared to 25% with no activation protocol. Moreover, in lamb oocytes, Catala *et al*. (2012) obtained similar blastocyst yield with PA and with IVF. Similar procedures were followed with goat oocytes with similar blastocyst yield (Lan *et al*., 2005).

2.5.4. Embryo culture and blastocyst production

The last stage of IVEP is the culture of the presumptive zygotes in culture media where they undergo a number of divisions until the blastocyst stage 6-7 days after *in vitro* fertilization in ruminant species (Gardner *et al.* 1994). This period has the greatest impact on the blastocyst quality (Rizos *et al.* 2002). Important developmental events take place: first cleavage division, embryonic genome activation, the compaction of the morula and the blastocyst formation. Within the blastocyst stage, the differentiation of two cell types occur: the inner cell mass (ICM), which after further differentiation gives rise to the fetus, and the trophectoderm (TE), which ultimately contributes to the placentation (Watson, 1992). Clearly, any modifications of the culture environment, which could affect any or all of these processes, could have a major effect on the quality of the embryo.

Three culture systems are routinely used (reviewed by Cognié *et al*. 2004, Lazzari *et al*. 2010, Paramio *et al*. 2014, de Souza *et al*. 2014): (1) IVC in the oviduct of temporary recipients, (2) *in vitro* co-culture with somatic cell support, and (3) semi-defined conditions in media designed to suit embryo requirements.

Currently, the most common media used during *in vitro* culture (IVC) of embryos is the Synthetic oviductal fluid (SOF: Tervit *et al*. 1972), with aminoacids (Walker *et al.* 1996), and the addition of 5 % to 10 % of FCS (Thompson *et al*., 1998) or BSA (Carolan, Lonergan, Van Langendonckt, & Mermillod, 1995). Most laboratories carry out IVC in groups in droplets of this medium (1–2 mL per embryo) under paraffin oil at 38.5ºC in 5% O_2, 5% CO_2, and 90% N_2 in humidified atmosphere.

2.6. Study of the oocyte

2.6.1. Meiosis: nuclear and cytoplasmic maturation

In mammals, oocytes are arrested for several weeks, months or years in prophase of the first meiotic division. During this long period, oocytes accumulate molecules of mRNA, proteins, lipids and sugars as well as they gradually increase in size. The accumulation of all necessary sources of energy and information during oocyte growth is essential for the final step of oogenesis: the oocyte maturation.

Maturation consists of two interlinked and mutually dependent processes: cytoplasmic and nuclear maturation. The cytoplasmic maturation of the oocyte includes cytoplasmic changes as organelle redistribution, micro and macro molecular changes that occur during oocyte maturation (Mermillod *et al*. 2006). These modifications contribute to the oocyte's ability to undergo: nuclear maturation, successful fertilization, cleavage and the development at least until the activation of the embryonic genome (Watson. 2007). Nuclear maturation includes chromatin changes during the oocyte maturation starting from germinal vesicle breakdown (GVBD) through Meiosis I and Meiosis II when the oocyte is finally arrested in the MII stage. At this moment the oocyte is physiologically prepared to complete the second meiotic division upon fertilization. Under *in vivo* conditions, only fully grown oocytes can resume meiosis which implies that cytoplasmic changes that occurs before maturation are essential for the acquisition of the developmental competence (reviewed by Marteil *et al*. 2009). However, when oocytes are removed manually before ovulation from an antraal follicle, the separation triggers a pseudo-maturation event leading in general to the completion of the first meiotic division and the arrest at the MII stage. This process has been called spontaneous maturation and is believed to be induced by the removal of the oocyte maturation inhibitor (OMI) present in the follicle where cAMP is involved (reviewed by Sirard 2011). A comparison between oocytes that were removed from the follicular environment and *in vitro* matured compared to *in vivo* matured oocytes, showed the same rates of nuclear maturation, fertilization and cleavage, but the percentage of blastocyst was significant lower on *in vitro* matured group [30% vs. 60%, reviewed by Sirard *et al*. (1996) indicating that the cytoplasmic competence must be different between the *in vitro* and the *in vivo* matured oocytes.

2.6.2. Mitochondria and ATP

Mitochondria are maternally inherited organelles that use oxidative phosphorylation to supply energy as adenosine triphosphate (ATP) to the cell (Stojkovic *et al*. 2001). This source of ATP, has a central role in the establishment of the developmental competence (Van Blerkom 2004 and 2011). While mitochondria are the most abundant organelles in the oocyte, little is known about how their different functions, including respiratory activity may be regulated during early development. In this regard, it has been proposed that levels of ATP generation are locally up or down regulated by endogenous factors (Van Blerkom 2004 and 2008).

The mitochondria distribution and activity changes during oocyte maturation and fertilization with the aim of bringing mitochondria to the region of the cell where a higher level of ATP (Van Blerkom *et al*. 1984) or calcium (Sousa *et al.* 1997) are required. Energy in the form of ATP is crucial; spindle formation and chromosome behavior depends on the expression and activity of motor proteins, which use ATP as their energy source. It have been proposed that mitochondria is influenced by the oocyte quality (Stojkovic *et al*. 2001) compactness of the cumulus (Torner *et al*. 2007) and cumulus apoptosis (Torner *et al*. 2004), GnRH (Dell'Aquila *et al.* 2009) and the microtubule cytoplasmic network (Brevini *et al*. 2005) affecting the early stages of the embryo (Tarazona *et al.* 2006). Therefore, several authors concluded that better quality oocytes contained significantly higher ATP levels and produced significantly higher blastocyst rates after fertilization (Van Blerkom *et al*. 1995, Stojkovic *et al*. 2001, Van Blerkom. 2004).

2.6.3. Gene expression

In the last few years, the study of mammalian genes has been the focus of much attention in the belief that a good expression pattern could derive in a successful oogenesis, folliculogenesis, fertilization and early embryonic development. In the course of acquiring the oocyte competencies and a good embryo development, the mRNA transcription is a crucial process occurring in the cytoplasm (Crozet *et al*. 1981, Gandolfi *et al*. 2001, Patel *et al*. 2007). The mRNA content in oocytes is affected by animal nutrition (Pisani *et al*. 2008), donor age (Hamatani *et al*. 2004, Zhang *et al*. 2013), follicle diameter (Caixeta *et al*. 2009, Bonnet *et al*. 2015), IVM culture media (Saadeldin *et al*. 2011, Salhab *et al*. 2011), *in vivo* and *in vitro* conditions (Wells *et al*. 2008, Adona *et al*. 2016), apoptosis (H. J. Li *et al*., 2009) and the cumulus cells (Adriaenssens *et al*. 2010, Paczkowski *et al*. 2013) among others.

In this thesis we are going to study the expression of nine genes that are shown to be altered if the cell is under stress, and which viability could be compromised.

Activating transcription factor 4 (ATF4) gene encodes a protein that belongs to a family of DNA-binding proteins that includes the AP-1 family of transcription factors, cAMP-response element binding proteins (CREBs) and CREB-like proteins. It induces the expression of genes involved in amino acid metabolism, anti-oxidation response and apoptosis. ATF4 is induced by stress signals including anoxia/hypoxia, endoplasmic reticulum stress, amino acid deprivation, and oxidative stress in various cells (Ameri *et al*. 2008, de Nadal *et al*. 2011). In oocytes, it is altered due to when lipotoxic effects are observed (Sutton-Mcdowall *et al*. 2016, Wu *et al*. 2010). DNA methyltransferase 1 (DNMT1) encodes an enzyme that transfers methyl groups to cytosine nucleotides of genomic DNA. This protein is an epigenetic modifier and it is responsible for maintaining methylation patterns following DNA replication. DNA methylation of CpG sites is one of the epigenetic modifications that regulates gene expression. DNMT1a is the main gene to maintain a normal methylation status in the oocyte (Russell *et al*. 2008, Heinzmann *et al*. 2011). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a metabolism related gene that encodes a protein that catalyses the sixth step of glycolysis, converting NAD^+ to NADH and thus generating reducing power. Even though it is widely used as a reference gene for relative quantification, it is found upregulated when energy metabolism is compromised (V. Van Hoeck *et al*., 2013) and it is even described as a regulator of cell death (Colell, Ricci, Green, & Ricci, 2009). Glutamate-cysteine ligase (GCL) is rate-limiting enzyme in glutathione synthesis, which in turn is a well-known antioxidant (de Matos *et al*., 2002). It is a heterodimer composed of a catalytic (GCLC) and a modifier (GCLM) subunit. Its expression is downregulated in follicular apoptosis (Luderer, Diaz, Faustman, & Kavanagh, 2003) and with oxidative stress (Franklin *et al*., 2009). Glutathione peroxidase 1 (GPX1/GSH-Px) encodes a protein that belongs to the glutathione peroxidase family, members of which catalyze the reduction of organic hydroperoxides and hydrogen peroxide (H2O2) by glutathione, and thereby protect cells against oxidative damage. It is extensively used as a marker of cell oxidative stress (Han *et al*. 2008, Salavati *et al*. 2012, Khalil *et al*. 2013, Van Hoeck *et al*. 2013). Heat shock protein family A (HSP70) member 5 (HSPA5/GPR78) is a chaperon protein which is localized in the lumen of the endoplasmic reticulum (ER), and is involved in the folding and assembly of proteins in the ER. Its expression is upregulated when the ER is under stress (Lee 2005, Xu *et al*. 2005), for example, due to lipotoxicity (Wu *et al*. 2010, Yang *et al*. 2012, Sutton-mcdowall *et al*. 2016). Ribosomal protein L19 (RPL19) encodes a ribosomal protein that is a component of the 60S subunit. It is located in the cytoplasm, and it is needed for ribosome assembly to catalyse protein synthesis.

RPL19 overexpression activates the unfolded protein response (UPR), which can induce pro-apoptotic cell death due to stress (Hong *et al*. 2014). Solute carrier family 2 member 1 (SLC2A1/GLUT1) encodes a glucose transporter that is found primarily in the cell membrane and on the cell surface. Its expression is used as a marker of cell stress, as it is upregulated with an increase of ROS (Sviderskaya *et al*. 1996, Van Hoeck *et al*. 2013). Superoxide dismutase 1 (SOD1/CuZnSOD) encodes for a soluble cytoplasmic protein that binds copper and zinc ions and is one of the two isozymes responsible for destroying free superoxide radicals in the cell. When there is an increase of superoxide species in the cell due to stress is found upregulated (Han *et al*. 2008, Salavati *et al*. 2012, Hou *et al*. 2016).

Relative expression of these genes was calculated against β-actin (ACTB) gene, described as a reference gene in goat studies (Frota *et al*. 2011, Zhang *et al*. 2013).

Chapter 3

Objectives

The present study has five main aims:

1- To determine the fatty acid profile of follicular fluid of goats.

2- To study the changes in fatty acid composition of goat follicle fluid according to the age of the donor, season of the year and size of the follicle

3- To identify potential markers of oocyte competence in Fatty Acids of Follicular Fluid

4- To study the oocyte competence of prepubertal goat oocytes *in vitro* matured with different concentration ratios of linoleic and linolenic acids.

5- To study the effect of linoleic and linolenic acid ratios on prepubertal goat oocyte quality by assessing mitochondrial distribution and activity, ATP concentration and relative gene expression

Chapter 4

Materials and methods

4.1. Chemicals and reagents

All the chemicals were purchased from Sigma-Aldrich Chemicals Co (St. Louis, USA) unless otherwise specified.

4.2. Ovary collection

Commercial goat meat in Catalonia and Spain is coming from suckling kids younger than 45 days old and with a live weight from 7 to 10 Kg. Ovaries from prepubertal goats were collected from a local slaughterhouse and transported within half an hour in Phosphate Buffered Saline (PBS) at 37ºC. Once in the laboratory, the ovaries were washed three times in PBS at 37ºC and kept at the same temperature in an incubator until use.

4.3. Fatty acid composition of follicular fluid

4.3.1. Time of follicular fluid extraction and animal conditions

Samples were collected by aspiration of follicles according to follicle diameter (larger and smaller than 3 mm) in two repetitions for each season of the year (spring, summer, autumn and winter) in Mediterranean climate in adult and prepubertal goats. Adult animals were kept in experimental indoor conditions and fed alfalfa ad libitum during all the experiment. Prepubertal one month old suckling goats were fed with maternal milk until slaughter.

4.3.2. Follicular fluid extraction of prepubertal goats by aspiration

Follicular fluid was recovered from large (≥ 3 mm) or small (< 3 mm) follicles using a 20G needle attached to a syringe. The samples were pooled and centrifuged at 10000 G during 20 minutes. Then, the supernatant was recovered and the pellet fraction was discarded. Finally, FF was kept at -80ºC until analysis.

4.3.3. Follicular fluid extraction of adult goats by laparoscopy

Animal ethics

All procedures were approved by the Ethical Commission of Animal and Human Experimentation (Spanish Government, Authorization number CEEAH 2656) under the auspices of the Ethical Commission of the Autonomous University of Barcelona.

Laparoscopy procedure

Two sessions of laparoscopy were carried out for each season of the year. Two hormonally unstimulated Murciano-Granadina goats were used for each repetition, and between one to four sessions were performed in each goat in separate seasons. Animals were deprived of food and water for 12 hours prior to laparoscopy. They were pre-medicated with midazolam (Dormicum, 0.2 mg Kg^{-1} body weight (BW) i.m.; Roche, Spain) and buprenorphine (Buprex, 0.01 mg Kg^{-1} BW i.m.; Schering-Plough S.A., United Kingdom) fifteen minutes before induction. General anaesthesia was induced with propofol (Lipurol 1%, 4 mg Kg^{-1} BW i.v.; B-Braun, Spain) and maintained with 2-2.5% isofluonate (Isoflo, Lab. Dr. Esteve S.A., Spain) in 100 % oxygen. An orogastric tube was inserted to prevent regurgitation and aspiration pneumonia. Laparoscopy was performed as it was previously described by Alberio *et al* (2002). The animals were restrained in dorsal recumbence; the head lower than the body on a 40º incline. The pneumoperitoneum was produced by injecting carbon dioxide through a Verress needle. A 10 mm trocar, associated to endoscope, was inserted approximately 10 cm cranial from the udder and 10 cm of the left side from the midline. A 5 mm trocar was introduced to the opposite side of the 10 mm trocar for the placement of the non-traumatic grasping forceps that was utilized to fix the ovary. A second 5 mm trocar was located to 2-3 cm from the midline and it was used to introduce the handmade follicular puncture set. The puncture set was made using a modified cannula constituted by a 21 G butterfly needle (Venofix, B. Braunn, Spain) without “wings” and final connection mounted into an Aspic of insemination (Aspic IVM Cassou, L’Aigle France) and all the system was introduced into 1 mL pipette to give rigidity to the system. The cannula was connected to a drainage line that ended in a 15 mL collection tube. The follicles were aspirated with a controlled-vacuum pump (Aspirator 3, Labotect GmbH, Germany), which maintained a vacuum pressure between 25 to 30 mmHg. Follicles within 2 to 9 mm of diameter were aspirated perpendicularly to the wall of the ovary, and divided into small follicles (<3 mm) and large follicles (≥3 mm). The cannula was then washed with 500 µL of PBS to collect all the fluid. At the end of the session, the ovaries were flushed with sterile heparinised saline solution (0.9% saline with 5 U mL-1 heparin) (Sodium heparin 5%, ROVI S.A, Spain). The goats received meloxicam (Metacam, 0.1 to 0.2 mg kg-1 BW i.m.; Boehringer Ingelheim, Spain) every 24 h and buprenorphine (Buprex, 0.01 mg kg-1 BW i.m) every 8 hours for the next three days. Antibiotherapy was performed with amoxicillin (Duphamox L.A., 22 mg kg-1 BW i.m; Fort Dodge Veterinaria, Spain) every 48 h during 5 days.

The samples were centrifuged at 10000 G during 20 minutes. Then, the supernatant was recovered and the pellet fraction was discarded. Finally, FF was kept at -80ºC until analysis.

4.3.3. FA extraction from follicular fluid and analysis

For the FA analysis Sukhija and Palmquist (1988) protocol was used with some modifications. Briefly, 200 µL of sample (100 µL FF + 100 µL PBS) with 450 µL toluene, 50 µL of nonadecanoic acid (C:19, 0.767 mg/mL in toluene) as internal standard, and 1 mL methanolic HCl (5%) was vortex for 60 seconds and then warmed in a water bath for 1 hour at 70ºC. Subsequently 1.25 mL K2CO3 (12%) and 500 µL toluene was added, vortex for 30 seconds and centrifuged for 5 min at 1000 g. Finally the supernatant (organic matter) was recovered and dried with Na2SO4. The extracted samples were maintained at -20°C until gas chromatographic analysis, on an Agilent 6890, with a chromatographic column Agilent DB23 60 m x 0.32 mm x 0.25 µm. For each sample, 2 µL were injected using pulsed splitless mode, with oven initial and final temperatures of 140 and 250 ºC in 93 min. Fatty acids were identified and quantified comparing the retention time of samples in the column in comparison with the ones of commercial standards Supelco 37 FAME Mix and Supelco cis-11-Vaccenic Methyl ester.

4.4. *In vitro* embryo production from prepubertal goats

4.4.1. Oocyte collection

Cumulus oocyte complexes (COCs) were recovered by slicing the surface of the ovary in a TCM-199 medium containing HEPES, calcium bicarbonate and antibiotic-antimicotic (AB, GIBCO, cat 14240-062) in a petri dish (Nunc, Roskilde, Denmark). COCs with homogeneous cytoplasm and three or more cumulus cells layers were selected for maturation (figure 8).

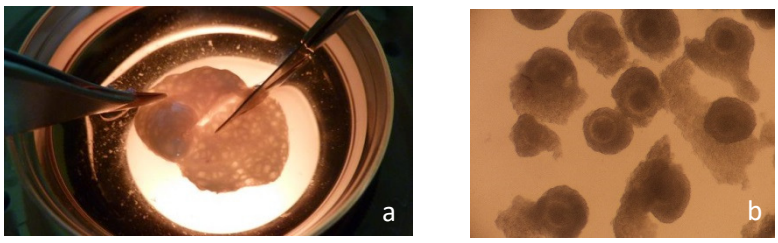

Figure 8. (a) Detailed image of the slicing technique (b) Oocytes selected for maturation.

4.4.2. *In vitro* maturation

Groups of 35 oocytes were washed twice in IVM medium and cultured for 24 h in a 4-well dish (Nunc, Roskilde, Denmark) containing 10 µL of medium for each oocyte at 38.5°C, in a 5% CO_2 humidified air atmosphere. Control maturation media (BSA TC) (adapted from Catalá *et al.* 2011) consisted of TCM-199 supplemented with 0.6 % w/v fatty acid free BSA, 5 µg/mL LH, 10 µg/mL FSH, 1 µg/mL 17β-E2, 10 ng/mL epidermal growth factor, 0.2 mM sodium pyruvate, 2 mM L-glutamine, 100 µM cysteamine. Linoleic acid (LA, 35 mM stock in water) and α-linolenic acid (ALA, 100 mM stock in dimethyl sulfoxide-DMSO) were added in the medium with the required LA:ALA ratio/concentrations (4:1/200:50 µM; 2:1/ 100:50 µM and 1:1/ 50:50 µM respectively). A second control group was used with 10 % of foetal calf serum (FCS) instead of BSA, as a lab internal control (IC).

4.4.3. *In vitro* fertilization

Fresh semen was obtained from two bucks with proven fertility. Only ejaculates with good mass motility were used. The selection of the motile sperm fraction was made by the swim-up method during 1 h in a 5% CO_2 humidified air atmosphere. Sperm was capacitated in a modified Defined Medium (mDM) containing heparin (final concentration 50 µg/mL) during 45 min at 38.5°C in a 5% CO_2 humidified air atmosphere.

Matured oocytes were washed twice and transferred to microdrops of fertilization medium (modified Tyrode's medium, TALP, from Parrish *et al* 1986), supplemented with 1 µg/mL hypotaurine and 0.3 mg/mL glutathione, covered with mineral oil. Oocytes were then co-cultured with a final concentration of 4 x 10^6 sperm/mL.

4.4.4. *In vitro* embryo culture

After 17 h, presumptive zygotes were completely denuded by gentle pipetting, washed twice to free them from spermatozoa, and cultured in 2 µL/zygote microdrops of culture media under mineral oil (Nidoil; Nidacon, Sweden). The culture media was synthetic oviductal fluid (SOF, Holm *et al* 1999) supplemented with 10 % (v/v) of FCS. Embryos were incubated at 38.5°C, 5% CO_2, and 5% O2 in a humidified atmosphere during 8 days. Embryo cleavage was assessed 48 h post fertilization. Culture media was changed at day 5.

4.4.5. Metaphase II of oocytes and pronuclear (PN) assessment of zygotes

To assess oocyte IVM and IVF, presumptive matured oocytes (24 h IVM) and presumptive zygotes (17 h post insemination) were completely denuded by pipetting, fixed in ethanol:acetic (3:1) for at least an hour and stained with 1 % of Orcein in 45 % acetic acid. Oocytes were categorized as matured if Metaphase II was observed. Zygotes were categorized as normally fertilized if one female and one male pronuclei were formed (2PN), polyspermic if 3 or more PN (3PN) were observed, and non-fertilized if no pronuclei or non oocyte sperm penetration was observed (in this case, we could observe oocytes in Metaphase II stage).

4.4.6. Parthenogenetic activation (PA)

In vitro matured oocytes were parthenogenically activated. Oocytes were denuded by gently pipetting and activated in mDPBS (DPBS + 10 % of FCS) containing 5 µM Ionomycin for 4 min. Immediately, oocytes were washed 3 times thoroughly in DPBS and placed in TCM199 containing 1.9 mM 6-DMAP (6-Dimetyl amino purine) for 3 hours. Calcium ionomycin is an ionophore that is used to raise the levels of intracellular Ca_{2+}, similar to the increase of calcium with the penetration of the sperm cell, and consequently activates the oocyte to undertake the cellular division. 6-DMAP prevents the oocyte from the extruding of the second polar body by inhibiting kinase, maintaining the cell diploid for blastocyst formation. After PA, presumptive activated oocytes were cultured *In vitro* in the same conditions as *in vitro* fertilized zygotes.

4.4.7. Blastocyst differential staining

Blastocyst differential staining protocol was adapted from Thouas *et al* (2001). Briefly, 9-day-old blastocysts were incubated for approximately 15 sec in a TCM199 solution with 1% (v/v) Triton X-100 and 100 µg/mL propidium iodide (PI). Immediately, they were transferred to an ethanol solution containing 25 µg/mL Hoechst 33258 (Invitrogen, Eugene, Oregon, USA) and kept overnight at 4ºC in the darkness. Stained blastocysts were then mounted on a glass slide with a drop of glycerol and flattened with a cover slip. Cells were counted under an Olympus BX50 fluorescent microscope with an ultraviolet lamp and a 460 nm excitation filter. The nuclei from the inner cell mass cells and trophectoderm cells were stained blue (Hoechst) and red (PI) respectively.

4.5. Oocyte molecular analysis from prepubertal goats

4.5.1. Mitochondrial activity assessment

Oocyte staining

Groups of 10 oocytes were used per group (immature 0h oocytes, and IVM with BSA, 50:50 and 200:50) in three repetitions. Mitochondria were stained with Mitotracker® Orange CMTMRos (Molecular Probes, Inc., Eugene, OR, USA), a selective probe that passively diffuse across the plasma membrane and accumulate in active mitochondria, depending on their oxidative activity. A 1 mM stock solution was prepared dissolving the lyophilized powder in DMSO. Mature or immature (0h) oocytes were totally denuded by pipetting and incubated in modified PBS (mPBS) with 3 % (m/v) BSA containing 200 nM Mitotracker under culture conditions for 30 minutes. Immediately after staining, oocytes were washed three times in mPBS 0.1 % BSA (w/v) and fixed for 60 minutes in 3 % paraformaldehyde at 38ºC. After fixation, oocytes were properly washed with mPBS 0.1 % BSA and stained for 5 minutes in 1 µg/mh Hoescht 33342 solution in mPBS 0.1 % BSA to determine nuclear maturation stage. Finally, they were mounted on poly-L-lysine treated coverslips fitted with a self-adhesive reinforcement ring and covered with a drop of Vectashield® mounting medium (Vector laboratories, Burlingame, Ca, USA). Slides were then sealed with nail varnish and stored at -20ºC for one (mature oocytes) or two (immature oocytes) days until their analysis using confocal laser microscopy.

Confocal analysis

The analysis was carried out only in the oocytes that present VG (0h: immature oocytes) or MII (24h post IVM). A Leica SP5 laser scanning confocal microscope (Leica, Mannheim, Germany) was used to examine active mitochondria (Mitotracker Orange CMTRos, excitation 552 nm) and chromatin (Hoescht; excitation 405 nm). Images were taken at 63X magnification under mineral oil. For mitochondrial intensity the microscope objective, pinhole, filters, offset, gain, pixels and laser potency were kept constant throughout the experiment. Thirty-five serial cuts in the region of major intensity were performed with a final image thickness of 50 µM. The 35 cuts of each oocyte were merged in a unique image and analysed with Metamorph® imaging software (Molecular devices, CA, USA). To express the fluorescence intensity, we used arbitrary unit of the average intensity divided by the area of the object. The mitochondrial distribution pattern was classified as homogeneous (fluorescence throughout the cytoplasm), semi-peripheral

(fluorescence throughout the cytoplasm but with less intensity in the middle of the cell), peripheral (fluorescence in the cortex of the oocyte) (Brevini, 2005) (Figure 9).

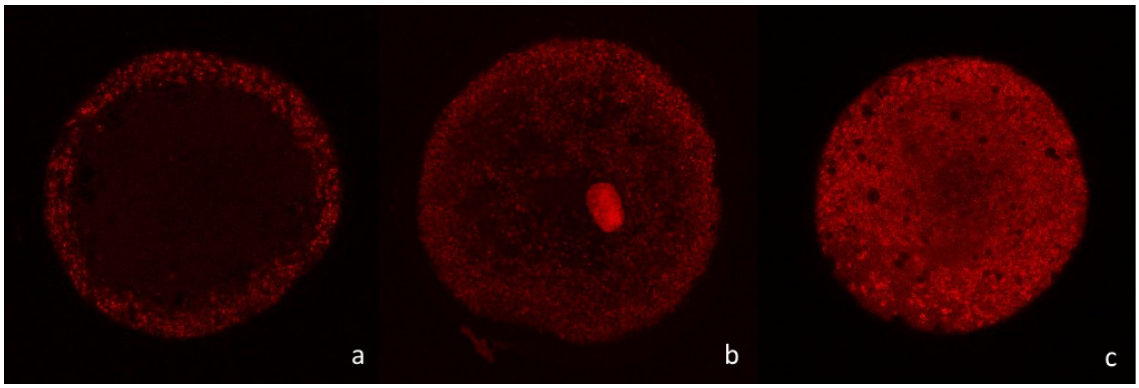

Figure 9. Representative images of active mitochondrial distribution on prepubertal goat oocytes. *Images taken by confocal microscope: (a) peripheral distribution; (b) semi-peripheral distribution; (c) homogeneous distribution.*

4.5.2. Adenosine 5'-triphosphate (ATP) analysis

The ATP content of oocytes was measured using a commercial assay kit based on the luciferin-luciferase reaction (fig 10) (Adenosine 5'-triphosphate (ATP) bioluminescent somatic cell assay kit (FLASC)), following manufacturer's protocol. Groups of 6 oocytes per treatment (immature 0h and mature 24h oocytes) were completely denuded by pipetting and placed in an Eppendorf with 200 µl of ultrapure water, snap frozen and stored at -80ºC until analysis.

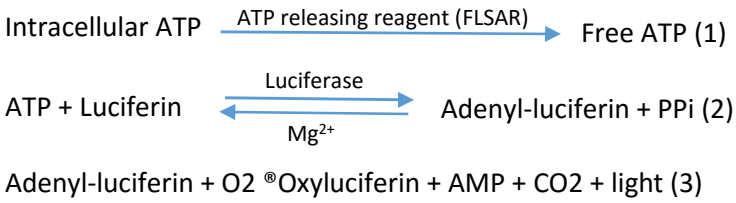

Figure 10. ATP measuring reactions (from sigma technical bulletin). *Reactions 1 and 3 are essentially irreversible and reaction 2 is reversible with the equilibrium lying far to the right. When ATP is the limiting reagent, the light emitted is proportional to the ATP present.*

Prior to analysis, 100 µl of ATP assay mix working solution (FLAAM; diluted 1:2 in assay mix buffer) was added to a reaction vial for 3 minutes to hydrolyse any endogenous ATP. In a separate vial, 50 µl of ultrapure water and 50 µl of the sample were mixed with 100 µl of somatic cell ATP releasing agent (FLSAR). Finally, 100 µl of this solution was transferred to the reaction vial and the light emitted was immediately measured in a Luminometer (Victor^3, Perkin Elmer, Waltham, USA). A 5 point standard curve (500, 1000, 2000, 4000, and 8000 fmol of ATP) and a

blank without sample was included in each essay. The ATP content of a single oocyte was calculated using the values of the standard curve and the blank.

4.5.3. RNA relative quantification

All the steps were done using RNase/DNase free lab ware.

4.5.3.1. Oocyte storage and RNA extraction and quantification

Groups of 15 oocytes per treatment (immature 0h oocytes, and IVM with BSA, 50:50 and 200:50; three replicates) were denuded by pipetting and washed thoroughly in PBS. Afterwards, they were snap-frozen in 15 µl of PBS-PVP 0.4 % (w/v) and stored at -80 ºC until RNA extraction, which was done on the same day for all the samples tested.

Total RNA was extracted using Qiagen RNeasy® mini kit (Qiagen, Ambion Inc., Austin, Texas) following manufacturer's protocol. Briefly, oocytes were lysed in 350 µl of RLT buffer (lysis buffer), vortex for 30 s, mixed with 350 µl of ethanol 70 %, transferred to the RNeasy spin columns and centrifuged. The flow-through was discarded and the spin column membrane was washed with several buffers as instructed by the manufacturer. Finally, the RNA was eluded in 30 µl of RNase-free ultrapure water (Qiagen).

RNA concentration (pg/µl) and integrity number (RIN) was measured using Agilent RNA 6000 pico chip on an Agilent 2100 bioanalyzer (Agilent technologies, Waldbronn, Germany).

4.5.3.1. Primer design

Goat (Capra spp) transcript sequences were obtained from NCBI Genbank (http://www.ncbi.nlm.nih.gov/gene). Intron/Exon positions were extrapolated comparing and aligning the goat transcript sequence with cow or sheep sequences obtained from Ensembl Genome browser (http://www.ensembl.org/index.html). The alignment was done using Multalin INRA application (http://multalin.toulouse.inra.fr/multalin/).

After calculating the exon position, primer design was performed using Primer Express® software (v 3.0.1, Applied biosystems, Foster City, CA, USA). Forward (FW) and reverse (RV) primers were placed on different exons and spanning exon-intron boundary as far as possible, in order to avoid amplification of any possible DNA contamination in the sample. To design the primers, some considerations were followed:

- **Primer size:** From 18-40. Optimal length: 20.
- **GC content (Guanidine & cytosine):** 60-70 %.
- **Melting temperature (Tm):** 58-60 ºC. It is important that FW and RV primers have the same or close Tm.
- **Amplicon size (bp):** 70-150

Table 4. Primer detailed information for each gene analysed.

Gene	Primer	Sequence (5'-3')	GenBank accession no.	Fragment size (bp)
ACTB	forward	TGCCCTGAGGCTCTCTTCCA	NC_022317.1	103
	reverse	TGCGGATGTCGACGTCACA		
RPL19	forward	AGATTGACCGCCACATGTATCAC	NC_022311.1	79
	reverse	TCCATGAGAATCCGCTTGTTTT		
SLC2A1	forward	GCCTGTGTACGCCACCATT	NC_022295.1	78
	reverse	GCTCGCTCCACCACAAACA		
ATF4	forward	CGCTTTTCACGGCATTCAG	NC_022297.1	74
	reverse	TTCGAGGAATGTGCTTAATTCG		
GPX1	forward	CCCGTGCAACCAGTTTGG	NC_022314.1	80
	reverse	CGCCTGGTCGGACGTACT		
HSPA5	forward	CATGGTTCTCACTAAAATGAAGGAAAC	NC_022303.1	73
	reverse	AGTAACAACTGCATGAGTAACCTTCTTT		
GAPDH	forward	GCAAGTTCCACGGCACAGTC	NC_022297.1	102
	reverse	CCCACTTGATGTTGGCAGGA		
DNMT1	forward	GGTGAAAAGGCTCTTCTTGGC	NC_022299.1	83
	reverse	AATAGTGGTGCGTACTCTGGGC		
GCLC	forward	CGTCTGTAGATGATAGAACTCGGGA	NC_022315.1	121
	reverse	TTCTCACCGCACTCAGATAAGTAACT		
SOD1	forward	ACCATCCACTTCGAGGCAAA	NC_022293.1	76
	reverse	CATGATCACCTTCAGTCAACCCT		

Reference gene: ACTB (Beta-actin); Quantified genes: RPL19 (ribosomal protein L19); SLC1A1 (facilitated glucose transporter 1); ATF4 (Activating transcription factor 4); GPX1 (Glutathione peroxidase 1); HSPA5 (Heat shock protein family A 70 KDa); GAPDH (Glyceraldehyde-3-phosphate dehydrogenase); DNMT1 (DNA metiltransferase 1); GCLC (Glutamate-cysteine ligase catalytic subunit); SOD1 (superoxide dismutase 1).

Once the primers were designed, were further analysed. First of all, the specificity and homology of the primers for each specific gene was evaluated using the Basic Local Alignment Search tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Afterwards, primers were tested using Oligo Analyzer tool (Integrated DNA technologies, Coralville, Iowa, USA). It is used to analyse possible hairpins, primer dimer (FW-FW RV-RV) or heterodimer (FW-RV) in temperatures similar to the

Tm, which can give amplification problems. Primer pairs were ordered lyophilized from Sigma-Aldrich Chemicals Co (St. Louis, USA) and reconstituted to a final concentration of 100 µM with RNase-free ultrapure water (Qiagen) and stored at -20º C until use. Table 4 shows the summarised information of the primers designed.

4.5.3.3. Reverse transcription

RNA reverse transcription (RT) was done using qPCRBIO cDNA synthesis kit (PCRbiosystems, London, UK) which contain random hexamers and anchored oligo(dT), in a total reaction volume of 30 µl per sample. All the samples analysed were reverse transcript at the same time to avoid inter-assay differences: three biological replicates for each treatment. A negative no reverse transcription control (-RT) was included, that consist in adding sample without adding enzyme to assess the absence of DNA in the sample. cDNA was synthetized following manufacturer's protocol. Briefly, 22,5 µl of the sample + 6 µl of the 5x cDNA synthesis mix + 1.5 µl 20x RTase were mixed for each reaction. The –RT control consisted in: 14 µl of the sample + 4 µl of 5x cDNA synthesis mix + 2 µl of RNAse free ultrapure water. The samples were then incubated for 30 min at 42 ºC and 10 min at 85 ºC in a Bio rad T100 thermal cycler (Bio Rad Laboratories, Hercules, CA, USA). After the incubation, the samples were used for RT-qPCR optimization or kept at -80 ºC until use.

4.5.3.4. RT-qPCR optimization: primer testing, melting curves and amplification

Expression levels of mRNA were determined by real time quantitative PCR (RT-qPCR) using SYBR® Select Master Mix (Applied biosystems, Foster City, CA, USA). The SYBR Green binds to the double-stranded DNA and emits light upon excitation. As the reaction proceeds and the PCR products accumulate, the fluorescence increases proportional to the amount of specific DNA present in the original sample.

For the optimization of the RT-qPCR, primers and cDNA were tested on an applied biosystems 7900HT fast real-time PCR system with SDS software (v 2.3). cDNA concentrations were tested by a 6 point standard curve (figure 11) : 1 – 1/2 – 1/4 - 1/8 – 1/16 – 1/32. Two negative controls were included to discard any DNA contamination: -RT control (from the –RT sample) and non template control (NTC) which consisted in adding water instead of cDNA on the reaction tubes. The PCR reaction was performed in a final volume of 20 µl: 10 µl Sybr select MM + 0,6 µl FW primer (10 µM) + 0,6 µl RV primer (10 µM) + 4,8 µl ultrapure water + 4 µl of cDNA (or water in the NTC). All the samples were measured by triplicates. PCR reactions were programmed as follows: initial holding stage at 50 ºC for 2 min and a denaturation step at 95 ºC for 10 min. PCR

stage or amplification stage at 95 ºC for 15 s and 60 ºC for 1 min repeated for 40 cycles. A final cycle of melting curve (figure 12) was done at 95 ºC for 15 s, 60 ºC for 1 min and 95 ºC for 15 s.

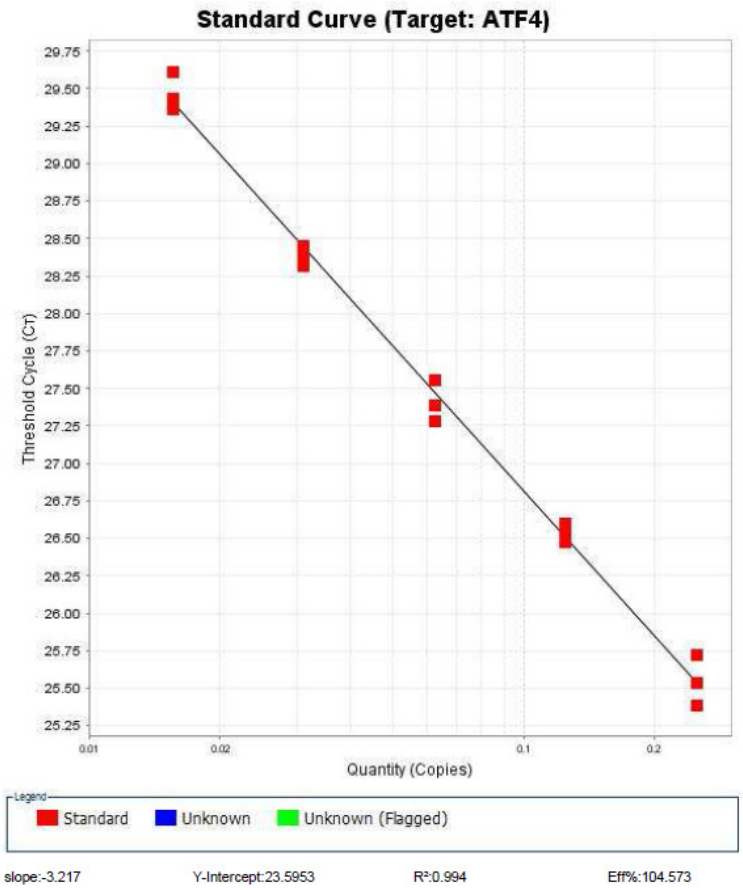

Figure 11. Sample of a standard curve for ATF4 gene.

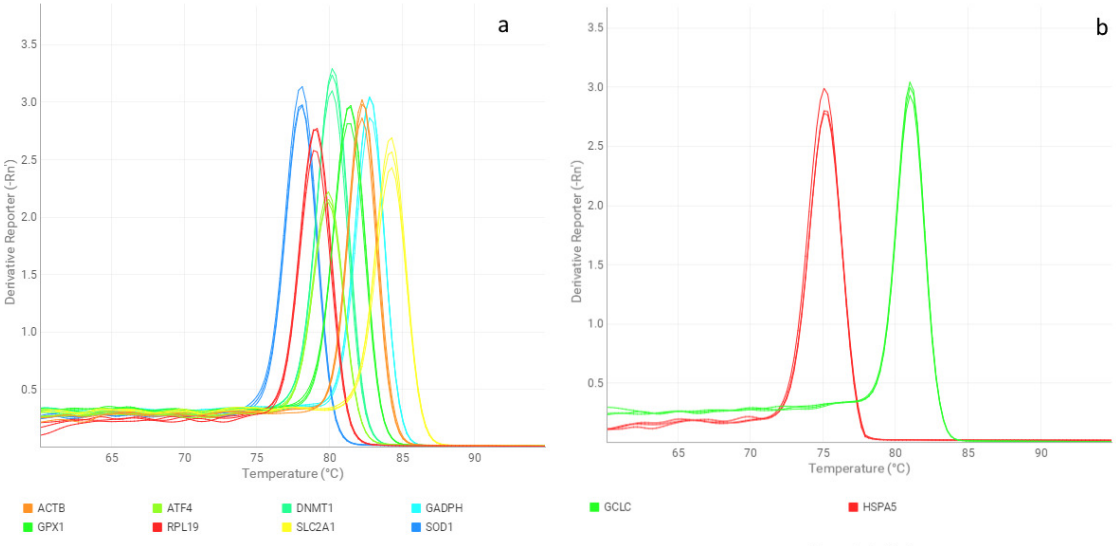

Figure 12. Melting curves of the genes analysed. *(a) genes: ACTB, ATF4, DNMT1, GAPDH, GPX1, RPL19,SLC2A1 and SOD 1; (b) GCLC AND HSPA5.*

4.5.3.5. RT-qPCR on samples

After the optimization with 7900HT, a final 7 point standard curve was done to calculate the efficiency of the PCR for each of the genes tested: 1 - 1/2 – 1/4 - 1/8 – 1/16 – 1/32 – 1/64. PCR reactions were done in a Quant Studio™ 12K Flex Real-time PCR system, with Quant studio 12K flex software (Applied biosystems, Foster City, CA, USA). The reaction was prepared in a 384 plate using Real time PCR pipetting robot Eppedorf epmotion 5075 (Eppendorf, Hamburg, Germany). The PCR reaction was performed in a final volume of 15 µl: 7,5 µl Sybr select MM + 0,45 µl FW primer (10 µM) + 0,45 µl RV primer (10 µM) + 5,1 µl ultrapure water + 1,5 µl of cDNA diluted at each concentration point (or water in the NTC). Initial holding, amplification and denaturation steps were programmed as in 7900HT experiments. The optimal cDNA concentration for RT-qPCR was 1/4. A final RT-PCR reactions were done for all the genes by triplicate for each biological repetition. For the Cq settings, the threshold was set at 0.4, and 3 and 15 for baseline start and end respectively. The ct values were corrected by the efficiency obtained with the standard curve. Figure 13 shows the amplification plots of the total of the genes analysed for one sample.

Relative quantification was calculated against the reference gene: ACTB, following Livak & Schmittgen (2001) method. All the calculations were done using the relative quantification RT app on the webpage tool on thermo fisher cloud (Thermo Fisher Scientific, Waltham, MA, USA).

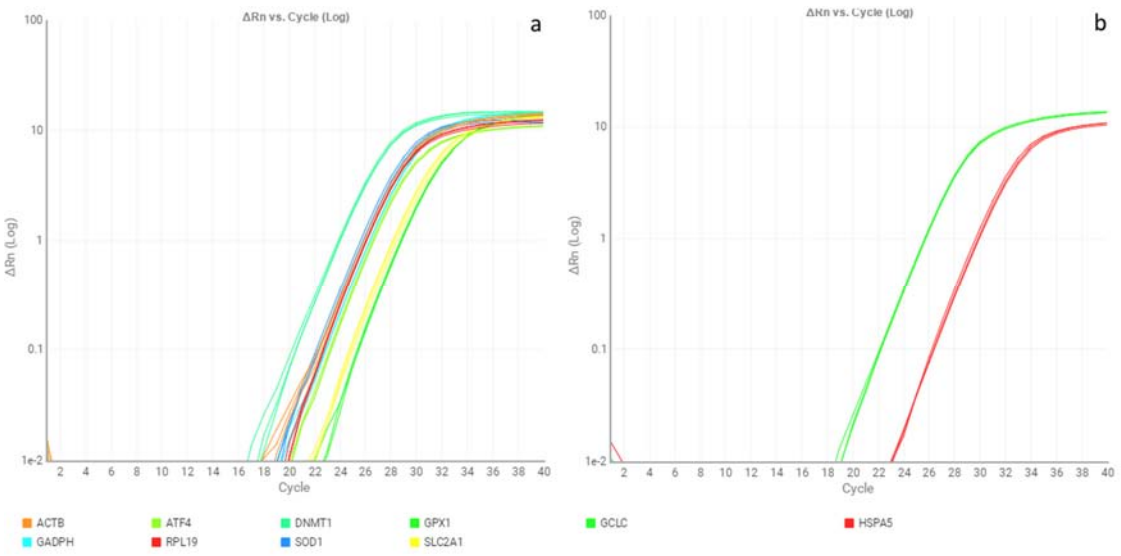

Figure 13. Amplification plots for the analysed genes of one sample. *(a) genes: ACTB, ATF4, DNMT1, GAPDH, GPX1, RPL19,SLC2A1 and SOD 1; (b) GCLC AND HSPA5.*

4.6. Statistical analysis

All statistical analysis were carried out with SAS/STAT® software v 9.3 (for windows, SAS institute Inc., Cary, USA).

Follicular fluid composition, embryo production, ATP and mitochondrial activity data were analysed using analysis of variance (ANOVA) through general linear model, with multiple pair-wise comparisons by Tukey post-hoc test. For FF composition, the model included, when necessary, the effects of size of the follicle, age or season and their interactions as fixed factors and replicate as random factor. For embryo production and total cell analysis, the model included the treatment, or treatment and technique respectively as fixed factors and replicate as random factor. Data with non-normal distribution were square root arcsine transformed prior to ANOVA, but results are reported as back-transformed. Results were considered significant when $P<0.05$ and tendencies at $P<0.1$.

Data of relative mRNA abundance was analysed by the RT app on thermo fisher cloud (Thermo Fisher Scientific, Waltham, MA, USA) through a model of integrated correlation. Results were considered significant when $P<0.05$ and tendencies at $P<0.1$.

4.7. Experimental design

Experiment 1: Fatty acid composition of follicular fluid

The aim of this experiment was to measure the fatty acid composition of follicular fluid in prepubertal and adult goats, in follicles of different sizes, and in different seasons of the year and try to relate the results to previous results on embryo production.

Experiment 2: *In vitro* embryo production

The aim of this experiment was to test the effect of the addition of different concentrations/ratios of LA/ALA on oocyte *in vitro* maturation media on subsequent embryo development. For this purpose, one month old suckling goat oocytes obtained by the slicing technique were matured in maturation media supplemented with 0.06 % BSA (w/v) and the LA:ALA (ω6:ω3) following ratios: 4:1 or 200:50 µM; 2:1 or 100:50 µM; 1:1 50:50 µM. Two groups of controls were added to the experimental design: maturation media supplemented with 0.06 % BSA (treatment control: TC) or supplemented with 10 % FCS (lab internal control: IC). Afterwards, they were *in vitro* fertilized or partenogenetically activated and *in vitro* cultured

until day 9. Maturation, fertilization, cleavage and embryo production rates and blastocyst cell number were registered.

Experiment 3: oocyte molecular analysis

The aim of this study was to analyse the prepubertal goat oocyte cytoplasm quality at the collection time (0h) and 24 hours post-maturation by molecular techniques (3 biological repetitions for each group). For this purpose, a total of three treatments were chosen after the results obtained on embryo production. Thus, prepubertal goat oocytes were matured in maturation media supplemented with 0.06 % BSA (control group) and the LA:ALA (ω6:ω3) following ratios: 4:1 or 200:50 µM; 1:1 50:50 µM.

Oocytes at collection time or after 24 hours of maturation were collected and treated depending on the developed technique: mitochondrial activity assessment, ATP measuring, and RNA relative expression. Table 5 lists the genes analysed and studies were it were found to be up or downregulated due to different alterations in the cell normal function.

Table 5. List of genes whom expression has been analysed in mature or immature prepubertal goat oocytes

GENE	FUNCTION	UP/DOWN REGULATED?	REFERENCES
ACTB	Essential component of the cytoesqueleton: Involved in cell migration, division and regulation of gene expression	Reference gene	Frota *et al.* (2011); Zhang *et al.* (2013)
RPL19	Ribosomal protein	RPL19 overexpression activates unfolded protein response (UPR), which can induce pro-apoptotic cell death due to stress.	Hong *et al.* (2014)
SLC2A1/GLUT1	Metabolism related: glucose transporter	Upregulated with the increase of ROS species	Sviderskaya *et al.* (1996); Van Hoeck *et al.* (2013)
ATF4	Transcription factor: induces expression of genes involved in amino acid metabolism, anti-ox response and apoptosis.	Upregulated when ER and cell are under stress, for example, due to lipotoxicity.	Xu *et al.* (2005); Ameri *et al.* (2008); Wu *et al.* (2010); de Nadal *et al.* (2011); Yang *et al*. (2012) Sutton-mcdowall *et al*. (2016)
GPX1/GSH-Px	Detoxification of hydrogen peroxide (ROS-related gene)	Upregulated when the cell is under stress	Han *et al.* (2008); Salavati *et al*. (2012); Khalil *et al*. (2013); Van Hoeck *et al.* (2013); Hou *et al.* (2016)
HSPA5/GRP78	Chaperon localized in the lumen of ER. Involved in folding and assembly of proteins.	Upregulated when the ER is under stress, for example, due to lipotoxicity.	Xu *et al.* (2005); Lee *et al.* (2005); Wu *et al.* (2010); Yang *et al*. (2012); Sutton-mcdowall *et al.* (2016)
GAPDH	Metabolism related: catalyses the sixth step of glycolysis, converting NAD^+ to NADH.	Upregulated when energy metabolism is compromised	Colell *et al.* (2009); Van Hoeck *et al.* (2013)
DNMT1	DNA methylation: main gene to maintain methylation status in the oocyte	Upregulated in cells with altered function and ROS and in IVM oocytes	Heinzmann *et al.* (2011); Zhang *et al*. (2013)
GCLC	Rate-limiting enzyme in glutathione (GSH) synthesis. GSH acts as an antioxidant	Downregulated in cells with compromised GSH synthesis and apoptotic cells	Luderer *et al.* (2003); Dickinson *et al.* (2004)
SOD1 (CuZnSOD)	ROS repairing enzyme: detoxification of free superoxide radicals.	Upregulated with an increase of superoxide species	Han *et al.* (2008); Hou *et al.* (2016)

List of genes: function in the cell and up or down-regulation due to altered function, with references to works from different laboratories that have studied the gene expression

Chapter 5

Results

5.1. Fatty acid composition of follicular fluid (experiment 1)

5.1.1. Follicular fluid fatty acid composition according to the age of the donor and follicular size

Fatty acid follicular fluid composition varied significantly according to the age of the donor (Table 6).

Table 6. Percentage of fatty acid composition of follicular fluid according to follicle size and age of the donor.

FATTY ACID	ADULT		PREPUBERTAL	
	LARGE (≥3mm)	SMALL (<3mm)	LARGE (≥3mm)	SMALL (<3mm)
C14:0 (myristic)	1.15 ± 0.17a*	1.60 ± 0.18ab	1.67 ± 0.07b	1.82 ± 0.08b
C15:0 (pentadecanoic)	0.75 ± 0.11a	1.08 ± 0.13a	0.33 ± 0.03b	0.34 ± 0.03b
C16:0 (palmitic)	24.59 ± 0.62	24.95 ± 0.84	23.01 ± 0.15	23.88 ± 0.26
C16:1 (palmitoleic)	1.41 ± 0.15	1.36 ± 0.22	1.41 ± 0.06	1.37 ± 0.06
C17:0 (margaric)	1.50 ± 0.08a	1.65 ± 0.11a	0.64 ± 0.03b	0.55 ± 0.02b
C18:0 (stearic)	22.66 ± 0.73a	22.97 ± 0.40a	17.21 ± 0.22b	15.72 ± 0.27b
C18:1n9c (oleic)	23.53 ± 1.00a	21.35 ± 0.67a	27.68 ± 0.34b	27.39 ± 0.36b
C18:1n11c (vaccenic)	2.47 ± 0.16a	2.10 ± 0.14a	3.05 ± 0.06b	3.94 ± 0.15c
C18:2n6c (LA)	10.01 ± 0.45a	10.60 ± 0.58a	12.67 ± 0.29b	11.38 ± 0.51ab
C18:3n3 (ALA)	2.22 ± 0.16a	2.32 ± 0.20a	1.22 ± 0.16b	0.91 ± 0.12b
C20:4n6 (arachidonic)	5.92 ± 0.27a	5.68 ± 0.32a	8.32 ± 0.23b	10.04 ± 0.24c
EPA	1.89 ± 0.18a	2.13 ± 0.27a	1.02 ± 0.13b	0.82 ± 0.12b
DHA	1.91 ± 0.16	2.24 ± 0.19	1.78 ± 0.12	1.85 ± 0.18
SFA	50.64 ± 0.94a	52.23 ± 1.19a	42.86 ± 0.30b	42.31 ± 0.34b
MUFAs	27.40 ± 1.20a	24.80 ± 0.76a	32.14 ± 0.39b	32.69 ± 0.39b
PUFAs	21.95 ± 0.87a	22.97 ± 1.15a	25.01 ± 0.15b	24.99 ± 0.26b
n-3 PUFAs	6.03 ± 0.46a	6.69 ± 0.62a	4.02 ± 0.42b	3.58 ± 0.41b
n-6 PUFAs	15.93 ± 0.59a	16.28 ± 0.73a	20.99 ± 0.42b	24.42 ± 0.57b
n6:n3	2.75 ± 0.18a	2.57 ± 0.22a	5.82 ± 0.83b	6.92 ± 1.22b

Values are represented as mean percentage ± SEM. Different letters in the same row (a-c) indicate significant differences (ANOVA P<0.05). Letters with (*) show tendency (P<0.1). SFA: saturated fatty acids: myristic, pentadecanoic, palmitic, margaric and stearic acids; MUFAs: Monounsaturated fatty acids: palmitoleic, oleic and vaccenic acids; PUFAs: Polyunsaturated fatty acids: LA, ALA, EPA, DHA and arachidonic acids; n-3 PUFAs: ALA, EPA, DHA; n-6 PUFAS: LA, arachidonic acid.

Whilst in adult goats the order of FA abundance was, from most to less abundant (in large vs small follicles): palmitic (24.59 vs 24.95 %); stearic (22.66 vs 22.97 %); oleic (23.53 vs 21.35 %);

linoleic (10.01 vs 10.60 %); arachidonic (5.92 vs 5.68 %) acids. In prepubertal goat was: oleic (27.68 vs 27.39 %); palmitic (23.01 vs 23.88 %); stearic (17.21 vs 15.72 %); linoleic (12.67 vs 11.38 %); arachidonic (8.32 vs 10.04 %) acids.

Regardless of the follicular size, we observed that adult females compared to prepubertal females had higher percentages of linolenic acid (ALA: 2.3 vs 1.1 % respectively), total saturated fatty acids (SFA: 51 vs 42 % respectively) and omega 3 PUFA (6.5 vs 4 % respectively). Contrarily, prepubertal females had, in comparison with adult females, higher percentages of linoleic acid (LA: 12 vs 10.3 % respectively), total monounsaturated FA (MUFAs: 32 vs 26 % respectively), total polyunsaturated FA (PUFAs: 25 vs 22 % respectively) and omega 6 PUFA (22 vs 16 % respectively). Thus, the ratio n6:n3 PUFA was higher in prepubertal females compared to its adult counterparts (6.3 vs 2.6 respectively).

In general, in adult goats there is no difference among the FA composition of large (≥3mm) and small follicles (<3mm), whereas in prepubertal goats there is a significant difference in the vaccenic and arachidonic acids between follicle sizes, being both of the FA higher in follicles smaller than 3 mm.

5.1.2. Follicular fluid fatty acid composition according to the season of the year and age of the donor

There is a variability between the results obtained when the FA composition is compared according to age of the animal and the season of the year or when it is compared according to the age and the size of the follicle. Even though in some of the fatty acids the main differences are due to the age of the female (for example, stearic acid), there are other FA that have differences according to the season of the year (table 7). The highest concentration of LA is found in follicular fluid from prepubertal females during autumn (13.08 %), being statistically different from the other seasons and adult counterparts. LA ranges from 10.67 to 13.08 % in young animals, while the lower concentration is found in adult animals during summer (9.12 %), ranging from 9.12 to 10.68 % among different seasons. Regarding ALA, it ranges from 1.87 to 2.97 in does, being higher in winter, and lower in summer, whilst in kids reach lower values: 0.57 to 1.50, being significantly lower in autumn. With respect to the SFA, MUFAs and PUFAs overall presence, it depends mainly on the season of the year instead of the age of the animal. However, if n-3 and n-6 PUFAs concentrations are analysed, there is a difference among seasons and age of the donor. As a consequence, the ratio n6:n3 varies, being significantly elevated in prepubertal animals in autumn (10.49 %) and lower in adult animals in winter (1.91 %). In fact,

this ratio is lower in adult animals (spring 3.09 %; summer 2.87 %; autumn 2.78 % and winter 1.91 %), in contrast with the results obtained in prepubertal animals (spring 5.11 %; summer 5.83 %; autumn 10.49 % and winter 4.04 %).

Table 7. Percentage of fatty acid composition of follicular fluid according to age of the donor and season of the year.

FATTY ACID	ADULT				PREPUBERTAL			
	SPRING	SUMMER	AUTUMN	WINTER	SPRING	SUMMER	AUTUMN	WINTER
C14:0 (myristic)	0.69 ± 0.03a	1.74 ± 0.17b	1.27 ± 0.25a*b*	1.42 ± 0.27a*b	1.57 ± 0.07b	1.68 ± 0.05b	1.74 ± 0.12b	2.00 ± 0.06b
C15:0 (pentadecanoic)	0.40 ± 0.02a	1.12 ± 0.07b	0.88 ± 0.18b	1.07 ± 0.21b	0.33 ± 0.01a	0.34 ± 0.01a	0.25 ± 0.04a	0.42 ± 0.03a
C16:0 (palmitic)	25.63 ± 0.87ab*	25.79 ± 0.70a	23.25 ± 0.97ab	22.57 ± 0.56b	23.40 ± 0.30ab	23.14 ± 0.37ab	23.02 ± 0.17a*b	24.21 ± 0.37ab
C16:1 (palmitoleic)	1.07 ± 0.11	1.71 ± 0.25	1.35 ± 0.18	1.33 ± 0.23	1.15 ± 0.02	1.38 ± 0.06	1.45 ± 0.03	1.57 ± 0.02
C17:0 (margaric)	1.16 ± 0.06a	1.72 ± 0.06bc	1.56 ± 0.06b	1.81 ± 0.01c	0.64 ± 0.03d	0.66 ± 0.04d	0.51 ± 0.03d	0.58 ± 0.02d
C18:0 (stearic)	23.90 ± 0.25a	22.20 ± 1.09a	22.86 ± 0.73a	22.28 ± 0.30a	17.28 ± 0.38b	16.40 ± 0.48b	16.18 ± 0.51b	16.01 ± 0.58b
C18:1n9c (oleic)	22.08 ± 1.45ab	23.69 ± 1.24a	22.68 ± 1.49ab	21.71 ± 1.11ab	27.30 ± 0.62a*b	28.12 ± 0.05b	28.01 ± 0.60a*b	26.70 ± 0.11ab
C18:1n11c (vaccenic)	2.24 ± 0.27	2.41 ± 0.24	2.36 ± 0.32	2.43 ± 1.19	3.23 ± 0.20	3.38 ± 0.18	3.61 ± 0.36	3.77 ± 0.36
C18:2n6c (LA)	10.68 ± 0.78ab	9.12 ± 0.47a	11.62 ± 0.41ab	10.46 ± 0.36ab	12.49 ± 0.62b	11.85 ± 0.50ab	13.08 ± 0.31b	10.67 ± 0.61ab
C18:3n3 (ALA)	2.06 ± 0.08acde	1.87 ± 0.12ade	2.62 ± 0.22ae	2.97 ± 0.16a	1.24 ± 0.17bd	0.97 ± 0.11bc*	0.57 ± 0.07b	1.50 ± 0.14be
C20:4n6 (arachidonic)	6.51 ± 2.24ac	5.32 ± 0.31a	5.76 ± 0.50ac	6.21 ± 0.48ac	8.46 ± 0.49bc	9.22 ± 0.46b	9.89 ± 0.50b	9.15 ± 0.67b
EPA	1.86 ± 0.15a	1.48 ± 1.14ac	2.10 ± 0.10a	3.02 ± 0.16b	1.02 ± 0.13cd	0.99 ± 0.14c*d	0.43 ± 0.08d	1.23 ± 0.06c
DHA	1.71 ± 0.22ab	1.80 ± 0.17ab	1.70 ± 0.57ab	2.73 ± 0.09a	1.90 ± 0.06ab	1.87 ± 0.25ab	1.27 ± 0.08b	2.20 ± 0.06ab
SFA	51.78 ± 0.96a	52.57 ± 1.41a	49.82 ± 1.93a	49.14 ± 1.00ac*	43.21 ± 0.15ac	42.21 ± 0.24bc	41.69 ± 0.61b	43.22 ± 0.20bc
MUFAs	25.39 ± 1.60ac	27.82 ± 1.53a	26.39 ± 1.82abc	25.47 ± 1.19abc	31.68 ± 0.69bc	32.87 ± 0.17b	33.07 ± 0.71b	32.04 ± 0.27bc
PUFAs	22.83 ± 0.86a*b	19.60 ± 0.96a	23.79 ± 0.43b	25.40 ± 0.83b	25.11 ± 0.54b	24.90 ± 0.17b	25.24 ± 0.21b	24.75 ± 0.13b
n-3 PUFAs	5.64 ± 0.36ad	5.16 ± 0.38ad	6.41 ± 0.53a	8.73 ± 0.32b	4.16 ± 0.32d	3.83 ± 0.48cd	2.27 ± 0.23c	4.93 ± 0.15ad
n-6 PUFAs	17.19 ± 0.72ac	14.45 ± 0.75a	17.38 ± 0.22abc	16.67 ± 0.62ac	20.95 ± 0.44bc	21.07 ± 0.60bc	22.97 ± 0.30b	19.82 ± 0.11bc
n6:n3	3.09 ± 0.22ad	2.87 ± 0.21ad	2.78 ± 0.27ad	1.91 ± 0.07a	5.11 ± 0.37bd	5.83 ± 0.88b	10.49 ± 1.25c	4.04 ± 0.14bd

Values are represented as mean percentage ± SEM. Different letters in the same row (a-d) indicate significant differences (ANOVA P<0.05). Letters with (*) show tendency (P<0.1). SFA: saturated fatty acids: myristic, pentadecanoic, palmitic, margaric and stearic acids; MUFAS: Monounsaturated fatty acids: palmitoleic, oleic and vaccenic acids; PUFAs: Polyunsaturated fatty acids: LA, ALA, EPA, DHA and arachidonic acids;; n-3 PUFAS: ALA, EPA, DHA; n-6 PUFAS: LA, arachidonic acid.

5.1.3. Fatty acid concentrations in prepubertal goat follicular fluid according to the size of the follicle

Comparing the FA concentrations in FF in large (≥ 3 mm) and small (< 3 mm) follicles (table 8), differences were found in SFA (993.88 µM vs 1144.74 µM), MUFAs (700.32 µM vs 829.17 µM), PUFAs (527.20 µM vs 608.97 µM) and n-6 PUFAs (445.32 µM vs 524.27 µM) in general.

Table 8. Fatty acid concentration (µM) in follicular fluid of 1 month old suckling goats, according to the size of the follicle.

FATTY ACID	FOLLICLE SIZE	
	LARGE (≥3mm)	SMALL (<3mm)
C14:0 (myristic)	45.21 ± 2.94a	57.61 ± 4.52b
C15:0 (pentadecanoic)	8.55 ± 0.87	10.04 ± 1.13
C16:0 (palmitic)	552.67 ± 19.74a	667.80 ± 34.00b
C16:1 (palmitoleic)	34.19 ± 2.12	38.92 ± 3.32
C17:0 (margaric)	14.64 ± 0.87	14.63 ± 1.00
C18:0 (stearic)	372.82 ± 14.30	394.66 ± 14.89
C18:1n9c (oleic)	602.29 ± 17.61a	694.08 ± 30.99b
C18:1n11c (vaccenic)	63.83 ± 2.50a	96.18 ± 6.70b
C18:2n6c (LA)	276.87 ± 6.09	287.40 ± 8.91
C18:3n3 (ALA)	27.34 ± 3.90	23.84 ± 3.49
C20:4n6 (arachidonic)	168.44 ± 7.66a	236.87 ± 13.96b
EPA	21.02 ± 2.98	19.86 ± 3.33
DHA	33.52 ± 2.92	41.00 ± 5.32
SFA	993.88 ± 37.26a	1144.74 ± 53.66b
MUFAS	700.32 ± 21.67a	829.17 ± 39.44b
PUFAS	527.20 ± 16.75a	608.97 ± 25.78b
n-3 PUFAS	81.88 ± 9.62	84.70 ± 11.91
n-6 PUFAS	445.32 ± 13.21a	524.27 ± 20.05b
n6:n3	6.16 ± 0.90	7.33 ± 1.30
LA:ALA	12.17 ± 2.13	15.00 ± 3.19

Values are represented as mean ± SEM. Different letters in the same row (a,b) indicate significant differences (ANOVA P<0.05). Letters with (*) show tendency (P<0.1). SFA: saturated fatty acids: myristic, pentadecanoic, palmitic, margaric and stearic acids; MUFAs: Monounsaturated fatty acids: palmitoleic, oleic and vaccenic acids; PUFAs: Polyunsaturated fatty acids: LA, ALA, EPA, DHA and arachidonic acids; n-3 PUFAs: ALA, EPA, DHA; n-6 PUFAS: LA, arachidonic acid.

If FA are compared singly, we found differences in the saturated: myristic (45.21 µM vs 57.61 µM) and palmitic (552.67 µM vs 667.80 µM) acids, being lower in large follicles than in small follicles. Concerning unsaturated fats, we found differences in: oleic (602.29 µM vs 694.08 µM), vaccenic (63.83 µM vs 96.18 µM) and arachidonic (168.44 vs 236.87) acids in large and small follicles respectively. No differences were found in n6:n3 or LA:ALA ratios.

5.1.4. Fatty acid concentrations in prepubertal goat follicular fluid according to season of the year

If we analyse the fatty acid concentration (µM) in follicular fluid of 1 month old suckling goat according to the season of the year (table 9), we found that the main statistical differences are related to omega-3 FA, and thus in the n6:n3 ratio. In autumn, the amount of n3 is drastically reduced compared to spring and summer (46.17 µM vs 81.84 µM and 88.70 µM respectively). But the highest difference is found when it is compared to winter, when the concentration is increased to 116.45 µM. A similar pattern was found when comparing DHA concentrations, with significant lower values where obtained (24.25 µM) compared to the rest of the seasons (35.08 µM, 40.82 µM and 48.90 µM for spring, summer and winter, respectively). Regarding EPA levels, there are significantly differences comparing autumn (9.02 µM) and the rest of the seasons, where there are no significantly different values, but a tendency to increase in winter (29.19 µM) vs spring and summer (20.23 µM and 23.33 µM, respectively). Moreover, when comparing ALA concentrations, 12.89 µM are found on autumn, 26.54 µM on winter, 24.56 µM on summer and 38.36 µM on winter, when the highest and significantly different values are obtained.

In view of the values obtained for the n3 PUFAs, and taking into account that the n6 PUFAs levels didn't vary among the different seasons, the n6:n3 and LA:ALA ratios are significantly increased on autumn (11.17 and 24.04, respectively) compared to spring (5.40 and 10.39), summer (6.17 and 12.75) and winter (4.23 and 7.16).

Concerning saturated FA levels, and similar to n3 PUFAs, there are significantly differences between pentadecanoic acid on autumn (6.53 µM) compared to spring (8.16 µM) and summer (9.98 µM) and with highest values on winter (12.50 µM). Besides, some differences were also found in myristic, palmitoleic and margaric acids, mainly comparing the values on winter (63.62 µM, 44.94 µM and 15.51 µM, respectively) with the rest of the seasons (from 41.72 µM to 52.10 µM for myristic, 27.46 µM to 38.33 µM for palmitoleic and 11.63 µM to 17.09 µM or margaric acids).

Table 9. Fatty acid concentration (µM) in follicular fluid of 1 month old suckling goats, according to the season of the year.

FATTY ACID	SEASON OF THE YEAR			
	SPRING	SUMMER	AUTUMN	WINTER
C14:0 (myristic)	41.72 ± 3.13a	52.10 ± 3.83ab	48.19 ± 6.74ab	63.62 ± 5.68b
C15:0 (pentadecanoic)	8.16 ± 0.40a	9.98 ± 0.51ab	6.53 ± 1.42a	12.50 ± 1.13b
C16:0 (palmitic)	553.78 ± 26.50	638.30 ± 35.32	560.85 ± 45.44	688.01 ± 58.84
C16:1 (palmitoleic)	27.46 ± 1.20a	38.33 ± 2.38b	35.51 ± 2.32ab*	44.94 ± 3.88b
C17:0 (margaric)	14.30 ± 0.50ab	17.09 ± 0.83a	11.63 ± 1.21b	15.51 ± 0.70ab
C18:0 (stearic)	367.53 ± 9.60	406.31 ± 15.76	355.02 ± 28.97	406.12 ± 16.38
C18:1n9c (oleic)	587.07 ± 34.16	703.57 ± 33.76	615.81 ± 34.67	686.29 ± 46.13
C18:1n11c (vaccenic)	66.98 ± 6.46	81.60 ± 8.17	76.72 ± 10.23	94.72 ± 15.52
C18:2n6c (LA)	268.50 ± 6.26	297.02 ± 6.96	289.48 ± 15.36	273.57 ± 8.87
C18:3n3 (ALA)	26.54 ± 2.60a	24.56 ± 3.07a	12.89 ± 2.06b	38.36 ± 1.63c
C20:4n6 (arachidonic)	169.41 ± 15.45	215.06 ± 19.09	204.60 ± 24.18	221.55 ± 31.89
EPA	20.23 ± 1.83a	23.33 ± 3.75a	9.02 ± 1.85b	29.19 ± 0.93a*
DHA	35.08 ± 1.79ab	40.82 ± 7.07a*b	24.25 ± 2.83a	48.90 ± 4.68b
SFA	985.49 ± 37.08	1123.77 ± 50.76	982.21 ± 81.64	1185.75 ± 80.65
MUFAs	681.51 ± 40.62	823.49 ± 43.53	728.04 ± 45.02	825.94 ± 65.25
PUFAs	519.75 ± 10.95	600.78 ± 29.87	540.24 ± 43.37	611.57 ± 41.52
n-3 PUFAs	81.84 ± 3.65a	88.70 ± 13.41ac	46.17 ± 6.59b	116.45 ± 4.85c
n-6 PUFAs	437.91 ± 14.55	512.08 ± 25.22	494.08 ± 38.96	495.12 ± 36.85
n6:n3	5.40 ± 0.40a	6.17 ± 0.93a	11.17 ± 1.34b	4.23 ± 0.15a
LA:ALA	10.39 ± 0.93a	12.75 ± 1.77a	24.04 ± 3.67b	7.16 ± 0.28a

Values are represented as mean ± SEM. Different letters in the same row (a-c) indicate significant differences (ANOVA P<0.05). Letters with (*) show tendency (P<0.1). SFA: saturated fatty acids: myristic, pentadecanoic, palmitic, margaric and stearic acids; MUFAs: Monounsaturated fatty acids: palmitoleic, oleic and vaccenic acids; PUFAs: Polyunsaturated fatty acids: LA, ALA, EPA, DHA and arachidonic acids; n-3 PUFAs: ALA, EPA, DHA; n-6 PUFAS: LA, arachidonic acid.

5.1.5. Fatty acid concentrations in prepubertal goat follicular fluid according to the season of the year and size of the follicle

If we analyse the fatty acid concentration (µM) in follicular fluid of 1 month old suckling goat, we don't find differences in most of the FA, as when compared with their adult counterparts. The main significant differences were found between small follicles, sometimes even in large follicles on winter (table 10). An example is the case of vaccenic acid, which has higher concentrations in the small winter follicle compared to the rest of the follicles on different

seasons (118 µM vs 57 to 94 µM). No differences were found in overall SFA, MUFAS, PUFAS, and n-6 PUFAS between different seasons or sizes of the follicle. However, there is a significant difference in n-3 PUFA concentration mainly on autumn (47.75 and 44.58 µM) and winter (111.82 and 121.08 µM) for both large and small follicles, increasing on winter and decreasing on autumn. Consequently, the ratio n6:n3 is affected by these variations in the concentration of n3 PUFAS, being significantly superior in small follicles on autumn (12.67) compared to winter (3.99 and 4.48 for large and small follicles, respectively).

Specifically, regarding LA (ω6) and ALA (ω3) levels, similar observations were made. No differences were observed in LA concentrations, whilst there were significant differences in ALA concentrations between autumn (13.92 and 11.87 µM for large and small follicles, respectively) and winter (40.88 and 35.83 µM for large and small follicles, respectively), making LA:ALA ratio significantly higher in small follicles on autumn (27.61) compared to the values obtained on winter (6.69 and 7.62 for large and small follicles, respectively).

Table 10 Fatty acid concentration (µM) in follicular fluid of 1 month old suckling goats, according to season of the year and the size of the follicle.

FATTY ACID	SPRING		SUMMER		AUTUMN		WINTER	
	LARGE (≥3mm)	SMALL (<3mm)	LARGE (≥3mm)	SMALL (<3mm)	LARGE (≥3mm)	SMALL (<3mm)	LARGE (≥3mm)	SMALL (<3mm)
C14:0 (myristic)	37.06 ± 0.32	46.38 ± 3.90	46.77 ± 3.16	57.44 ± 4.62	42.42 ± 8.15	53.97 ± 11.82	54.59 ± 0.41	72.65 ± 5.51
C15:0 (pentadecanoic)	7.95 ± 0.59	8.37 ± 0.71	9.72 ± 0.81	10.24 ± 0.89	5.59 ± 1.20	7.47 ± 2.99	10.93 ± 1.62	14.08 ± 0.24
C16:0 (palmitic)	517.12 ± 22.13a	590.45 ± 32.17ab	577.64 ± 11.29ab	698.95 ± 0.41ab	513.31 ± 66.01a	608.39 ± 59.25ab	602.60 ± 2.25ab	773.42 ± 78.61b
C16:1 (palmitoleic)	26.65 ± 1.28a	28.26 ± 2.39a	36.51 ± 4.26ab	40.16 ± 3.05ab	33.73 ± 3.37ab	37.29 ± 3.82ab	39.88 ± 0.14ab	49.99 ± 6.27b
C17:0 (margaric)	14.70 ± 0.92	13.91 ± 0.62	17.28 ± 0.27	16.91 ± 2.00	11.81 ± 2.29	11.46 ± 1.88	14.79 ± 0.08	16.23 ± 1.39
C18:0 (stearic)	363.82 ± 20.13	371.25 ± 10.99	391.83 ± 25.14	420.78 ± 20.96	343.94 ± 55.12	366.09 ± 41.88	391.70 ± 4.34	420.54 ± 34.28
C18:1n9c (oleic)	560.85 ± 36.43	613.29 ± 65.58	649.29 ± 20.65	757.85 ± 22.78	576.17 ± 43.37	655.45 ± 46.76	622.87 ± 3.55	749.71 ± 68.62
C18:1n11c (vaccenic)	56.97 ± 4.83a	77.00 ± 5.16a	68.70 ± 1.39a	94.50 ± 8.12a*b	59.20 ± 3.53a	92.24 ± 1.24ab	70.48 ± 0.56a	118.97 ± 16.40b
C18:2n6c (LA)	271.57 ± 8.19	265.43 ± 12.23	287.41 ± 6.66	306.64 ± 7.84	274.94 ± 27.62	304.03 ± 15.18	273.63 ± 3.11	273.52 ± 21.50
C18:3n3 (ALA)	30.31 ± 2.51ab	22.78 ± 2.47ab*	24.25 ± 5.18ab	24.88 ± 5.44ab	13.92 ± 3.35a	11.87 ± 3.48a	40.88 ± 0.31b	35.83 ± 1.77b
C20:4n6 (arachidonic)	145.85 ± 12.56a	192.97 ± 12.79ab	182.17 ± 0.28ab	247.95 ± 4.89ab	172.98 ± 27.25ab	236.21 ± 27.66ab	172.75 ± 2.36ab	270.35 ± 36.52b
EPA	22.61 ± 2.87ab	17.85 ± 0.64ab	22.43 ± 6.36ab	24.23 ± 6.53ab	10.05 ± 3.33ab	8.00 ± 2.70a	28.99 ± 0.51ab	29.39 ± 2.20b
DHA	34.13 ± 0.41	36.03 ± 4.15	34.24 ± 9.90	47.39 ± 12.83	23.79 ± 4.47	24.71 ± 4.98	41.95 ± 0.83	55.86 ± 5.81
SFA	940.63 ± 43.44	1030.35 ± 48.37	1043.23 ± 40.66	1204.32 ± 28.88	917.06 ± 132.76	1047.37 ± 117.81	1074.60 ± 4.65	1296.91 ± 119.55
MUFAS	644.47 ± 42.54	718.55 ± 73.13	754.49 ± 26.29	892.50 ± 33.94	669.10 ± 50.27	796.98 ± 51.82	733.22 ± 3.96	918.67 ± 91.29
PUFAS	504.46 ± 15.78	535.04 ± 1.61	550.49 ± 14.15	651.07 ± 12.07	495.67 ± 66.31	584.82 ± 53.99	558.19 ± 7.11	664.95 ± 67.80
n-3 PUFAS	87.04 ± 4.97ab	76.65 ± 1.05ab	80.91 ± 18.53ab	96.49 ± 24.79ab	47.75 ± 16.18a	44.58 ± 11.15a	111.82 ± 1.65b	121.08 ± 9.78b
n-6 PUFAS	417.42 ± 20.75	458.40 ± 0.57	469.58 ± 6.39	554.58 ± 12.72	447.92 ± 54.87	540.24 ± 42.84	446 ± 5.47	543.87 ± 58.02
n6:n3	4.83 ± 0.51a	5.98 ± 0.07a	6.14 ± 1.49ab*	6.19 ± 1.72ab*	9.66 ± 1.16ab	12.67 ± 2.21b	3.99 ± 0.01a	4.48 ± 0.12a
LA:ALA	9.05±1.0a	11.73±0.7ab*	12.48±2.9ab	13.02±3.2ab	20.46±2.9ab	27.61±6.8b	6.69±0.0a	7.62±0.2a

Follicle size: Large (≥3mm) or small (<3mm). Values are represented as mean ± SEM. Different letters in the same row (a,b) indicate significant differences (ANOVA P<0.05).Letters with (*) show tendency (P<0.1). SFA: saturated fatty acids: myristic, pentadecanoic, palmitic, margaric and stearic acids; MUFAS: Monounsaturated fatty acids: palmitoleic, oleic and vaccenic acids; PUFAS: Polyunsaturated fatty acids: LA, ALA, EPA, DHA and arachidonic acids; n-3 PUFAS: ALA, EPA, DHA; n-6 PUFAS: LA, arachidonic acid.

5.2. *In vitro* embryo production (experiment 2)

5.2.1. Embryo development of prepubertal goat oocytes matured with different ratios of LA:ALA: FIV and PA

A total number of 2056 oocytes were matured and used to assess nuclear maturation (229 oocytes; 3 repetitions), fertilized (1249 oocytes; 6 repetitions) and parthenogenetically activated (578 oocytes; 3 repetitions). Assessing oocytes at Metaphase II after 24 h of IVM, no differences were found among groups FCS (94.8 ± 2.6 %; N=36), BSA (89.7 ± 1.9 %; N=50), 50:50 (93.5 ± 3.85 %; N=45), 100:50 (93.3 ± 3.85 %; N=49) and 200:50 (92.9 ± 3.86 %; N= 49). Contrarily, compared to the other groups of treatment, 200:50 group had significantly higher rate of un-fertilised oocytes (41.82 % compared to ≈ 15 %), low normal fertilization (2PN zygotes, 1.82 % compared to ≈ 77 %) and high rate of polyspermic zygotes (58.18 % compared to 5-11 %) (table 11).

***Table 11. In vitro* fertilisation at 17 hours post insemination of prepubertal goat oocytes *in vitro* matured with different Acid Linoleic (LA) and Acid Linolenic (ALA) ratio (LA:ALA) and concentrations.**

TREATMENT	N OOCYTE	UN-FERTILISED (%)	FERTILISED	
			2PN (%)	PS (%)
FCS (IC)	53	9 (16.98)	40 (75.47)[a]	4 (7.55)[a]
BSA (TC)	57	9 (15.79)	45 (78.95)[a]	3 (5.26)[a]
50:50	56	7 (15.00)	46 (82.14)[a]	3 (5.36)[a]
100:50	60	9 (15.00)	44 (73.33)[a]	7 (11.67)[a]
200:50	55	23 (41.82)	1 (1.82)[b]	32 (58.18)[b]

Different letters in the same column (a,b) indicate significant differences (ANOVA P<0.05). *2PN: Normal Fertilised; PS: Polyspermic ; N: number of IVF-oocytes.*

Furthermore, after 8 days of *in vitro* culture, blastocyst production was not significantly different among controls (FCS lab internal control, and BSA treatment control), 50:50 and 100:50 groups (12.83 %; 11.41 %; 13.68 %; 13.33 % respectively). However, embryo production was significantly lower in 200:50 treatment group compared to all other groups (2.63 %)(Table 12).

Table 12. In vitro embryo development of prepubertal goat oocytes in vitro matured with different Acid Linoleic (LA) and Acid Linolenic (ALA) ratio (LA:ALA) and concentrations.

TREATMENT	N OOCYTE	CLEAVED (%)	BLASTOCYSTS (%/N)	BLASTOCYSTS (%/CL)
FCS (IC)	187	123 (65.78)	24 (12.83)[a]	24 (19.51)[a]
BSA (TC)	184	104 (56.52)	21 (11.41)[a]	21 (20.19)[a]
50:50	212	142 (66.98)	28 (13.68)[a]	29 (20.42)[a]
100:50	195	127 (65.13)	26 (13.33)[a]	26 (20.47)[a]
200:50	190	102 (53.68)	5 (2.63)[b]	5 (4.90)[b]

Different letters in the same column (a,b) indicate significant differences (ANOVA P<0.05). N: number of IVF-oocytes; CL: cleavage.

In contrast to the results obtained on *in vitro* fertilised oocytes, and despite there is a reduction on the number of embryos obtained in the 200:50 group (9.6 vs ≈ 21 %), there were no significant differences on embryo production of parthenogenetically activated oocytes (table 13).

Table 13. Blastocyst development at 8 after parthenogenetic activation (PA) of prepubertal goat oocytes in vitro matured with different Acid Linoleic (LA) and Acid Linolenic (ALA) ratio (LA:ALA) and concentrations.

TREATMENT	N OOCYTES	CLEAVED (%)	BLASTOCYSTS (%/N)	BLASTOCYSTS (%/CL)
FCS (IC)	103	90 (87.38)	23 (22.33)	23 (25.56)
BSA (TC)	107	92 (85.98)	21 (19.63)	21 (22.83)
50:50	124	112 (90.32)	28 (22.58)	28 (25.00)
100:50	119	107 (89.92)	25 (21.01)	25 (23.36)
200:50	125	106 (84.80)	12 (9.60)	12 (11.32)

Values expressed as N (%). N: number of oocytes activated: CL: cleaved embryos.

5.2.2. Embryo quality assessed by differential staining of the blastocysts

No differences were found when the blastocyst quality was assessed by the number of blastomeres in the inner cell mass and trophectoderm, from embryos produced by IVF or PA (table 14).

Besides, no differences were found on total cell number, as well as on trophectoderm cell number, ICM:TE percentages and ICM of IVF produced embryos. The only difference observed is in the ICM cell number of PA oocytes, being the 200:50 group the one with the highest number

of cells (60.00 ± 5.00), compared to FCS (35.23 ± 4.91), BSA (21.42 ± 2.51) and 100:50 (22.38 ± 3.60) groups of treatment.

Table 14. Number of Cells (mean ± SEM) of In vitro Fertilized (IVF) and Parthenogenic activated (PA) blastocyst of prepubertal goat oocytes in vitro matured with different Acid Linoleic (LA) and Acid Linolenic (ALA) ratio (LA:ALA) and concentrations.

TREATMENT	Total Blastocysts		Total cell number		ICM (Inner Cell Mass)		TE (Trophectoderm)		ICM:TE (%)	
	IVF	PA	IVF	PA	IVF	PA	IVF	PA	IVF	PA
FCS (IC)	11	13	201.60 ± 33.77	200.15 ± 25.47	33.27 ± 5.08	35.23 ± 4.91^{b}	161.18 ± 28.01	164.92 ± 21.37	22.87	21.77
BSA (TC)	8	19	162.25 ± 31.98	129.00 ± 15.37	31.75 ± 6.60	21.42 ± 2.51^{b}	130.50 ± 26.63	105.74 ± 12.60	24.75	26.41
50:50	11	18	134.22 ± 20.55	189.67 ± 31.66	23.82 ± 3.17	29.06 ± 4.17^{ab}	97.09 ± 16.80	160.61 ± 27.83	29.21	20.79
100:50	8	16	151.88 ± 31.10	131.14 ± 17.79	29.75 ± 6.17	22.38 ± 3.60^{b}	122.13 ± 26.13	100.06 ± 13.54	27.58	24.01
200:50	2	2	116.50 ± 37.50	289.00 ± 8.00	25.50 ± 8.50	60.00 ± 5.00^{a}	91.00 ± 29.00	229.00 ± 13.00	27.88	26.41

Values are represented as mean number of cells ± SEM. Different letters in the same column (a,b) indicate significant differences (ANOVA P<0.05). IVF: in vitro fertilized; PA: partenogenetically activated.

5.3. Oocyte molecular analysis from prepubertal goats (experiment 3)

5.3.1. Mitochondrial activity and distribution in oocytes

In relation to the study of mitochondrial activity, we found that there is a significant increase of active mitochondria between oocyte collection time (0h) and after 24h of maturation (0.40 ± 0.06 vs 0.88 ± 0.05 to 1.38 ± 0.05, respectively) (figure 14). Moreover, there is a significant increase in mitochondrial activity between treatments, being higher in the 200:50 treatment group (1.38 ± 0.05) compared to BSA (control: 0.88 ± 0.05) and 50:50 treatment groups (0.88 ± 0.05 and 1.00 ± 0.05, respectively). Note the differences in intensity shown in figure 16.

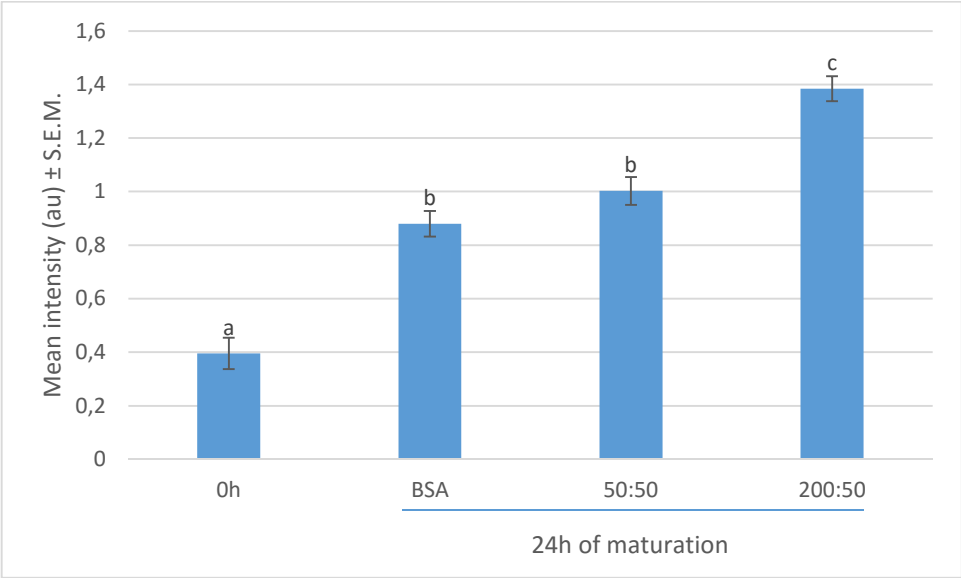

Figure 14. Mitochondrial activity in oocytes at collection time (0h) and after 24h of maturation (BSA; LA:ALA 50:50 µM and 200:50 µM groups). *Values represented as MEAN intensity in arbitrary units (au) ± SEM. Different letters indicate significant differences (a-c) (ANOVA P<0.05).*

Besides, as it was observed on mitochondrial activity, there is also a significant difference on the distribution of these organelles between oocyte collection point and after 24 hours of maturation in all groups (figure 15). At the GV stage (0h), 86.77 % of the oocytes presented peripheral, 4.76 % semi-peripheral and 8.47 % homogeneous distribution. On the contrary, after maturation, the peripheral distribution decreased (8.40 %, 2.27 % and 2.27 % for BSA, 50:50 and 200:50 groups, respectively) and semi-peripheral distribution increased (91.60 %, 97.73 % and 87.45 % for BSA, 50:50 and 200:50 groups, respectively). Only 10.28 % of the oocytes presented homogeneous distribution in the 200:50 group.

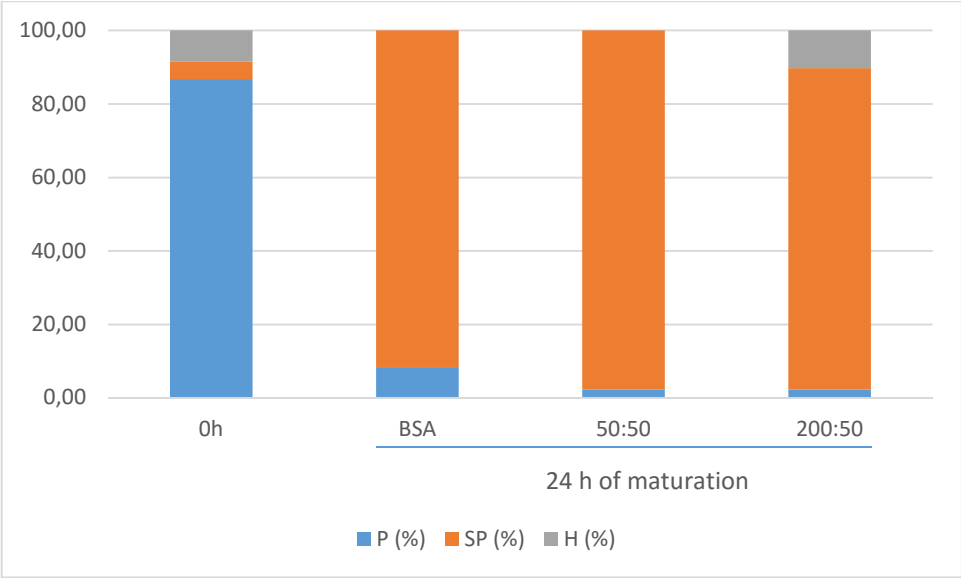

Figure 15. Mitochondrial distribution in prepupertal goat oocyte at collection time (0h) and after 24h of maturation (BSA control group; LA:ALA 50:50 µM and 200:50 µM groups). *Values are represented as percentages. P: Peripheral; SP: Semi-peripheral; H: Homogeneous.*

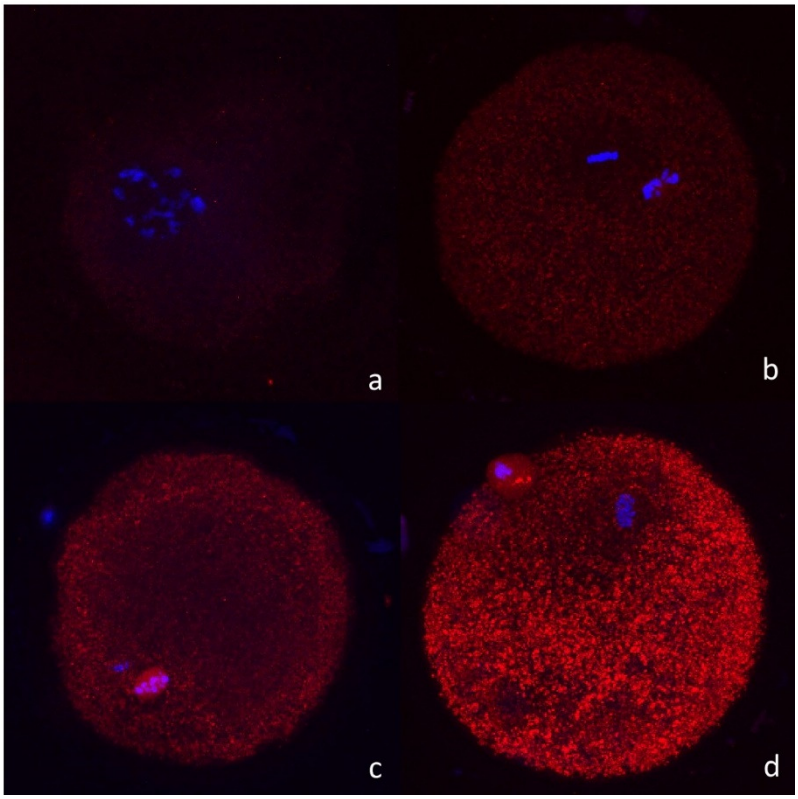

Figure 16. Oocyte mitochondrial activity at 0h at collection time (0h) and at 24h of maturation (BSA, 50:50, 200:50 groups). *Images obtained with confocal microscope. Stained with mitotracker red (red: active mitochondria) and hoescht (blue: nucleus). (a) oocyte at GV (collection time); (b-d) oocytes at MII after 24 h of maturation with BSA (b), LA:ALA 50:50 µM (c) and LA:ALA 200:50 µM (d).*

5.3.2. ATP concentration in oocytes

There is a significant increase of the ATP levels between immature (0h: 691.12 ± 70.72) and mature oocytes (BSA, 1229.01 ± 50.75; 50:50, 1126.38 ± 137.56; and 200:50, 1077.37 ± 97.09), but not between control treatment (BSA) and oocytes treated with LA:ALA ratios 50:50 µM and 200:50 µM (figure 17).

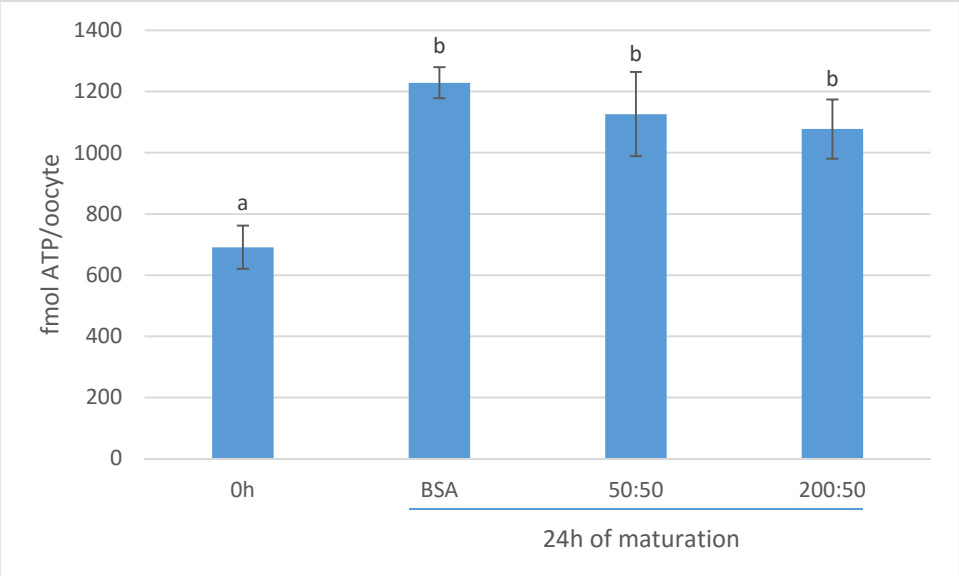

Figure 17. ATP concentration in oocytes at the collection time (0h) and at 24 hours after IVM (BSA control treatment; LA:ALA 50:50 µM and 200:50 µM groups). *Values are represented as MEAN ± SEM. Different letters indicate significant differences (a-b) (ANOVA P<0.05).*

5.3.3. mRNA relative expression

As shown in figure 18, oocytes exposed to LA:ALA during maturation don't have different levels of expression of the analysed genes. However, there is a decrease of expression of GPX1, RPL19 and SOD1 after oocyte maturation (BSA, 50:50 and 200:50 groups), compared to the expression at oocyte collection time.

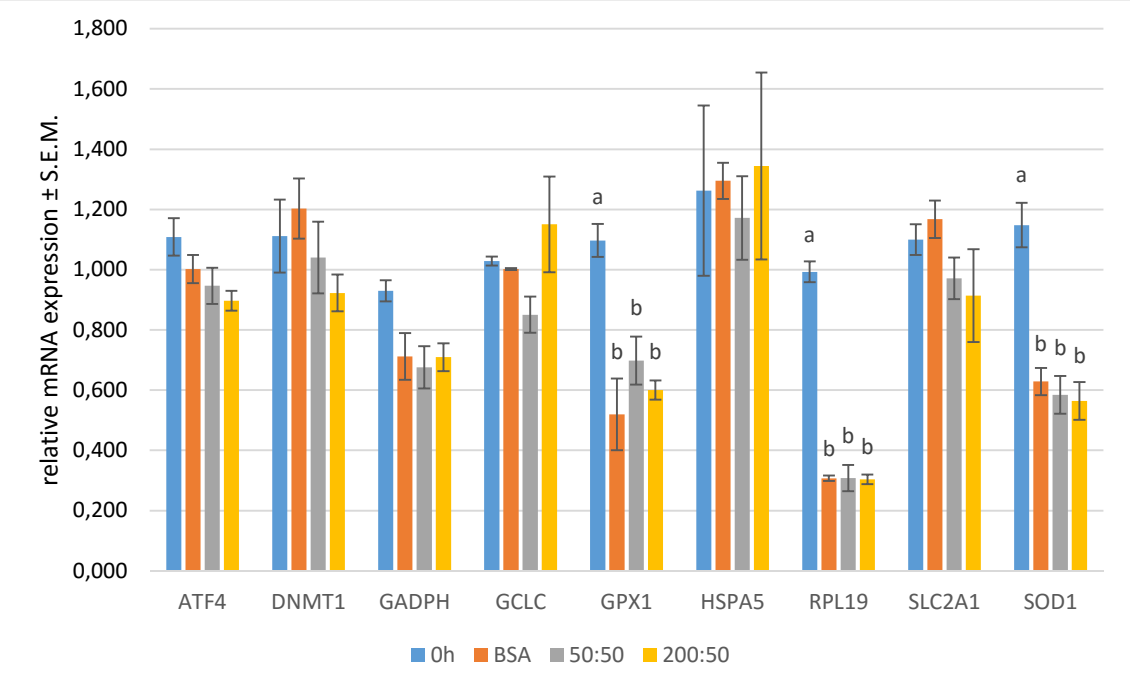

Figure 18. Relative mRNA expression of ATF4, DNMT1, GAPDH, GCLC, GPX1, HSPA5, RPL19, SLC2A1 and SOD1 transcripts before (0h) and after IVM (BSA, 50:50 and 200:50 groups) of prepubertal goat oocytes. *Values expressed as mean RQ ± S.E.M. Different letters indicate significant differences (a-b) (ANOVA P<0.05).*

Chapter 6

General discussion

Fatty acids are an important source of energy for the growing and developing oocytes. Thus, the study of the follicular fluid, where these oocytes grow and mature could be of special interest as it could help to determine their developmental capacity. Additionally, it is widely accepted that oocytes from adult animals have better competence than those from its prepubertal counterparts. As mentioned in the introduction, in previous studies in our lab, it was demonstrated that oocytes from prepubertal goats recovered from large follicles (≥ 3 mm) have better competence that the oocytes recovered from small follicles (< 3 mm) (Romaguera *et al.* 2010) and the same competence as oocytes from adult females (Romaguera *et al*. 2011). Moreover, Catalá *et al*. 2015 showed a differential embryo production in goats among the seasons of the year, being significantly lower in autumn and higher in winter.

Our hypothesis was that the FA profile of FF from adult and prepubertal goats, as well as from different follicular sizes and seasons of the year could give us information to compare with oocyte developmental competence. Therefore, our objective was to determine if we could use the FA profile as a molecular marker for oocyte quality. Moreover, we wanted to give an explanation on how the follicular content could influence the lower developmental capacity of prepubertal oocytes.

The present thesis was divided in three experiments, with the following results in general terms: in experiment 1, among the 13 FA analysed, 9 FA and combinations of them were different according to the age of the donor. We observed higher percentages of total PUFA and n6 PUFA and lower n3 PUFA percentages on FF from prepubertal goats in comparison with adult goats, resulting in a higher n6:n3 ratio in prepubertal females. When comparing adult and prepubertal animals, the size of the follicle or the season of the year had no effect, and the differences observed were due to the age of the donor. However, when comparing FF FA concentrations in prepubertal animals, some differences were found among the follicle size and the season of the year mainly for n3 and n6 PUFA. In experiment 2, we found that maturing oocytes with 200:50 µM LA:ALA ratio had a detrimental effect on oocyte developmental competence compared to the control groups (BSA and FCS) and the rest of the treatments (100:50 and 50:50 µM) in IVF oocytes but not in parthenogenetically activated oocytes. Thus, in the 200:50 µM group there was a decrease in blastocyst production (2.63 %) compared to the other groups (≈ 13 %) after IVF. Moreover, in experiment 3, after 24h of IVM with 200:50 LA:ALA µM we found an increase of mitochondrial activity compared to the rest of the groups (0h collection time, BSA and 50:50 µM). Assessing mitochondrial activity, active mitochondria distribution and ATP concentration in the oocyte, we found that there was a change in this parameters when they were analysed on immature oocytes (collection point) or on IVM oocytes (after 24 h of maturation). Concerning

mRNA relative expression, from the 9 genes analysed, 3 had significant differences when comparing immature and IVM oocytes, but not between groups of treatment.

The aim of the first experiment was to determine the FA profile of goat FF and changes in this fluid according to age of the donor, size of the follicle and season of the year. Among the FA analysed, our results show that the most abundant FA in adult and prepubertal goat FF were, respectively: palmitic, oleic, stearic, linoleic and arachidonic acids, followed by linoleic acid. This results are similar to previous studies in terms of the type of FA, but there were differences on the abundance of these FA. As it was found in goats, similar profiles were observed in sheep (Zeron *et al*. 2002) and woman (Jungheim *et al*. 2011, O'Gorman *et al*. 2013), being palmitic acid the most abundant FA in FF. However, in cows the most abundant FA is linoleic acid, followed by oleic, stearic, palmitic and linolenic (Sinclair *et al*. 2008, Bender *et al*. 2010, Matoba *et al*. 2014, Aardema *et al*. 2015), being slightly different from goats.

In our study, adult females were fed alfalfa ad libitum and kept indoor during all the experimental procedure to avoid variability in the FF due to changes in the diet. Concerning prepubertal goat ovaries, although they were recovered from the slaughterhouse, these animals are traditionally fed with maternal milk in Spanish farms. Castro-Gómez *et al*. (2014) observed that palmitic, oleic, capric, stearic and myristic acids were the most prevalent FA in Spanish goat milk (27.69 %, 21.70 %, 9.71 %, 9.66 % and 9.63 % respectively). Hellîn *et al*. (1998) measured FA in murciano granadina goat milk and found that palmitic (34.8 %), myristic (12.8 %) and oleic (13.3 %) acids were the most prevalent, followed by capric (10.4 %), stearic (6.8 %), linoleic (3.6 %) and linolenic (0.9 %) acids. In previous studies it was shown that FA in plasma, milk and follicular fluid were related to the diet, or animal metabolic conditions (Leroy *et al*. 2005, Childs *et al*. 2008, Fouladi-Nashta *et al*. 2009, Zachut *et al*. 2010, Leroy *et al*. 2014). However, the FA concentration in FF seems to be affected by a selective uptake (Fouladi-Nashta *et al*. 2009, Wonnacott *et al*. 2010, Jungheim *et al*. 2011). This was in accordance with the results obtained for prepubertal goats fed with maternal milk, as differences in percentages between the milk content and the values of FA in FF were observed.

When comparing the FA of FF according to the age of the donor, we found that there were significant differences among the percentages of the majority of the fatty acids measured. Specifically, comparing adult and prepubertal goats, total SFA and n-3 PUFA were significantly higher in adult goats, while total MUFA, PUFA n-6 PUFA n-6:n-3 PUFA ratio were significantly higher in prepubertal goats. Similar results were found by Bender *et al*. (2010), comparing FA percentages between dairy cows and heifers. Thus, in poor fertility cows the FA composition

was higher PUFA, n-6 PUFA and n-6:n-3 PUFA ratio, while in good fertility heifers, the composition of SFA and n-3 PUFA were higher. No differences were found in total MUFA. In conclusion good oocyte competence seems be positively related to the composition of Total SFA and n-3 PUFA and the low ratio n6:n3 PUFA.

When analysing the interaction age-follicle size, no differences in the percentages of FA were found due to the size of the follicle. However, if we focus in prepubertal females, we found significant differences in palmitic, oleic, vaccenic, arachidonic, total SFA, MUFA, PUFA and n6 PUFA according to the follicular size. Moreover, total FA were higher in small follicles than in large follicles (2583 µM vs 2221 µM). In the study of Matoba *et al*. (2014), total fatty acids found in poor fertility cows were higher than those found in heifers (2104.06 µM vs 928.02 µM respectively). In a previous study in our lab, Romaguera *et al*. (2010) demonstrated that oocytes from large follicles (≥ 3mm) had more competence than the ones from small follicles (< 3mm) in prepubertal goats. Several authors highlighted the negative effect that an increase of total FA in FF has on oocyte developmental competence (Yang *et al*. 2012, Sessions-Bresnahan *et al*. 2016) in relation to obesity (Wu *et al*. 2010 and 2012 , Purcell *et al*. 2011) and consequent poor COC morphology (Jungheim *et al*. 2011). Our results demonstrate that small follicles have more concentration of FA than large follicles, which could be correlated with the lower blastocyst yield in oocytes from small follicles by Romaguera *et al*. (2010).

When analysing the interaction age-season of the year, no differences in the percentages of FA were found due to the season of the year. However, if we focus in prepubertal females, we observed differences mainly in n3 PUFAS. Thus, ALA, DHA and total n3 PUFA concentrations were significantly lower in autumn than in winter, while values on spring and summer were not significantly different when compared to values on winter. Consequently, n6:n3 and LA:ALA ratios were significantly higher on autumn, than on winter, spring or summer. This is positively correlated with the results on embryo production observed in our lab by Catalá *et al*. (2015), as during autumn there was a significant decrease in embryo production. Cleavage rates were significantly reduced on autumn, compared to spring, summer and winter (34 % vs 73.7 %, 50 % and 52.7% respectively). Moreover, blastocyst rates (blastocysts/oocytes) were lower on autumn compared to summer (4.7 % vs 10.4 %) and both were lower compared to winter and spring (15.8 % and 16.2 %). If blastocyst rates were compared among the cleaved zygotes, the highest value was obtained on winter (29.9 %) in comparison with spring (22 %), and summer (20.8 %) and the lowest value was obtained on autumn (14 %) (Unpublished data). Similarly, Matoba *et al*. (2014) in a study in cows found significant differences in FF from oocytes that developed to blastocyst compared to the ones that degenerate in ALA (25.11 µM vs 14.15 µM

respectively), and no differences were found in LA (121.1 µM and 114.1 µM respectively), making n6:n3 and LA:ALA ratios higher in FF from oocytes that degenerate compared with the ones that develop up to blastocyst. In a study in human FF, O'Gorman *et al*. (2013) found significant differences in n6:n3 PUFA ratio in FF from embryos that cleaved compared with those that failed to cleave after fertilization (4.79 vs 6.84 respectively). In conclusion, taking into account that prepubertal goats are a model of poor oocyte quality, the knowledge that dairy cows have a decline in fertility and the results obtained by several authors on embryo production, we can correlate the increase in n6:n3 ratio as a candidate for a decrease in oocyte competence.

The aim of the second experiment was to study the effect of the addition of different n6:n3 ratios on IVM media of prepubertal goat oocytes and their subsequent development up to blastocyst stage. For this purpose, LA and ALA were used as representatives of omega 6 and omega 3 families respectively, with the following ratios: 4:1,2:1 and 1:1, or 200:50 µM, 100:50 µM and 50:50 µM. No differences were found among groups when nuclear maturation (MII) was assessed, as it was nearly 90 % in all the groups. Nevertheless, we found that oocytes matured with 200:50 µM LA:ALA ratio had significantly higher rates of polyspermy (58.18 %) compared with control groups, 50:50 and 100:50 treatment groups. Moreover, the rate of normal fertilised oocytes was significantly lower in 200:50 µM compared to control and treatment groups. An increase of un-fertilised percentage was also observed in 200:50 µM, but it wasn't significantly different. When embryo development was evaluated, we found no differences in the cleavage rates but there was a decrease of blastocyst rate from the total of oocytes *in vitro* matured (blastocysts/oocytes) or from the number of cleaved embryos (blastocyst/cleavage) in the 200:50 µM treated oocytes (2.63 % and 4.90 % respectively). Although a decrease of blastocyst rate was observed in 200:50 µM, no significant differences were found neither in cleavage nor in blastocyst rates among all the groups when oocytes were parthenogenetically activated. Concerning the blastocyst quality assessed by differential staining, no differences were found between IVF or PA oocytes or between groups of treatment, although the number of cells of the ICM was higher in PA oocytes from the 200:50 µM group compared to the rest of the groups. In cows, Marei *et al*. (2009) observed that the treatment of COCs with 50 µM of ALA during IVM increased the percentage of oocytes at the MII stage, cleaved embryos and blastocyst rate, but higher doses were detrimental. In an another study, they observed that the treatment of COCs with 50, 100 and 200 µM of LA significantly reduced the percentage of MII oocytes in a dose dependent manner, and that there was a decrease of cleavage and blastocyst rates using 50 or higher LA concentrations (Marei *et al*. 2010). In our study, we did not find any negative effect

of LA at 50 and 100 µM concentrations on MII oocytes and blastocyst output. However, at the 200:50 group we found a significant reduction in the percentage of blastocyst development. In cows, Van Hoeck *et al*. (2011) demonstrated that exposing bovine oocytes during IVM to elevated levels of palmitic, oleic and stearic acids caused significant detrimental effects on embryo production but, this negative effect was not observed when the fatty acids were supplemented individually. In human embryos, those that developed further had higher concentrations of linoleic and oleic (unsaturated fatty acids), and lower total saturated fatty acids, than those that did not (Haggarty *et al*. 2006). In our study, the reduction of blastocyst rate in 200:50 group could be explained by the effect of the addition of both LA and ALA or a different response to the treatment in prepubertal goat oocytes, as oocytes from prepubertal animals have different performance than those from adult animals (Armstrong, 2001), cows (Revel *et al*. 1995, Torres *et al*. 2015), sheep (O'Brien *et al*. 1996, Fang *et al*. 2016) and pigs (Peters *et al*. 2001). In suckling 1 month old goats FF, LA concentration ranged from 265 µM to 306 µM and ALA concentration ranged from 11.9 µM to 40.9 µM among the different seasons of the year. In cattle there were differences reported on LA and ALA concentrations in preovulatory follicles. Moreover, LA and ALA FF concentrations ranged from 71 µM to 710 µM and from 36 µM to 72 µM respectively in cattle according to the diet (Childs *et al*. 2008) or from 170.38 µM to 782.31 µM and from 53.54 µM to 107.16 µM respectively according to age (Bender *et al*. 2010, for cows and heifers). Therefore, the concentrations of LA tested in the *in vitro* studies were under or within the range of physiological concentrations, suggesting that the effect that these concentrations had on IVM oocyte could be due to the nature of the FA in culture or for the consequences of *in vitro* culture by itself compared to *in vivo* (Rizos *et al*. 2002, Dunning *et al*. 2014). In conclusion the highest ratio LA:ALA (200:50) presented a negative effect on blastocyst development compared to other groups.

In order to explain the results of the experiment 2, different molecular parameters were analysed in the oocyte. For this purpose, 50:50 µM and 200:50 µM LA:ALA concentration ratios were added to the IVM to further analyse the active mitochondria, ATP concentration and the mRNA expression to determine differences in the oocyte cytoplasm.

Mitochondria are maternally inherited organelles and are important for the oocyte because they supply the ATP needed for further embryo development (Stojkovic *et al*. 2001, Dumollard *et al*. 2004, Wang *et al*. 2009, Van Blerkom, 2009 and 2011), yet mitochondrial dysfunction causes impaired oocyte development competence (Jansen *et al*. 2004, Schatten *et al*. 2014). Mitochondrial activity and distribution of active mitochondria were evaluated in oocytes at the

GV stage (0h: collection time) and after 24h of maturation. The distribution of mitochondria changes during oocyte maturation and fertilisation with the aim of bringing these organelles to the region of the cell where a higher level of ATP (Van Blerkom *et al*. 1984, Yu *et al*. 2010) or calcium (Sousa *et al*. 1997) are required. Stojkovic *et al*. (2001) showed that mitochondrial reorganisation was different between morphologically good and poor quality oocytes. In our study, mitochondria migrated throughout the IVM process. We found that there is a significant increase in mitochondrial activity between GV stage and MII oocytes (24 h after maturation). Additionally, there were differences on active mitochondria distribution between GV oocytes and MII oocytes, as at the collection time 87.7 % of active mitochondria were peripheral, whilst in matured oocytes 90-97 % were semi-peripheral. In pigs, Torner *et al*. (2004) found that mitochondria moved from a homogeneous to a peripheral distribution; however, Brevini *et al*. (2005) showed a peripheral distribution in immature pig oocytes that became diffused after IVM, as it was observed in our study. It has been demonstrated that mitochondrial function and the cytoplasmic ATP level can affect fertilisation, resulting in a significant increase in blastocyst rates or their total failure after IVF (Van Blerkom *et al*. 1995, Liu *et al*. 2000). Mitochondrial distribution and activity are modified during oocyte *in vitro* maturation (IVM) and this differs among species such as cattle (Stojkovic *et al*. 2001, Tarazona *et al*. 2006), dogs (Valentini *et al*. 2010), horses (Torner *et al*. 2007), humans (Van Blerkom *et al*. 1995, Dell'Aquila *et al*. 2009, Takahashi *et al*. 2016), mice (Calarco, 1995) and pigs (Sun *et al*. 2001, Torner *et al*. 2004, Brevini *et al*. 2005). Using the fluorescence probe MitoTracker Green, Sun *et al*. (2001) concluded that *in vitro* matured pig oocytes present changes in the distribution of mitochondria causing the incomplete movement of mitochondria into the inner cytoplasm affecting the cytoplasmic maturation. In our laboratory, it was shown that there were differences in the distribution pattern of mitochondria between adult and prepubertal goat oocytes (Velilla *et al*. 2006) and between BCB selected prepubertal sheep oocytes (Catala *et al*. 2011). Moreover, Leoni *et al*. (2015) found a differential mitochondrial activity and ATP production in prepubertal and adult animals in a sheep model. They observed that in the majority of prepubertal oocytes, the active mitochondria were homogenously distributed, while in adults they were aggregated in big clusters. Additionally, ATP production was reduced in prepubertal female oocytes compared to oocytes from adult females. Similar findings were observed in cows by Jeseta *et al*. (2014) comparing oocytes with greater or lesser developmental competence. In cows, Marei *et al*. (2012) found that 100 µM of LA added in IVM delayed the mitochondrial redistribution during the first hour of maturation and decreased mitochondrial activity, whilst 50 µM of ALA in IVM had no effect. In our study, the addition of 200:50 at IVM modified the mitochondrial activity of these oocytes being higher compared with the other treatment groups, but no changes were

observed in the active mitochondria distribution. In relation to our results concerning the mitochondrial activity, there was an increase of the levels of ATP in *in vitro* matured oocytes compared to immature oocytes. ATP levels within the oocyte is positively correlated to developmental competence (Van Blerkom *et al*. 1995). Moreover, the increase in the ATP levels after oocyte maturation was previously reported in our lab in sheep (Catalá *et al*. 2012) and in another studies in pig (Brevini *et al*. 2005), cattle (Stojkovic *et al*. 2001) and mouse (Yu *et al*. 2010).

Our results showed that mitochondrial activity was higher according to the increasing concentration of LA and ALA in the maturation medium. It is known that high fat availability could increase the mitochondrial fatty acid oxidation and thus, its activity in cardiac cells (Cole *et al*. 2011) and muscle cells (Turner *et al*. 2007, Li *et al*. 2016). However, an increase on FA availability due to obesity (Montgomery *et al*. 2015, Hou *et al*. 2016) or due to culture conditions (Seifert *et al*. 2010) leads to a mitochondrial dysfunction. Moreover, it was shown that FA metabolism, evaluated through β-oxidation increased in *in vitro* matured mouse COCs (Dunning *et al*. 2010). Fatty acid oxidation (FAO) plays an important role in oocyte developmental competence (Gulliver *et al*. 2012, Dunning *et al*. 2014a), as it has been demonstrated that a reduction in this process is one of the mechanisms why *in vitro* matured oocytes have less competence than *in vivo* matured oocytes (Dunning *et al*. 2014b). Our results suggest that an increase of the availability of FA is positively correlated to an increase of FAO, and thus, to an increase of active mitochondria in the 200:50 group compared to 50:50 group. However, no differences were found in the ATP concentration between these two groups.

The genes analysed in this work were related to: energy metabolism (SLC2A1/GLUT1 and GAPDH), cell detoxifying or antioxidant mechanisms (GPX1/GSH-Px, GCLC and SOD1/CuZnSOD), transcription factor (ATF4), folding and assembly of proteins (HSPA5/GPR78), ribosomal protein (RPL19) and DNA methylation (DNMT1). Differences were found in GPX1, RPL19 and SOD1 relative expression comparing prepubertal goat oocytes before (0h) and after maturation (24h), but no differences were found between treatments, in spite of differences on embryo development between groups. It is widely known that despite having antioxidant mechanisms, mitochondria is the main producer of ROS in the cell as a consequence of its activity (Turrens, 2003). An increase of ROS production is related to an increase of oxidative stress and compromised oocyte developmental capacity (Guerin *et al*. 2001, Combelles *et al*. 2009) that could lead to apoptosis (Burton *et al*. 2002). As reviewed by Schönfeld *et al*. (2008), long chain fatty acids and derivatives could cause oxidative stress by stimulating ROS production in various

cell types. PUFAs in oocyte culture have shown an increase of ROS production. In cows, Marei *et al*. (2010) observed that the treatment of COCs with 50 µM of LA during IVM increased ROS levels within the oocyte, associated with a lower maturation rate. This effect could be alleviated with the addition of antioxidants on the media (Nonogaki *et al*. 1994). Contrarily, Sutton-Mcdowall *et al*. (2016) demonstrated that adding NEFAS to IVM of mice oocytes resulted in significantly lower levels of ROS production, reduced levels of anti-oxidants and an increase of mitochondrial membrane potential, suggesting that the negative effects that these FA have on oocyte development were due to endoplasmic reticulum (ER) stress. In line with these observations, Wu *et al*. (2010) showed that ER stress, mitochondrial dysfunction and decreased fertilization rates in an *in vivo* mouse model was caused by lipotoxicity due to an increase of FA, ultimately inducing apoptosis in the cell. Lipotoxicity has an impact on oocyte development both *in vivo* and *in vitro* (reviewed by Alves *et al*. 2015). Yang *et al*. (2012) also found an impaired oocyte maturation when exposing mice oocytes to women lipid-rich follicular fluid due to ER stress. Moreover, Van Hoeck *et al*. (2013), treating oocytes with NEFA found that while there was an increase of mitochondrial membrane potential, it was not related to an increase of oxygen consumption, which suggested a regulation on the mitochondrial respiratory chain and thus, a regulation on ROS production by mitochondria. The relative expression of SLC2A1/GLUT1 (Sviderskaya *et al*. 1996), GPX1/GSH-Px (Hou *et al*. 2016), DNMT1 (Heinzmann *et al*. 2011) and SOD1 (Salavati *et al*. 2012) is up-regulated, and GCLC is down-regulated (Luderer *et al*. 2003, Dickinson *et al*. 2004) when there REDOX state of the cell is compromised. It is described that when ER is under stress, some genes are up-regulated: ATF4 (Xu *et al*. 2005, Ameri *et al*. 2008, de Nadal *et al*. 2011), HSPA5/GPR78 (Lee 2005, Xu *et al*. 2005). GAPDH is up-regulated when energy metabolism is compromised (Colell *et al*. 2009). RPL19 over-expression activates unfolded protein response (UPR), which can induce pro-apoptotic cell death due to stress (Hong *et al*. 2014). In cow, Van Hoeck *et al*. (2013) maturing oocytes with high NEFAs, showed an up-regulation of GAPDH and GPX1. GPX1 over-expression was also observed by Khalil *et al*. (2013) on LA treated oocytes, concluding that it was due to an increase of ROS produced by this FA. In mice, Wu *et al*. (2010), Yang *et al*. (2012) and in cows, Sutton-Mcdowall *et al*. (2016) found an up-regulation of ER stress markers ATF4, HSPA5/GRP78 in oocytes matured with high concentrations of FA. We did not found differences in the expression of the genes studied in the oocytes from prepubertal goats. From this perspective, we couldn't conclude that the treatment of prepubertal goat oocytes with FA impairs oocyte development by an increase of ROS or ER stress.

In this study, the addition of 200:50 LA:ALA in the IVM medium has affected negatively the blastocyst production after IVF, but not after Parthenogenetic Activation (PA). After 24 of IVF the produced zygotes of this group presented significantly higher percentage of polyspermy and lower normal fertilization (2PN). The analysis of the mitochondrial activity shows higher activity of this group compared with the other treatments. However, the ATP content of these oocytes was not different from the other groups. Moreover, the relative expression of the studied genes showed no differences in the 200:50 group compared to the other groups.

Taking all these results into account, we conclude that the negative effect of the highest LA:ALA ratio on oocyte competence from prepubertal goats was not related to an increase in ROS production nor ER stress according to the relative expression of the studied genes. Additionally, due to the increase in polyspermy or non-fertilized oocytes in this group, and the lack of negative effects in blastocyst development after PA, we hypothesize that the impairment of the oocyte development could be due to a change in the membrane structure. When LA is available in cell culture, it is actively incorporated into the membrane phospholipid phosphatidylcholine replacing monounsaturated fats. This was observed in keratinocytes (Dunham *et al*. 1996) and other epithelial cell lines (Bryan *et al*. 2001, Subbaiah *et al*. 2011). In another experiment *in vivo*, (Pacetti *et al*. 2016) observed a change in FA composition with an incorporation of LA in human erythrocytes after supplementing diets with oils containing this FA. Homa *et al*. (1986) reported that immature pig oocyte phospholipids were rich in n6 PUFA linolenic and arachidonic acids. Additionally, Carro *et al*. (2013) showed that LA (9 and 43 µM) was rapidly incorporated to oocyte plasma membrane increasing the levels of unsaturation of membrane phospholipids improving the tolerance of the oocytes to cryopreservation. This was also observed in an *in vivo* sheep model, supplementing diets with fats (Zeron *et al*. 2002). Besides, the incorporation of FA into membrane phospholypids requires ATP (Oliveira *et al*. 1962). In conclusion, in the 200:50 group, while there was an increase of mitochondrial activity, an increase of ATP was not found, probably due to cell regulation and its partial consume to incorporate LA into the membrane.

Chapter 7

General conclusions

1- The most abundant fatty acid in follicular fluid of goat were: palmitic, oleic, stearic, linoleic and arachidonic acids. This profile is similar to the follicular fluid in cows, sheep and woman.

2- The differences on the fatty acid composition of follicular fluid in goats was mainly due to the age of the female.

3- We had shown the relationship between oocyte developmental competence and follicular size and season of the year in prepubertal animals. With the results obtained in this study we can conclude that the n6:n3 PUFA ratio in follicular fluid could be a biomarker of oocyte competence.

4- The highest ratio LA:ALA has had a detrimental effect on blastocyst production of prepubertal goat oocytes produced by IVF but not by parthenogenetic activation.

5- The negative effect of the highest LA:ALA ratio on oocyte competence was not related to impaired mitochondrial function, and ROS production or ER stress according to the relative expression of the studied genes.

Chapter 8

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Capítol final

Agraïments

Per fi ha arribat el moment d'escriure l'últim capítol d'aquesta tesi... però no el menys important! De la mateixa manera que quan estàs escrivint la memòria i penses: m'estaré deixant alguna informació? Ara penso.. espero no deixar-me a ningú! Perquè tots amb els que he compartit aquest temps han deixat el seu granet de sorra per tal que això sigui una realitat.

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