Impact of free fatty acid composition on oocyte developmental competence in dairy cows

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Cover: Fluorescent image of cumulus-oocyte-complex performed with confocal microscopy by Hilde Aardema at the CCB facility from the Faculty of Veterinary Medicine, Utrecht University. In blue DNA cumulus cells, in red DNA oocyte and in green lipid droplets.
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Impact of free fatty acid composition on oocyte developmental competence in dairy cows

Impact van vrije vetzuursamenstelling op de ontwikkelingscompetentie van de eicel in melkkoeien
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Chapter 1

General introduction

INTRODUCTION

During the last four decades, the fertility of high-producing-dairy cows has declined markedly, from calving rates of around 55% per insemination in the eighties to as low as 40% in recent surveys [1-6]. The concomitant increase in milk production by these cows and the resultant negative energy balance (NEB) in the early post-partum period are thought to be responsible for this reduction in fertility [3-7]. A reduced calving rate after insemination can essentially originate from either a reduced fertilization rate, an increase in the incidence of early or late embryonic death, or a combination of all three [1]. In this respect, the fertilization rates of modern dairy cows appear to be undiminished compared to 40 years ago with around 85-90% of inseminations resulting in successful fertilization in both high and moderate producing dairy cows [2, 8]. Therefore, failure of fertilization does not appear to be a significant contributor to the decline in fertility. By contrast, early embryonic death rates have increased from an estimated 28% of successful conceptions in 1980 to around 43% in 2006 [1]. Moreover, the incidence of early embryonic death appears to be much higher in lactating cows, which is in line with the relatively high proportion of morphologically poor quality embryos recovered on day 7 post-insemination [2, 9]. The incidence of late embryonic loss has remained unchanged over the same time interval [1]. In short, the major contributor to the decline in dairy cow fertility during the last four decades is the increased incidence of early embryonic death. There is no indication that genetic factors play a direct role in the reduced fertility of high-producing dairy cows, given that the fertility of heifers has remained high and undiminished throughout the period in question [2]. More likely, the impaired early embryo quality reflects reduced oocyte quality which, in turn, may be a result of environmental factors (e.g. metabolites, hormones) influencing *in vivo* oocyte maturation [10-13]. In this respect, the direct environment of the oocyte may be altered during the period of negative energy balance (NEB) that high-producing dairy cows experience during the early post-partum period, and this may contribute to the apparent compromised oocyte quality and impaired fertility [3-7]. The NEB is itself a direct consequence of the impressive increases in milk production among the top dairy cows that have taken place as a result of breeding programmes aimed primarily at genetic improvement for milk yield. A major characteristic of this NEB is the increase in free fatty acid concentrations in the blood, due to the mobilization of body fat reserves. The energy demand required to enable this supra-physiological milk production results in a net loss of energy that cannot

be compensated by dietary energy intake in the first few weeks post calving [3, 4, 14]. The glucose-driven, insulin-independent, production of milk results in decreased glucose concentrations in the blood and a decreased insulin/glucagon ratio that triggers the mobilization of body fat reserves. A low insulin/glucagon ratio stimulates the rate-limiting enzyme of lipid breakdown in adipose tissue, hormone-sensitive lipase (HSL), which induces lipolysis and the release of fatty acids from adipose tissue. The "free fatty acids" released form a complex with albumin within which they are transported to the cells [15].

The liberated fatty acids are valuable as an alternative energy source and are required because of the inadequate supply of carbohydrates, but elevated levels of, in particular, saturated free fatty acids can induce lipotoxic events in several somatic cell types [16-22]. Given the temporal coincidence of elevated free fatty acid levels during the period in which the oocytes destined for fertilization are developing, it is essential to investigate in further detail how fatty acid exposure influences oocyte maturation and developmental competence. Indeed, aberrant metabolic conditions, including NEB, obesity and diabetes, have all been associated with impaired fertility characteristics such as delayed or failed ovulation, reduced conception rates, a delay in the establishment of pregnancy and an increased incidence of miscarriage, and can result in permanent abnormalities of growth in the fetus and resulting offspring [23-32]. The primary goal of the studies described in this thesis was therefore to investigate the impact of elevated free fatty acid concentrations on fertility, with a specific focus on the maturation and the developmental competence of the oocytes that are exposed to aberrant metabolic conditions.

A model to investigate the impact of elevated free fatty acids on oocyte quality

In this thesis, the impact of elevated free fatty acids on the developing follicle and its contained oocyte was investigated using the 'fasting heifer model' [26]. This experimental model uses a four day period of food withholding to stimulate the mobilization of body fat reserves and a rise in blood free fatty acid concentrations comparable to those encountered during the post-partum, lactation-induced NEB. The major advantage of this model is that it is standardized, in contrast to NEB in early post-partum cows, and can be induced for use at a specific time. In the studies presented in this thesis, the fasting heifer model was used to expose follicles and oocytes to elevated free fatty acid levels during the final phase of oocyte maturation. In addition, the cows were super-stimulated with gonadotrophic hormones to increase the number of dominant follicles available for examination, since the cow is mono-ovulatory and normally

ovulates only a single oocyte during each oestrus. A potential disadvantage of super-stimulation is that the gonadotrophins are able to 'rescue' follicles that have already committed to atresia and are likely to contain developmentally incompetent oocytes. To avoid the inclusion of material from incompetent follicles in the data set, concentrations of 17β-oestradiol and progesterone in follicular fluid were used to select only follicles that were in the growing phase and that could be considered 'competent'.

Oogenesis in ovarian follicles

The ovary is the reproductive organ responsible for oogenesis and the release of viable oocytes on the one hand and, following ovulation, on the other hand the synthesis of progesterone to prepare the uterus for its role in nourishing an anticipated pregnancy. The follicle is the ovarian structure that surrounds the oocyte from the onset, beginning during *in utero* ovarian development and continuing up to the moment of ovulation of the matured oocyte by a follicle 'selected' for final development, or atresia and degeneration of follicles that were not selected. At puberty, the primordial follicles, that consist of an oocyte arrested in prophase-1 of meiosis and surrounded by a single layer of flat squamous pre-granulosa cells [33], start to develop in groups. The recruitment of resting primordial follicles into the 'growing pool' triggers the transformation of the flat pre-granulosa cells into cuboidal granulosa cells and results in growth of both the granulosa cells and the oocyte. At this stage, the follicle is termed a primary follicle [34]. The primary follicle develops further to the secondary follicle stage, which is characterized by three to six layers of granulosa cells and the formation of a theca cell layer from the interstitial stromal cells. In addition, a glycoprotein layer known as the zona pellucida is formed around the oocyte [34]. After these pre-antral follicular stages, the fluid secreted by the granulosa and theca cells coalesces to form a follicular 'antrum' that separates the theca and the granulosa cells from the oocyte and its layer of specialized granulosa cells, the cumulus cell layer [35]. Figure 1 presents the different stages of follicular growth. The oocyte and cumulus cell layers together are referred to as the cumulus-oocyte-complex (COC). During each estrous cycle, one or more dominant antral follicles are 'selected' for maturation and the release, during ovulation, of a metaphase-II stage oocyte. During the antral stages, the COC is separated from the blood by several cell layers that constitute the so-called "blood-follicle barrier". The blood-follicle-barrier is made up of the capillary endothelium, sub-endothelial basal lamina, vascular theca interna, follicular basement membrane and the non-vascular granulosa layer [35-37]. The cumulus cells form an extra cell

complex around the oocyte that helps to support and nurture the oocyte during maturation, and is crucial for proper oocyte development [38, 39].

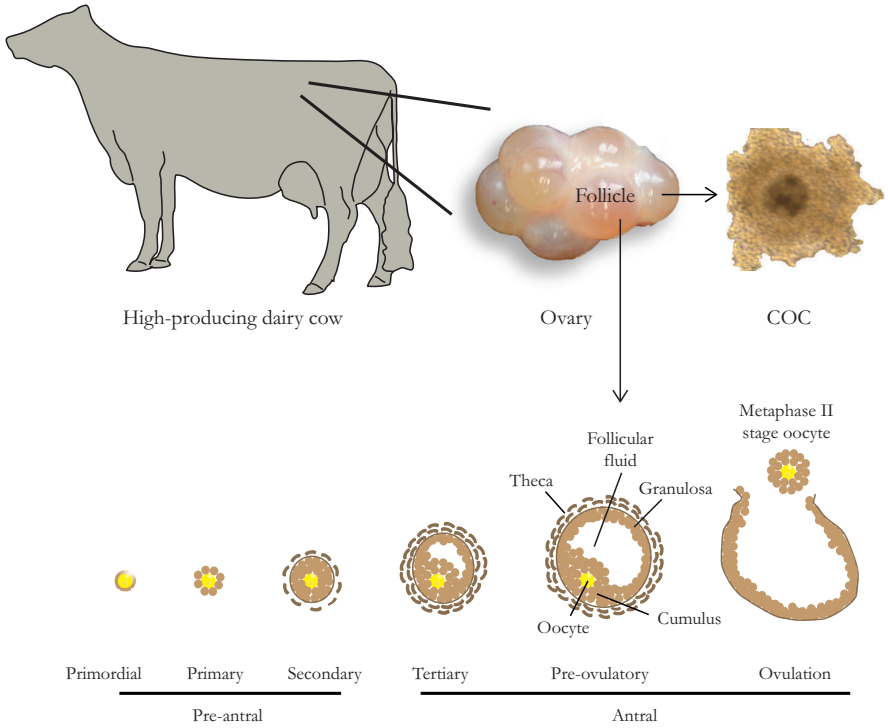

Figure 1. Schematic picture of the different follicular growth stages in the cow and pictures of a super-stimulated ovary at 22 hours after the LH surge and a COC (cumulus-oocyte-complex) retrieved from cows that participated in the *in vivo* experiments of the study.

The cumulus cells are tightly connected to the oocyte by gap junctions that project through the zona pellucida in the form of 'trans-zonal processes' and enable cytosolic transport of small signaling molecules and metabolites (in general <1 kDa) between these two cell types [40]. The gap junctions continue to form a link between oocyte and cumulus until the retraction of the cumulus cell extensions at final maturation, in response to the pre-ovulatory LH surge [38, 39, 41]. The LH surge is the result of increasing synthesis of 17β-estradiol by the granulosa cells of the growing dominant follicle until circulating concentrations reach the threshold level that triggers 'surge' release of LH from the pituitary gland. The LH peak results in morphological and molecular changes in the oocyte (cytoplasmic maturation;[42]), and the resumption of meiosis from the arrested prophase-I stage (germinal vesicle stage) to the Metaphase-II stage. The LH peak also initiates molecular changes in the follicle wall that trigger the ovulation process [42].

The first sign that an oocyte has undergone meiotic resumption is germinal vesicle breakdown, which occurs approximately 6 h after the LH peak [43, 44]. The first meiotic division (meiosis I) culminates in the extrusion of one set of chromosomes, in the first polar body. The oocyte then enters the second meiotic division (meiosis II) but arrests at the metaphase stage (Metaphase-II). Reactivation is triggered by fertilization of the ovulated metaphase-II stage oocyte and results in extrusion of one set of the sister-chromatids in the second polar body, leaving a haploid nucleus that will fuse with the haploid paternal nucleus introduced by the fertilizing sperm to form a diploid zygote. Simultaneously, the LH surge triggers the transformation of the theca interna and granulosa cells into luteal cells that will organize into the progesterone producing corpus luteum that is essential for the maintenance of pregnancy, and is either destroyed at luteolysis or prolonged as a result of conceptus signaling during the 'maternal recognition of pregnancy' [35, 45]. At the start of final oocyte maturation, testosterone produced by the theca cells is converted to 17β-estradiol by the granulosa cells of the selected presumptive dominant follicle [46]. At the end of final follicular maturation, close to the time of ovulation, the hormone synthesis in the follicle shifts towards progesterone synthesized by luteal cells [46]. Previous studies have indicated that this shift from 17β-estradiol to progesterone production is an important indicator of proper follicle maturation and may be related to the generation of a functional, developmentally competent oocyte [46, 47]. If there was a clear correlation between hormone concentrations in follicular fluid and the developmental capacity of the oocyte, hormone concentrations could be used to predict and select competent oocytes after super-stimulation. This could in turn help to reduce the unpredictability with regard to the number of embryos produced from oocytes recovered from pre-ovulatory follicles after gonadotrophin stimulation to induce multiple follicle development [47, 48]. **Chapter 2** investigates how the concentrations of 17β-estradiol and progesterone in follicular fluid correlate with and predict the developmental competence of the contained oocyte.

Fatty acids in follicular fluid

The impact of elevated circulating free fatty acid concentrations on the oocyte may largely depend on the developmental stage of the follicle, due to the major structural rearrangements in the follicle that can affect the manner in which blood-borne factors are transported to the oocyte. For instance, the cellular barrier between the blood and the oocyte changes during folliculogenesis, from a single layer of granulosa cells in the primordial follicle to several cell

layers and a considerable volume of follicular fluid by the late antral stage. The hydrophobic (water-insoluble) fatty acids are transported in the blood in the free form, complexed to albumin, or esterified as triacylglycerols or cholesteryl esters within lipoprotein particles. There are different classes of lipoproteins that can be distinguished on the basis of their content and size. Classes of lipoproteins that have been identified in the blood include chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL) and high-density lipoproteins (HDL). Chylomicrons are formed by intestinal cells and are packed with dietary lipids; they are channeled towards the liver where they are transformed into VLDLs. VLDLs mainly contain TAG and a small amount of cholesteryl esters. LDL and HDL are remnants of the VLDLs and are characterized by a reduced TAG content and an enhanced level of cholesteryl-esters. Fatty acids from the blood that enter the follicular fluid need to pass through the various cell layers that compose the blood-follicle barrier. The theca cells that constitute the outer layer of the follicle, from the primary follicular stage onwards, express the fatty acid transporter CD36, fatty-acid-binding protein (H-FABP) and lipoprotein-lipase (LPL) which enable the uptake of free fatty acids and esterified fatty acids from lipoproteins [49, 50]. HDLs are the most abundant lipoproteins present in follicular fluid, although low levels of LDLs and VLDLs appear in some follicles close to the time of ovulation as a result of the increased vascular permeability of the follicle [51-53]. The absence of chylomicrons and VLDLs, IDLs and LDLs in follicular fluid is due to their inability to cross the blood-follicle-barrier, since this barrier only allows the passage of low molecular weight proteins up to 500 kDa, and is both size and charge selective [36, 37, 54]. Follicular fluid also contains free fatty acids [23, 55, 56] with which the cumulus comes into direct contact; indeed the cumulus cells represent the only barrier between free fatty acids in the follicular fluid and the oocyte. Cumulus cells express the fatty acid transporter CD36, indicating that they are able to incorporate fatty acids from the environment [32, 56]. When fatty acids enter the cell they are activated by the attachment of a CoA group by acyl CoA synthetase and are further distributed within the cell [57].

Fatty acid composition of the oocyte

Intracellular fatty acids can be stored in lipid droplets, which act as organelles that can store or release lipids depending on the metabolic condition of the cell. Lipid droplets are thought to be formed in the hydrophobic interior of the membrane leaflet of the endoplasmic reticulum (ER) from which they bud

off as lipid droplets after accumulation of neutral lipids [58, 59]. The newly formed lipid droplets then have a hydrophobic neutral lipid core containing predominantly TAG and cholesteryl-esters, surrounded by a monolayer surface consisting of amphipathic lipids such as cholesterol and phospholipids. The lipid droplet monolayer surface is coated with proteins from the perilipin family, which regulate the storage and release of fatty acids depending on cellular need [58, 59]. It was not known whether lipid droplets in oocytes contain perilipin or how the lipid droplet features of oocytes may change during maturation; this was investigated in **Chapter 3**.

The fatty acids in a cell can originate from extracellular sources or from *de novo* synthesis. The origin of the fatty acids in the oocyte is unknown. **Chapter 3** investigated whether the oocyte is able to incorporate fatty acids from its environment. This is of particular interest as fatty acid incorporation may link the NEB-induced increase in extracellular fatty acid concentrations to altered fatty acid pools and metabolism inside the oocyte. The fatty acids stored in the oocyte are mainly present in the esterified form as TAG in lipid droplets [60-62]. Of the 24 molecular species of fatty acids esterified into TAG the saturated palmitic (C16:0) and stearic (C18:0), and mono-unsaturated oleic acids (C18:1 n-9) are the three most abundant in human, pig, cattle, sheep and rabbit oocytes [56, 60-63, 63-65]. The composition of stored fatty acids in the oocyte has been correlated with oocyte developmental competence [61]. Oocytes from COCs ranked A (highest quality) or B (moderate or low quality) all had palmitic acid as the most abundant fatty acid; however, the second most abundant fatty acid in A-ranked COCs was oleic acid as opposed to stearic acid in B-ranked COCs [61]. This finding confirms the hypothesis that altered exposure to environmental fatty acids, selective uptake of free fatty acids or an altered metabolic use of certain fatty acids may influence oocyte maturation and developmental competence.

Fatty acid metabolism in oocytes

Liberated free fatty acids are an important energy source for most cells, because of their high energetic value. After intracellular uptake the fatty acids are activated by the attachment of a CoA group, and after activation they can be completely oxidized by mitochondria and be converted to chemical energy via the β-oxidation pathway and subsequent oxidative phosphorylation. However, the amount of lipid droplets in the oocyte largely differs among mammals, such as the pig, mouse and cow [42, 43, 66, 67]. One hypothesis is that the larger amounts of lipids in bovine and porcine oocytes, as compared to, for example, murine and human oocytes, are important in enabling the longer time between

fertilization and embryo implantation in cattle and pigs; i.e. they provide a store of energy for pre-implantation development [62, 66, 68]. The first indication that intra-cellular lipid depots can be used as an energy supply by oocytes came from the observation that lipid droplets and mitochondria clustered together to form "metabolic units" and the potential of the fertilized oocyte to develop to the blastocyst stage without external nutrient sources [42, 43, 69]. A subsequent study confirmed a close association of lipid droplets and mitochondria, and demonstrated their co-localization on a molecular scale by detection of fluorescence resonance energy transfer after specific fluorescent staining of both organelles [67]. The activation of hormone sensitive lipase and the reduction of the amounts of TAG during oocyte maturation, fertilization and early embryonic development all indicate that stored fat is consumed to generate energy during these processes [61, 70-73]. The enzymes involved in lipid metabolism and *de novo* synthesis of fatty acids, both at the transcript and protein level, are indeed expressed in oocytes [73-75]. In addition, activated fatty acids can enter mitochondria for breakdown via a carnitine shuttle involving the rate-limiting enzyme of β-oxidation, carnitine-palmitoyl transferase I (CPT-I). Interestingly, when CPT-I is inhibited by etomoxir or methyl-palmoxirate, and the transfer of fatty acids into mitochondria for β-oxidation is limited, the competence of an oocyte to develop into an embryo is reduced [67, 76-80]. In the maturing oocyte, high oxygen consumption is related to fatty acid breakdown which is, in turn, related to the relocalization of lipid droplets and mitochondria to the oocyte's periphery (also known as cytoplasmic maturation) thereby maximizing the cytosolic oxygen and carbon dioxide diffusion required for high respiratory activity [72, 76]. Oocytes are large cells with a diameter of 100-140 μm and have a higher oxygen tension near their outer membranes [42, 68]. Indeed a higher mitochondrial membrane potential has been demonstrated in the peri-cortical region of oocytes compared with that of central mitochondria, indicating increased activity of the peripheral mitochondria [81, 82]. Stimulation of β-oxidation by exposure to carnitine has been related to increased developmental competence of treated oocytes and was sufficient to maintain the developmental competence of oocytes in the absence of carbohydrates [77, 80, 83]. However, one has to take into account that the carnitine concentrations used were relatively high compared to the physiological situation and that carnitine may have also acted as an anti-oxidant or as a protein source in the oocyte, rather than only by inducing fatty acid breakdown and energy production from fatty acids. Apart from their use as an energy source in the oocyte and early embryo, fatty acids are also crucial for the formation of phospholipids in cell membranes. In particular,

for the rapidly cleaving early embryo with an estimated increase in membrane area of 33% and 74% from the oocyte to the 2-cell and 4-cell stages, respectively, a source of these phospholipids is essential [84].

Elevated concentrations of free fatty acids impair follicular cell activity

When theca and granulosa cells were exposed *in vitro* to elevated levels of the types of saturated free fatty acids that are most dominant in blood, reduced cell proliferation and apoptosis were observed [85-87]. Indeed the survival rate of granulosa cells was reduced to below 20% after exposure to 300 μM of saturated fatty acids for 3 days [85]. The potential negative impact of elevated free fatty acid concentrations has also been illustrated by higher expression of the ER stress marker ATF4, an indicator of lipotoxic stress, in granulosa cells of obese women [31]. Saturated palmitic acid can also reduce cumulus cell quality and can induce an ER stress response resulting in poor cumulus cell expansion, due to a reduced secretion of the crucial extracellular matrix protein, pentraxin 3 [82]. Furthermore, exposure to saturated fatty acids resulted in increased apoptosis of cumulus cells [55]. These data indicate that elevated free fatty acid concentrations in the blood can be hazardous for theca, granulosa and cumulus cells. It also suggests that elevated free fatty acid concentrations may compromise follicle function. Interestingly, the survival of granulosa cells was much higher when they were exposed to unsaturated oleic or linoleic acid rather than saturated fatty acids [85]. This indicates that the impact of elevated free fatty acid concentrations depends to a large extent on the type of fatty acid to which the cells are exposed. This is in accordance with the observations in other somatic cell types where saturated fatty acids are lipotoxic whereas mono-unsaturated fatty acids are not [16-22]. The toxicity of saturated fatty acids has been attributed to the generation of specific pro-apoptotic lipid species that initiate apoptotic pathways, something that unsaturated fatty acids do not do. The main apoptotic cascades that are induced by saturated fatty acids appear to be cell type-dependent, but include *de novo* ceramide synthesis, the induction of reactive oxygen species (ROS), down-regulation of Bcl-2 expression (an apoptosis inhibitor), nitrogen-oxide formation, and accumulation of cytochrome-c and of diacylglycerol (DAG; glycerol backbone esterified with two fatty acids) [16, 17, 19-22, 88] in the cytoplasm. Apart from the induction of apoptotic pathways, saturated fatty acids can also impair cell survival by a primary lipotoxic effect on mitochondria and the ER [89, 90]. The data from theca, granulosa and cumulus cells suggest that elevated free fatty acid concentrations can be hazardous to the oocyte. It is important to understand

the impact of increased free fatty acid concentrations around the maturing oocyte, as it may have broad implications for the quality of the oocyte and, therefore, fertility. The follicular fluid is dominated by free fatty acids; namely, saturated palmitic acid (C16:0) and stearic acid (C18:0) and mono-unsaturated oleic acid (C18:1 n-9) [23, 55, 56]. The impact of these three predominant free fatty acids on the oocyte is investigated in **Chapter 3**, in which *in vitro* maturing oocytes were exposed to different concentrations of palmitic or stearic acid and/or oleic acid in order to examine the effects on the developmental competence of the oocyte. However, while such an *in vitro* model can be a useful indicator of the effects of free fatty acids, *in vivo* experiments can be more informative when attempting to understand the impact of elevated free fatty acid levels on the follicle and its oocyte during periods of metabolic stress. In **Chapter 4,** the impact of *in vivo* elevations in free fatty acid levels in the blood on the follicle and its contents were investigated, to determine whether and how elevated free fatty acid levels affect the developmental competence of the oocyte in a cow. Since it is extremely difficult to perform standardized experiments with physiological NEB in postpartum dairy cows, an experimental model, the fasting heifer model [26], was used to elevate circulating free fatty acid concentrations by means of a 4 day period of fasting. These experiments and their results are described in **Chapter 4**. In addition, the free fatty acid composition of blood and follicular fluid from the dominant follicles of post-partum cows that experienced NEB was investigated longitudinally at days 55, 80 and 105 post-partum in **Chapter 5**. The schematic picture of the pre-ovulatory follicle in figure 2 demonstrates the different levels of the follicle that may be affected by elevated free fatty acid levels in blood and that were investigated in this thesis.

Scope of this thesis

Fatty acids are crucial substrates in all living animals for membrane formation, signaling processes and as an energy source. However, metabolic conditions characterized by elevated circulating free fatty acid concentrations, like the period of negative energy balance experienced by post-partum dairy cows, have been associated with impaired fertility. Furthermore, elevated levels of saturated free fatty acids appear to induce lipotoxic responses in several somatic cell types. Understanding of the impact of elevated free fatty acids on the maturing oocyte and the effects on developmental competence is limited. This thesis aimed to improve our understanding of the impact of elevated free fatty acid levels on fertility with a particular focus on its effects on the developing oocyte and follicle.

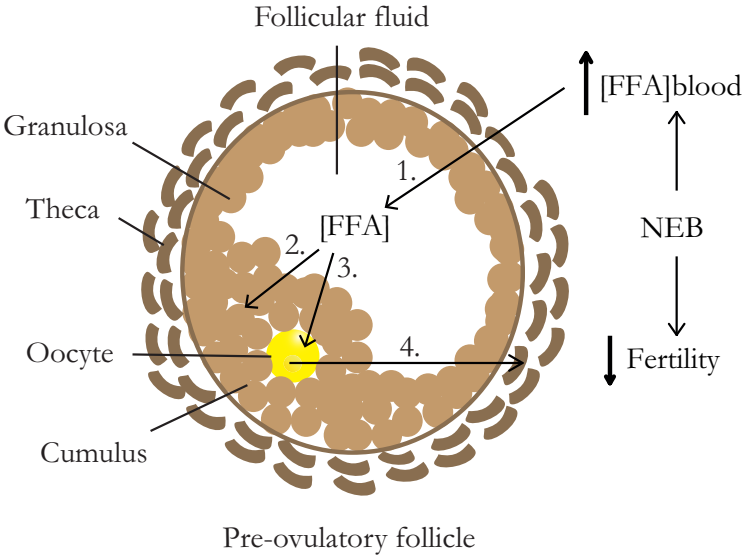

Figure 2. Schematic picture of a pre-ovulatory follicle showing the different levels of the follicle that may be affected by elevated free fatty acid concentrations in blood. The numbers represent questions that were addressed in this thesis. 1. Do elevated free fatty acid levels in blood affect the free fatty acid composition of follicular fluid. 2. Are cumulus cells affected by the free fatty acid levels in follicular fluid. 3. Is the oocyte able to incorporate free fatty acids and do free fatty acids affect the lipid composition of the oocyte. 4. Is there an impact of elevated free fatty acid levels on the developmental competence of the oocyte. NEB = negative energy balance. FFA = free fatty acid.

REFERENCES

1. Diskin MG, Murphy JJ, Sreenan JM. Embryo survival in dairy cows managed under pastoral conditions. Anim Reprod Sci 2006; 96:297-311.
2. Sartori R, Sartor-Bergfelt R, Mertens SA, Guenther JN, Parrish JJ, Wiltbank MC. Fertilization and early embryonic development in heifers and lactating cows in suμMer and lactating and dry cows in winter. J Dairy Sci 2002; 85:2803-2812.
3. Walters AH, Bailey TL, Pearson RE, Gwazdauskas FC. Parity-related changes in bovine follicle and oocyte populations, oocyte quality, and hormones to 90 days postpartum. J Dairy Sci 2002; 85:824-832.
4. van Knegsel AT, van den Brand H, Dijkstra J, TaμMinga S, Kemp B. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. Reprod Nutr Dev 2005; 45:665-688.
5. Britt JH. Impacts of early postpartum metabolism on follicular development and fertility. Bov Proc 1992; 24:39-43.
6. Butler WR. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livest Prod Sci 2003; 83:211-218.
7. Kawashima C, Matsui M, Shimizu T, Kida K, Miyamoto A. Nutritional factors that regulate ovulation of the dominant follicle during the first follicular wave postpartum in high-producing dairy cows. J Reprod Dev 2012; 58:10-16.
8. Diskin MG, Morris DG. Embryonic and early foetal losses in cattle and other ruminants. Reprod Domest Anim 2008; 43 Suppl 2:260-267.
9. Wiebold JL. Embryonic mortality and the uterine environment in first-service lactating dairy cows. J Reprod Fertil 1988; 84:393-399.
10. Lonergan P, Rizos D, Ward F, Boland MP. Factors influencing oocyte and embryo quality in cattle. Reprod Nutr Dev 2001; 41:427-437.
11. Dieleman SJ, Hendriksen PJ, Viuff D, Thomsen PD, Hyttel P, Knijn HM, Wrenzycki C, Kruip TA, Niemann H, Gadella BM, Bevers μM, Vos PL. Effects of in vivo prematuration and in vivo final maturation on developmental capacity and quality of pre-implantation embryos. Theriogenology 2002; 57:5-20.
12. Knijn HM, Wrenzycki C, Hendriksen PJ, Vos PL, Herrmann D, van der Weijden GC, Niemann H, Dieleman SJ. Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived in vitro or in vivo. Reproduction 2002; 124:365-375.
13. Gad A, Hoelker M, Besenfelder U, Havlicek V, Cinar U, Rings F, Held E, Dufort I, Sirard MA, Schellander K, Tesfaye D. Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions. Biol Reprod 2012; 87:100.
14. Rukkwamsuk T, Kruip TA, Wensing T. Relationship between overfeeding and overconditioning in the dry period and the problems of high producing dairy cows during the postparturient period. Vet Q 1999; 21:71-77.
15. Contreras GA, Sordillo LM. Lipid mobilization and inflaμMatory responses during the transition period of dairy cows. Comp IμMunol Microbiol Infect Dis 2011; 34:281-289.
16. Cnop M, Hannaert JC, Hoorens A, Eizirik DL, PipeleersDG. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. Diabetes 2001; 50:1771-1777.

17. Listenberger LL, Han X, Lewis SE, Cases S, Farese RV,Jr, Ory DS, Schaffer JE. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc Natl Acad Sci U S A 2003; 100:3077-3082.
18. Mishra R, Simonson MS. Saturated free fatty acids and apoptosis in microvascular mesangial cells: palmitate activates pro-apoptotic signaling involving caspase 9 and mitochondrial release of endonuclease G. Cardiovasc Diabetol 2005; 4:2.
19. Coll T, Eyre E, Rodriguez-Calvo R, Palomer X, Sanchez RM, Merlos M, Laguna JC, Vazquez-Carrera M. Oleate reverses palmitate-induced insulin resistance and inflaμMation in skeletal muscle cells. J Biol Chem 2008; 283:11107-11116.
20. Henique C, Mansouri A, Fumey G, Lenoir V, Girard J, Bouillaud F, Prip-Buus C, Cohen I. Increased mitochondrial fatty acid oxidation is sufficient to protect skeletal muscle cells from palmitate-induced apoptosis. J Biol Chem 2010; 285:36818-36827.
21. Listenberger LL, Ory DS, Schaffer JE. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. J Biol Chem 2001; 276:14890-14895.
22. Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, Donath MY. Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. Diabetes 2001; 50:69-76.
23. Jungheim ES, Macones GA, Odem RR, Patterson BW, Lanzendorf SE, Ratts VS, Moley KH. Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during in vitro fertilization. Fertil Steril 2011; 95:1970-1974.
24. Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE, Moley KH. Diet-induced obesity model: abnormal oocytes and persistent growth abnormalities in the offspring. Endocrinology 2010; 151:4039-4046.
25. Metwally M, Saravelos SH, Ledger WL, Li TC. Body mass index and risk of miscarriage in women with recurrent miscarriage. Fertil Steril 2010; 94:290-295.
26. Jorritsma R, Wensing T, Kruip TA, Vos PL, Noordhuizen JP. Metabolic changes in early lactation and impaired reproductive performance in dairy cows. Vet Res 2003; 34:11-26.
27. Gesink Law DC, Maclehose RF, Longnecker MP. Obesity and time to pregnancy. Human Reproduction 2007; 22:414-420.
28. Rich-Edwards JW, Spiegelman D, Garland M, Hertzmark E, Hunter DJ, Colditz GA, Willett WC, Wand H, Manson JE. Physical activity, body mass index, and ovulatory disorder infertility. Epidemiology 2002; 13:184-190.
29. Kawashima C, Sakaguchi M, Suzuki T, Sasamoto Y, Takahashi Y, Matsui M, Miyamoto A. Metabolic profiles in ovulatory and anovulatory primiparous dairy cows during the first follicular wave postpartum. J Reprod Dev 2007; 53:113-120.
30. Robker RL, Wu LL, Yang X. InflaμMatory pathways linking obesity and ovarian dysfunction. J Reprod IμMunol 2011; 88:142-148.
31. Wu LL, Dunning KR, Yang X, Russell DL, Lane M, Norman RJ, Robker RL. High-fat diet causes lipotoxicity responses in cumulus-oocyte complexes and decreased fertilization rates. Endocrinology 2010; 151:5438-5445.
32. Yang X, Wu LL, Chura LR, Liang X, Lane M, Norman RJ, Robker RL. Exposure to lipid-rich follicular fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus-oocyte complexes. Fertil Steril 2012; 97:1438-5.
33. Picton H, Briggs D, Gosden R. The molecular basis of oocyte growth and development. Mol Cell Endocrinol 1998; 145:27-37.

34. Fair T, Hulshof SC, Hyttel P, Greve T, Boland M. Oocyte ultrastructure in bovine primordial to early tertiary follicles. Anat Embryol (Berl) 1997; 195:327-336.
35. Hennet ML, Combelles CM. The antral follicle: a microenvironment for oocyte differentiation. Int J Dev Biol 2012; 56:819-831.
36. Hess KA, Chen L, Larsen WJ. The ovarian blood follicle barrier is both charge- and size-selective in mice. Biol Reprod 1998; 58:705-711.
37. Shalgi R, Kraicer P, Rimon A, Pinto M, Soferman N. Proteins of human follicular fluid: the blood-follicle barrier. Fertil Steril 1973; 24:429-434.
38. Albertini DF, Combelles CM, Benecchi E, Carabatsos MJ. Cellular basis for paracrine regulation of ovarian follicle development. Reproduction 2001; 121:647-653.
39. Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum Reprod Update 2008; 14:159-177.
40. de Loos F, Kastrop P, Van Maurik P, Van Beneden TH, Kruip TA. Heterologous cell contacts and metabolic coupling in bovine cumulus oocyte complexes. Mol Reprod Dev 1991; 28:255-259.
41. Hyttel P. Bovine cumulus-oocyte disconnection in vitro. Anat Embryol (Berl) 1987; 176:41-44.
42. Hyttel P, Fair T, Callesen H, Greve T. Oocyte growth, capacitation and final maturation in cattle. Theriogenology 1997; 47:23-32.
43. Kruip TAM, Cran DG, Beneden TH, Dieleman SJ. Structural changes in bovine oocytes during final maturation in vivo. Gamete Res 1983; 8:29-47.
44. de Loos FA, Bevers μM, Dieleman SJ, Kruip TA. Morphology of preovulatory bovine follicles as related to oocyte maturation. Theriogenology 1991; 35:527-535.
45. Komar CM, Berndtson AK, Evans AC, Fortune JE. Decline in circulating estradiol during the periovulatory period is correlated with decreases in estradiol and androgen, and in messenger RNA for p450 aromatase and p450 17alpha-hydroxylase, in bovine preovulatory follicles. Biol Reprod 2001; 64:1797-1805.
46. Dieleman SJ, Bevers μM, Poortman J, van Tol HT. Steroid and pituitary hormone concentrations in the fluid of preovulatory bovine follicles relative to the peak of LH in the peripheral blood. J Reprod Fertil 1983; 69:641-649.
47. de Loos FA, Bevers μM, Dieleman SJ, Kruip TA. Follicular and oocyte maturation in cows treated for superovulation. Theriogenology 1991; 35:537-546.
48. Callesen H, Greve T, Hyttel P. Preovulatory endocrinology and oocyte maturation in superovulated cattle. Theriogenology 1986; 25:71-86.
49. Iseki S, Amano O, Fujii H, Kanda T, Ono T. IμMunohistochemical localization of two types of fatty acid-binding proteins in rat ovaries during postnatal development and in iμMature rat ovaries treated with gonadotropins. Anat Rec 1995; 241:235-243.
50. Camps L, Gafvels M, Reina M, Wallin C, Vilaro S, Olivecrona T. Expression of lipoprotein lipase in ovaries of the guinea pig. Biol Reprod 1990; 42:917-927.
51. Simpson ER, Rochelle DB, Carr BR, MacDonald PC. Plasma lipoproteins in follicular fluid of human ovaries. J Clin Endocrinol Metab 1980; 51:1469-1471.
52. Le Goff D. Follicular fluid lipoproteins in the mare: evaluation of HDL transfer from plasma to follicular fluid. Biochim Biophys Acta 1994; 1210:226-232.
53. Volpe A, Coukos G, Uccelli E, Droghini F, Adamo R, Artini PG. Follicular fluid lipoproteins in preovulatory period and their relationship with follicular maturation and progesterone production by human granulosa-luteal cells in vivo and in vitro. J Endocrinol Invest 1991; 14:737-742.

54. Cran DG, Moor RM, Hay MF. Permeability of ovarian follicles to electron-dense macromolecules. Acta Endocrinol (Copenh) 1976; 82:631-636.

55. Leroy JL, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G, Van Soom A. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. Reproduction 2005; 130:485-495.

56. Aardema H, Lolicato F, van de Lest CH, Brouwers JF, Vaandrager AB, van Tol HT, Roelen BA, Vos PL, Helms JB, Gadella BM. Bovine Cumulus Cells Protect Maturing Oocytes from Increased Fatty Acid Levels by Massive Intracellular Lipid Storage. Biol Reprod 2013; 88:164.

57. Watkins PA, Ellis JM. Peroxisomal acyl-CoA synthetases. Biochim Biophys Acta 2012; 1822:1411-1420.

58. Brasaemle DL, Wolins NE. Packaging of Fat: An Evolving Model of Lipid Droplet Assembly and Expansion. Journal of Biological Chemistry 2012; 287:2273-2279.

59. Walther TC, Farese RV,Jr. Lipid droplets and cellular lipid metabolism. Annu Rev Biochem 2012; 81:687-714.

60. Homa ST, Racowsky C, McGaughey RW. Lipid analysis of iμMature pig oocytes. J Reprod Fertil 1986; 77:425-434.

61. Kim JY, Kinoshita M, Ohnishi M, Fukui Y. Lipid and fatty acid analysis of fresh and frozen-thawed iμMature and in vitro matured bovine oocytes. Reproduction 2001; 122:131-138.

62. McEvoy TG, Coull GD, Broadbent PJ, Hutchinson JS, Speake BK. Fatty acid composition of lipids in iμMature cattle, pig and sheep oocytes with intact zona pellucida. J Reprod Fertil 2000; 118:163-170.

63. Khandoker MAMY, Tsujii H, Karasawa D. Fatty acid analysis of oocytes, oviductal and uterine fluids of rabbit Anim Sci Technol 1996; 67:549-4.

64. Matorras R, Ruiz JI, Mendoza R, Ruiz N, Sanjurjo P, Rodriguez-Escudero FJ. Fatty acid composition of fertilization-failed human oocytes. Hum Reprod 1998; 13:2227-2230.

65. Khandoker MAMY, Tsujii H, Karasawa D. Fatty acid compositions of oocytes, follicular, oviductal and uterine fluids of pig and cow 1997; 10:523-4.

66. Genicot G, Leroy JL, Soom AV, Donnay I. The use of a fluorescent dye, Nile red, to evaluate the lipid content of single maμMalian oocytes. Theriogenology 2005; 63:1181-1194.

67. Sturmey RG, O'Toole PJ, Leese HJ. Fluorescence resonance energy transfer analysis of mitochondrial:lipid association in the porcine oocyte. Reproduction 2006; 132:829-837.

68. Sturmey RG, Reis A, Leese HJ, McEvoy TG. Role of fatty acids in energy provision during oocyte maturation and early embryo development. Reprod Domest Anim 2009; 44 Suppl 3:50-58.

69. Kane MT. Minimal nutrient requirements for culture of one-cell rabbit embryos. Biol Reprod 1987; 37:775-778.

70. Ferguson EM, Leese HJ. Triglyceride content of bovine oocytes and early embryos. J Reprod Fertil 1999; 116:373-378.

71. Abe H, Yamashita S, Itoh T, Satoh T, Hoshi H. Ultrastructure of bovine embryos developed from in vitro-matured and -fertilized oocytes: comparative morphological evaluation of embryos cultured either in serum-free medium or in serum-supplemented medium. Mol Reprod Dev 1999; 53:325-335.

72. Sturmey RG, Leese HJ. Energy metabolism in pig oocytes and early embryos. Reproduction 2003; 126:197-204.

73. Auclair S, Uzbekov R, Elis S, Sanchez L, Kireev I, Lardic L, Dalbies-Tran R, Uzbekova S. Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes. Am J Physiol Endocrinol Metab 2013; 304:E599-613.

74. Cetica P, Pintos L, Dalvit G, Beconi M. Involvement of enzymes of amino acid metabolism and tricarboxylic acid cycle in bovine oocyte maturation in vitro. Reproduction 2003; 126:753-763.

75. Cetica P, Pintos L, Dalvit G, Beconi M. Activity of key enzymes involved in glucose and triglyceride catabolism during bovine oocyte maturation in vitro. Reproduction 2002; 124:675-681.

76. Ferguson EM, Leese HJ. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. Mol Reprod Dev 2006; 73:1195-1201.

77. Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ, Robker RL. Beta-Oxidation Is Essential for Mouse Oocyte Developmental Competence and Early Embryo Development. Biol Reprod 2010;

78. Downs SM, Mosey JL, Klinger J. Fatty acid oxidation and meiotic resumption in mouse oocytes. Mol Reprod Dev 2009; 76:844-853.

79. Paczkowski M, Silva E, Schoolcraft WB, Krisher RL. Comparative importance of Fatty Acid Beta-oxidation to nuclear maturation, gene expression, and glucose metabolism in mouse, bovine, and porcine cumulus oocyte complexes. Biol Reprod 2013; 88:111.

80. Sutton-McDowall ML, Feil D, Robker RL, Thompson JG, Dunning KR. Utilization of endogenous fatty acid stores for energy production in bovine preimplantation embryos. Theriogenology 2012; 77:1632-1641.

81. Preis KA, Seidel GE,Jr, Gardner DK. Reduced oxygen concentration improves the developmental competence of mouse oocytes following in vitro maturation. Mol Reprod Dev 2007; 74:893-903.

82. Wu LL, Russell DL, Norman RJ, Robker RL. Endoplasmic Reticulum (ER) Stress in Cumulus-Oocyte Complexes Impairs Pentraxin-3 Secretion, Mitochondrial Membrane Potential ($\Delta\Psi m$), and Embryo Development. Mol Endocrinol 2012; 26:562-573.

83. Dunning KR, Akison LK, Russell DL, Norman RJ, Robker RL. Increased beta-oxidation and improved oocyte developmental competence in response to l-carnitine during ovarian in vitro follicle development in mice. Biol Reprod 2011; 85:548-555.

84. Pratt HPM, George MA. Organisation and assembly of the surface membrane during early cleavage of the mouse embryo. Roux's Arch Dev Biol 198:170-8.

85. Mu YM, Yanase T, Nishi Y, Tanaka A, Saito M, Jin CH, Mukasa C, Okabe T, Nomura M, Goto K, Nawata H. Saturated FFAs, palmitic acid and stearic acid, induce apoptosis in human granulosa cells. Endocrinology 2001; 142:3590-3597.

86. Vanholder T, Lmr Leroy J, Van Soom A, Maes D, Coryn M, Fiers T, de Kruif A, Opsomer G. Effect of non-esterified fatty acids on bovine theca cell steroidogenesis and proliferation in vitro. Anim Reprod Sci 2006; 92:51-63.

87. Vanholder T, Leroy JL, Soom AV, Opsomer G, Maes D, Coryn M, de Kruif A. Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation in vitro. Anim Reprod Sci 2005; 87:33-44.

88. Maedler K, Oberholzer J, Bucher P, Spinas GA, Donath MY. Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. Diabetes 2003; 52:726-733.

89. Borradaile NM, Han X, Harp JD, Gale SE, Ory DS, Schaffer JE. Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. J Lipid Res 2006; 47:2726-2737.

90. Schrauwen P, Hesselink MK. Oxidative capacity, lipotoxicity, and mitochondrial damage in type 2 diabetes. Diabetes 2004; 53:1412-1417.

Chapter 2

Follicular 17β-estradiol and progesterone concentrations and degree of cumulus cell expansion as predictors of in vivo-matured oocyte developmental competence in superstimulated heifers

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ABSTRACT

The quality of an oocyte is crucial for successful generation of offspring, but few selection parameters have been identified that reliably predict oocyte developmental competence. The objective of the present study was to determine whether the developmental competence of in vivo-matured oocytes derived from superstimulated heifers could be predicted by 17β-estradiol and progesterone concentrations in follicular fluid, degree of cumulus cell expansion, and follicular diameter. Cumulus oocyte complexes were individually collected from follicles ≥ 8 mm 22 hours after an induced LH peak and individually fertilized and cultured in vitro. Only oocytes that originated from follicles with 17β-estradiol ≤ 0.25 μM and progesterone ≥ 0.26 μM developed into blastocysts. When a combination of these cutoff values was evaluated as a predictor of oocyte competence, the sensitivity, specificity, positive predictive value, and negative predictive value were 100%, 75%, 49%, and 100%, respectively. Hormone concentrations in follicular fluid were also associated with the degree of cumulus cell expansion and only cumulus oocyte complexes with full expansion developed into blastocysts; sensitivity, specificity, positive predictive value, and negative predictive value were 100%, 71%, 45%, and 100%, respectively, when full expansion was used as the predictive criterion for blastocyst production. Follicular diameter was not a good predictor of oocyte competence. In conclusion, concentrations of 17β-estradiol and progesterone in the preovulatory follicle and the degree of cumulus cell expansion are predictors of blastocyst production in superstimulated heifers and can be used as selection markers for oocyte developmental competency.

INTRODUCTION

The ovarian follicle creates a microenvironment in which the oocyte grows and develops until its final release at the moment of ovulation. Complex extra- and intraovarian factors that drive the oocyte toward developmental competence have been identified [1–3]. Several attempts have been made to define specific markers that are related to oocyte developmental competence, using analysis of specific contents in follicular fluid or mRNA expression of cumulus cells for example, but a marker that reliably predicts oocyte viability has not been identified [4–7]. Sex steroids present in follicular fluid might be key factors that determine the fate of the oocyte, in particular because there is a prominent shift in the concentrations of 17β-estradiol and progesterone during the phase of final oocyte maturation [8,9]. Until the LH surge, the concentration of 17b-estradiol, synthesized by granulosa cells after aromatization of testosterone (or 4-androstendione) from theca cells, is high in follicular fluid [10]. The LH surge marks the start of sudden changes within the preovulatory follicle, involving the oocyte that resumes meiosis and the follicle that prepares for ovulation and formation of the corpus luteum. The LH surge stimulates the transformation of theca and granulosa cells into luteal cells that start to produce progesterone instead of 17b-estradiol, accompanied by a decline in mRNA expression of the enzymes P450 17α-hydroxylase (the rate-limiting enzyme for testosterone synthesis) and P450 aromatase (the rate-limiting enzyme for 17β-estradiol synthesis) [11–14]. Consequently, the steroid levels in the preovulatory follicle switch from 17bestradiol predominance near the time of the LH surge toward progesterone predominance near the time of ovulation. This phenomenon is not only observed in cows [8], but has also been described in other mammalian species including primates [15]. Estrogen-receptor knockout mice exhibit arrested folliculogenesis, demonstrating that 17β-estradiol is essential during follicular growth [16,17]. However, high concentrations of 17β-estradiol during final maturation induce oocyte nuclear aberrations and inhibit meiosis progression [18,19]. Progesterone receptor knockout mice show normal follicular growth but fail to ovulate and when cumulus oocyte complexes (COCs) were cocultured with follicular wall fragments, by which resumption of meiosis is inhibited, progesterone induced nuclear maturation of oocytes in a concentration-dependent manner [20,21]. An optimal balance in the hormone levels of follicular fluid during final maturation seems crucial for oocyte development. However, little is known about the relation between 17β-estradiol and progesterone concentrations in follicular fluid and the developmental competence of the oocytes from those

follicles. Two other potential parameters that might be predictive of oocyte competence are follicular diameter and expansion of the cumulus cells. It has been demonstrated that, in nonstimulated cows, a large-sized preovulatory follicle at the moment of ovulation is related to a higher pregnancy rate [22–24]. In addition, proper cumulus cell expansion has been correlated with oocyte developmental competence [25,26]. The objective of the present study was to determine whether the developmental competence of in vivo matured oocytes derived from superstimulated heifers could be predicted by 17β-estradiol and progesterone concentrations in follicular fluid, degree of cumulus cell expansion, and follicular diameter.

MATERIAL AND METHODS

Chemicals

Unless stated otherwise, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental design

This experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Utrecht University. Clinically healthy, non-lactating Holstein-Friesian heifers (N=8) were selected on the basis of normal ovarian cyclicity based on blood progesterone concentrations measured three times a week for four successive weeks during the pre-experimental period. Heifers were fed ad libitum with grass silage supplemented with 1 kg concentrate to meet the nutritional requirements and had unlimited access to water. Estrus was synchronized using a combination of an intravaginal progesterone device (CIDR; Pfizer Animal Health, New York, NY, USA; 1.38 g progesterone) and prostaglandin (PGF2α; Enzaprost; Ceva Sante Animale, Libourne, France; 25 mg dinoprost, im). On Day 8 of the synchronized cycle (estrus=Day 0), dominant follicles (≥ 8 mm) were removed using transvaginal follicle aspiration [27] to induce a new follicular wave [28]. Superstimulation treatments were initiated on Day 10 and consisted of the administration of FSH (Folltropin-V; Bioniche Animal Health, Belleville, Ontario, Canada) twice daily in decreasing doses during the following 4 days (40, 30, 20, and 10 mg, respectively; 200 mg total, im) [29].

Heifers received an intravaginal progesterone device (CIDR) to avoid a spontaneous LH surge between days 10 and 14 and PGF2α (Enzaprost) was administered simultaneously with the fifth dose of FSH on Day 12. Gonadotropin

releasing hormone (GnRH; Fertagyl; Intervet International B.V., Boxmeer, The Netherlands; 1 mg gonadorelin, im) was administered concomitantly with CIDR removal on Day 14 to induce a timed LH peak [30]. Ovaries were collected 24 hours after GnRH treatment (or 22 hours after induced LH peak) by ovariectomy through a flank incision made using local anesthesia. Blood samples were collected from the jugular vein using a vacutainer system and heparin-coated tubes hourly for 4 hours after GnRH treatment to determine circulating LH concentrations [31].

Follicular fluid and COC collection

Ovaries were placed in 0.9% (wt/vol) NaCl at 30 °C and immediately transported to the laboratory. At the laboratory, the content of follicles ≥ 8 mm (evaluated with a ruler) were individually aspirated using an 18-gauge winged infusion set needle attached to a 15-mL polystyrene conical tube under low pressure from a suction pump. Follicular fluid volume was determined using comparison with a standardized series of volumes ranging from 100 to 2500 μL. Follicular diameter was estimated from the follicular fluid volume (diameter = 2 x cube root [volume/1.3333 x π]). The number of follicles ≥ 8 mm was 24 ± 10 (mean ± SD) and the COC recovery rate was 57 ± 18% per heifer. Cumulus cell expansion was evaluated using a stereoscope and scored as (1) absent (compact multilayered cumulus investment); (2) moderate (less compact cumulus investment); or (3) full expansion (expanded cumulus investment in an orderly radial way) according to criteria adapted from de Loos et al. [32]. Follicular fluid was kept on ice until centrifugation at 3000 x g for 10 minutes at 4 °C and stored at -20 °C until steroid analysis.

Hormone analyses

Circulating LH concentrations were determined with a validated homologous double-antibody radioimmunoassay (RIA) in duplicate aliquots of 100 μL of plasma, according to Dieleman et al. [8]. Bovine LH (bLH-7981) was used for iodination and standards and rabbit anti-bLH (8103J; dilution 1:800,000) as antiserum. The concentrations of progesterone and 17β-estradiol in follicular fluid were determined in aliquots of 1 to 25 μL fluid, depending on the type of hormone and size of the follicle. Follicular fluid was analyzed using solid-phase [125I] RIA (Coat-A-Count; TKPG and TKE2 respectively; Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) according to the manufacturer [31] with slight modifications. Briefly, follicular fluid samples were extracted with 2 mL diethyl ether (Scharlau, Barcelona, Spain) and an internal standard

of 167 Bq [3H] steroid was added before extraction in order to determine and correct for the efficiency of extraction. After evaporation of the organic solvent, the samples and efficiency series were dissolved in 250 μL borate buffer (for 17β-estradiol) or in 250 μL zero plasma from the manufacturer (for progesterone). Duplicate volumes of the samples were then incubated in the antibody-coated tubes. Calculation of hormone concentrations was performed by applying the approximation for the standard series from RIA Smart (Packard Instruments Company, Meriden, CT, USA). The concentrations calculated using RIA differed <4% from the defined concentrations of the standards used to build the reference curves. The intra and interassay coefficients of variation were <10% for all assays.

In vitro fertilization and culture

In vitro fertilization and embryo culture were performed according to a standard protocol [33], except that COCs were handled individually during the entire process; IVF was performed at 39 °C in a humidified atmosphere with 5% CO_2 in air and embryo culture was performed at 39 °C in a humidified atmosphere with 7% O_2, 5% CO_2, and 88% N_2. Cumulus oocyte complexes were washed in 500 μL oocyte culture medium (MP Biomedicals, Eindhoven, The Netherlands) and placed into a microwell of a tissue culture plate (Terasaki Microwell Plate; Nalgene Nunc International, Rochester, NY, USA) with fertilization droplets consisting of 10 mL fertilization medium. Frozen-thawed sperm, originating from one batch of the same bull, were added to the fertilization medium to a final concentration of 1 x 106 sperm per mL. After 20 hours of coincubation, presumptive zygotes were denuded using repeated pipet- ting through a narrow-bored pipette and placed in micro- wells with synthetic oviductal fluid medium. Each microwell plate contained the presumed zygotes of one heifer. Microwells were covered with medium (25 μL per zygote allowing medium contact by "medium bridges") and the plate was covered with 9 mL light mineral oil. On Day 5 of embryo culture, embryonic developmental stage was determined and defined as noncleaved, less than eight-cell, and eight-cell or later stage embryos; cleaved embryos were transferred to fresh synthetic oviductal fluid medium. Blastocyst production rates were determined on Day 8 of embryo culture. As a control, 50 in vitro-matured COCs originating from slaughterhouse ovaries were fertilized and cultured according to the same procedures as a group. The control blastocyst production rate for different replicates was38 ± 8%.

Statistical analysis

Statistical analysis was performed using SPSS statistics version 20.0. Hormone concentration data were log-transformed to attain normality and analyzed using a linear mixed model with cow as random effect and Bonferroni post hoc test. Differences in hormone concentrations were evaluated using final embryonic developmental stage, follicular diameter, and cumulus expansion category as fixed effects. Receiver operating characteristic curves were used to determine the optimal cutoff values for hormone concentrations and follicular diameter as predictors of blastocyst production.

RESULTS

All heifers showed an LH surge after GnRH treatment; maximum circulating LH concentration was observed 2 hours after treatment (Fig. 1).

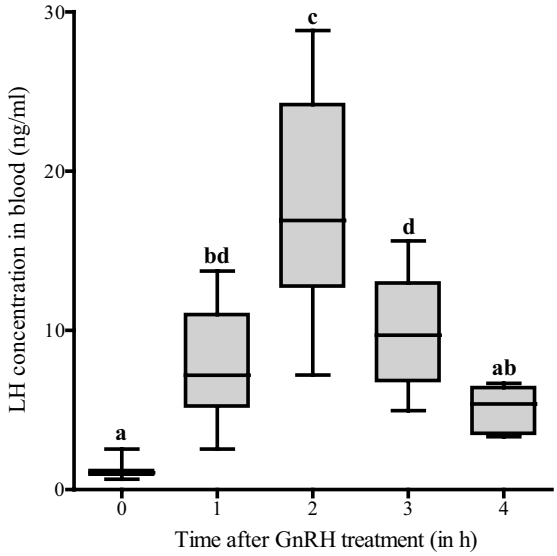

Figure 1. Box and whisker plot of circulating LH concentrations according to time after GnRH treatment in heifers (N= 8). Values with different letters (a–d) differ ($P < 0.005$).

Oocytes that developed into an embryo of eight-cell stage or larger, or into a blastocyst originated from follicles with lower ($P < 0.001$) 17β-estradiol concentration and estradiol:progesterone ratio and higher ($P < 0.001$) progesterone concentration than oocytes that did not cleave or did not develop to an eight-cell embryo (Fig. 2A–C).

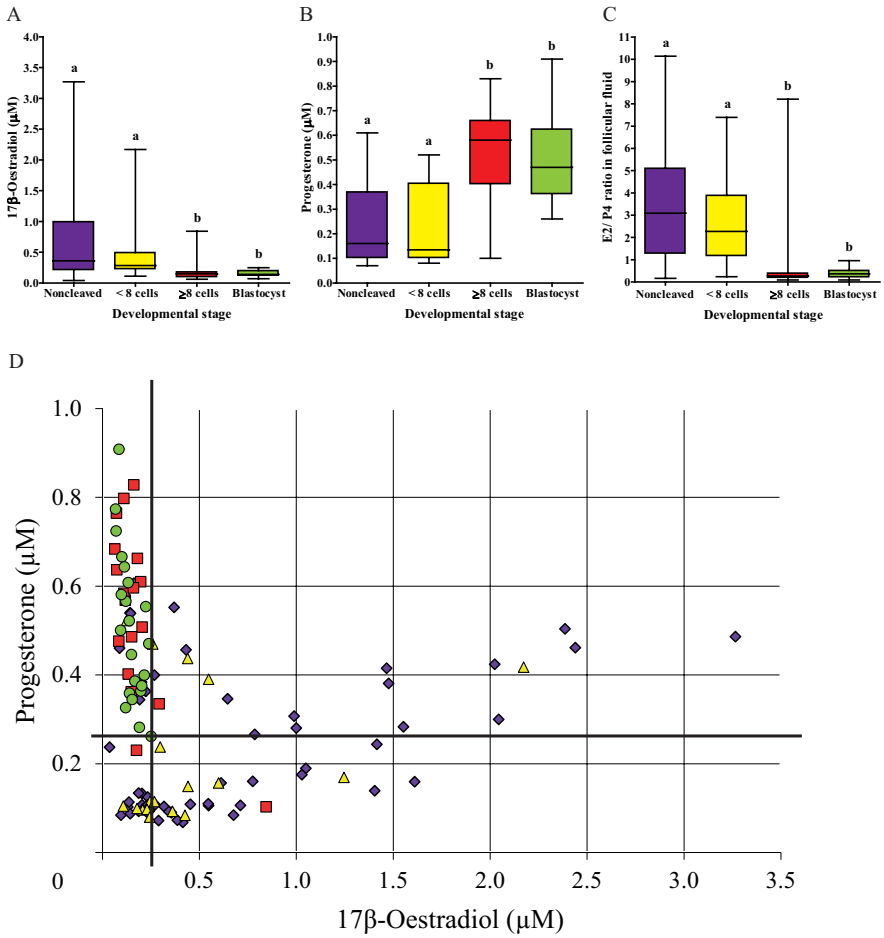

Figure 2. Box and whisker plots of follicular 17β-estradiol (A) and progesterone (B) concentrations and (C) 17b-estradiol:progesterone ratio according to oocyte developmental competence (noncleaved, N = 56; embryos <8 cells, N = 18; embryo ≥ 8 cells, N = 18; blastocyst, N = 22). Values with different letters (a and b) differ ($P < 0.001$). (D) Scatter plot of 17β-estradiol and progesterone concentrations for individual follicles according to oocyte developmental competence; noncleaved (diamonds), embryos <8 cells (triangles), embryos ≥ 8 cells (squares), and blastocysts (circles). The lines mark the concentrations of 17β-estradiol 0.25 μM and progesterone 0.26 μM. E2, 17β-estradiol; P4, progesterone.

Oocytes that developed into a blastocyst originated exclusively from follicles with 17β-estradiol ≤ 0.25 μM and progesterone ≥ 0.26 μM; the same was observed for most oocytes that developed to 8-cell stage or larger embryo (15/18; Fig. 2D). These hormone concentrations had the best predictive values for blastocyst production (Table 1). Most noncleaved oocytes (49/56) and embryos less than eight- cell stage (17/18) originated from follicles with nonoptimal steroid concentrations (Fig. 2D).

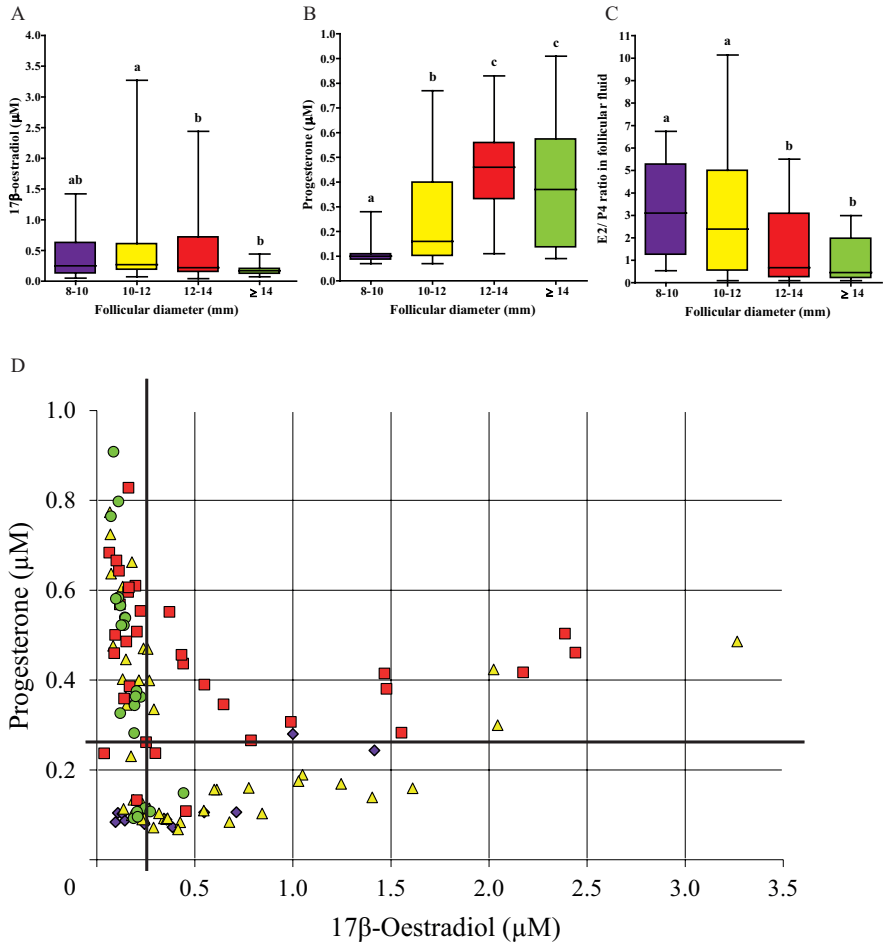

Figure 3. Box and whisker plots of follicular 17β-estradiol (A) and progesterone (B) concentrations and (C) 17b-estradiol:progesterone ratio according to follicular diameter (8–10 mm, N = 12; 10–12 mm, N = 47; 12–14 mm, N = 33; ≥ 14 mm, N = 22). Values with different letters (a–c) differ ($P < 0.005$). (D) Scatter plot of 17β-estradiol and progesterone concentrations for individual follicles according to follicular diameter; 8 to 10 mm (diamonds), 10 to 12 mm (triangles), 12 to 14 mm (squares), and ≥ 14 mm (circles). The lines mark the concentrations of 17β-estradiol 0.25 μM and progesterone 0.26 μM. E2, 17b-estradiol; P4, progesterone.

Follicles ≥ 12 mm had lower ($P < 0.005$) 17β-estradiol concentration and estradiol:progesterone ratio and higher ($P < 0.005$) progesterone concentration compared with smaller follicles (Fig. 3A–C). Optimal follicular fluid steroid concentrations were also more often observed in larger follicles: 0% (0/12) in follicles <10 mm, 28% (13/47) in follicles 10 to 12 mm, 48% (16/33) in follicles 12 to 14 mm, and 73% (16/22) in follicles ≥ 14 mm (Fig. 3D). However, diameter was a nonspecific predictor of oocyte develop- mental competence because oocytes

that developed into a blastocyst originated from all follicular size categories, except from follicles <10 mm (Table 1).

Table 1. Cut-off values of the potential predictors for oocyte developmental competence.

Best cut-off value	*Sensitivity*	*Specificity*	*Positive predictive value*	*Negative predictive value*
≤ 0.25 μM 17β-oestradiol	1	0.522	0.33	1
≥ 0.26 μM Progesterone	1	0.533	0.34	1
≤ 1.0 E2/P4 ratio	1	0.674	0.42	1
≤ 0.25 μM E2, ≥ 0.26 μM P4	1	0.75	0.49	1
Full cumulus cell expansion	1	0.71	0.45	1
≥ 10 mm follicular diameter	1	0.13	0.22	1

Cutoff values of the potential predictors for oocyte developmental competence. Predictors of oocyte developmental competence (blastocyst production). The cutoff values for steroid concentrations and follicular diameter were identified through receiver operating characteristic analysis. Cumulus cell expansion was categorized into absent, moderate, or full. Abbreviations: E2, 17β-estradiol; P4, progesterone.

COCs with fully expanded cumulus cells originated from follicles with lower ($P < 0.005$) 17β-estradiol concentration and estradiol:progesterone ratio and higher ($P < 0.005$) progesterone concentration than COCs with absent or moderate cumulus cell expansion (Fig. 4A–C). Most COCs with fully expanded cumulus cells originated from follicles with optimal steroid concentrations (43/49; Fig. 4D). All blastocysts (22/22) and most embryos at the eight-cell stage or larger (16/18) originated from COCs with full cumulus cell expansion. Oocytes that did not cleave or did not develop beyond the eight-cell stage originated mostly from COC without full cumulus cell expansion (49/56 and 14/18, respectively). Consequently, the degree of cumulus cell expansion was a good predictor of blastocyst production (Table 1).

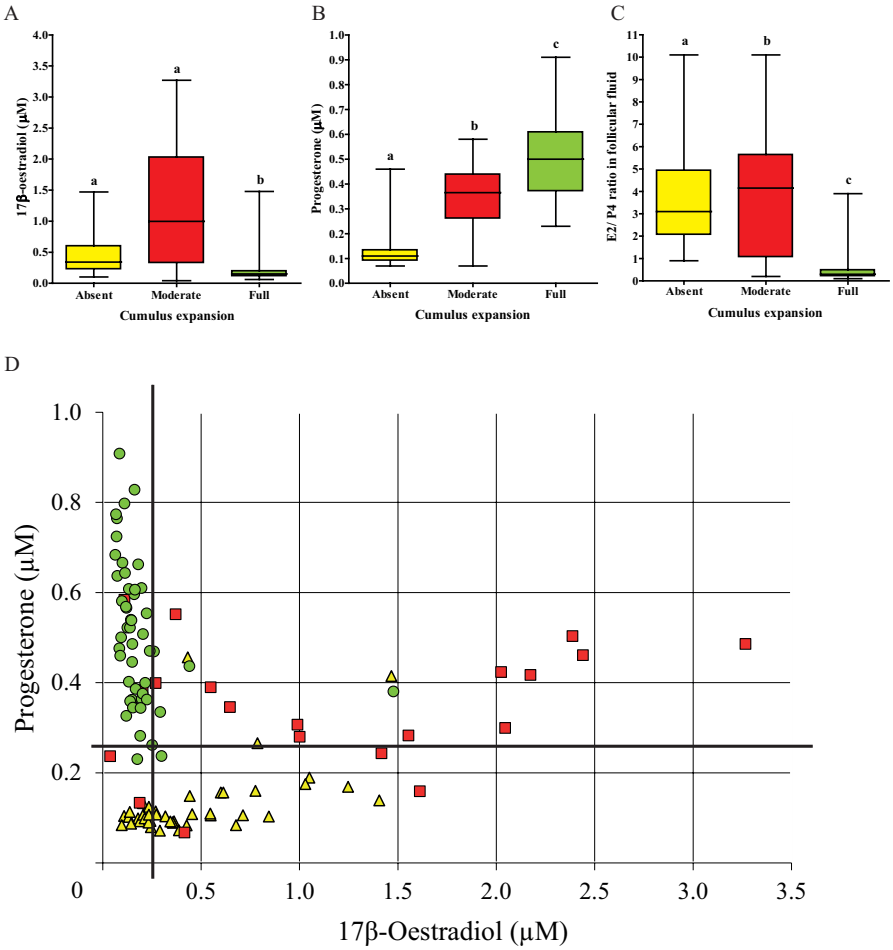

Figure 4. Box and whisker plots of follicular 17β-estradiol (A) and progesterone (B) concentrations and (C) 17b-estradiol:progesterone ratio according to cumulus cell expansion (absent, N = 45; moderate, N = 20; full, N = 49). Values with different letters (a–c) differ (P < 0.005). (D) Scatter plot of 17β-estradiol and progesterone concentrations for individual follicles according to cumulus cell expansion; absent (triangles), moderate (squares), and full (circles). The lines mark the concentrations of 17β-estradiol 0.25 μM and progesterone 0.26 μM. E2, 17β-estradiol; P4, progesterone.

DISCUSSION

With the use of individual in vitro fertilization and embryo culture, we demonstrated for the first time that the competence of in vivo-matured oocytes is associated with follicular fluid steroid concentrations and the degree of cumulus cell expansion. A combination of 17β-estradiol ≤ 0.25 μM and progesterone ≥ 0.26 μM or full cumulus cell expansion were both good predictors of blastocyst

production, with high sensitivity and negative predictive values, good specificity, and moderate positive predictive values. Conversely, follicular diameter was a poor predictor of oocyte developmental competence.

Near the time of ovulation, the fluid of preovulatory follicles of nonstimulated and superstimulated cows have decreased 17β-estradiol and elevated progesterone con- centrations [8,34]. The defined optimal steroid levels are in the same range as the levels from another study of superstimulated cows, but appear to be lower than the levels measured in the preovulatory follicles of nonstimulated cows [9,14,35]. When the reported optimal steroid levels of nonstimulated cows were used as criteria for selection of COC from the superstimulated heifers in our study, the ability to predict blastocyst development was dimini- shed because of the differences in absolute concentration levels. These observations demonstrate that independent hormone criteria are needed for superstimulated cows to select follicles with optimal steroid concentrations from the total pool of follicles and improve the ability to predict the number of produced embryos [36,37].

Full cumulus cell expansion was observed almost exclusively in COCs from follicles with optimal steroid concentrations, suggesting that hormonal changes in follicular fluid are associated with cumulus expansion. Receptors for 17β-estradiol and progesterone are expressed on theca, granulosa, and cumulus cells, and the oocyte [19,38–40]. The expression levels of progesterone receptors change dynamically during the maturation of bovine COCs [38]. In the rat, a short and rapid increase in progesterone receptor expression occurs in granulosa cells, induced by the LH peak, indicating a role for progesterone during follicular and oocyte maturation [38,41]. Granulosa cells of progesterone- receptor knockout mice show reduced mRNA and protein levels of epidermal growth factor-like factors that play key roles in cumulus cell expansion [42]. The dependence on progesterone for cumulus cell expansion was also demonstrated by reduced expansion observed after inhibition of progesterone synthesis during in vitro maturation of bovine COCs [38]. The correlation of full cumulus cell expansion with high progesterone and low 17β-estradiol levels in follicular fluid indicates that optimal steroid levels are important for proper cumulus cell expansion. The crucial role of cumulus cells to support the oocyte during maturation is well known [1]. Whether the absence of proper cumulus cell expansion accounted for the impaired development of oocytes in the nonoptimal steroid conditions is unknown.

Interestingly, the development until the eight-cell stage was a discriminating point in our study with all blastocysts and almost all embryos of eight cells or more developing from COCs that originated from follicles with optimal steroid

conditions. This is of specific interest because the embryonic genome is activated at the eight-cell stage in bovine embryos [43,44]. Blastocyst development did not occur when oocytes originated from follicles with a 17β-estradiol concentration of >0.25 μM and a progesterone concentration <0.26 μM. This is in line with the observation that oocytes exposed to 17β-estradiol during maturation exhibited nuclear aberrances and arrested development [18,19]. Likewise, elevated concentrations of 17β-estradiol in follicular fluid might have resulted in impaired meiosis and thus in reduced developmental competence in these oocytes. However, a progesterone concentration <0.26 μM might also have negatively influenced the developmental competence of the oocytes. The presence of a minimal threshold level of progesterone for optimal development of oocytes is in accordance with data of other studies in which progesterone affected germinal vesicle breakdown and nuclear maturation in porcine and bovine oocytes [21,45]. Furthermore, reduced germinal vesicle breakdown and blastocyst development after inhibition of progesterone synthesis during maturation of COCs can be counteracted by supplementation of progesterone [38,45]. The causative relation among steroid concentrations in follicular fluid, cumulus cell expansion, and oocyte developmental competence certainly needs further inves- tigation. The oocyte itself might be a driving force in this delicate relation because oocytes secrete factors that modulate steroidogenesis and differentiation of granulosa cells and promote cumulus cell expansion [1,46].

CONCLUSION

Oocytes that developed into blastocysts after fertilization originated from preovulatory follicles with low 17β-estradiol and high progesterone concentrations and COCs with full cumulus expansion. Steroid levels in follicular fluid and cumulus cell expansion are predictors of blastocyst formation in superstimulated heifers and can be used as selection markers for oocyte competency.

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REFERENCES

1. Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum Reprod Update 2008;14:159–77.
2. Fair T. Mammalian oocyte development: checkpoints for competence. Reprod Fertil Dev 2010;22:13–20.
3. Scaramuzzi RJ, Baird DT, Campbell BK, Driancourt MA, Dupont J, Fortune JE, et al. Regulation of folliculogenesis and the determination of ovulation rate in ruminants. Reprod Fertil Dev 2011;23: 444–67.
4. Assidi M, Dufort I, Ali A, Hamel M, Algriany O, Dielemann S, et al. Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate in vitro. Biol Reprod 2008;79: 209–22.
5. Sturmey RG, Bermejo-Alvarez P, Gutierrez-Adan A, Rizos D, Leese HJ, Lonergan P. Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. Mol Reprod Dev 2010;77:285–96.
6. Wallace M, Cottell E, Gibney MJ, McAuliffe FM, Wingfield M, Brennan L. An investigation into the relationship between the metabolic profile of follicular fluid, oocyte developmental potential, and implantation outcome. Fertil Steril 2012;97:1078–84.
7. O'Shea LC, Mehta J, Lonergan P, Hensey C, Fair T. Developmental competence in oocytes and cumulus cells: candidate genes and networks. Syst Biol Reprod Med 2012;58:88–101.
8. Dieleman SJ, Bevers μM, Poortman J, van Tol HT. Steroid and pituitary hormone concentrations in the fluid of preovulatory bovine follicles relative to the peak of LH in the peripheral blood. J Reprod Fertil 1983;69:641–9.
9. Callesen H, Greve T, Hyttel P. Preovulatory endocrinology and oocyte maturation in superovulated cattle. Theriogenology 1986;25: 71–86.
10. Fortune JE, Quirk SM. Regulation of steroidogenesis in bovine preovulatory follicles. J Anim Sci 1988;66:1–8.
11. Chaffin CL, Dissen GA, Stouffer RL. Hormonal regulation of steroidogenic enzyme expression in granulosa cells during the periovulatory interval in monkeys. Mol Hum Reprod 2000;6:11–8.
12. Komar CM, Berndtson AK, Evans AC, Fortune JE. Decline in circulating estradiol during the periovulatory period is correlated with decreases in estradiol and androgen, and in messenger RNA for p450 aromatase and p450 17alpha-hydroxylase, in bovine preovulatory follicles. Biol Reprod 2001;64:1797–805.
13. Murphy BD. Models of luteinization. Biol Reprod 2000;63:2–11.
14. Walsh SW, Mehta JP, McGettigan PA, Browne JA, Forde N, Alibrahim RM, et al. Effect of the metabolic environment at key stages of follicle development in cattle: focus on steroid biosynthesis. Physiol Genomics 2012;44:504–17.
15. Chaffin CL, Hess DL, Stouffer RL. Dynamics of periovulatory steroidogenesis in the rhesus monkey follicle after ovarian stimulation. Hum Reprod 1999;14:642–9.
16. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci U S A 1993;90:11162–6.

17. Couse JF, Lindzey J, Grandien K, Gustafsson JA, Korach KS. Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. Endocrinology 1997;138:4613–21.
18. Beker AR, Colenbrander B, Bevers μM. Effect of 17beta-estradiol on the in vitro maturation of bovine oocytes. Theriogenology 2002;58: 1663–73.
19. Beker-van Woudenberg AR, van Tol HT, Roelen BA, Colenbrander B, Bevers μM. Estradiol and its membrane-impermeable conjugate (estradiol-bovine serum albumin) during in vitro maturation of bovine oocytes: effects on nuclear and cytoplasmic maturation, cytoskeleton, and embryo quality. Biol Reprod 2004;70:1465–74.
20. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery Jr CA, et al. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev 1995;9:2266–78.
21. Siqueira LC, Barreta MH, Gasperin B, Bohrer R, Santos JT, Buratini Jr J, et al. Angiotensin II, progesterone, and prostaglandins are sequential steps in the pathway to bovine oocyte nuclear maturation. Theriogenology 2012;77:1779–87.
22. Lopes AS, Butler ST, Gilbert RO, Butler WR. Relationship of preovulatory follicle size, estradiol concentrations and season to pregnancy outcome in dairy cows. Anim Reprod Sci 2007;99:34–43.
23. Perry GA, Smith MF, Lucy MC, Green JA, Parks TE, MacNeil MD, et al. Relationship between follicle size at insemination and pregnancy success. Proc Natl Acad Sci U S A 2005;102:5268–73.
24. Vasconcelos JL, Sartori R, Oliveira HN, Guenther JG, Wiltbank MC. Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. Theriogenology 2001;56:307–14.
25. Chen L, Russell PT, Larsen WJ. Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. Mol Reprod Dev 1993;34: 87–93.
26. Furnus CC, de Matos DG, Moses DF. Cumulus expansion during in vitro maturation of bovine oocytes: relationship with intracellular glutathione level and its role on subsequent embryo development. Mol Reprod Dev 1998;51:76–83.
27. Pieterse MC, Vos PL, Kruip TA, Wurth YA, van Beneden TH, Willemse AH, et al. Transvaginal ultrasound guided follicular aspiration of bovine oocytes. Theriogenology 1991;35:857–62.
28. Bungartz L, Niemann H. Assessment of the presence of a dominant follicle and selection of dairy cows suitable for superovulation by a single ultrasound examination. J Reprod Fertil 1994;101: 583–91.
29. Knijn H, Fokker W, van der Weijden G, Dieleman S, Vos P. Effects of superovulation with oFSH and Norgestomet/GnRH-controlled release of the LH surge on hormone concentrations, and yield of oocytes and embryos at specific developmental stages. Reprod Domest Anim 2012;47:177–83.
30. Vos PL, Bevers μM, Willemse AH, Dieleman SJ. Effects of suppression by a progesterone-releasing intravaginal device and subsequent induction by GnRH of the preovulatory LH surge on follicular function in PMSG/PG-treated heifers. J Reprod Fertil 1994;101:43–9.
31. Dieleman SJ, Bevers μM. Effects of monoclonal antibody against PMSG administered shortly after the preovulatory LH surge on time and number of ovulations in PMSG/PG-treated cows. J Reprod Fertil 1987;81:533–42.

32. de Loos F, van Maurik P, van Beneden T, Kruip TA. Structural aspects of bovine oocyte maturation in vitro. Mol Reprod Dev 1992;31:208– 14.

33. van Tol HT, van Eerdenburg FJ, Colenbrander B, Roelen BA. Enhancement of bovine oocyte maturation by leptin is accompanied by an upregulation in mRNA expression of leptin receptor isoforms in cumulus cells. Mol Reprod Dev 2008; 75:578–87.

34. de Loos FA, Bevers μM, Dieleman SJ, Kruip TA. Follicular and oocyte maturation in cows treated for superovulation. Theriogenology 1991;35:537–46.

35. Dieleman SJ, Kruip TA, Fontijne P, de Jong WH, van der Weyden GC. Changes in oestradiol, progesterone and testosterone concentrations in follicular fluid and in the micromorphology of preovulatory bovine follicles relative to the peak of luteinizing hormone. J Endocrinol 1983;97:31–42.

36. Guilbault LA, Grasso F, Lussier JG, Rouillier P, Matton P. Decreased superovulatory responses in heifers superovulated in the presence of a dominant follicle. J Reprod Fertil 1991;91:81–9.

37. Kafi M, McGowan MR. Factors associated with variation in the superovulatory response of cattle. Anim Reprod Sci 1997;48:137–57.

38. Aparicio IM, Garcia-Herreros M, O'Shea LC, Hensey C, Lonergan P, Fair T. Expression, regulation, and function of progesterone receptors in bovine cumulus oocyte complexes during in vitro maturation. Biol Reprod 2011;84:910–21.

39. Akison LK, Robker RL. The critical roles of progesterone receptor (PGR) in ovulation, oocyte developmental competence and oviductal transport in mammalian reproduction. Reprod Domest Anim 2012;47:288–96.

40. Tománek M, Pisselet C, Monget P, Madigou T, Thieulant M-, Monniaux D. Estrogen receptor protein and mRNA expression in the ovary of sheep. Mol Reprod Dev 1997;48:53–62.

41. Park OK, Mayo KE. Transient expression of progesterone receptor messenger RNA in ovarian granulosa cells after the preovulatory luteinizing hormone surge. Mol Endocrinol 1991;5: 967–78.

42. Shimada M, Hernandez-Gonzalez I, Gonzalez-Robayna I, Richards JS. Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor. Mol Endocrinol 2006;20:1352–65.

43. Memili E, Dominko T, First NL. Onset of transcription in bovine oocytes and preimplantation embryos. Mol Reprod Dev 1998;51: 36–41.

44. Meirelles FV, Caetano AR, Watanabe YF, Ripamonte P, Carambula SF, Merighe GK, et al. Genome activation and developmental block in bovine embryos. Anim Reprod Sci 2004;82–83: 13–20.

45. Yamashita Y, Shimada M, Okazaki T, Maeda T, Terada T. Production of progesterone from de novo-synthesized cholesterol in cumulus cells and its physiological role during meiotic resumption of porcine oocytes. Biol Reprod 2003;68:1193–8.

46. Vanderhyden BC, Macdonald EA. Mouse oocytes regulate granulosa cell steroidogenesis throughout follicular development. Biol Reprod 1998;59:1296–301.

Chapter 3

Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence

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ABSTRACT

Mobilization of fatty acids from adipose tissue during metabolic stress will increase the amount of free fatty acids in blood and follicular fluid and, thus, may affect oocyte quality. In this in vitro study, the three predominant fatty acids in follicular fluid (saturated palmitic and stearic acid and unsaturated oleic acid) were presented to maturing oocytes to test whether fatty acids can affect lipid storage of the oocyte and developmental competence postfertilization. Palmitic and stearic acid had a dose-dependent inhibitory effect on the amount of fat stored in lipid droplets and a concomitant detrimental effect on oocyte developmental competence. Oleic acid, in contrast, had the opposite effect, causing an increase of lipid storage in lipid droplets and an improvement of oocyte developmental compe- tence. Remarkably, the adverse effects of palmitic and stearic acid could be counteracted by oleic acid. These results suggest that the ratio and amount of saturated and unsaturated fatty acid is relevant for lipid storage in the maturing oocyte and that this relates to the developmental competence of maturing oocytes.

INTRODUCTION

High-yielding dairy cows face metabolic stress during the early postpartum period. This results in a negative energy balance (NEB) due to energy loss by milk production that cannot be compensated by energy intake [1–3]. The NEB is believed to impair the fertility of these cows [2–5]. During periods of metabolic stress, massive body fat mobilization increases the free fatty acid concentration (fatty acid com- plexed to albumin) in both blood and follicular fluid [5–11]. The increase in and changed composition of free fatty acids may impair fertility by affecting oocyte quality due to transport of fatty acid into the oocyte [9–11].

After fatty acids are taken up by somatic cells, they are esterified into triacylglycerols (TAG) and cholesterol-esters and stored as neutral lipids in lipid droplets. In the oocyte it has been suggested that neutral lipids fulfil an important function in supplying energy and in biosynthesis of membranes during early embryonic development [12–16]. The esterification of fatty acids and storage into lipid droplets may also protect the oocyte against fatty acid-induced lipotoxicity [17]. In line with this, accumulation of neutral storage lipids in oocytes has been related to improved developmental competence [18, 19]. The fatty acid composition of esterified lipids in porcine, cow, and sheep oocytes is dominated by palmitic, oleic, and stearic acid and mirrors the composition of free fatty acids present in blood and follicular fluid [8, 14, 20]. Furthermore, these fatty acid compositions are comparable with those of the adipose tissue from which they are liberated, suggesting a dynamic fatty acid exchange [21]. The fatty acid composition of high- and low- quality oocytes differs: high-quality oocytes contain more oleic, linoleic, and arachidonic acid [15]. This indicates that the fatty acid composition of oocytes and their environment influences development competence. Moreover, in vitro studies have demonstrated differences in the tolerance somatic cells have of different fatty acids, saturated fatty acids being toxic and unsaturated fatty acids being relatively harmless [17, 22– 25]. To examine the impact of saturated and unsaturated fatty acids on bovine oocytes, they were exposed to saturated palmitic or stearic acid and unsaturated oleic acid during in vitro maturation. After 23 h of maturation, the lipid droplets and postfertilization developmental competence of oocytes were examined. Exposure to saturated palmitic or stearic acid was compared with exposure to unsaturated oleic acid, since these fatty acids are the most prominent fatty acids in follicular fluid of early postpartum cows [8].

MATERIAL AND METHODS

Reagents and antibodies

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Collection of oocytes

Bovine ovaries were collected from a local abattoir and transported to the laboratory within 2 h after withdrawal. Ovaries were washed in physiological saline (0.9% NaCl) and kept in physiological saline with 0.1% (v/v) penicillin-streptomycin (Gibco BRL, Paisley, U.K.) at a temperature of 30°C. Follicles ranging from 3 to 8 mm were aspirated under low vacuum by a suction pump with a 19-gauge needle and allocated to a 50-mL conical tube. Cumulus oocyte complexes (COCs) with a minimum of three layers of cumulus were selected and first washed in HEPES-buffered M199 (Gibco BRL) and subsequently washed and cultured in M199 maturation medium (Gibco BRL) supplemented with 2.2 mg/mL NaHCO3.

Selected COCs were cultured in four-well culture plates (Nunc A/S, Roskilde, Denmark) containing maturation medium (M199 supplemented with 0.02 IU/mL follicle-stimulating hormone [Sioux Biochemical Inc., Sioux Center, IA]), 0.02 IU/mL luteinizing hormone (Sioux Biochemical Inc.), 7.71 μg/mL cysteamine, 10 ng/mL epidermal growth factor in 0.1% (w/v) fatty acid- free bovine serum albumin (BSA) and 1% (v/v) penicillin-streptomycin (Gibco BRL). The oocytes were matured in groups of 35 COCs in 500 μl and incubated under a humidified atmosphere of 5% CO2 in air for 23 h at 39°C.

In vitro maturation media with palmitic, stearic, andlor oleic acid

COCs were exposed to palmitic, stearic, or oleic acid in a concentration of 0 (control group), 100, 250, or 500 μM fatty acid during the entire maturation period of 23 h. Two maturation groups of COCs were exposed to either a combination of 250 μM palmitic and 250 μM oleic acid or 250 μM stearic and 250 μM oleic acid. Fatty acid-free BSA was prepared after charcoal treatment according to Chen [26] to liberate all fatty acids (> 99.9%; own data not shown) as well as contaminating metabolic components such as remnant citrate, pyruvate, and lactate [27]. The resulting fatty acid-free BSA (2.35 mM) was complexed to 12 mM fatty acid in 20% KOH to obtain a 10.7-mM solution of fatty acid (albumin has five binding pockets for fatty acid, and the conditions were set to saturate albumin with the fatty acid of choice). A concentration of either 10

mM palmitic, stearic, or oleic acid was bound to 10% (w/v) fatty acid-free BSA (fatty acid:BSA ratio of 5:1). The used concentrations of fatty acid in this study were based on in vivo-measured individual and total fatty acid concentrations in follicular fluid at Day 16 after parturition, during the NEB of the cow [8].

Determination of uptake and incorporation of radio- labeled palmitic or oleic acid
COCs were matured in 500 µl maturation medium and exposed to a concentration of 100 µM palmitic and 2.5 µCi [9, 10 (n) - 3H] palmitic (60 Ci/ mmol; NEN, Boston, MA) or 100 µM oleic acid and 2.5 µCi [9, 10 (n) - 3H] oleic acid (7 Ci/ mmol; Amersham Pharmacia Biotech, Little Chalfont, U.K.) for 23 h. In this study a total of 180 COCs was used in two independent runs. After maturation COCs were denuded via vortexing and washed four times in PBS. Lipid extraction was performed on 45 oocytes per sample. Lipids were extracted with chloroform-methanol according to the method of Bligh and Dyer [28]. Briefly, chloroform and methanol (2:1, v/v) were added to the oocytes with 0.8 mL PBS and mixed. After separation of the alcoholic phase by centrifugation (5 min, 3000 x g), the chloroform phase was collected. This procedure was repeated three times, and the chloroform phase was evaporated. The lipid extract was redissolved in chloroform and methanol (2:1, v/v) for thin-layer chromatography. Thin-layer chromatography was performed on prefab silica plates (HPTLC grade; Merck, Darmstadt, Germany) in a solvent system of hexane/diethyl ether/acetic acid 80:20:2 (v/v/v) at room temperature for characterization of neutral lipids. References of TAG, cholesterol-ester, diacylglycerol, and cholesterol were run in the same analysis to determine the exact position of the different fractions on the plate. Appropriate non-labeled pure lipid extract was added to the oocyte lipids to provide enough material for detection on the thin-layer plates. [3H] containing spots were scraped off, and radioactivity in the spots was measured by liquid scintillation counting.

Lipid droplet staining of oocytes
After maturation, oocytes were fixed and stained with a specific neutral lipid stain [29] for lipid droplet analysis for each oocyte. Matured COCs were washed twice in PBS and denuded by vortexing in PBS with 0.05 mg/mL fatty acid-free BSA for 4 min. Denuded oocytes were then washed again in PBS and fixed in 4% (v/v) paraformaldehyde (PF; Electron Microscopy Sciences, Hatfield, PA) at 37°C for 1 h and stored in 1% (v/v) PF at 48C for a maximum of 1 wk. Oocytes were washed twice in PBS with 0.3% (w/v) polyvinylpyrrolidone (PVP), permeabilized for 30 min in PBS with 0.1% (w/v) saponin (PBS-S; Riedel-de Haën, Seelze,

Germany) and 0.1 M glycin (Merck) and washed in PBS-S. To determine the maturational stage, oocytes were stained with 10 µg/mL TO-PRO-3 (Molecular Probes, Eugene, OR) for 20 min and subsequently three times washed in PBS-S. After this, neutral lipids in lipid droplets were stained according to a modified protocol of Chinese hamster ovary cells [30]. Lipid droplets were stained with the specific neutral lipid stain BODIPY 493/503 (Molecular Probes) in PBS (20 µg/mL, 1 h), and oocytes were washed three times in PBS with 3 mg/mL PVP. Oocytes were then mounted in a 0.12-mm eight-well Secure-Seal Spacer (Molecular Probes) on a glass slide (Superfrost Plus; Menzel, Braunschweig, Germany), covered in Vectashield (Vector Laboratories, Burlingame, CA), and sealed with a microscope slide (Superfrost Plus). In this study a total of 1340 COCs was used for lipid droplet analysis in at least three independent runs.

Immunofluorescent staining for adipose differentiation-related protein

Immunolabeling was performed on oocytes as previously described [31]. Rabbit polyclonal antibodies against adipose differentiation-related protein (ADRP) were purchased from Abcam (Ab52355; Cambridge, U.K.). Both primary and secondary antibodies were diluted in blocking buffer and centrifuged at 100000 x g for 1 h before use to prevent inclusion of aggregated antibodies in the immunostaining procedure. Oocytes were incubated with primary antibody overnight at 48C. As negative controls, purified mouse or rabbit IgG (BD Biosciences, San Jose, CA) matching the host species of primary antibodies was used, and the dilutions of negative controls were identical to the dilutions of the primary antibodies used in the same experiment. Oocytes were rinsed three times in PBS after primary antibody incubation. ADRP-labeled oocytes were subsequently incubated with Alexa- 568-conjugated goat anti-rabbit IgG (Molecular Probes) for 1 h at room temperature, followed by the above-described neutral lipid and DNA staining.

Fluorescent imaging of oocytes

Confocal microscopy was performed by using a Bio-Rad Radiance 2100 MP setup (Zeiss/Bio-Rad, Hertfordshire, U.K.) attached to a Nikon Eclipse TE300 inverted microscope (Nikon, Badhoevedorp, The Netherlands) at a magnification of 403 (1.25 NA). BODIPY 493/503, TO-PRO-3, and conjugated Alexa-568 were sequentially excited by argon laser (488 nm), red-helium neon diode laser (637 nm), and green 568-nm line. Images were acquired using LaserSharp 2000 software (Zeiss/Bio-Rad). Nuclear stage of oocytes was determined and oocytes were classified as germinal vesicle, metaphase-I (from germinal vesicle

breakdown up to metaphase-I plate), or metaphase-II (from anaphase-I up to metaphase-II plate). The middle part of the oocyte was determined by defining the top and bottom position of the oocyte with LaserSharp 2000 software measurements, and three defined slices of the oocyte (middle part included) were measured. The middle part was investigated to ensure comparable images for each oocyte, representing the middle region of the oocyte. The three slices at a distance of 10 μm were highly correlated for lipid droplet number and mean lipid droplet size (in μm2), with a stack of images at a distance of 5 μm through the whole oocyte ($R \geq 0.8$), secured to give a reliable picture of the whole oocyte.

Lipid droplet analysis

The 8-bit grayscale images of the three slices per oocyte were imaged with ImageJ (NIH; http://rsb.info.nih.gov/ij/) software. From the matured groups only metaphase II stage oocytes were analyzed. Lipid droplets were analyzed from a size of 0.3 μm2 (equal to four pixels); this minimal threshold was set to overcome false positive counting from background pixels. From this the number of lipid droplets and the size per individual droplet (in μm2) from the three slices per oocyte could be calculated. Metaphase-II oocytes from the control medium of the experiments with oleic and/or palmitic acid differed in the number of lipid droplets from metaphase-II oocytes, compared to the control medium of the experiments with stearic acid and with the combination of stearic and oleic acid.

Immunoblotting

Cumulus-free oocytes were obtained as previously described and directly lysed in an appropriate amount of lithium dodecyl dulfate loading buffer (Invitrogen, Carlsbad, CA) in the presence of 0.1 M dithiothreitol. For ADRP protein detection, 50 oocytes were used per sample (corresponding to approximately 6 μg of total protein [32]). The sample was heated for 5 min at 1008C prior to immunoblotting. Proteins were separated in a 4% stacking and 12% running SDS-PAGE gel and wet blotted onto polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, U.K.). After blocking for 1 h with ReliaBLOT (Bethyl Laboratories, Inc., Montgomery, TX) at room temperature, blots were incubated with primary antibodies diluted in PBS with 0.2 % v/v Tween-20 (PBS-T) and 1% BSA overnight at 48C. After washing the blot in PBS-T, secondary antibodies were added for 1 h. After rinsing with PBS-T, protein was visualized using chemiluminescence (ECL-detection kit; Supersignal West Pico; Pierce, Rockford, IL). The ADRP antibody was raised against a human

peptide fragment with full amino acid sequence homology. Monoclonal mouse a- tubulin antibody (clone DM1A) was obtained from Sigma Chemical Co.

In vitro embryo production

Following maturation, COCs were fertilized in vitro in groups of 35. Procedures for in vitro fertilization were performed as described by Parrish et al. [33] with minor modifications [34]. Briefly, sperm cells were added to the fertilization medium (modified Tyrode's medium also called Fert-TALP [33], without glucose and 1% (v/v) penicillin-streptomycin instead of gentamycin [34]) to a final concentration of 0.25 x 106 sperm cells/ml in the presence of 10 μg/ml heparin, 20 μM d-penicillamine, 10 μM hypotaurine, and 1 μM epinephrine (t 1⁄4 0). After 20 h of incubation, cumulus cells of presumptive zygotes were removed by vortexing for 3 min and groups of 35 presumptive zygotes were transferred to 500 μl synthetic oviductal fluid medium supplemented with essential and nonessential amino acids and 0.1% BSA (w/v) (SOF medium; [35]) at 398C in a humidified atmosphere of 5% CO2 and 7% O2 in air. At 5 days postfertilization, all cleavage stages were transferred to fresh SOF medium, and the proportion of cleavage stages was scored per group. At 8 days postfertilization, the number of blastocysts was scored. Note that the whole culture was performed in the absence of fetal calf serum [35]. In total, 5300 COCs were used for the culture experiments in at least three independent runs.

Scoring of oocyte developmental competence after in vitro maturation with palmitic, stearic, andlor oleic acid

At 5 days postfertilization, the number of cleaved embryos, and embryos with 8 or more cells (≥ 8-cell embryos) were scored. At 8 days postfertilization, the number of blastocysts was determined. Results presented here are described as the percentage rates of cleavages, ≥ 8-cell embryos, or blastocysts from the original metaphase-II oocytes used for in vitro embryo production.

Statistical analysis

Statistical analysis was performed in SPSS version 16.0 (SPSS Inc., Chicago, IL) with condition and experimental run as fixed factors. Analysis of lipid droplet size and number was done by general linear modeling with Bonferroni correction. The experiments with stearic acid were analyzed apart fromoleic and palmitic acid since lipid droplets in the pool of oocytes differed. Lipid droplet size was analyzed after transformation to the natural logarithm to achieve a normal distribution. Analysis of oocyte developmental competence (maturation

rate, cleavage, ≥ 8-cell embryos and number of blastocysts) was performed with logistic regression for grouped data. $P < 0.05$ was considered statistically significant.

RESULTS

Maturing oocytes actively take up and metabolize fatty acids

In order to determine the capacity of oocytes to incorporate fatty acids in their lipid droplets, maturing oocytes were exposed to radio-labeled palmitic or oleic acid (total fatty acid concentration was 100 μM). After lipid extraction and thinlayer chromatographic separation, the radioactivity was measured in the isolated lipid fractions of the oocytes. Under both conditions almost all radioactivity was detected in TAG (storage lipid) as well as in phospholipids/ fatty acid oxidation products, showing active uptake and metabolism of the fatty acids by the oocytes (Table 1). Only < 5% of the incorporated radioactivity was recovered in the free fatty acid fraction, whereas > 95% of the fatty acids were metabolized (Table 1). The oocytes took up a smaller amount of [^{3}H] palmitic acid than [^{3}H] oleic acid, with a difference of 30 ± 5%.

Table 1. Fatty acids taken up by maturing bovine oocytes are nearly completely metabolized.

Oocytes	Radioactivity (%)	
Lipid fraction	100μM + [^{3}H] palmitic acid	100μM + [^{3}H] oleic acid
Cholesterol-esters	2 ± 0.7	2 ± 0.5
Triacylglycerol	50 ± 0.4	36 ± 0.4
Free fatty acid	5 ± 0.6	4 ± 0.8
Diacylglycerol	12 ± 1.6	14 ± 1.1
Phospholipids and oxidation products of fatty acids	31 ± 4.3	45 ± 1.0

Distribution of radioactivity in different lipid fractions from oocytes after 23 hours of maturation with 100μM unlabeled [9, 10 (n) - ^{3}H] palmitic acid or 100μM unlabeled [9, 10 (n) - ^{3}H] oleic acid. As is indicated most of the taken up fatty acids are actively metabolized by the maturing oocyte. Results are presented as mean ± standard deviation.

Lipid droplets are identified with a specific neutral lipid stain

Lipid droplets, the storage reservoir of esterified fatty acids (stored either as TAG or as cholesterol esters) in cells, were identified with a neutral lipid stain. To determine the specificity of the neutral lipid stain for lipid droplets, oocytes were coimmunolabeled with an antibody against the lipid dropletspecific protein ADRP [36]. Figure 1 shows the presence of lipid droplets in a metaphase II oocyte. The merges depict that the BODIPY-neutral, lipid-stained structures clearly show red immunolabeling on their surfaces for ADRP; this validates that these structures are lipid droplets (Fig. 1A). The ability of the ADRP antibody to recognize bovine ADRP was demonstrated by immunoblotting (Fig. 1B).

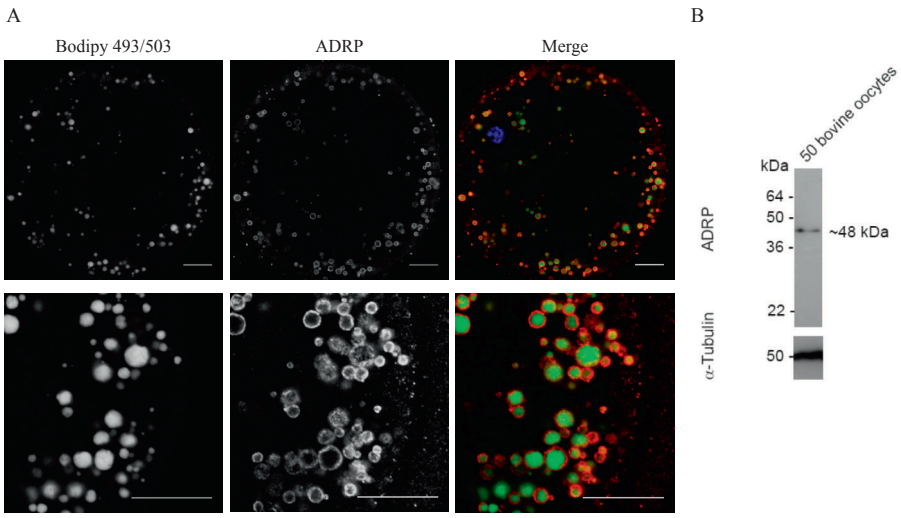

Figure 1. Confocal images of mature oocytes at metaphase II stage after maturation in control medium (A). Merges show lipid droplets in green, ADRP in red, and DNA in blue. Bar = 20 µm. ADRP coats lipid droplets in bovine oocytes and colocalizes with BODIPY 493/503 labeling. Western blot (B). Fifty cumulus-free oocytes were used per sample loading lane. The ADRP observed band at ;48 kDa corresponds to the predicted size according to the manufacturer. α-Tubulin was used as loading control.

High levels of palmitic and stearic acid induce a reduction of lipid storage in maturing oocytes

Given the fact that maturing oocytes took up fatty acids from their environment and that they actively metabolized these fatty acids, we have investigated whether exposure to low, middle, and high fatty acid levels (100, 250, and 500 µM, respectively) affected oocytes' lipid storage and developmental capacity. Oocytes were matured in vitro, and lipid droplets were identified with the above-

described specific neutral lipid stain while a DNA stain was used to determine the stage of oocyte maturation. In control matured oocytes, the number of lipid droplets was slightly but significantly increased in metaphase II-stage oocytes compared to the germinal vesicle stage, but the size of the lipid droplets remained unchanged (Fig. 2).

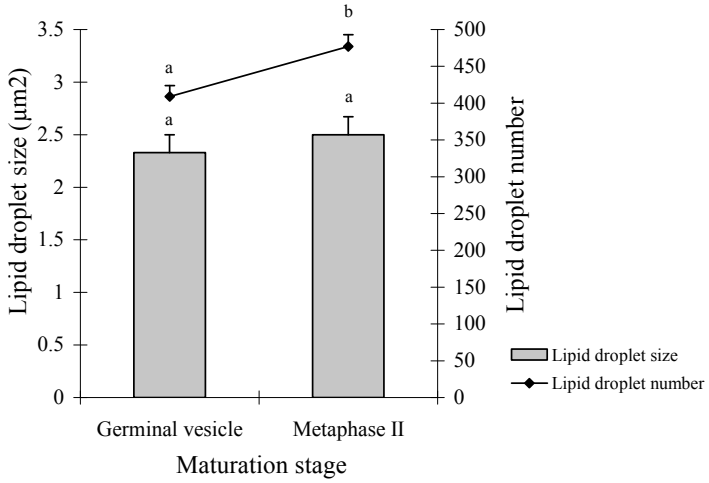

Figure 2. Lipid droplet size (µm2) and total number of lipid droplets per immature and mature oocyte (metaphase-II stage) after maturation in control medium. The number of lipid droplets increased slightly but significantly during maturation in control medium, whereas size of lipid droplets was unaffected. Results are presented as mean ± SEM. Data with different letters differ significantly ($P \leq 0.05$).

Additional exposure to oleic acid (500 µM) resulted in a significantly increased number and size of lipid droplets in oocytes after maturation (Figs. 3 and 4, A and D). In contrast, the number and size of lipid droplets in oocytes that were exposed to either palmitic or stearic acid during maturation were significantly reduced or tended ($P = 0.059$ and $P = 0.056$) to reduce (Figs. 3 and 4, B, C, E, and F). Remarkably, oocytes matured in the presence of a combination of palmitic (250 µM) and oleic acid (250 µM) tended to have a higher number of lipid droplets ($P = 0.074$) compared to palmitic acid alone (250 µM; Fig. 4E). Oocytes matured in the presence of a combination of stearic and oleic acid (250 µM) had a significantly increased lipid droplet size compared to stearic acid alone (250 µM; Fig. 4C).

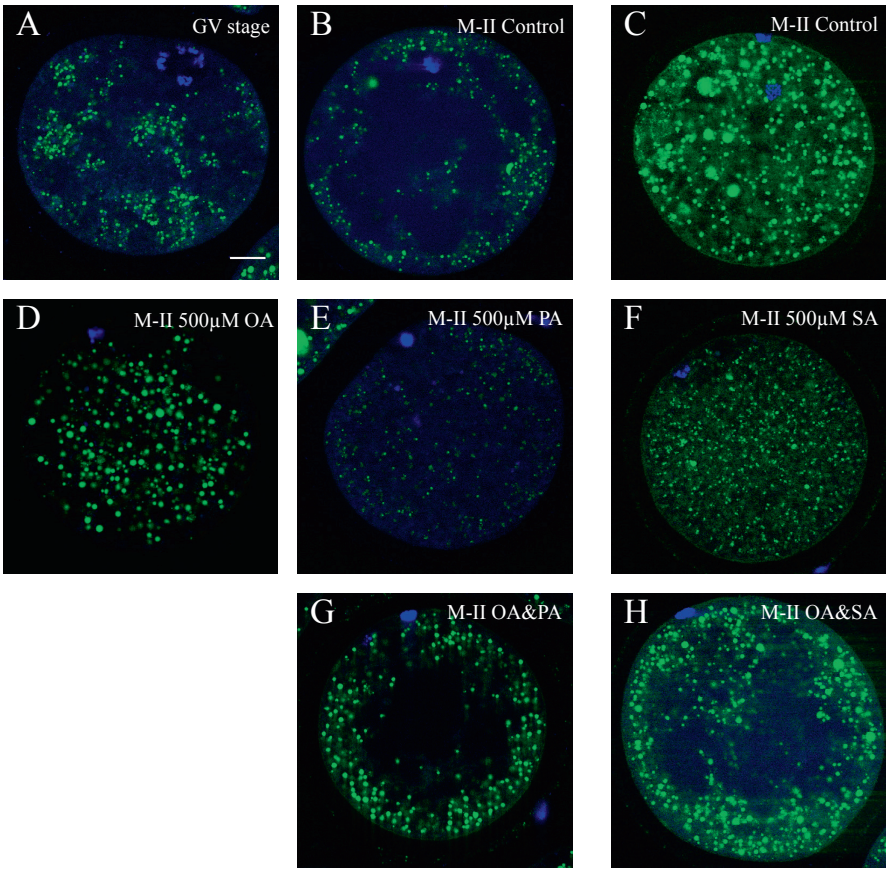

Figure 3. Confocal images of immature oocytes at the germinal vesicle stage (A) and mature oocytes at the metaphase II stage after maturation in control medium during experiments of oleic and palmitic acid (B) and maturation in control medium during experiments with stearic acid (C). Mature oocytes at metaphase II stage after exposure to 500 µM oleic (D), 500 µM palmitic (E), 500 µM stearic acid (F), a combination of 250 µM palmitic and oleic acid (G), or a combination of 250 µM oleic and stearic acid during maturation (H). Merges show lipid droplets in green and DNA in blue. Bar = 20 µm. Note the reduced amount of BODIPY 493/503 labeling in E and the increased labeling in D. PA, palmitic acid; SA, stearic; OA, oleic acid; MII, metaphase-II oocytes.

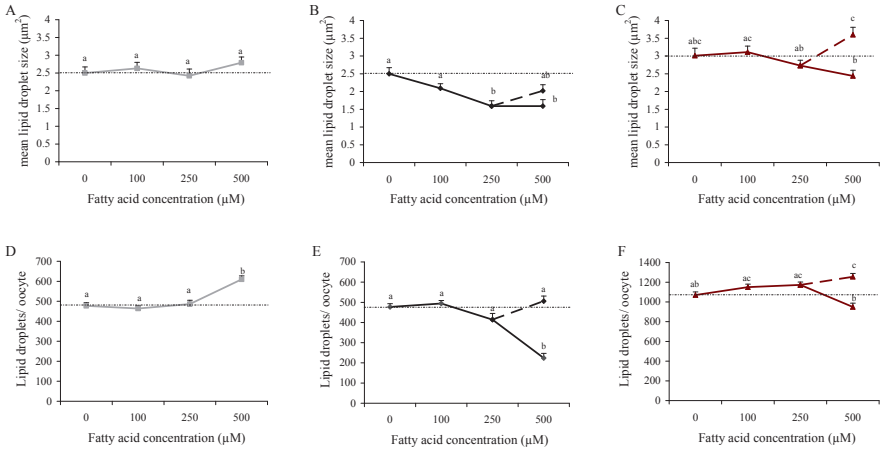

Figure 4. The mean size (A–C) and number of lipid droplets (D–F) in oocytes after exposure to different concentrations of oleic acid (A and D), palmitic acid (B and E), a combination of palmitic and oleic acid (dashed line in B and E) and stearic acid (C and F), or a combination of stearic and oleic acid (dashed line in C and F) during maturation. Note the decrease in both number and size of lipid droplets after palmitic acid or stearic acid exposure (P = 0.056 and P = 0.059) and the compensation of this effect by combining palmitic or stearic acid with oleic acid. Unexpectedly, the number of lipid droplets in the batch of oocytes (including control oocytes) used for the stearic acid experiments was higher than the batch used for palmitic and oleic acid experiments (F). Although we cannot explain these differences, they can be attributed to a number of environmental influences (such as season). Results are presented as mean ± SEM. Data with different letters differ significantly (P ≤ 0.05).

Palmitic and stearic acid exposure impairs postfertilization developmental competence

Oocytes were exposed to the above-mentioned fatty acid conditions during maturation. After this exposure, the progress of oocyte nuclear maturation and the post-fertilization developmental competence was studied. In all conditions a similar percentage of oocytes reached the metaphase-II stage (approximately 80%), indicating that the fatty acid exposures did not affect oocyte nuclear maturation (Fig. 5, A–C). The post-fertilization development, however, was significantly and dose-dependently reduced after exposure to palmitic or stearic acid during oocyte maturation (Fig. 5, E, F, H, and I). In contrast to palmitic acid and stearic acid, oleic acid did not adversely affect the post-fertilization development and at highest dose even showed a moderate increase in the number of blastocysts at Day 8 of culture (Fig. 5, D and G). Remarkably, the negative effects of exposure to palmitic or stearic acid were completely counteracted by simultaneous exposure to equimolar levels of oleic acid during maturation (both at a concentration of 250 µM) (Fig. 5, E, F, H, and I).

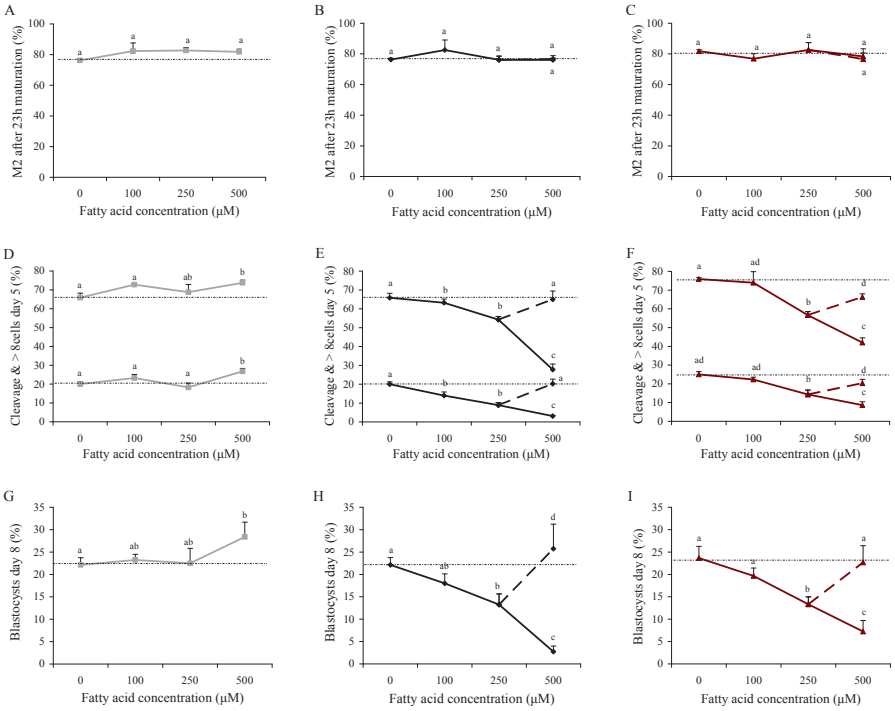

Figure 5. Percentage of metaphase II stage oocytes (A–C), cleavage, and ≥ 8 cells at Day 5 of culture (D–F), and blastocysts at Day 8 of culture (G–I) from oocytes exposed to 100, 250, or 500 µM oleic acid (A, D, and G), palmitic acid (B, E, and H), 250 µM palmitic and oleic acid (dashed line, B, E, and H) and stearic acid (C, F, and G), or 250 µM stearic and oleic acid (dashed line in C, F, and G) during 23 h of maturation. The reduction of developmental competence of oocytes by palmitic or stearic acid exposure and compensation of this adverse effect by oleic acid coincides with the effects noted on lipid droplets (Fig. 4). Results are presented as mean ± SEM. Data with different letters differ significantly ($P \leq 0.05$).

DISCUSSION

The concentration of free fatty acids in blood and follicular fluid increases in cows during the NEB in the early postpartum period due to the mobilization of storage fat in adipose tissue [5–11]. The increased fatty acid levels may cause the observed decrease in fertility. It has been suggested that the changes in fatty acid content of follicular fluid will affect oocyte quality, possibly by influencing its lipid metabolism [9–11]. In line with this we here show that mammalian oocytes indeed efficiently incorporate and metabolize external fatty acids. It has been observed that embryos are capable of taking up fatty acids from the environment [37–39], but our data show that lipid uptake occurs already at an earlier stage. Unfertilized and even immature oocytes are capable of incorporating fatty acids

in their neutral lipid and phospholipid fractions. Therefore, it is possible that the differential NEB-associated fatty acid exposure directly influences the oocyte rather than the reported indirect effects on granulosa and cumulus cells [8, 40]. The amount and size of lipid droplets in oocytes in our experiment were changed after exposure to fatty acids during maturation. This also suggests that fatty acids from the medium directly affect the oocyte. However, the effects on lipid droplets in oocytes largely depended on the type and concentration of the fatty acids to which the oocytes were exposed during maturation. Our study shows that the adverse effects of palmitic and stearic acid as well as the compensatory effects of oleic acid to both saturated fatty acids were comparable in both batches. Exposure to higher concentrations of palmitic or stearic acid during maturation resulted in smaller droplets and at 500 μM to a significantly reduced, or a tendency for a reduced, number of lipid droplets, whereas 500 μM oleic acid resulted in more and larger lipid droplets. Interestingly, the reduction of stored neutral lipids after palmitic or stearic acid exposure was accompanied by a severely impaired postfertilization development of oocytes. Poor incorporation of saturated palmitic acid and efficient incorporation of mono-unsaturated oleic acid in TAG has been described for somatic cell types and may explain the observed difference in lipotoxicity between the saturated and unsaturated fatty acids [17, 22–24]. The ability to efficiently store esterified fatty acids in lipid droplets might deter a rise in lipotoxic effects that could be derived from fatty acids in the cell. This is in line with our observation that a high concentration of oleic acid resulted in an increased storage of neutral lipids and did not affect the developmental competence of exposed oocytes. Accordingly, lipid-rich oocytes were indeed shown to possess better developmental competence [18, 19]. The incorporation of fatty acid in triglycerides can be a method to store energy for the preimplantation development [37, 41–43]. The use of lipids from endogenous reserves during early embryonic development has been indicated by a) a decrease in triglyceride, b) the necessity of b oxidation of fatty acids by mitochondria during development, and c) the potential to develop in the complete absence of exogenous nutrients [13, 15, 16, 37–39, 41, 44–46]. Interestingly, the negative effect of palmitic and stearic acid on oocyte developmental competence was completely counteracted by oleic acid and, moreover, this combination of fatty acids tended to increase the number of lipid droplets and the size of lipid droplets in comparison to only palmitic or stearic acid. The observed increase in neutral lipids was not due to an increase of the fatty acid concentration, since 500 μM of palmitic or stearic acid alone did not increase neutral lipid storage. In addition to the possibility that oleic acid uptake by the maturing oocyte on

its own restored neutral lipid storage, it is also possible that coexposure to the fatty acids caused an oleic acid-dependent metabolic channeling of palmitic and stearic acid into the lipid droplets. The incorporation of palmitic acid in lipid droplets in the presence of oleic acid has been shown in Chinese hamster ovary cells and could explain the improved development of the oocytes, since palmitic acid is channeled away from palmitic acid-induced cell-dependent apoptotic pathways [17, 22, 24, 25, 40, 47]. Mono-unsaturated fatty acids have been reported to prevent palmitate-induced apoptosis by the induction of Bcl-2 and the prevention of mitochondrial release of cytochrome c, or by the competition between fatty acids for transport into the cell or cell metabolism [17, 22–24, 47]. Further studies should clarify the precise mode of action by which palmitic and stearic acid mediate their deleterious effect during maturation of oocytes and the mechanism by which oleic acid prevents the postfertilization lipotoxicity. In conclusion, we have shown that exposure to external fatty acids modulates lipid storage in maturing oocytes. Palmitic and stearic acid caused a decrease in lipid storage and reduced postfertilization developmental competence. Oleic acid had no adverse effect at high dosage but caused a slight increase in lipid storage and postfertilization development. Oleic acid was also capable of compensating for the adverse effects of palmitic and stearic acid. In accordance, this implies that not only the concentration, but more importantly the ratio of saturated and unsaturated fatty acid in follicular fluid affects the developmental competence of the oocyte.

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REFERENCES

1. Rukkwamsuk T, Kruip TA, Wensing T. Relationship between overfeeding and overconditioning in the dry period and the problems of high producing dairy cows during the postparturient period. Vet Q 1999; 21:71–77.
2. Walters AH, Bailey TL, Pearson RE, Gwazdauskas FC. Parity-related changes in bovine follicle and oocyte populations, oocyte quality, and hormones to 90 days postpartum. J Dairy Sci 2002; 85:824–832.
3. van Knegsel AT, van den Brand H, Dijkstra J, Tamminga S, Kemp B. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. Reprod Nutr Dev 2005; 45:665–688.
4. Britt JH. Impacts of early postpartum metabolism on follicular development and fertility. Bov Proc 1992; 24:39–43.
5. Butler WR. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livestock Prod Sci 2003; 83:211–218.
6. van den Top AM, Wensing T, Geelen MJ, Wentink GH, van't Klooster AT, Beynen AC. Time trends of plasma lipids and enzymes synthesizing hepatic triacylglycerol during postpartum development of fatty liver in dairy cows. J Dairy Sci 1995; 78:2208–2220.
7. Jorritsma R, de Groot MW, Vos PL, Kruip TA, Wensing T, Noordhuizen JP. Acute fasting in heifers as a model for assessing the relationship between plasma and follicular fluid NEFA concentrations. Theriogenology 2003; 60:151–161.
8. Leroy JL, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G, Van Soom A. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. Reproduction 2005; 130:485–495.
9. Roche JF. The effect of nutritional management of the dairy cow on reproductive efficiency. Anim Reprod Sci 2006; 96:282–296.
10. Fouladi-Nashta AA, Gutierrez CG, Gong JG, Garnsworthy PC, Webb R. Impact of dietary fatty acids on oocyte quality and development in lactating dairy cows. Biol Reprod 2007; 9–17.
11. Leroy JL, Van Soom A, Opsomer G, Goovaerts IG, Bols PE. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? Part II: mechanisms linking nutrition and reduced oocyte and embryo quality in high-yielding dairy cows. Reprod Domest Anim 2008; 43:623–632.
12. Kruip TAM, Cran DG, Beneden TH, Dieleman SJ. Structural changes in bovine oocytes during final maturation in vivo. Gamete Res 1983; 8:29–47.
13. Ferguson EM, Leese HJ. Triglyceride content of bovine oocytes and early embryos. J Reprod Fertil 1999; 116:373–378.
14. McEvoy TG, Coull GD, Broadbent PJ, Hutchinson JS, Speake BK. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. J Reprod Fertil 2000; 118:163–170.
15. Kim JY, Kinoshita M, Ohnishi M, Fukui Y. Lipid and fatty acid analysis of fresh and frozen-thawed immature and in vitro matured bovine oocytes. Reproduction 2001; 122:131–138.
16. Sturmey RG, O'Toole PJ, Leese HJ. Fluorescence resonance energy transfer analysis of mitochondrial:lipid association in the porcine oocyte. Reproduction 2006; 132:829–837.

17. Listenberger LL, Han X, Lewis SE, Cases S, Farese RV Jr, Ory DS, Schaffer JE. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc Natl Acad Sci U S A 2003; 100:3077–3082.
18. Nagano M, Katagiri S, Takahashi Y. Relationship between bovine oocyte morphology and in vitro developmental potential. Zygote 2006; 14:53–61.
19. Jeong WJ, Cho SJ, Lee HS, Deb GK, Lee YS, Kwon TH, Kong IK. Effect of cytoplasmic lipid content on in vitro developmental efficiency of bovine IVP embryos. Theriogenology 2009; 72:584–589.
20. Homa ST, Racowsky C, McGaughey RW. Lipid analysis of immature pig oocytes. J Reprod Fertil 1986; 77:425–434.
21. Rukkwamsuk T, Geelen MJ, Kruip TA, Wensing T. Interrelation of fatty acid composition in adipose tissue, serum, and liver of dairy cows during the development of fatty liver postpartum. J Dairy Sci 2000; 83:52–59.
22. Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. Diabetes 2001; 50:1771–1777.
23. Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, Donath MY. Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. Diabetes 2001; 50:69–76.
24. Mishra R, Simonson MS. Saturated free fatty acids and apoptosis in microvascular mesangial cells: palmitate activates pro-apoptotic signaling involving caspase 9 and mitochondrial release of endonuclease G. Cardiovasc Diabetol 2005; 4:2.
25. Listenberger LL, Ory DS, Schaffer JE. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. J Biol Chem 2001; 276:14890–14895.
26. Chen RF. Removal of fatty acids from serum albumin by charcoal treatment. J Biol Chem 1967; 242:173–181.
27. Hanson RW, Ballard FJ. Citrate, pyruvate, and lactate contaminants of commercial serum albumin. J Lipid Res 1968; 9:667–668.
28. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; 37:911–917.
29. Gocze PM, Freeman DA. Factors underlying the variability of lipid droplet fluorescence in MA-10 Leydig tumor cells. Cytometry 1994; 17:151–158.
30. Testerink N, van der Sanden MH, Houweling M, Helms JB, Vaandrager AB. Depletion of phosphatidylcholine affects endoplasmic reticulum morphology and protein traffic at the Golgi complex. J Lipid Res 2009; 50:2182–2192.
31. Holzenspies JJ, Stoorvogel W, Colenbrander B, Roelen BA, Gutknecht DR, van Haeften T. CDC2/SPDY transiently associates with endoplasmic reticulum exit sites during oocyte maturation. BMC Dev Biol 2009; 9:8.
32. Grealy M, Diskin MG, Sreenan JM. Protein content of cattle oocytes and embryos from the two-cell to th elongated blastocyst stage at day 16. J Reprod Fertil 1996; 107:229–233.
33. Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988; 38:1171–1180.
34. Izadyar F, Zeinstra E, Colenbrander B, Vanderstichele HM, Bevers MM. In vitro maturation of bovine oocytes in the presence of bovine activin A does not affect the number of embryos. Anim Reprod Sci 1996; 45:37–45.
35. van Tol HT, van Eerdenburg FJ, Colenbrander B, Roelen BA. Enhancement of bovine oocyte maturation by leptin is accompanied by an upregulation in mRNA expression of leptin receptor isoforms in cumulus cells. Mol Reprod Dev 2008; 75:578–587.

36. Listenberger LL, Brown DA. Fluorescent detection of lipid droplets and associated proteins. Curr Protoc Cell Biol 2007; Chapter 24:Unit 24.2.
37. Hillman N, Flynn TJ. The metabolism of exogenous fatty acids by preimplantation mouse embryos developing in vitro. J Embryol Exp Morphol 1980; 56:157–168.
38. Khandoker MAMY, Tsujii H. Metabolism of exogenous fatty acids by preimplantation rabbit embryos. Jpn J Fertil Steril 1998; 43:195–201.
39. Khandoker MAMY, Tsujii H. The metabolism of exogenous fatty acid by preimplantation rat embryos. Asian-Aus J Anim Sci 1999; 12:1181–1187.
40. Mu YM, Yanase T, Nishi Y, Tanaka A, Saito M, Jin CH, Mukasa C, Okabe T, Nomura M, Goto K, Nawata H. Saturated FFAs, palmitic acid and stearic acid induce apoptosis in human granulosa cells. Endocrinology 2001; 142:3590–3597.
41. Ferguson EM, Leese HJ. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. Mol Reprod Dev 2006; 73:1195–1201.
42. Sturmey RG, Hawkhead JA, Barker EA, Leese HJ. DNA damage and metabolic activity in the preimplantation embryo. Hum Reprod 2009; 24:81–91.
43. Sturmey RG, Reis A, Leese HJ, McEvoy TG. Role of fatty acids in energy provision during oocyte maturation and early embryo development. Reprod Domest Anim 2009; 44(suppl 3):50–58.
44. Kane MT. Minimal nutrient requirements for culture of one-cell rabbit embryos. Biol Reprod 1987; 37:775–778.
45. Hewitson LC, Martin KL, Leese HJ. Effects of metabolic inhibitors on mouse preimplantation embryo development and the energy metabolism of isolated inner cell masses. Mol Reprod Dev 1996; 43:323–330.
46. Sturmey RG, Leese HJ. Energy metabolism in pig oocytes and early embryos. Reproduction 2003; 126:197–204.
47. Maedler K, Oberholzer J, Bucher P, Spinas GA, Donath MY. Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. Diabetes 2003; 52:726–733.

Chapter 4

Bovine Cumulus Cells Protect Maturing Oocytes from Increased Fatty Acid Levels by Massive Intracellular Lipid Storage

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ABSTRACT

Metabolic conditions characterized by elevated free fatty acid concentrations in blood and follicular fluid are often associated with impaired female fertility. Especially elevated saturated fatty acid levels can be lipotoxic for several somatic cell types. The aim of this study was to determine the impact of elevated free fatty acid concentrations in follicular fluid on neutral lipids (fatty acids stored in lipid droplets) inside cumulus cells and oocytes and their developmental competence. To this end, cows were exposed to a short-term fasting period during final oocyte maturation. This resulted in elevated, but distinct, free fatty acid concentrations in blood and follicular fluid and a rise in the concentrations of in particular fatty acids with a chain length of 14–18 carbon atoms. Interestingly, elevated free fatty acid concentrations in follicular fluid resulted in a massive increase in the level of neutral lipids in cumulus cells, whereas the level of neutral lipid in oocytes was hardly affected. Furthermore, competence of oocytes to develop to the blastocyst stage after fertilization and culture of cumulus-oocyte-complexes of the experimental and control group was not different. In conclusion these data suggest that short-term elevated free fatty acid concentrations in follicular fluid do not harm oocyte developmental competence. We propose that the involvement of high levels of mobilized oleic acid in follicular fluid in combination with the induced lipid storage in cumulus cells serves to prevent harmful saturated fatty acid exposure to the oocyte.

INTRODUCTION

Metabolic rich or poor conditions, like obesity or a negative energy balance, are related to reduced female fertility and share the metabolic characteristic of elevated free fatty acid levels in blood [1–4]. Free fatty acids in blood and follicular fluid are transported as dynamic fatty acid complexes that form the basis of fatty acid supply from adipose tissue (donor of fatty acids to albumin) to peripheral cells or tissues (acceptor of fatty acids provided by albumin). Major functions of fatty acids derived from either transport, temporally stored neutral lipids or de novo-synthesis, are the use as energy source or the formation of membranes in the cell [5]. However, increased free fatty acid concentrations by enhanced lipolysis of adipose tissue do not only serve as a valuable source for tissues, but can induce lipotoxic effects at the cellular level accompanied by impaired cell function and even cell death in for example the liver, creating fatty liver syndrome [6]. Furthermore, increased levels of free fatty acids in blood result in an increase of free fatty acid concentrations in the follicular fluid, which may affect cumulus-oocyte complex (COC) morphology and embryo quality [1, 3, 7].

Especially an increase in saturated fatty acid levels can result in lipotoxicity by induction of ceramide formation, mitochondrial release of cytochrome-c and caspase activation [8–12]. In contrast high levels of unsaturated fatty acids do not induce such effects in a variety of somatic cell types and even can prevent the lipotoxic effects imposed by increased cellular amounts of saturated fatty acids by stimulating lipid storage and β-oxidation [8–14].

Interestingly, oocytes exposed to saturated fatty acids during in vitro maturation faced a decrease of their postfertilization developmental competence, while in presence of the mono-unsaturated oleic acid a normal developmental competence remained [7, 15, 16]. Oleic acid contains one *cis* C=C atom bond between carbon atoms 9 and 10 and has a chain length of 18 carbon atoms. Oleic acid is thus further designated as C18:1; other fatty acid species likewise will be designated with carbon atom chain length and amount of *cis* C=C atom bonds. Simultaneous exposure of oocytes with saturated palmitic acid (C16:0) or stearic acid (C18:0) and oleic acid (C18:1) counteracted the adverse effects of saturated fatty acids on developmental competence [15]. This strongly indicates that the composition and balance of saturated and unsaturated free fatty acids in follicular fluid is crucial for postfertilization developmental competence of oocytes.

The cumulus cell layer that surrounds the oocyte, and which together with the oocyte forms the COC, is in direct contact with the follicular fluid. The cumulus cells nourish the developing oocyte through gap-junctional cell-cell contacts. The gap-junctions allow bidirectional transport of small metabolites and are instrumental for oocyte maturation and the acquisition of developmental competence [17, 18]. The cumulus cell layer is the first metabolic area that is affected by altered free fatty acid levels in follicular fluid. Indeed, exposure to saturated fatty acids in vitro induces apoptosis in cumulus cells but not in the oocyte [7, 19].

The oocyte that is surrounded by the cumulus cell layer is able to incorporate fatty acids from the medium [15]. Subsequently, the oocyte can store the fatty acids as neutral lipids into lipid droplets. A sign of this storage is the increase in size and amount of lipid droplets in oocytes after exposure to unsaturated free fatty acids [15]. Presumably, after fertilization the stored neutral lipids are important as energy source and as precursors for de novo membrane synthesis during early embryo development [20–22]. Increased free fatty acid concentrations in follicular fluid may alter lipid storage properties of the oocyte, which may adversely influence its later developmental competence.

The aim of this study was to determine the impact of elevated free fatty acid concentrations in follicular fluid on the neutral lipid of cumulus cells and the oocyte and on their developmental competence. To this end, a short-term elevation of free fatty acid concentrations in blood and follicular fluid was induced by a short-term fasting period [23]. We used the cow as animal model because the negative energy balance in the high producing dairy cow is characterized by elevated free fatty acid concentrations and is linked to reduced fertility [24–27]. Like humans, the cow is mono-ovulatory and oocytes of both species are of similar size and have a broadly comparable energy metabolism [28].

We induced the short-term elevation of free fatty acid concentrations during the final growth and maturation phase of oocytes until just before ovulation. The data of this study show the impact of short-term elevated free fatty acid levels in follicular fluid on the lipid composition of the maturing cumulus-oocyte-complex and the impact on developmental competence of the oocyte postfertilization.

MATERIALS AND METHODS

Chemicals

Unless stated otherwise, all chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available. Solvents (acetone, acetonitrile, chloroform, methanol, and hexane) were of high-performance liquid chromatography (HPLC) grade (Labscan, Dublin, Ireland).

Experimental Design

The two independent in vivo experiments in which cows were super-stimulated and experimental cows were exposed to a short-term fasting period during the final maturation period were carried out as approved by the Ethical Committee of the Faculty of Veterinary Medicine of Utrecht University.

The first in vivo experiment was performed to investigate the role of short-term elevated free fatty acid concentrations in the blood on the free fatty acid composition in follicular fluid and the neutral lipid stored in lipid droplets of cumulus cells and oocytes. The second in vivo experiment was performed to investigate the role of short-term elevated free fatty acid concentrations on the developmental competence of in vivo matured COCs and their subsequent in vitro fertilization and culture in a single embryo culture system.

We performed additional in vitro maturation experiments with COCs derived from slaughterhouse ovaries to further investigate the impact of elevated free fatty acid concentrations (based on free fatty acid levels in follicular fluid of control and fasted heifers) on i) neutral lipid stored in lipid droplets of COCs and on ii) the developmental competence of oocytes without cumulus during the last 6 h of the 23 h maturation period. To overcome the potential absence of significant differences among the control and fasted group by the relatively low number of animals, we iii) exposed a high number of COCs in vitro to maturation conditions based on the in vivo conditions and cultured them until the blastocyst stage.

Experimental procedures of animals

Clinically healthy nonlactating Holstein-Friesian heifers (n = 16 per in vivo experiment; n = 32 in total) were selected on normal ovarian cyclicity based on three times weekly blood progesterone concentrations for 4 weeks during the pre-experimental period. Heifers were fed ad libitum with grass silage supplemented with minerals to meet the nutritional requirements and had unlimited access to water. On the basis of back fat thickness [29] and body

condition score (scale 1–5) the heifers were pseudo at random divided among the control (n = 8 per in vivo experiment) and experimental (n = 8 per in vivo experiment) group. Heifers were ranked in order from high till low conditions and in that sequence of order each next heifer was placed in the other group, to allow for groups of heifers with comparable conditions at the start of the experiment. Animals were presynchronized during 7 days with an intravaginal progesterone device (1.38 g progesterone; CIDR; Pfizer Animal Health, New York, NY). One day before removal of the progesterone (P4) device (Day −3), prostaglandin (PGF2α; 25 mg dinoprost i.m.; Enzaprost, Ceva Sante Animale, Libourne, France) was administered to ensure complete regression of a present corpus luteum. On Day 8 of the synchronized cycle (estrus = Day 0) and 2 days before the start of the super-stimulation protocol, predominant follicles (>8 mm) were removed by transvaginal ultrasound-guided follicle aspiration [30], to avoid the inhibitory effects of follicular dominance on the super-stimulation response [31]. Four heifers from the first in vivo experiment were excluded (2 heifers from the control and 2 from the experimental group) because of failed cycle synchronization. At Day 10, heifers received an intravaginal progesterone device and twice daily follicle stimulating hormone (FSH; Folltropin-V; Bioniche Animal Health, Belleville, ON, Canada) in decreasing doses (40, 30, 20, and 10 mg i.m., in total 200 mg of Folltropin-V) during the following 4 days according to our super-stimulation protocol [32]. $PGF_2\alpha$ (25 mg, dinoprost i.m.) was administered together with the fifth dose of FSH. P4 device removal after 4 days at Day 14 was combined with an injection of gonadotropin releasing hormone (GnRH; 1 mg gonadorelin i.m. Fertagyl, Intervet International BV, Boxmeer, The Netherlands) to induce the LH peak [33]. Ovaries were collected at 22 h after the LH peak, just before ovulation, by ovariectomy through a flank incision under local anesthesia (Alfasan Nederland BV, Woerden, The Netherlands). One control heifer of the second in vivo experiment was excluded for further analysis because of a remaining corpus luteum and high P4 level (>2 ng/ml) at the moment of ovariectomy (Day 15). From the second day of super-stimulation onwards until ovariectomy, the experimental group was fasted for 4 days [23] to induce elevated free fatty acid concentrations in blood and follicular fluid during the final follicular growth and maturation phase. Blood was collected from the jugular vein using a Vacutainer system and heparin-coated tubes (Becton Dickinson and Co., Franklin Lakes, NJ) every 2 days throughout the experimental period, on a daily basis during synchronization and super-stimulation treatment for hormonal analysis, and hourly in the first 8 h after GnRH injection to determine the LH concentration in blood [34]. All heifers

showed a peak of the LH concentration at 2h after the GnRH injection. Blood samples for metabolic analysis were collected at the start of the new cycle (Day 0) and at Days 8, 11, 12, 13, 14, and the day of ovariectomy (Day 15) in sodium-fluoride tubes (Vacutainer; Beckton Dickinson) for glucose and serum tubes for β-hydroxybutyric acid (Ranbut kit; Randox Laboratories Ltd., Crumlin, U.K.), total free fatty acid analyses (FA 115 kit, Randox) and high-density lipoprotein (HDL; HDL cholesterol method, Synchron CX System, Beckman Coulter, Galway, Ireland) measurement.

Follicular fluid collection and analysis

Ovaries were collected in 0.9% (w/v) NaCl at 30°C and immediately transported to the laboratory. At the laboratory the content of each follicle was individually aspirated under vacuum using an 18 gauge winged infusion set needle attached to a 15-ml polystyrene conical tube under low pressure by means of a suction pump. Follicles with a diameter of >8 mm, estimated from the volume of follicular fluid, were selected and the concomitant COCs were individually collected from follicular fluid under a stereomicroscope. Follicular fluid was kept on ice at 4°C and was centrifuged at 3000 × *g* for 10 minutes at 4°C and partly stored at −20°C until analysis for steroids and at −80°C for further analysis of metabolites. We selected only those follicles, retrospectively, with intrafollicular steroid concentrations of 17β-estradiol (E2) and P4 that were representative for dominant follicles in unstimulated cycles around the time of ovulation (P4 ≥0.5 μM and E2/P4 <1) [35]. Concentrations of P4 and E2 in follicular fluid were determined in aliquots of 1–25 μl of fluid dependent of the hormone and the size of the follicle by solid phase ^{125}I-labeled radioimmunoassay (RIA; Coat-A-Count, TKPG and TKE2, respectively; Siemens Medical Solution Diagnostics, Los Angeles, CA) according to the manufacturer, as described previously [34] with slight modifications. Briefly, the follicular fluid samples were extracted with 2 ml diethyl ether (Scharlau, Barcelona, Spain). An internal standard of 167 Bq of ^{3}H-labeled steroid was added before extraction in order to determine and correct for the efficiency of extraction. After evaporation of the organic solvent the samples and efficiency series were dissolved in 250 μl of borate buffer (for E2) or in 250 μl of zero plasma of the manufacturer. Duplicate volumes of the samples were then incubated in the antibody-coated tubes (Coat-A-Count). Specificity of the RIA was high as indicated by low cross-reactivity for other steroid hormones of physiological importance (for details see the Coat-A-Count manual). Calculation of all hormone results was done applying the approximation for the standard series from RIA Smart (Packard Instruments

Company, Meriden, CT). The calculated doses were <4% different from the defined doses over the entire range. The intra- and interassay coefficients of variation were <10% for all assays.

Collection of COCs for lipid analysis and single IVF and IVC

For lipid analysis, in vivo matured COCs were rinsed twice in 500 µl of phosphate-buffered saline. Cumulus cells were removed by continuous pipetting after incubation for one minute in PBS containing 15 IU/ml hyaluronidase (Hyason, Organon, Oss, The Netherlands), 0.021 mM trypsin and 0.48 mM EDTA (Gibco BRL, Paisley, U.K.). Denuded oocytes were checked for remaining cumulus cells and washed three times with PBS and stored individually at −80°C until lipid analysis was performed.

For assessment of the developmental competence of the oocytes, COCs were individually collected and washed in 500 µl of oocyte culture medium (MP Biomedicals, Eindhoven, the Netherlands) before they were subjected to the single in vitro fertilization (IVF) and embryo culture. Individual COCs were introduced into a microwell of a tissue culture Terasaki Microwell plate (Nalge Nunc International, Rochester, NY) with fertilization drops consisting of 10 µl of fertilization medium (Fert-Talp) containing 1.8 IU/ml heparin, 20 µM D-penicillamine, 10 µM hypotaurine, 1 µM epinephrine, and 10^6 spermatozoa/ml from frozen-thawed semen washed over a Percoll gradient. All wells were covered with light mineral oil (Irvine Scientific, Santa Ana, CA). Fertilization took place at 38.6°C in a humidified atmosphere with 5% CO_2 in air. After 20 h of co-incubation, presumptive zygotes were denuded by repeated pipetting through a narrow-bored pipette and placed in Terasaki plate microwells with synthetic oviduct fluid medium supplemented with essential and nonessential amino acids and 0.4% (w/v) BSA (SOF medium; [36]). Each microwell contained one presumptive zygote (25 µl of SOF medium/zygote). From Day 1 of culture a medium layer connected the fluid of individual microwells [37]. The plates, all of which contained a separate group of wells for a control and an experimental animal, were covered with 9 ml light mineral oil and incubated at 38.6°C in a humidified atmosphere with 7% O_2, 5% CO_2 and 88% N_2. On Day 5 of embryo culture, cleavage rates and ≥8 cell stage embryos (based on the original number of oocytes) were determined and the cleaved embryos were transferred to fresh SOF medium, according to the standard protocol for embryo culture [36]. From Day 5 onwards, the medium of microwells was connected among embryos of different origin, to ascertain normal developmental conditions irrespective of the number of embryos per animal. Blastocyst rates (based on the original

number of oocytes) were determined on Day 8 of embryo culture. Blastocysts were collected and individually stored in PBS at −80°C. The single embryo culture system results in blastocyst rates comparable to standard group culture, as is routinely validated. As a control for the culture conditions during the single embryo culture, a group of 50 in vitro-matured COCs, originating from slaughterhouse ovaries, was fertilized and cultured in the same media as the individual cultures according to the standard protocol, described below.

IVM with fatty acid mixtures and subsequent fertilization and culture

The in vivo free fatty acid conditions in follicular fluid of control and fasted heifers were mimicked in vitro during the maturation of COCs, derived from slaughterhouse ovaries, followed by fertilization and culture according to the standard protocol [15]. COCs were exposed to the standard maturation medium without free fatty acids or to one of the two fatty acid mixtures during the 23 h maturation period; the first representing the in vivo control condition in follicular fluid with 80 μM C16:0, 70 μM stearic C18:0 and 100 μM C18:1; the second representing the in vivo fasting condition with 150 μM C16:0, 100 μM C18:0 and 200 μM C18:1. Fatty acids (10 mM) were bound to 10% delipidified bovine serum albumin (BSA; in a FA:BSA ratio of 5:1). At Day 5 of embryo culture the number of cleaved zygotes and ≥8 cell stage embryos and at Day 8 the number of blastocysts was scored. In total, 1200 COCs were used for the in vitro maturation experiments, in four independent experimental runs.

Lipid Droplet Staining of COCs after In Vitro Maturation with Fatty Acid Mixtures

Neutral lipid present in lipid droplets of COCs was stained with BODIPY 493/503 (Molecular Probes, Eugene, OR) and DNA with TO-PRO-3 (Molecular probes) according to our standard protocol [15]. Confocal microscopy was performed by using a model TCS SPE-II setup (Leica Microsystems GmbH, Wetzlar, Germany) attached to an inverted semiautomated DMI4000 microscope (Leica) at 20× magnification. BODIPY 493/503 and TO-PRO-3 were sequentially excited by an Ar laser (488 nm) and a red HeNe diode laser (649 nm). Emitted light was selected with emission filters for 530 nm (BODIPY) and >700 nm for TO-PRO-3 and detected on photomultiplier tubes. Image reconstruction was done with LAS_AF software (Leica). The BODIPY fluorescence (AU) in the cumulus cell layer and oocyte was determined after selection of the cumulus cell layer, oocyte and the background region in the middle of each COC (n = 25 per group). The mean amount of BODIPY pixels was determined per area (μm^2) after background correction.

Cumulus Cell Removal and Exposure to Free Fatty Acids after 16 h of Maturation
The cumulus cells of a part of the COCs were removed (denuded oocytes; DOs) after 16h of standard maturation conditions. Subsequently, during the last 6h of maturation DOs and intact COCs were exposed to the standard maturation medium without free fatty acids or the defined elevated free fatty acid concentrations based on the free fatty acid levels in follicular fluid of fasted heifers. 23 h after the start of maturation, DOs and COCs were fertilized and cultured according to the standard protocol described above. At Day 5 of embryo culture the number of cleaved zygotes and ≥8 cell stage embryos and at Day 8 the number of blastocysts was scored. In total, 1825 COCs were used in three independent experiments.

RNA isolation and cDNA synthesis of blastocysts from control and fasted heifers
Total RNA isolation combined with on-column DNase digestion was performed using the RNeasy micro kit and the RNase-free DNase set (Qiagen, Valencia, CA) according to the manufacturer's instructions. The elution of the RNA from the column was done with 18 µl of RNase-free water. Reverse transcription was performed in a total volume of 20 µl of containing 10 µl of RNA sample, 4 µl of 5× reverse transcriptase buffer (Gibco BRL), 0.036 units/µl random primers (Invitrogen, Breda, The Netherlands), 8 units/µl RNA-sin (Promega Benelux, Leiden, The Netherlands), and 150 units of Superscript III reverse transcriptase (Invitrogen) and final concentrations 10 mM DTT and 0.5 mM of each dNTP (Promega). The mixtures were incubated for 45 min at 50°C, for 10 min at 80°C and stored at −20°C.

Primer design and quantitative PCR
The primers used for analysis of the relative mRNA expression of the genes cathepsin B (*CTSB*), placenta-specific 8 (*PLAC8*), and prostaglandin-endoperoxide synthase 2 (*PTGS*) as markers for blastocyst quality [38, 39], and of the reference genes H_2A histone family, member Z (*H2AFZ*) and phosphoglycerokinase (*PGK*) are summarized in Table 1. Each primer of a pair was located on a separate exon. Quantitative Real Time PCR (qPCR) was performed in duplicate on a 96-well plate and the samples were quantified simultaneously using a real time PCR detection system (MyiQ Single color real-time PCR detection system; Bio-Rad Laboratories, Hercules, CA). Standard curves were 3-fold serial dilutions of cDNA from 52 blastocysts synthesized as described above. The reaction mixture (20 µl) contained 0.5 µl (*H2AFZ* and *PGK*) or 1 µl (*PLAC8*, *PTGS*, and *CTSB*) cDNA, 0.5 µM of each primer (Eurogentec,

Seraing, Belgium) and 10 µl of iQ SYBR Green Supermix (Bio-Rad). After an initial denaturation step at 95°C for 3 min, 40 cycles were carried out, each consisting of 95°C for 10 sec, the primer-specific annealing temperature for 10 sec, and 72°C for 20 sec. Melting curves were plotted to determine the purity of the product. The provided application software produced standard curves by plotting the log of the starting amount versus the threshold cycle for detection. The standard curves were subsequently used to calculate the relative starting quantity for each experimental sample by interpolation.

Table 1. Details of primers used for QRT-PCR

Code	Gene	Primer sequence (5' -> 3')	Position	Anneal Temp (°C)	Gene bank accession no.
PLAC8	Placenta-specific 8	GACTGGCAGACTGGCATCTT CTCATGGCGACACTTGATCC	210 – 229 330 - 349	60	NM_001025325.2
PTGS2	Prostaglandin-endoperoxide synthase 2	TGGCTACGGGAACACAACA TGATACTTTCTCTACTGCGACTGG	980 – 998 1388 - 1411	65	NM_174445.2
CTSB	Cathepsin B	GCAACTCCTGGAACACTGAT ACTGATGAGTGCACGGCATT	890 – 909 984 - 1003	63	NM_174031.2
H2AFZ	H2A histone family, member Z	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	162 – 185 347 - 370	60	NM_174809.2
PGK1	Phospho-glycerokinase	CTGGACAAGCTGGATGTGAA AACAGCAGCCTTGATCCTCT	72 – 91 160 - 179	61	BT021601.1

Extraction and isolation of neutral lipids

The total lipid fraction from either in vivo derived oocytes or from their corresponding cumulus cells was extracted according to the method of Bligh and Dyer starting from 200 µl of aqueous phase [40]. The internal standard for triacylglycerol (TAG) and cholesterol, respectively, tripentadecanoin (Larodan Fine Chemicals, Malmö, Sweden), and [25,26,26,26,27,27,27-$^{2}H_{7}$] cholesterol, were included during extraction (1 pmol/oocyte of each standard, 6 pmol per sample of 6 pooled oocytes or pool of corresponding cumulus cells) to calculate recovery and absolute concentrations of extracted lipids. Subsequently, TAG and diacylglycerol (DAG) (further referred to as the neutral lipid fraction) were separated from phospholipids by solid-phase extraction on house made silica gel 60 mini columns (Merck, Darmstadt, Germany) according to Rouser et al. [41]. The neutral lipid fraction was eluted with 3 volumes of acetone [42, 43], dried under a constant stream of nitrogen gas at 40°C, and stored at −20°C until use. For a detailed description of these methods see references [41] and [44].

HPLC mass spectrometry analysis of neutral lipids

Isolated neutral lipids were dissolved in 25 µl of methanol/chloroform (1:1, v/v) and 20 µl was injected on a Halo C8 (150 × 3.0 mm, particle size of 2.7 µm) HPLC column (Advanced Material Technology, Inc., Wilmington, DE) maintained at 40°C. Lipids were eluted by a linear gradient from methanol/water (50:50, v/v) to methanol-2-propanol (80:20, v/v) in 5 min, followed by isocratic elution with the latter solvent for 20 min and regeneration of the column for 5 min, all at a flow rate of 0.3 ml/min. The column effluent was introduced by an atmospheric pressure chemical ionization (APCI) interface (AB Sciex Instruments, Toronto, ON, Canada) into a 4000 QTRAP mass spectrometer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Source temperature was set to 450°C, and nitrogen was used as curtain gas. The declustering potential was set to 100 V and the needle current to 3 µA. Scans were recorded in ion trapping mode. Ions were identified by the automated acquisition of product ion spectra (from *m*/*z* 200 to *m*/*z* 650 amu) in a separate HPLC run using collision energy of 32 V. Mass spectrometer settings were optimized with cells from slaughterhouse ovaries and recorded in full scan positive ion mode (scan range, *m*/*z* 250-1100 amu) to obtain representative lipid profiles of bovine oocytes and their cumulus cells. Based on these results (data not shown), the mass spectrometer was then operated in multiple reaction monitoring (MRM) mode at unit mass resolution and selected ion transitions of the most representative neutral lipid species were monitored. Peaks were identified by comparison of retention time and (product) mass spectra with authentic standards and calibration curves of synthetic analogs of identified compounds.

Extraction and isolation of free fatty acids

The total lipid fraction from 200 µl of blood or 100 µl of follicular fluid was extracted according to the method of Bligh and Dyer [40] and evaporated under a constant stream of nitrogen gas at 40°C. Deuterated palmitic acid [7,7,8,8-$^{2}H_{4}$] (Cambridge Isotopes Laboratories, Inc., MA) was added as internal standard to the samples (10 nmol/sample in follicular fluid, 20 nmol sample in blood) at the beginning of the lipid extraction procedure in order to calculate the recovery and absolute concentrations. Isolation of the free fatty acid pool was obtained according to [45] without the heating step and on ice to avoid the hydrolysis of the free fatty acids and was performed as follows: 1 ml of 0.3 M NaOH in methanol/water (9:1, v/v) was added to a evaporated lipid mixture and unsaponifiables were extracted three times with 1 ml of hexane. After subsequent acidification of the aqueous methanol phase, free fatty acids were extracted with three portions

of hexane, evaporated under a constant stream of nitrogen gas at 40°C and stored at −20°C until analyzed.

HPLC mass spectrometry analysis of free fatty acids

Free fatty acids were dissolved in 100 µl of a methanol/acetonitrile/chloroform/water (46:20:17:17, v/v) solution and injected on a Halo C18 (150 × 3.0 mm, particle size of 2.7 µm) HPLC column (Advanced Material Technology). The injection volume was 40 µl, the temperature of the column was maintained at 40°C. Lipids were eluted by a linear gradient from acetonitrile/methanol/water (6:9:5, v/v) 2.5 mM ammonium acetate to acetone/methanol (4:6, v/v) 2.5 mM ammonium acetate in 15 min, followed by isocratic elution with the latter solvent for 10 min and regeneration of the column for 5 min, all at a flow rate of 0.6 ml/min. Mass spectrometry of free fatty acids was performed using electro spray ionization (ESI) on a 2000 QTRAP system (Applied Biosystems). Source temperature was set to 450°C and nitrogen was used as curtain gas. The declustering potential was set to −40 V. Full scans were performed in negative mode in the m/z range from 225–400 amu. Peaks were identified by comparison of retention time and mass spectra with authentic standard and calibration curves were generated to correct for differences in response factors.

Data processing

Lipid data were recorded with Analyst version 1.4.2 software (MDS Sciex, Concord, ON, Canada) and exported to an mzXML format. Peak detection, integration and alignment were performed using the open source software package XCMS running on R software [46]. A correlation matrix of the combined data sets was calculated in R. Principal component discriminant analysis was performed with Markerview version 1.1 software (MDS Sciex), using Pareto scaling, where each value is subtracted by its average in all samples and divided by the square root of the standard deviation. In this way, data maintains a dimension (units), and peaks with a good signal noise ratio will gain importance, without having intense peaks dominating the analysis.

Statistics

Statistical analysis was performed using R statistical software version 2.15.0 (R development Core Team, 2011). For lipid data, a linear mixed model (http://cran.r-project.org/web/packages/nlme/index.html [47]) was applied with animal (blood and follicular fluid data) or pool (cumulus and oocyte data) as random effect to take the correlation between observations into account.

Explanatory variables are GROUP and MATERIAL and the interaction between both variables. For lipid species detected in either cumulus or oocytes, a two-sample *t*-test for normally distributed data of TAG and DAG molecular species with fatty acid molecular species estrified to glycerol designated with carbon atom length and amount of *cis* C=C carbon bonds as explained for FFA in the *Introduction* with GROUP as random effect was applied or the Mann-Whitney *U* test for the not normally distributed data (TAG species: C15:0-C18:2-C18:1; C16:0-C16:0-C18:0; C16:0-C18:1-C18:0; C16:0-C20:3-C20:3; C17:1-C18:3-C18:1; C17:1-C20:2-C18:1; C18:0-C18:1-C16:1; C18:0-C18:1-C18:1; C18:1-C18:2-C18:1; and DAG species: C15:0-C18:1; C16:0-C16:1; C16:0-C18:3; C16:0-C20:2; C16:0-C20:3; C16:1-C18:1; C16:1-C18:2; C17:1-C18:1; C18:1-C18:1; C18:1-C18:3; C18:2-C18:2). All data except glucose, β-hydroxybutyric acid, high-density lipoprotein (HDL), TAG species; C14:0-C20:2-C18:1; C15:0-C18:1-C16:0; C16:0-C16:1-C16:0; C16:0-C18:2-C16:0; C16:0-C20:1-C16:0; C16:0-C20:4-C20:2; C18:0-C18:2-C18:2; C18:0-C18:3-C18:1; C18:1-C20:4-C20:1; and DAG species: C16:0-C17:1; and C18:0-C18:3 were ^{e}log transformed to achieve normality. Residual plots were used to study the model assumptions. The Akaike information criterion was used to select the best model. In vitro culture data of in vivo-derived oocytes were analyzed with a McNemar test and culture and BODIPY fluorescence data from in vitro matured oocytes by a one-way ANOVA residuals were normally distributed. All measures are reported as means ± SD. A P value of <0.05 was considered statistically significant.

RESULTS

Short-term fasting results in a rise of free fatty acid levels in blood and follicular fluid

The short-term fasting period resulted in a slight but significant decrease of the glucose concentration in the blood (Fig. 1A). A drop in glucose levels in the blood normally results in increased levels of glucagon and this triggers the body to mobilize body fat reserves as an alternative energy source. Our experimental model indeed resulted in a major rise of the free fatty acid levels in the blood and concomitantly elevated β-hydroxybutyric acid concentrations, indicating an induced ketogenesis of the liver (Fig. 1, B and C). As expected, the glucose and β-hydroxybutyric acid concentrations in follicular fluid were comparable to the concentrations in blood, as these metabolites can freely diffuse through the follicular wall (Fig. 1, A and C). The levels of the free fatty acids, which are transported by albumin also increased in follicular fluid in response to the elevated levels in the blood, but did not reach the levels in blood (Fig. 1B). The second abundant lipid fraction in follicular fluid, next to the free fatty acid fraction, the high-density lipoprotein (HDL) was not affected by short-term fasting (Fig. 1D). The levels of the other lipid proteins, VLDL and LDL were low (below 0.14 mM in both conditions) in follicular fluid (data not shown). This indicates that the lipid changes in follicular fluid induced by short-term fasting were restricted to the elevated levels of free fatty acids in follicular fluid.

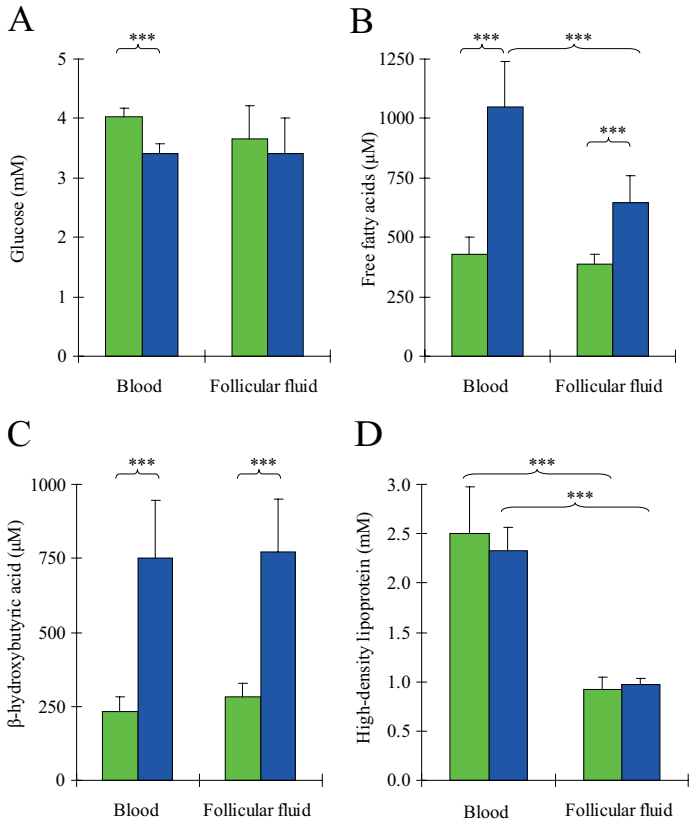

Figure 1. Short-term fasting alters metabolic profiles in blood and follicular fluid. The short-term fasting period resulted in a decreased concentration of glucose (A) and an increase of the concentrations of free fatty acids (B) and β-hydroxybutyric acid (C) in the blood of the experimental animals (blue) compared to the control animals (green). The metabolic profile of blood was reflected in the follicular fluid and fasting resulted in a major rise of the free fatty acid concentrations in follicular fluid in comparison to the control situation (B). Short-term fasting did not alter the concentrations of HDL in blood and follicular fluid (D). Values are means ± SD. Asterisks indicate a significant effect compared to control (***$P \leq 0.001$).

High concentration of oleic acid in follicular fluid

The specific rise in the free fatty acid concentrations in blood and follicular fluid was almost exclusively due to a rise in the free fatty acid concentrations with a chain length of 14–18 carbon atoms in blood and follicular fluid (Fig. 2). Follicular fluid reflected the increase of free fatty acid levels in the blood, but the free fatty acid compositions in blood and follicular fluid were distinct particularly in the case of the lower concentrations of C16:0 and C18:0 in follicular fluid (Fig. 2).

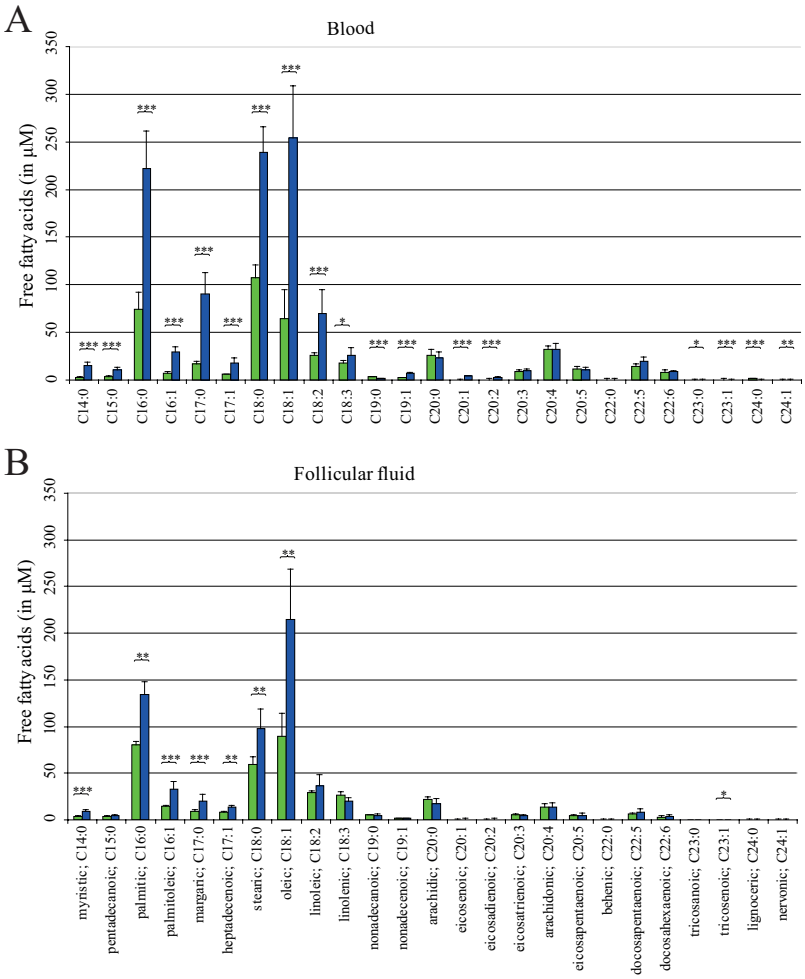

Figure 2. C18:1 and C16:0 and C18:0 dominate blood and follicular fluid. Short-term fasting (blue bars) resulted in a massive increase in the levels of free fatty acids with a carbon length of 14–18 both in the blood (A) and in follicular fluid (B) in comparison to the control condition (green bars). Saturated free fatty acid concentrations in follicular fluid remained lower than the concentrations measured in the blood. Remarkably the concentration of C18:1 was comparable to that of blood and as a consequence the C18:1 was relatively highly presented in follicular fluid. n = 6 per group. Values are means ± SD. Asterisks indicate a significant difference compared to control (*$P \leq 0.05$; **$P \leq 0.01$, and ***$P \leq 0.001$).

Of note is that the rise of the linoleic acid (C18:2) concentration in blood was not reflected in the follicular fluid of the experimental animals which indicates that only a small portion of linoleic acid is transported to the follicular fluid compartment over the follicle wall. In contrast a relatively large proportion of C18:1 infiltrated into the follicular fluid of fasted animals.

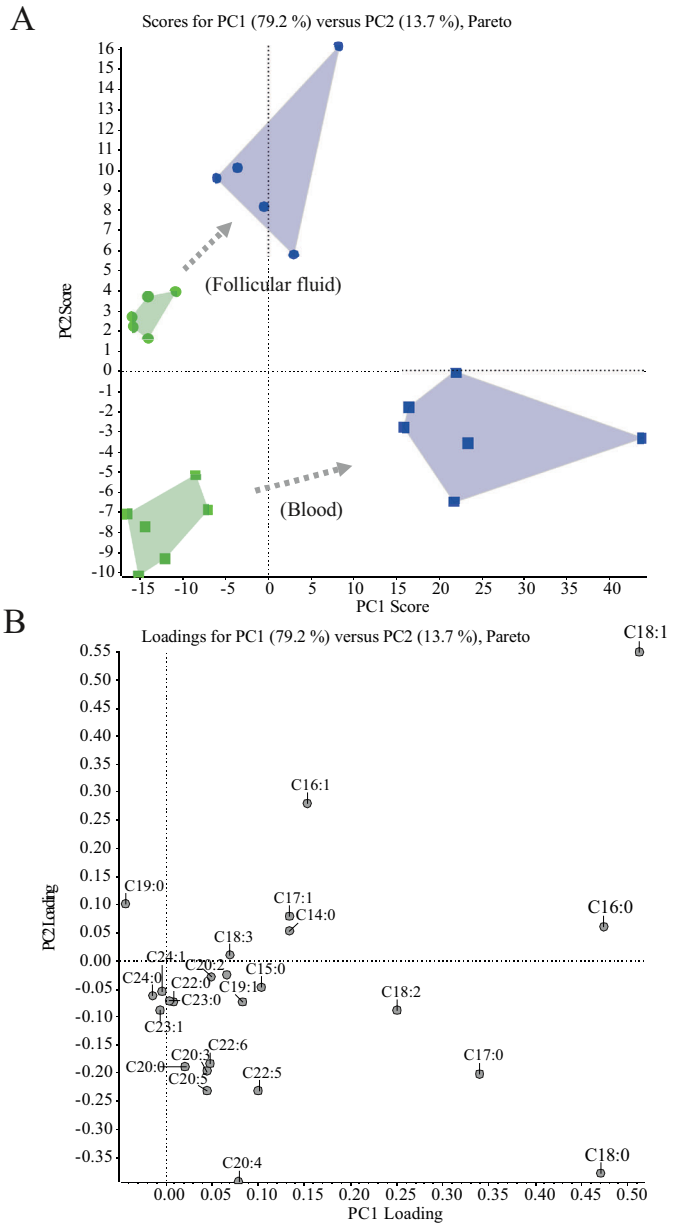

Figure 3. Distinct free fatty acid compositions in blood and follicular fluid of control and fasted animals. The principal component analysis revealed characteristic free fatty acid compositions between the control (green) and experimental group (blue) in blood (squares) and follicular fluid (circles) and as shown by the different clustering of each set of samples in the score plot (A). The major contributors for the distinct free fatty acid compositions were C18:1, C16:0, and C18:0, as they had coordinates with the highest absolute values in the loading plot (B). Arrows indicate the shift induced by short-term fasting.

Distinct free fatty acid concentrations in blood and follicular fluid

A principal component analysis performed on the free fatty acid data of blood and follicular fluid retained no less than 93% of the data set's original variance (Fig. 3). The first principal component explained 79%, the second 14% of original variance. The score plot of the principal components showed a distinct clustering of samples per group and for samples originating from either blood or follicular fluid, indicating a characteristic free fatty acid composition for each set of samples from blood and follicular fluid of control and experimental animals (Fig. 3A). The loading plot indicates that C18:1, C16:0 and C18:0 were the most important fatty acids to explain the original variance and thus characteristic compositions of the fluids, as the coordinates of these fatty acids have the highest absolute values (Fig. 3B). In control fluids the enrichment with longer chain free fatty acids (≥C19) was most predictive. Control blood contained a more complex fatty acid spectrum than control follicular fluid with more (poly) unsaturated long chain fatty acids, whereas relatively more nonadecanoic acid (C19:0) was detected in follicular fluid (Fig. 3B). The short-term fasting period resulted for both blood and follicular fluid in an under representation of long chain (poly) unsaturated fatty acids. In addition, a relative overrepresentation of C16:0, palmitoleic acid (C16:1) and C18:1 was observed in follicular fluid while margaric acid (C17:0), C18:0 and C18:2 were overrepresented in blood (Figs. 2 and 3B). These data demonstrate that the free fatty acid compositions of blood and follicular fluid are different and that a short-term fasting period alters the free fatty acid composition of blood and follicular fluid.

Oocyte developmental competence is not impaired after in vitro exposure to elevated free fatty acid levels

In vitro maturing COCs were exposed to a mixture of the most prominent free fatty acids (C18:1, C16:0 and C18:0) in concentrations similar to those measured in the follicular fluid of control or fasted animals (Figs. 2B and 3B). The free fatty acid levels for fasted animals resulted in a major increase in the neutral lipid of the cumulus cells of the COCs compared to control free fatty acid levels or to free fatty acid depleted conditions (Fig. 4, A and B). The developmental competence of the oocytes as determined by the cleavage and blastocyst rate after fertilization was not altered under these three conditions (Fig. 4, C–E).

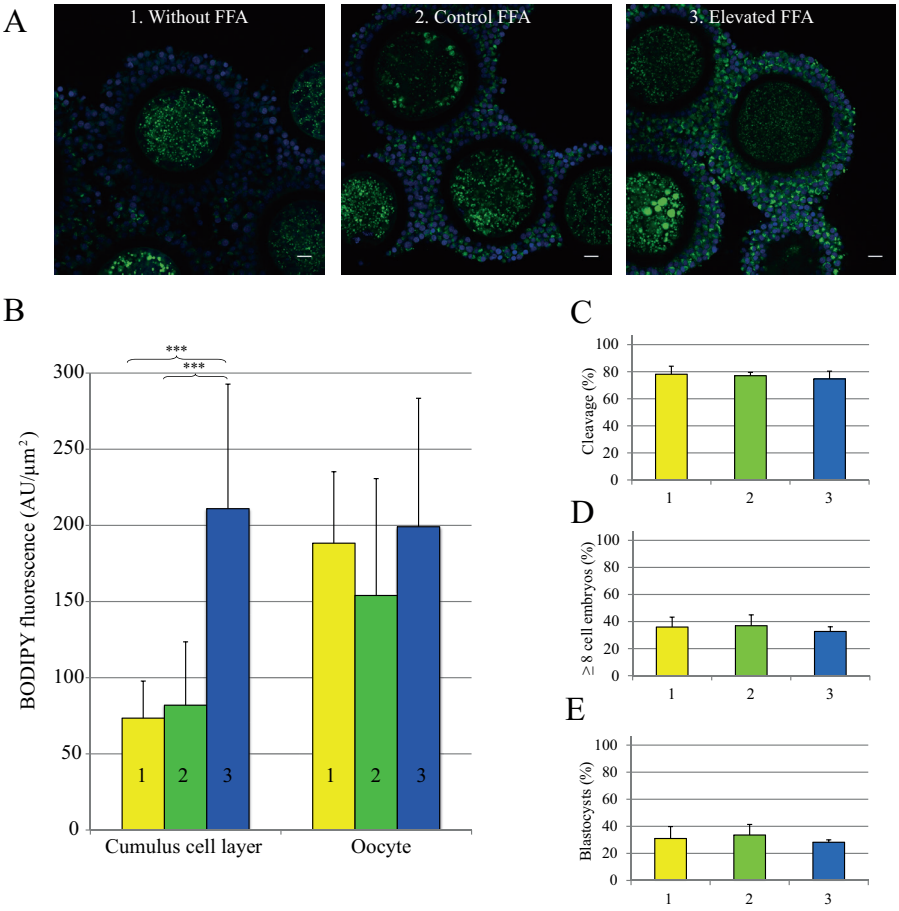

Figure 4. Exposure to the most abundant free fatty acids in follicular fluid does not impair oocyte developmental competence. In vitro maturation with medium without FFA (1), with control FFA levels; 80 µM C16:0, 70 µM C18:0, 100 µM C18:1 (2) or with elevated FFA levels; 150 µM C16:0, 100 µM C18:0, 200 µM C18:1 (3) representing their predetermined levels in follicular fluids (conform figure 2). (A) Neutral lipid staining with BODIPY 493/503 (green) showed an increase in neutral lipid in lipid droplets of cumulus cells after exposure to the elevated FFA condition; nuclei of cells were counterstained with ToPro3 (blue), the distance bar represents 20 µm. (B) Mean BODIPY fluorescence in the cumulus cell layer and oocyte per area. (C–E) Elevated FFA concentrations did not alter cleavage (C), ≥8 cell embryos at Day 5 (D), and blastocyst rates at Day 8 of culture (E). Values are means ± SD. Asterisks indicate a significant effect among the groups (***$P \leq 0.001$). FFA: free fatty acids. (B–E) Yellow bars numbered 1 refer to the without FFA condition, green bars numbered 2 refer to control FFA condition and blue bars numbered 3 refer to elevated FFA condition as described above.

In order to further investigate the protective function of cumulus cells oocytes were exposed to fatty acids in the absence of cumulus cells. Since bovine oocytes do not mature in the absence of a cumulus cells layer, the cumulus was only removed from COCs after 16 h maturation. The resulting denuded oocytes (DOs) as well as intact COCs were exposed to either a medium without or with elevated free fatty acid concentrations for the final 6 h of maturation. No differences in developmental competence were found between COCs and DOs exposed to standard medium (Fig. 5). A tendency for a lower blastocyst rate was observed for DOs exposed to elevated free fatty acids compared with intact COCs exposed to elevated free fatty acids (P = 0.07) (Fig. 5), while the developmental competence of COCs was unaffected after exposure to elevated free fatty acid levels during the final 6h or the entire maturation period.

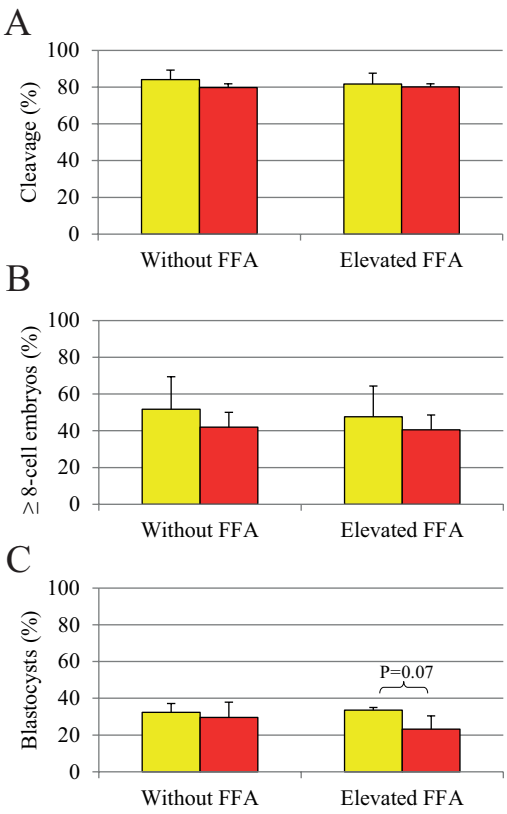

Figure 5. Cumulus cell removal of COCs and FFA exposure during the last 6 h of maturation. Removal of cumulus cells (red bars, DOs) from COCs did not affect the developmental competence of the DOs compared to intact COCs (yellow bars). The culture conditions are defined in Figure 4. Developmental competence was measured as cleavage (A), ≥8 cell embryos at Day 5 (B), and blastocyst rates at Day 8 of culture (C). Values are means ± SD. FFA, free fatty acids.

Short-term elevated free fatty acid levels in follicular fluid result in massive lipid changes of in vivo cumulus cells

The next step was to investigate the fatty acid composition of neutral lipid from cumulus cells of COCs after in vivo maturation in the presence of elevated free fatty acid concentrations. Interestingly, elevated levels of free fatty acids in follicular fluid had a major impact on the cumulus cells and resulted in a massive TAG accumulation in the cumulus cells (Figs. 6A, 7A, and 8A).

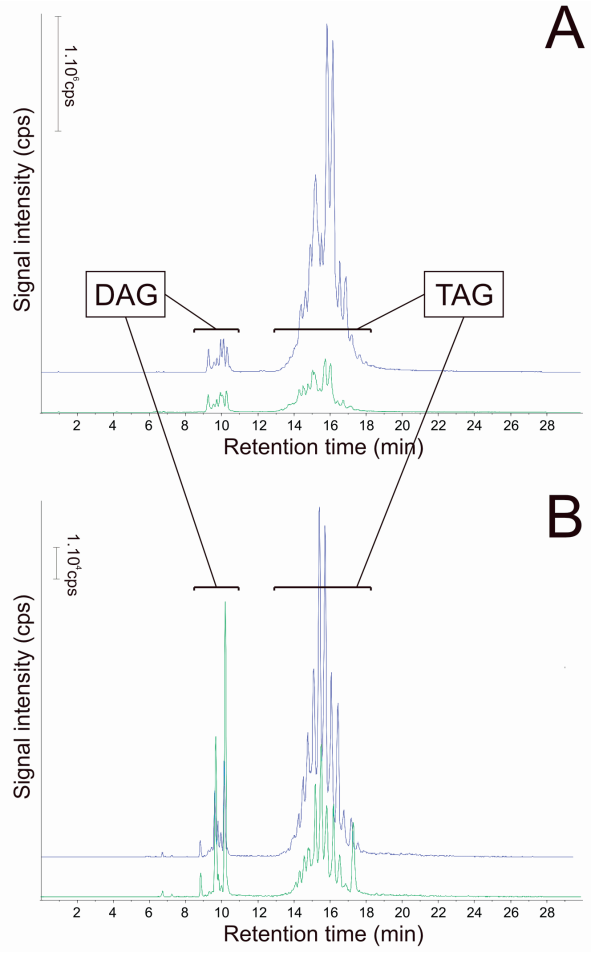

Figure 6. Intensity HPLC chromatograms of ionized neutral lipids in cumulus cells and in oocytes measured by mass spectrometry. The intensity of ionized neutral lipids from in vivo matured cumulus cells (A) and oocytes (B) were measured after control (green line) or experimental (blue line) conditions. Cumulus cells and oocytes originating from the same 6 COCs were pooled separately and extracted for lipids and their intensities were recorded. The intensity of ions entering (in counts per second, cps) the mass spectrometer equipment after reverse-phase HPLC separation is expressed as function of retention time (min) on the column. Ion intensities were normalized by an internal standard added prior to lipid extraction. Cps, ion counts per second.

The total TAG amount in cumulus cells increased 6-fold upon exposure to short-term fasting, while the amount of DAG was not affected (Figs. 6A, 7A, and 9A). The substantial increase of TAG and constant DAG amount in cumulus cells resulted in a sharp decrease of the DAG/TAG molar ratio of 0.06 for the fasted group versus 0.29 for the control group. The elevated levels of free fatty acids in follicular fluid resulted in a major accumulation of TAG species with one or more acylations with C18:1 (Figs. 8A and 10A). The second most abundant fatty acid was C16:0 in TAG species (Figs. 8A and 10A). Overall almost all the fatty acids incorporated in TAG of cumulus cells increased upon fasting, but in particular C18:1 and C16:0 massively increased.

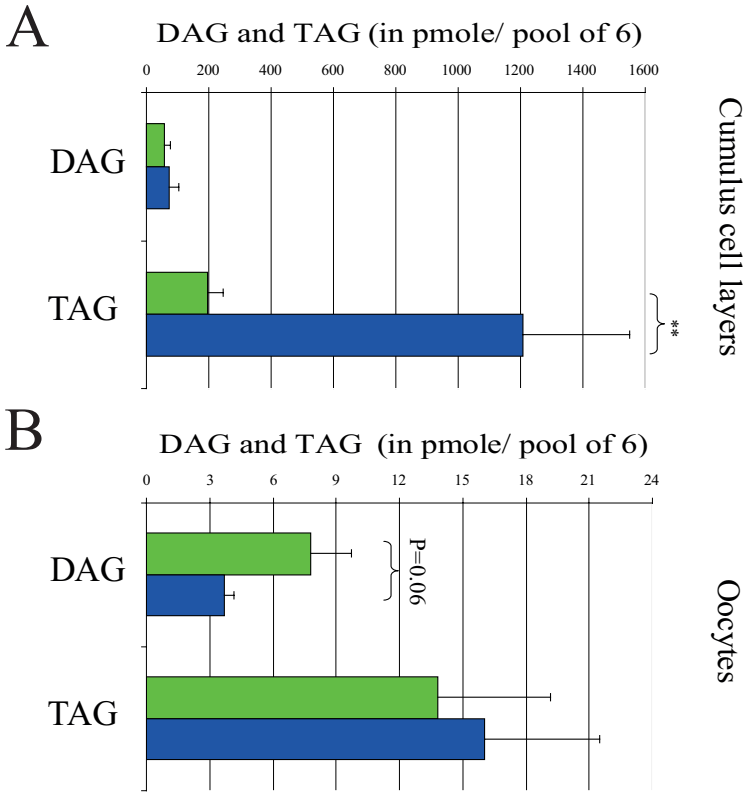

Figure 7. Elevated free fatty acid concentrations induce massive lipid accumulation in cumulus cells and minor lipid alterations in oocytes. The cumulus cells (pool of cumulus cells from 6 oocytes per sample) that surrounded the oocytes during exposure to short-term fasting (blue bars) showed a massive increase in the TAG fraction in comparison to the cumulus cells that surrounded oocytes of the control group (green bars) (A). In contrast to the cumulus cells, short-term fasting did not change the TAG amount in oocytes (n = 6 per sample) (B). Values are means ± SD. Asterisks indicate a significant effect compared to control (**$P \leq 0.01$). Note the different scale of A and B.

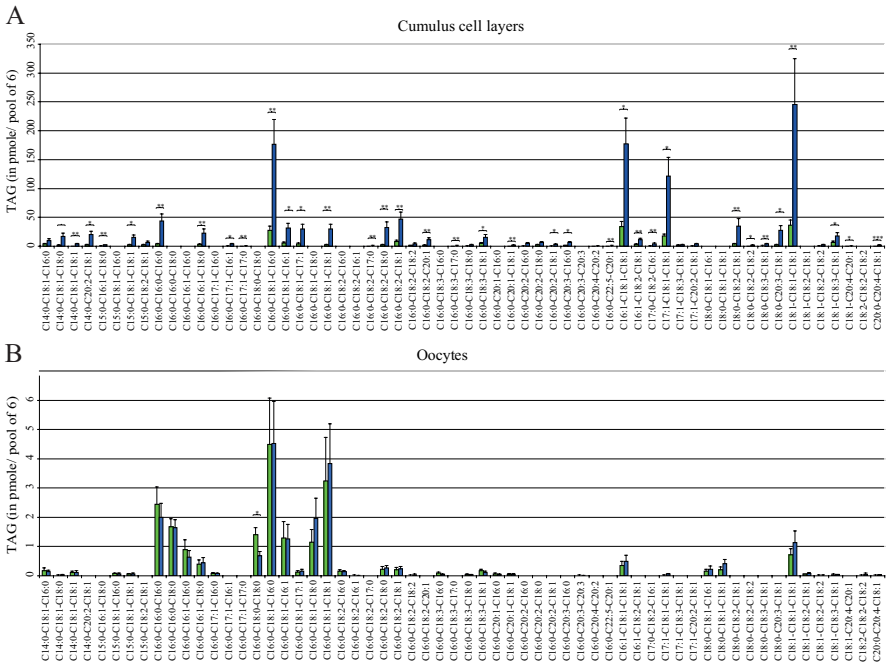

Figure 8. The level of TAG species with C18:1 massively increases in cumulus cells exposed to elevated levels of free fatty acids in follicular fluid. Especially the concentrations of TAG species in cumulus cells (pool of cumulus cells originating from 6 COCs) with one or more acylations with C18:1 heavily increased upon exposure to short-term fasting (blue bars) in comparison to cumulus cells from control animals (green bars) (A). TAG species of oocytes (n = 6) were marginally affected in the experimental group (B). Values are means ± SD. Asterisks indicate a significant effect compared to control (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$). Note the different scales of A and B.

Short-term elevated free fatty acid levels in follicular fluid do not change the lipid composition of oocytes

Remarkably, the neutral lipid concentrations of oocytes exposed to fasting were hardly affected by the elevated free fatty acid levels in follicular fluid. Both the composition and amount of fatty acids incorporated in neutral lipid was maintained in the oocytes (Figs. 6B and 7B), except for one TAG (C16:0–C18:0–C18:0) (Fig. 8B) and DAG (C16:0–C18:0) (Fig. 9B) species that decreased upon exposure to short-term fasting (Fig. 10B). The neutral lipid of oocytes was, in contrast to the lipid present in their cumulus cells, nearly unaltered upon exposure to a short-term fasting period.

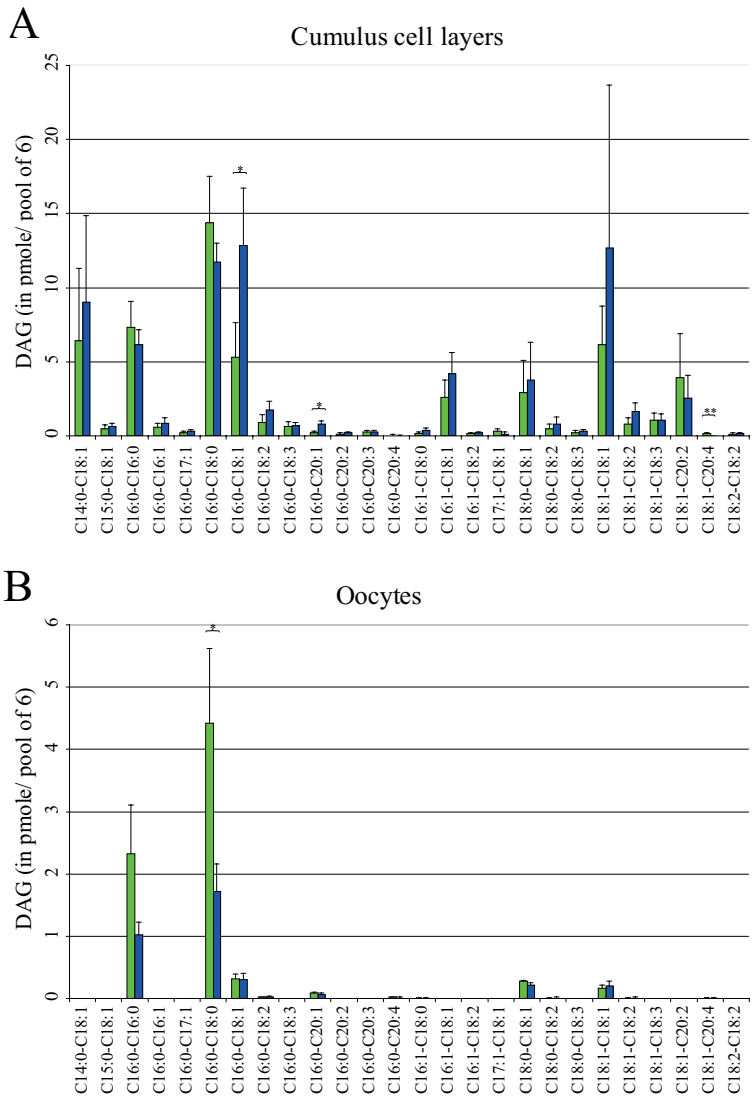

Figure 9. The levels of DAG species in cumulus cells and oocytes. The concentrations of DAG species of cumulus cells (pool of cumulus cells originating from 6 COCs) were unaltered upon exposure to short-term fasting (blue bars) in comparison to cumulus cells of the control group (green) (A). In contrast to cumulus cells, the DAG composition of oocytes (n = 6 per sample) was significantly altered upon exposure to short-term fasting (blue bars) and resulted in a reduction of the DAG species C16:0-C18:0 in comparison to oocytes from the control group (green bars) (B). Values are means ± SD. Asterisks indicate a significant effect compared to control (*$P \leq 0.05$ and **$P \leq 0.01$). Note the different scales of A and B.

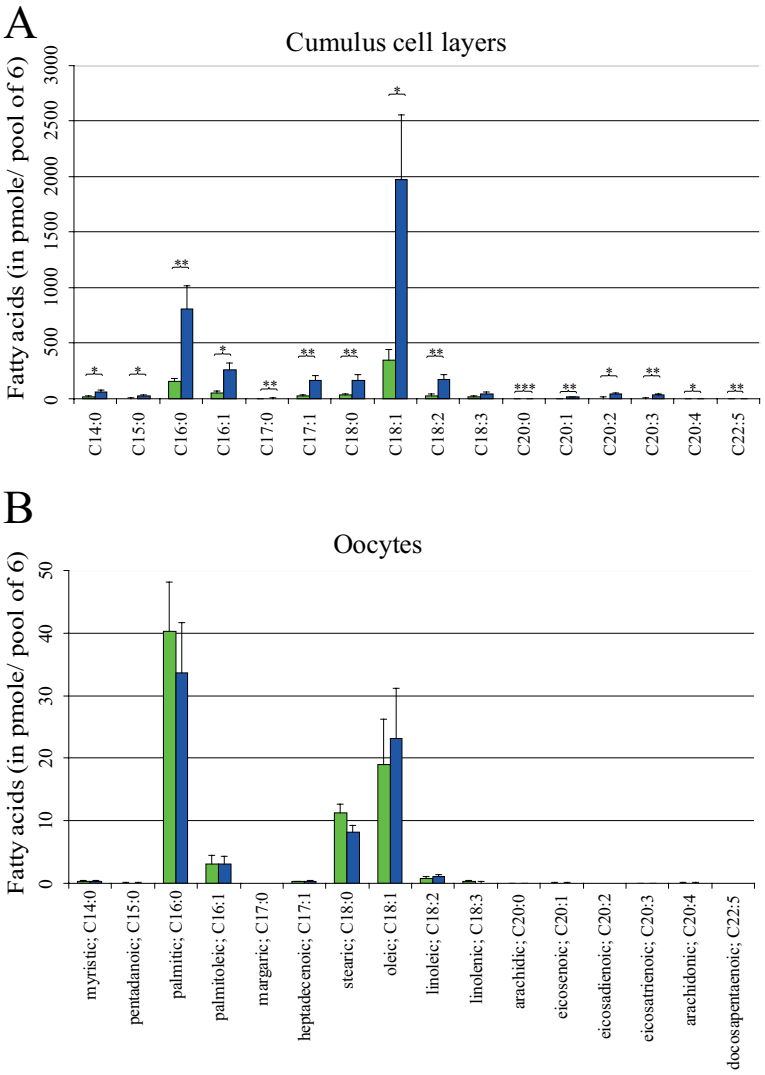

Figure 10. The total amount of individual fatty acids present in DAG and TAG. Almost all the different fatty acids present in the lipid of cumulus cells (pool of cumulus cells 6 originating from 6 COCs) significantly increased upon exposure to short-term fasting (blue bars) compared with cumulus cells of the control condition (green bars) (A). The fatty acid composition of oocytes (n = 6 per sample) only marginally changed in the experimental group (blue bars) compared to the oocytes from the control group (green bars) (B). In contrast to cumulus cells (A), the oocytes (B) had a relatively large amount of saturated fatty acid (B). Values are means ± SD. Asterisks indicate a significant effect compared to control (*$P \leq 0.05$ and **$P \leq 0.01$). Note the different scale of A and B.

Short-term elevated free fatty acid levels in follicular fluid during maturation of oocytes do not harm their developmental competence

Finally, we investigated in an independent in vivo experiment whether short-term fasting affected the developmental competence of maturing oocytes. To this end we collected COCs at 22h after the induced LH peak from control and experimental super-stimulated animals that were short-term fasted during the period of final oocyte maturation. The in vivo matured COCs were individually fertilized and cultured in a single embryo culture system. Interestingly, individual COCs of the experimental group yielded cleavage and blastocyst rates comparable to those of the control group (Fig. 11. A–C). Furthermore, the relative mRNA expression levels of three genes (*CTSB, PLAC8* , and *PTGS*) considered to be markers for blastocyst quality were quantified by q-PCR on individual blastocysts formed from in vivo matured oocytes of the control and fasted group (Fig. 11D). No significant differences in the mRNA expression of the genes were observed between the blastocysts from fasted and control heifers. This further demonstrates that the imposed free fatty acid levels by fasting had neither altered the developmental competence of oocytes nor the quality of the produced blastocysts.

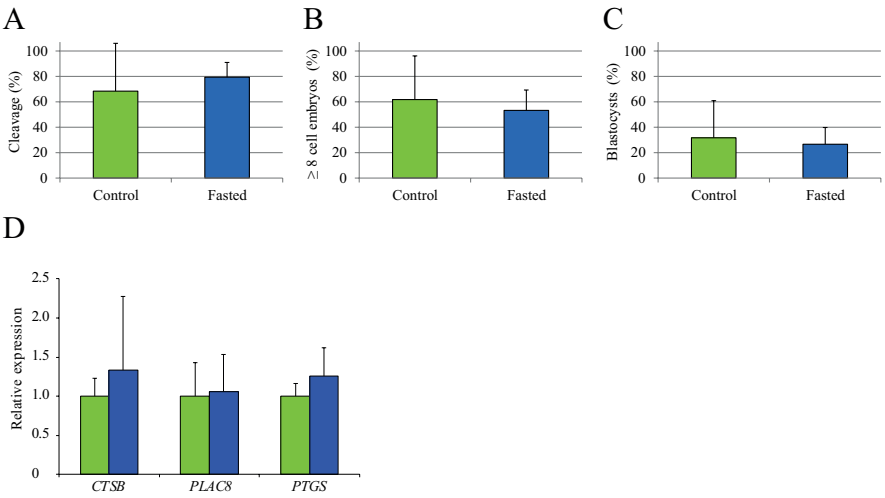

Figure 11. Short-term fasting during final oocyte maturation does not impair the postfertilization developmental competence of the oocytes. The developmental competence of in vivo matured COCs originating from control (green bars) and fasted (blue bars) cows was measured by determining the cleavage (A), ≥8 cell embryos at Day 5 (B), and blastocyst rates at Day 8 of culture (C). The relative expression of the putative quality markers *CTSB, PLAC8* , or *PTGS* in blastocysts formed from in vivo matured oocytes of the control (green bars) and fasted group (blue bars), whereby the expression levels of the control group is set at 1 (D). Values are means ± SD.

DISCUSSION

This study shows that short-term elevated free fatty acid concentrations in blood and follicular fluid have a direct impact on the COC. The rise in the levels of free fatty acids in follicular fluid resulted in a massive lipid accumulation in the cumulus cell layer that surrounds the oocyte throughout maturation. Interestingly, the oocytes were largely unaffected after exposure to elevated free fatty acids and development to the blastocyst stage and the quality of blastocysts was unaffected.

The short-term fasting period resulted in a decrease in the glucose level in the blood and increased free fatty acid concentrations, most likely by lipolysis of body fat reserves. The rise in the level of free fatty acids resulted in a concomitant rise of the concentration of β-hydroxybutyric acid indicating enhanced ketogenic activity of the liver. This combination of the metabolic concentrations of glucose (low), free fatty acids (high) and β-hydroxybutyric acid (high) is typical for a period of energy scarcity and for example the negative energy balance of cows during the early postpartum period [48]. The glucose and β-hydroxybutyric acid levels in follicular fluid were comparable to the levels in blood as these metabolites can freely diffuse through the follicular wall. In follicular fluid the rise in the concentration of free fatty acids, which are transported by albumin, was not as high as in the blood. HDL was the other main lipid fraction present in follicular fluid and was not affected by fasting. This indicates that the lipid changes in follicular fluid induced by the short-term fasting period are restricted to the free fatty acid changes.

The short-term fasting period resulted in a concentration rise of free fatty acids with a chain length of 14–18 carbon atoms; this represents the fatty acid species that are stored in adipose tissue and are mobilized during energy deprivation [49]. Free fatty acid levels in follicular fluid were not as high as the levels measured in the blood, in accordance with other studies [3, 7]. The levels of the poly unsaturated free fatty acids, including C18:2 and linolenic acid (C18:3), remained unaltered in follicular fluid despite the concentration rise of these fatty acids in the blood. Blood and follicular fluid had a distinct free fatty acid composition in particular due to saturated C16:0 and C18:0 and mono-unsaturated C18:1. Interestingly, the levels of the saturated free fatty acids did not follow the rise of the concentrations in the blood, while mono-unsaturated free fatty acids were comparable to the levels in the blood and relatively highly represented in follicular fluid. Apart from selective uptake of fatty acids or metabolic activity of cells of the follicular wall or COC, the relatively high

concentrations of mono-unsaturated free fatty acids in follicular fluid may be due to desaturation of saturated fatty acids into mono-unsaturated fatty acids by the enzyme stearoyl-CoA-desaturase (Δ9 desaturase) which is expressed in granulosa cells [50]. The cause for the distinct free fatty acid compositions in blood and follicular fluid certainly needs further investigation. The enrichment in C18:1 in follicular fluid is of special interest as C18:1 is known for its counteracting activity against potential toxic effects of saturated fatty acids on cells in vitro [9–13, 15]. Previously, two mechanisms were described by which C18:1 rescues cells from potential toxic effects of saturated fatty acids either by redistributing fatty acids towards lipid storage in lipid droplets or towards β-oxidation of fatty acids in mitochondria but away from pathways leading to apoptosis [9, 11, 12]. In this study, cumulus cells massively stored lipids upon exposure to elevated free fatty acid concentrations in follicular fluid. Cumulus cells are in direct gap-junctional contact with the oocyte and are crucial for establishing the developmental competence of oocytes by supplying nutrition and metabolites [17, 18, 51]. Furthermore, cumulus cells protect the oocyte against reactive oxygen species and cell damage with intrinsic oantixidant enzymes like superoxide dismutase and their presence is crucial during oocyte maturation [52, 53]. The massive lipid accumulation in cumulus cells in response to elevated free fatty acid levels in follicular fluid as described in this study may safeguard the oocyte from lipotoxic effects induced by increased fatty acid levels.

A protective function of cumulus cells is further suggested by the marginal lipid changes that were observed in oocytes and the unaffected developmental competence of the oocytes upon exposure to the increased free fatty acid concentrations in follicular fluid. In fact removal of the cumulus and exposure to elevated fatty acid levels during the last 6h of maturation already resulted in a tendency for a lower blastocyst rate in the denuded COCs. We have not denuded COCs before 16 h of maturation as for this period the presence of a cumulus layer is considered to be essential for maintenance of developmental competence [52, 54].

Elevated concentrations of saturated fatty acids often result in deleterious effects on cell survival by the induction of an apoptotic signaling cascade, while mono-unsaturated C18:1 is reported to be harmless even at high concentrations [9–14]. Previously, we and others have shown that in vitro exposure to C16:0 and C18:0 during maturation of COCs resulted in endoplasmic reticular stress, mitochondrial damage, increased apoptosis in cumulus cells and impaired oocyte developmental competence, while C18:1 was harmless even at high concentrations [9–15, 39, 55]. In addition our group established that C18:1 can

compensate lipotoxic effects of saturated fatty acids on oocyte developmental competence [15]. Similar results were reported in various somatic cell types [9–13]. To this end, the relatively high abundance of C18:1 in follicular fluid of the fasted animals is of special interest as this fatty acid, in accordance with the in vitro data, could have compensated the potential negative impact of the elevated levels of saturated free fatty acids. We propose that C18:1 may have prevented lipotoxicity by inducing the observed TAG accumulation in cumulus cells.

Relevant for this study is that we induced an increase of free fatty acid concentrations by fasting during a short period during the final follicular growth and maturation phase. This is in contrast to a few studies that investigated the impact of obesity and elevated free fatty acid concentrations during a prolonged period of time. Diet-induced obesity (induced by feeding a high-fat diet to female mice) resulted in aberrant oocytes with dysfunctional mitochondria, endoplasmic reticular stress and apoptosis in cumulus cells, which may culminate in impaired embryonal development competence or in abnormalities in the offspring [16, 56]. The high fat diet resulted in increased glucose levels and elevated levels of free fatty acids in the blood of these mice, similar to what has been observed during obesity. Unfortunately, these studies do not give information on the free fatty acid concentrations present in follicular fluid which makes comparison among their and our study of free fatty acid concentrations difficult. The diet-induced obesity model resulted in significant changes in the oocytes of these mice, while elevated free fatty acid levels in our study did not impair the developmental competence of oocytes. One major difference between the two conditions is that the level of glucose was increased in the obese condition, while it was moderately but significantly decreased in response to our fasting model. Glucose is a major indicator for the metabolic status of an animal and is the driving force for insulin (high in an energy rich anabolic condition) and glucagon (high in an energy poor catabolic condition) concentrations in the blood. The metabolic conditions may therefore have had a different impact on the cells to cope with elevated levels of free fatty acids. Another difference is that in our study oocytes were exposed to acute metabolic stress for 4 days during the final follicular growth and maturation phase, while oocytes in the diet-induced obesity studies were chronically exposed to elevated free fatty acids during a prolonged period of time and thus also during the earlier stages of folliculogenesis. Oocytes may be less protected against elevated free fatty acid concentrations during early folliculogenesis by the presence of a small layer of cumulus cells and absence (in preantral follicles) and small volume of follicular fluid which may have resulted in the different outcomes of our study and others [24].

Our findings indicate that short time exposure to elevated levels of free fatty acids in follicular fluid during final maturation does not harm the oocyte. Elevated levels of free fatty acids in follicular fluid resulted in a massive lipid accumulation in cumulus cells, but did not affect the lipid composition and developmental competence of the oocyte. The high abundance of C18:1 in follicular fluid may compensate the potential toxic effects of increased concentrations of saturated fatty acids. In addition, the surrounding cumulus cell layer of the oocyte presumably protects the oocyte by storing the elevated levels of free fatty acids from follicular fluid as neutral lipids in cumulus cells.

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REFERENCES

1. Metwally M, Cutting R, Tipton A, Skull J, Ledger WL, Li TC. Effect of increased body mass index on oocyte and embryo quality in IVF patients. Reprod Biomed Online 2007; 15:532–538.
2. Robker RL, Wu LL, Yang X. Inflammatory pathways linking obesity and ovarian dysfunction. J Reprod Immunol 2011; 88:142–148.
3. Jungheim ES, Macones GA, Odem RR, Patterson BW, Lanzendorf SE, Ratts VS, Moley KH. Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during in vitro fertilization. Fertil Steril 2011; 95:1970–1974.
4. Yang X, Wu LL, Chura LR, Liang X, Lane M, Norman RJ, Robker RL. Exposure to lipid-rich follicular fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus-oocyte complexes. Fertil Steril 2012; 97:1438–1443.
5. Walther TC. Farese RV Jr. Lipid droplets and cellular lipid metabolism. Annu Rev Biochem 2012; 81:687–714.
6. Cusi K. Role of obesity and lipotoxicity in the development of nonalcoholic steatohepatitis: pathophysiology and clinical implications. Gastroenterology 2012; 142:711–725. e6.
7. Leroy JL, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G, Van Soom A. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. Reproduction 2005; 130:485–495.
8. Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. Diabetes 2001; 50:1771–1777.
9. Listenberger LL, Han X, Lewis SE, Cases S, Farese RVJr, Ory DS, Schaffer JE. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc Natl Acad Sci U S A 2003; 100:3077–3082.
10. Mishra R, Simonson MS. Saturated free fatty acids and apoptosis in microvascular mesangial cells: palmitate activates pro-apoptotic signaling involving caspase 9 and mitochondrial release of endonuclease G. Cardiovasc Diabetol 2005; 4:2.
11. Coll T, Eyre E, Rodriguez-Calvo R, Palomer X, Sanchez RM, Merlos M, Laguna JC, Vazquez-Carrera M. Oleate reverses palmitate-induced insulin resistance and inflammation in skeletal muscle cells. J Biol Chem 2008; 283:11107–11116.
12. Henique C, Mansouri A, Fumey G, Lenoir V, Girard J, Bouillaud F, Prip-Buus C, Cohen I. Increased mitochondrial fatty acid oxidation is sufficient to protect skeletal muscle cells from palmitate-induced apoptosis. J Biol Chem 2010; 285:36818 36827.
13. Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, Donath MY. Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. Diabetes 2001; 50:69–76.
14. Listenberger LL, Ory DS, Schaffer JE. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. J Biol Chem 2001; 276: 14890–14895.
15. Aardema H, Vos PL, Lolicato F, Roelen BA, Knijn HM, Vaandrager AB, Helms JB, Gadella BM. Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. Biol Reprod 2011; 85:62–69.

16. Wu LL, Dunning KR, Yang X, Russell DL, Lane M, Norman RJ, Robker RL. High-fat diet causes lipotoxicity responses in cumulus-oocyte complexes and decreased fertilization rates. Endocrinology 2010; 151: 5438–5445.
17. Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum Reprod Update 2008; 14:159–177.
18. Albertini DF, Combelles CM, Benecchi E, Carabatsos MJ. Cellular basis for paracrine regulation of ovarian follicle development. Reproduction 2001; 121:647–653.
19. Mu YM, Yanase T, Nishi Y, Tanaka A, Saito M, Jin CH, Mukasa C, Okabe T, Nomura M, Goto K, Nawata H. Saturated FFAs, palmitic acid and stearic acid, induce apoptosis in human granulosa cells. Endocrinology 2001; 142:3590–3597.
20. Ferguson EM, Leese HJ. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. Mol Reprod Dev 2006; 73:1195–1201.
21. Sturmey RG, O'Toole PJ, Leese HJ. Fluorescence resonance energy transfer analysis of mitochondrial:lipid association in the porcine oocyte. Reproduction 2006; 132:829–837.
22. Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ, Robker RL. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. Biol Reprod 2010;
23. Jorritsma R, de Groot MW, Vos PL, Kruip TA, Wensing T, Noordhuizen JP. Acute fasting in heifers as a model for assessing the relationship between plasma and follicular fluid NEFA concentrations. Theriogenology 2003; 60:151–161.
24. Britt JH. Impacts of early postpartum metabolism on follicular development and fertility. The Bovine Proceedings 1992; 24:39–43.
25. Walters AH, Bailey TL, Pearson RE, Gwazdauskas FC. Parity-related changes in bovine follicle and oocyte populations, oocyte quality, and hormones to 90 days postpartum. J Dairy Sci 2002; 85:824–832.
26. van Knegsel AT, van den Brand H, Dijkstra J, Tamminga S, Kemp B. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. Reprod Nutr Dev 2005; 45:665–688.
27. Butler WR. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livestock Production Science 2003; 83:211–218.
28. Thompson JG, Partridge RJ, Houghton FD, Cox CI, Leese HJ. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. J Reprod Fertil 1996; 106:299–306.
29. Schroder UJ, Staufenbiel R. Invited review: methods to determine body fat reserves in the dairy cow with special regard to ultrasonographic measurement of backfat thickness. J Dairy Sci 2006; 89:1–14.
30. Pieterse MC, Vos PL, Kruip TA, Wurth YA, van Beneden TH, Willemse AH, Taverne MA. Transvaginal ultrasound guided follicular aspiration of bovine oocytes. Theriogenology 1991; 35:857–862.
31. Bungartz L, Niemann H. Assessment of the presence of a dominant follicle and selection of dairy cows suitable for superovulation by a single ultrasound examination. J Reprod Fertil 1994; 101:583–591.
32. Knijn H, Fokker W, van der Weijden G, Dieleman S, Vos P. Effects of superovulation with oFSH and norgestomet/GnRH-controlled release of the LH surge on hormone concentrations, and yield of oocytes and embryos at specific developmental stages. Reprod Domest Anim 2012; 47: 177–183.

33. Vos PL, Bevers MM, Willemse AH, Dieleman SJ. Effects of suppression by a progesterone-releasing intravaginal device and subsequent induction by GnRH of the preovulatory LH surge on follicular function in PMSG/ PG-treated heifers. J Reprod Fertil 1994; 101:43–49.
34. Dieleman SJ, Bevers MM. Effects of monoclonal antibody against PMSG administered shortly after the preovulatory LH surge on time and number of ovulations in PMSG/PG-treated cows. J Reprod Fertil 1987; 81: 533–542.
35. Dieleman SJ, Bevers MM, Poortman J, van Tol HT. Steroid and pituitary hormone concentrations in the fluid of preovulatory bovine follicles relative to the peak of LH in the peripheral blood. J Reprod Fertil 1983; 69:641–649.
36. van Tol HT, van Eerdenburg FJ, Colenbrander B, Roelen BA. Enhancement of Bovine oocyte maturation by leptin is accompanied by an upregulation in mRNA expression of leptin receptor isoforms in cumulus cells. Mol Reprod Dev 2008; 75:578–587.
37. Goovaerts IG, Leroy JL, Jorssen EP, Bols PE. Noninvasive bovine oocyte quality assessment: possibilities of a single oocyte culture. Theriogenology 2010; 74:1509–1520.
38. Balboula AZ, Yamanaka K, Sakatani M, Hegab AO, Zaabel SM, Takahashi M. Intracellular cathepsin B activity is inversely correlated with the quality and developmental competence of bovine preimplantation embryos. Mol Reprod Dev 2010; 77:1031–1039.
39. Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PE, Leroy JL. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. PLoS One 2011; 6:e23183.
40. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; 37:911–917.
41. Rouser G, Fleischer S, Yamamoto A. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 1970; 5:494–496.
42. Manini P, Andreoli R, Careri M, Elviri L, Musci M. Atmospheric pressure chemical ionization liquid chromatography/mass spectrometry in cholesterol oxide determination and characterization. Rapid Commun Mass Spectrom 1998; 12:883–889.
43. Brouwers JF, Gadella BM, van Golde LM, Tielens AG. Quantitative analysis of phosphatidylcholine molecular species using HPLC and light scattering detection. J Lipid Res 1998; 39:344–353.
44. Aitzemüller K. Recent progress in the high performance liquid chromatography of lipids. Prog Lipid Res 1982; 21:171–193.
45. Kates M. Techniques of lipidology: isolation, analysis and identification of lipids In: Burdon RH, and Van Knippenberg PH (eds.), Laboratory Techniques in Biochemistry and Molecular Biology, 2nd ed. Amsterdam: Elsevier; 1986; 125.
46. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal Chem 2006; 78: 779–787.
47. Pinheiro J, Bates D, DebRoy S, Sarkar D, the R Development Core Team. Linear and Nonlinear Mixed Effects Models. R Package Version 3.1101. 2011.
48. Jorritsma R, Wensing T, Kruip TA, Vos PL, Noordhuizen JP. Metabolic changes in early lactation and impaired reproductive performance in dairy cows. Vet Res 2003; 34:11–26.

49. Rukkwamsuk T, Geelen MJ, Kruip TA, Wensing T. Interrelation of fatty acid composition in adipose tissue, serum, and liver of dairy cows during the development of fatty liver postpartum. J Dairy Sci 2000; 83:52–59.

50. Moreau C, Froment P, Tosca L, Moreau V, Dupont J. Expression and regulation of the SCD2 desaturase in the rat ovary. Biol Reprod 2006; 74: 75–87.

51. Fragouli E, Wells D. Transcriptomic analysis of follicular cells provides information on the chromosomal status and competence of unfertilized oocytes. Expert Rev Mol Diagn 2012; 12:1–4.

52. Fatehi AN, Roelen BA, Colenbrander B, Schoevers EJ, Gadella BM, Bevers MM, van den Hurk R. Presence of cumulus cells during in vitro fertilization protects the bovine oocyte against oxidative stress and improves first cleavage but does not affect further development. Zygote 2005; 13:177–185.

53. Tatemoto H, Sakurai N, Muto N. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during In vitro maturation: role of cumulus cells. Biol Reprod 2000; 63:805–810.

54. Merton JS, de Roos AP, Koenen EP, Roelen BA, Vos PL, Mullaart E, Knijn HM. Bovine OPU-derived oocytes can be matured in vitro for 16– 28 h with similar developmental capacity. Reprod Domest Anim 2012; 47: 1037–1042.

55. Wu LL, Russell DL, Norman RJ, Robker RL. Endoplasmic reticulum (ER) stress in cumulus-oocyte complexes impairs pentraxin-3 secretion, mitochondrial membrane potential (DWm), and embryo development. Mol Endocrinol 2012; 26:562–573.

56. Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE, Moley KH. Diet-induced obesity model: abnormal oocytes and persistent growth abnormalities in the offspring. Endocrinology 2010; 151: 4039–4046.

Chapter 5

Free fatty acid composition in the fluid of dominant follicles during the preferred insemination period is not distinct between dairy cows with an early or delayed first postpartum ovulation

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ABSTRACT

The fertility of high-yielding dairy cows has declined during the last 3 decades, in association with a more profound negative energy balance (NEB) during the early weeks postpartum. One feature of this NEB is a marked elevation in circulating free fatty acid concentrations. During the early postpartum period (≤ day 42) circulatory free fatty acids were measured weekly, and progesterone concentrations and the ultrasongraphically determined diameter of the dominant follicle were determined thrice weekly. Cows that ovulated within 35 days postpartum were grouped as 'normal ovulating' cows, and the others were grouped as 'delayed ovulating' cows. In both groups, high total free fatty acid levels (> 500 μM) were evident immediately postpartum. Interestingly, cows with a delayed ovulation had higher plasma free fatty acid concentrations in the first weeks postpartum than normal ovulating cows. The free fatty acid composition of fluid from the dominant follicles of postpartum cows was measured at potential insemination points, days 55, 80 and 105 postpartum. No differences were found between the normal and delayed ovulating cows with respect to follicular fluid free fatty acid composition. In both groups at each postpartum time-point, the concentration of mono-unsaturated oleic acid was higher, and that of saturated stearic acid lower, in follicular fluid than in blood, indicating that free fatty acid metabolism in developing follicles was similar in the two groups. Taken together this indicates that a more severe NEB early postpartum is related to a delay in the first postpartum ovulation, but does not affect the free fatty acid composition of the follicular fluid in dominant follicles at the time of insemination.

INTRODUCTION

The reproductive performance of high yielding dairy cows has declined markedly during the last four decades, from a calving rate of around 55% per insemination in the eighties to 40% nowadays [1-6]. In contrast, the fertility of non-lactating heifers has remained undiminished throughout this period [2]. During the period of declining fertility, the milk production of cows has also increased appreciably with the consequence that cows experience a more profound period of negative energy balance (NEB) in the first months postpartum. This NEB is considered to be, at least in part, responsible for the drop in fertility [3-7]. Cows with a more severe NEB, and consequent more dramatic loss of body condition during the peri-partum period, show compromised reproductive performance in terms of a delayed resumption in ovarian activity and reduced ovulation rate from the first follicular wave postpartum, compared to cows with less severe body condition loss [8, 9]. Britt hypothesized that exposure of follicles to the unfavorable metabolic conditions of NEB during early follicular growth, based on the premise that growth of a follicle from the primordial to peri-ovulatory stage takes around 60-80 days in the cow, could have a latent impact on the quality of the follicle and its contained oocyte and may explain reduced fertility in cows several weeks to months after the NEB [4].

A major metabolic characteristic of NEB is the elevation in circulating free fatty acid concentrations. Free fatty acids are released into the circulation from adipose tissue during NEB and periods of glucose-deprivation, here they are complexed to albumin to enable uptake by tissues and cells as an alternative energy source [10]. Beyond their role as an alternative energy source, however, free fatty acids (especially the saturated species) have been shown to induce lipotoxic stress in several somatic cell types [11-17]. The ovary is also exposed to the elevated free fatty acid levels in the blood, and it has been shown that *in vitro* elevated free fatty acids reduce cell proliferation and increase apoptosis in both granulosa and theca cells, the cells that form the inner and outer layers of the follicle from the early stages of follicular development and up until ovulation [18-20]. Elevated free fatty acid concentrations in the blood are reported to be reflected in follicular fluid composition and can therefore affect the cumulus-oocyte complex more directly (Leroy et al., 2005; Aardema et al., 2013). The cumulus cell layer that is in direct contact with the follicular fluid, and surrounds the oocyte during the antral follicular stages, also shows signs of lipotoxic stress when exposed to saturated free fatty acids *in vitro* [21, 22]. Exposure of the cumulus-oocyte-complex (COC) to saturated free fatty acids during final

maturation results in oocytes with a diminished mitochondrial membrane potential and impaired capacity to develop into a blastocyst [21-23]. In short, it appears that a disturbance of the free fatty acid composition of follicular fluid around the time of insemination may have important consequences for oocyte developmental competence.

In general, cows are inseminated from around 60 days postpartum, to achieve a desired calving interval of around 12-13 months [24]. Dominant follicles present at the time of the first postpartum inseminations therefore started their development during the NEB period and were exposed to elevated levels of free fatty acids during maturation. In this study we investigated whether the early postpartum elevation in free fatty acid concentrations was reflected in the dominant follicle by monitoring the free fatty acid composition of follicular fluid, as well as in blood, at potential postpartum insemination points, namely days 55, 80 and 105 postpartum. In addition, we examined the extent of free fatty acid elevation in the early postpartum period and its potential impact on reproductive activity by monitoring follicular growth and development and the timing of the first postpartum ovulation.

MATERIALS AND METHODS

Chemicals

Unless stated otherwise, all chemicals were obtained from Sigma Chemical Co (St. Louis, MO, USA) and were of the highest available purity. Solvents (acetone, acetonitrile, chloroform, methanol and hexane) were of high-performance liquid chromatography grade (Labscan, Dublin, Ireland).

Experimental procedures on animals

All animal experiments were approved by Utrecht University's Animal Experimental Procedures Committee. Clinically healthy pregnant Holstein-Friesian heifers (n=10) were included in the experiment from the time of calving until day 105 postpartum. The heifers were fed 10 kg maize, ½ kg soybean hulls, *ad libitum* grass silage daily, supplemented with 2 kg concentrated feed (Synchro-optimaal; De Heus Voeders BV, Ede, The Netherlands) on the day of calving rising to 8 kg concentrate on day 14 post-calving and until the end of the experiment. The heifers had unlimited access to water and, once a week, their body condition score (scale 1-5) was determined. From day 14 postpartum, per rectum ultrasonography was performed 3 times a week using a 240 Parus scanner (Pie Medical, Maastricht, The Netherlands) equipped with a 7.5 MHz

linear array transducer, and the diameters of ovarian follicles (> 5mm) were recorded. Cows with a first ovulation before day 35 postpartum were grouped as 'normally ovulating'; cows without a first ovulation before day 35 postpartum were grouped as 'delayed ovulating'. The cut-off point of day 35 postpartum was based on a previous study [25]. The first ovulation was considered to have taken place when 3 successive blood progesterone measurements exceeded 2 ng/ml, combined with ultra-sonographic evidence of corpus luteum formation. The follicular fluid was collected individually from follicles ≥ 14 mm in diameter by trans-vaginal ultrasound guided follicle aspiration [26], on discrete days from day 42 to 105 postpartum. Follicular fluid from individual follicles was centrifuged for 10 min at 3000 g and the supernatant was stored at -80 °C. Blood for progesterone analysis was collected 3 times a week from the jugular vein using a vacutainer system and heparin coated tubes (Becton Dickinson and Co.: Franklin Lakes, NJ, USA). Plasma progesterone concentrations were analyzed using solid-phase [125I] RIA (Coat-A-Count, TKPG; Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) as described previously [27]. Blood samples for free fatty acid analysis were collected weekly from the day of parturition until day 42 (FA 115 kit; Randox Laboratories Ltd., Crumlin, UK).

Extraction and isolation of free fatty acid

The blood and follicular fluid samples collected on day 7 (blood only), 55, 80 and 105 post-calving were selected to isolate and analyze the free fatty acid fractions, as described previously [27]. The total lipid fraction from 100 µL blood or follicular fluid was extracted according to the method of Bligh and Dyer [28] and evaporated under a constant stream of nitrogen gas at 40 ºC. Deuterated palmitic acid [7,7,8,8-$^2H_4$] (Cambridge Isotopes Laboratories, Inc., MA, USA) was added as an internal standard to the samples (10 nmol/sample) at the beginning of the lipid extraction procedure to allow calculation of recovery efficiency and absolute concentrations. Isolation of the free fatty acid fraction was performed as described by Kates, [29] without the heating step, and maintaining the samples on ice to avoid hydrolysis of the free fatty acid. The protocol was as follows: 1 mL of 0.3 M NaOH in methanol/water (9:1, v/v) was added to the evaporated lipid mixture and the polar phase was washed 3 times with 1 mL of hexane in order to purify the free fatty acid fraction in the resulting aqueous methanol phase. This was subsequently acidified and the free fatty acids were extracted using three portions of 1 ml of hexane, evaporated under a constant stream of nitrogen gas at 40ºC and stored in an atmosphere of 100 % nitrogen at -20ºC until analysis.

Analysis of free fatty acids by high-performance liquid chromatography mass spectrometry

Free fatty acids were dissolved in 100 µL methanol/acetonitrile/chloroform/water (46:20:17:17, v/v/v/v) and injected into a HALO C18 (150 x 3.0 mm; particle size of 2.7 µm) HPLC column (Advanced Material Technology, Inc.; Wilmington, DE, USA). The injection volume was 40 µL, the temperature of the column was maintained at 40ºC. Lipids were eluted using a linear gradient, from acetonitrile/methanol/water (6:9:5, v/v/v) 2.5 mM ammonium acetate to acetone/methanol (4:6, v/v) 2.5 mM ammonium acetate for 15 min, followed by isocratic elution with the latter solvent for 10 min and regeneration of the column for 5 min, all at a flow rate of 0.6 mL/min. Mass spectrometry of free fatty acids was performed using electrospray ionization (ESI) on a 2000 QTRAP system (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Source temperature was set to 450°C and nitrogen was used as curtain gas. The declustering potential was set to -40 V. Full scans were performed in negative mode in the m/z range from 225-400 amu. Peaks were identified by comparison of retention time and mass spectra with authentic standard, and calibration curves were generated to correct for differences in response factors.

Data processing

Lipid data were recorded using Analyst version 1.4.2 software (MDS Sciex; Concord, ON, Canada) and exported in mzXML format. Peak detection, integration and alignment were performed using the open source software package, XCMS, running under "R" [30]. A correlation matrix of the combined data sets was calculated in "R". Principal component discriminant analysis was also performed with R using Pareto scaling, in which the value for each peak is transformed by subtraction of the mean for all samples and division by the square root of the standard deviation. In this way, data maintains a dimension (units), and peaks with a good signal to noise ratio will gain importance without allowing intense peaks to dominate the analysis.

Statistics

The statistical analysis was performed using R statistical software version 3.0.2 (R development Core Team, 2013). Longitudinal comparison of the total free fatty acid concentrations and comparison of the individual free fatty acid concentrations in blood and follicular fluid across the groups was performed using a linear mixed model [31]*; R package version 3.1-101* 'nlme') with days postpartum and group as fixed effects and cow as a random effect.

For the longitudinal comparison of blood and follicular fluid free fatty acid concentrations a linear mixed model [31]*; R package version 3.1-101* 'nlme') was applied with days postpartum and blood and follicular fluid as full factorial fixed effects and animal as a random effect, so that the correlation between observations was accounted for. Unless stated otherwise, all measures are reported as mean ± SD. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Cows with delayed ovulation have higher plasma total free fatty acid concentrations

The total free fatty acid concentration in the blood plasma of 10 cows was followed weekly during the first 6 weeks postpartum. In addition, the time of ovulation was monitored 3 times a week by *per rectum* ultrasound detection of follicle size and collapse, and by detection of elevated progesterone concentrations in the blood. Half of the cows had a first postpartum ovulation within 35 days of calving (Fig. 1A-E) and were grouped as 'normally ovulating' cows, while the other 5 cows showed a first ovulation after 35 days postpartum (Fig. 1F-I) and were assigned to the 'delayed ovulation' group. Both groups showed follicular growth from as early as 14 days postpartum, the time of the first postpartum ultrasound examination, and developed follicles to a size compatible with ovulation (Fig. 1). However, most of the dominant follicles in the delayed ovulation group became atretic (Fig. 1F-I). Interestingly, 2 out of 5 cows in the group with a delayed ovulation developed follicles with a larger diameter than the follicles ovulated before day 35 in the 'normal' group (Fig. 1). Cows with a delayed first postpartum ovulation had overall significantly higher total free fatty acid concentrations in blood during the early postpartum period than the cows with a first ovulation before day 35 ($P= 0.0011$; Fig. 2A). The rate of the concentration decrease (K; for the formula of the lines see legend Fig. 2) was comparable among the groups and the overall higher concentration of free fatty acids in the group with a delayed ovulation was due to higher starting values of free fatty acids (Fig. 2A). There were no significant differences in the free fatty acid levels between the groups at individual time points. The main differences in the free fatty acid concentrations among the groups were at days 0, 7 and 14; for delayed ovulating cows the mean (± SD) total free fatty acid concentration was 988 ± 297 μM, 600 ± 307 μM and 500 ± 121 μM compared to 732 ± 207 μM, 424 ± 167 μM and 280 ± 74 μM for cows that ovulated before day 35 (Fig. 2A).

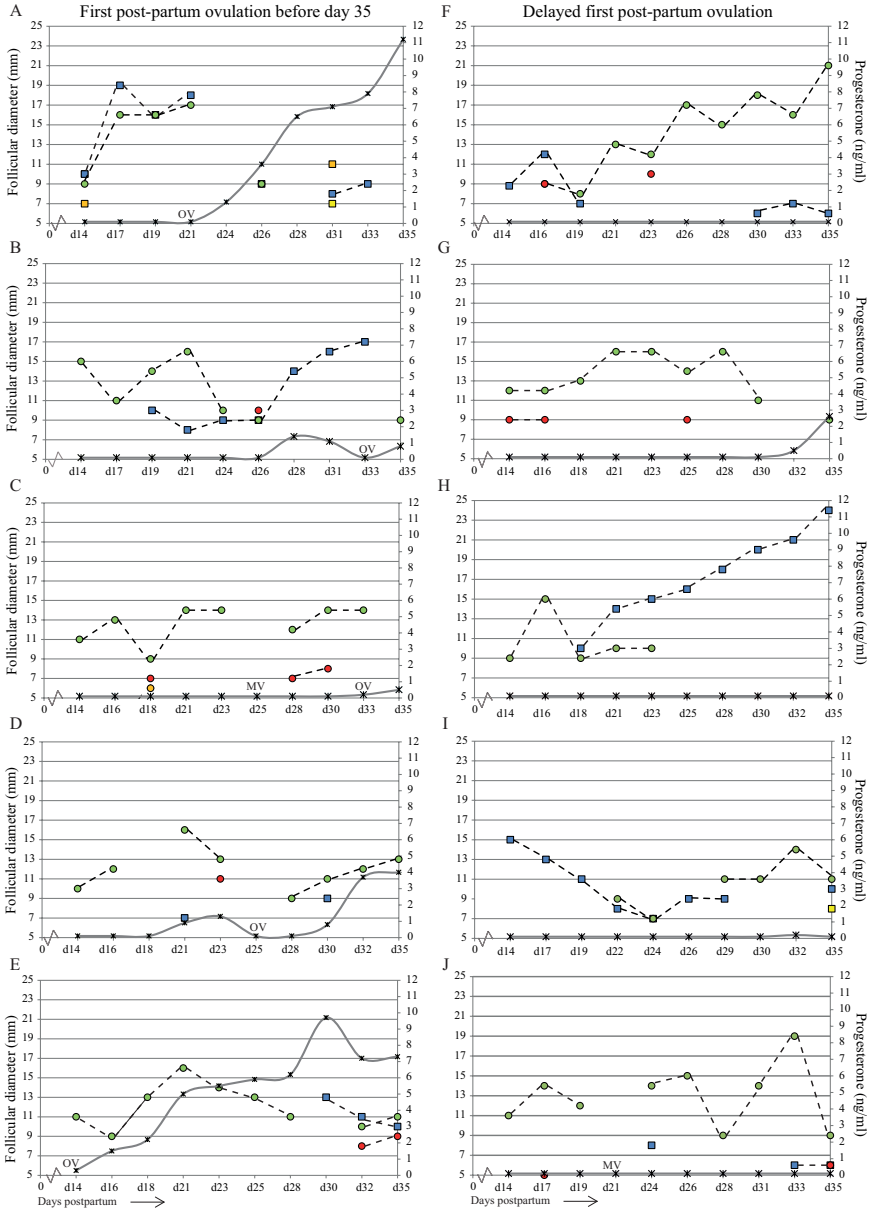

Figure 1. Ovarian follicular growth patterns during the early postpartum period (day 14- 35). The diameter of follicles present on the left (squares) and right (circles) ovaries in cows that had their first postpartum ovulation before (A-E) or after day 35 postpartum (F-J). The slight progesterone rise (grey line) in panel B and C was followed by 3 subsequent measurements with a progesterone value of ≥ 2 ng/ml and resulted in an estimated time of ovulation before day 35, the cow in panel G had a low value of progesterone at the subsequent measurement after day 35 and was defined as delayed ovulating cow. The moment of ovulation was retrospectively defined and based on at least 3 subsequent measurements with a progesterone value of ≥ 2 ng/ml. Day of parturition is t=0. Per rectum ultrasound was performed thrice weekly on Monday, Wednesday and Friday. Note that the y-axis starts from a diameter of 5mm. OV= estimated day of ovulation. MV = missing value.

The composition of free fatty acid molecular species in the blood at day 7 postpartum was comparable in both groups (Fig. 2B), indicating that the increase in total free fatty acids in the delayed ovulating group was primarily a factor of accelerated mobilization of body fat, rather than mobilization of specific FA containing lipid pools (Fig. 2B). From 21 days postpartum, the free fatty acid levels in both groups were back to basal levels (around 250 μM, Fig. 2A).

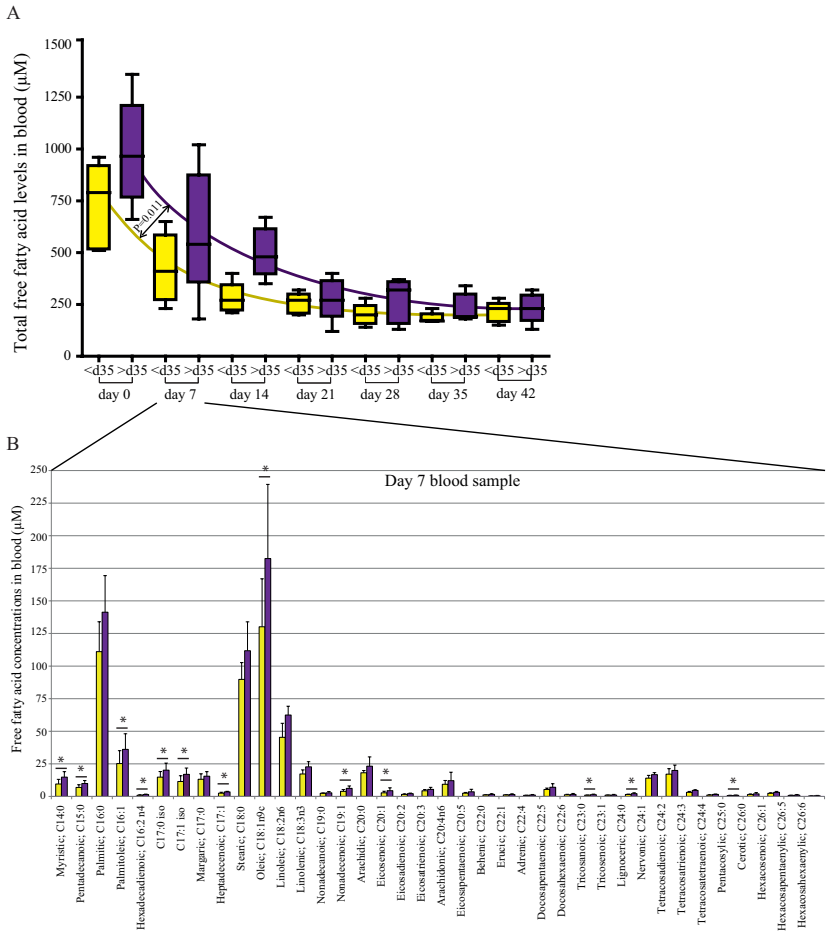

Figure 2. Total free fatty acid concentrations in blood and follicular fluid of postpartum cows. The total free fatty acid concentrations in blood from day 0, the day of parturition, until day 42 (A) and free fatty acid profile at day 7 postpartum (B) are presented for cows with a first ovulation before day 35 postpartum (yellow) and with a delayed first ovulation postpartum (purple). The boxes in panel A represent the median (horizontal line in the middle of the boxes) and interquartile ranges, the bottom of the boxes indicates the lower quartile and the top indicates the upper quartile. The whiskers include all cases. The data were fitted to a line with the formula [free fatty acid] = baselevel + ([free fatty acid$_{t=0}$]–baselevel)*exp(-K*(t $_{day}$)) as shown by the yellow and purple line in panel A. In panel B the values are means ± SD. Asterisks indicate a significant difference ($P < 0.05$).

Normal and delayed ovulating cows have similar free fatty acid compositions in blood and follicular fluid

In most commercial breeding programs, dairy cows are not inseminated before 55 days postpartum. For this reason, we monitored the blood free fatty acid levels and composition in cows with a normal and those with a delayed first ovulation at 55, 80 and 105 days postpartum (Fig. 3).

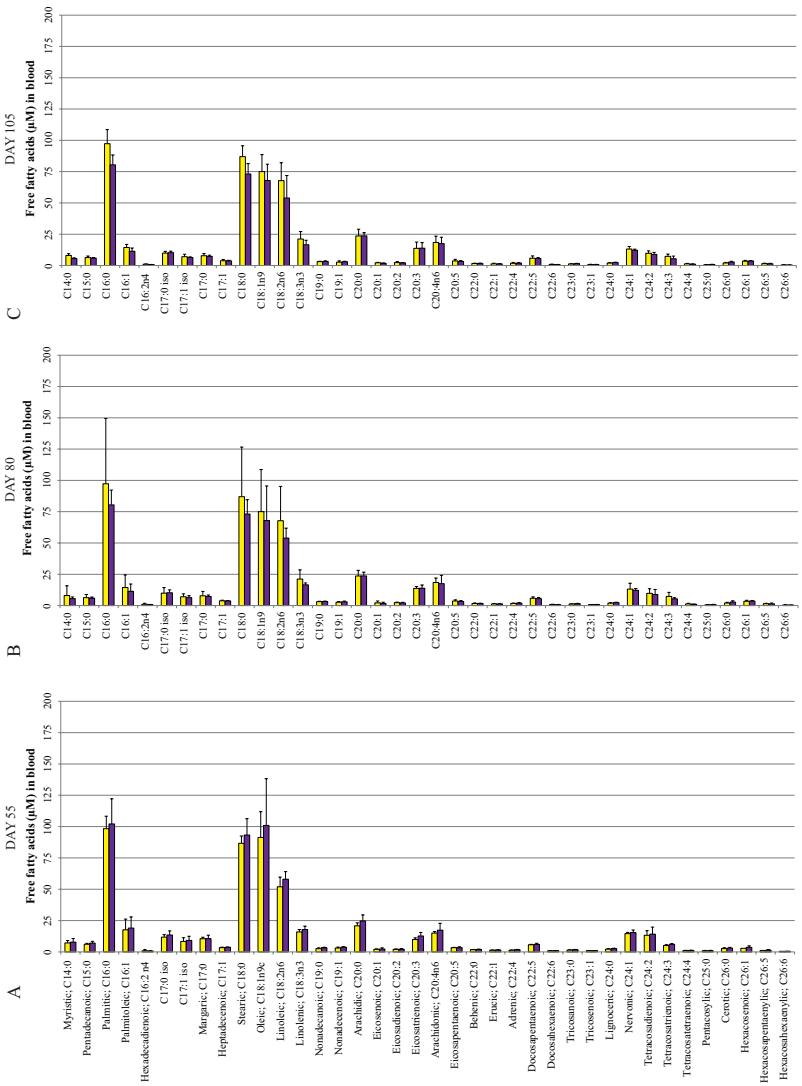

Figure 3. The free fatty acid composition in the blood of postpartum dairy cows. The absolute concentrations of individual free fatty acids are shown for cows with a first ovulation before day 35 (yellow) ,and cows with a delayed first ovulation postpartum (purple) at days 55 (A), 80 (B) and 105 (C). Values are means ± SD.

The groups did not differ in free fatty acid concentrations, or molecular species composition thereof. Likewise the fluid recovered from dominant follicles on days 55, 80 and 105 postpartum showed no differences in free fatty acid concentrations or molecular species composition between normal and delayed first postpartum ovulation cow groups (Fig. 4).

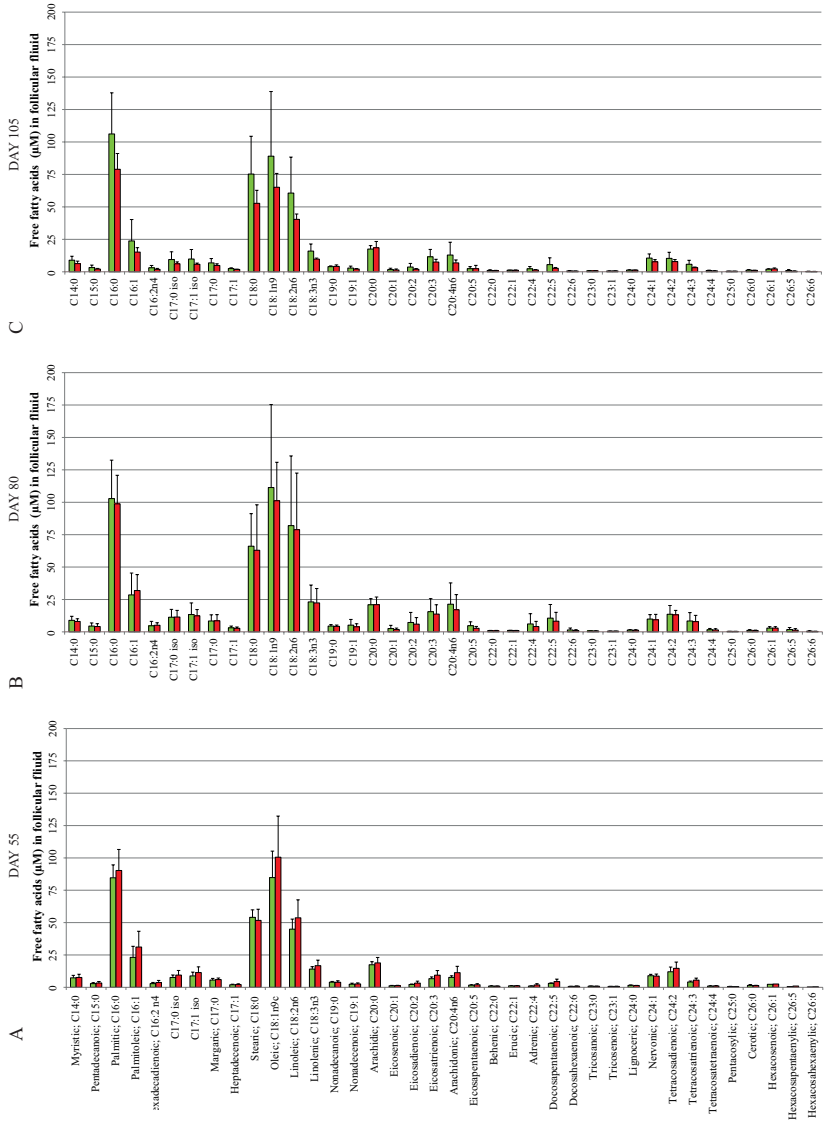

Figure 4. The free fatty acid composition of the follicular fluid of postpartum cows. The absolute concentrations of individual free fatty acids are shown for cows with a first ovulation before day 35 postpartum (green) and cows with a delayed postpartum first ovulation (red) at days 55 (A), 80 (B) and 105 (C). Values are means ± SD.

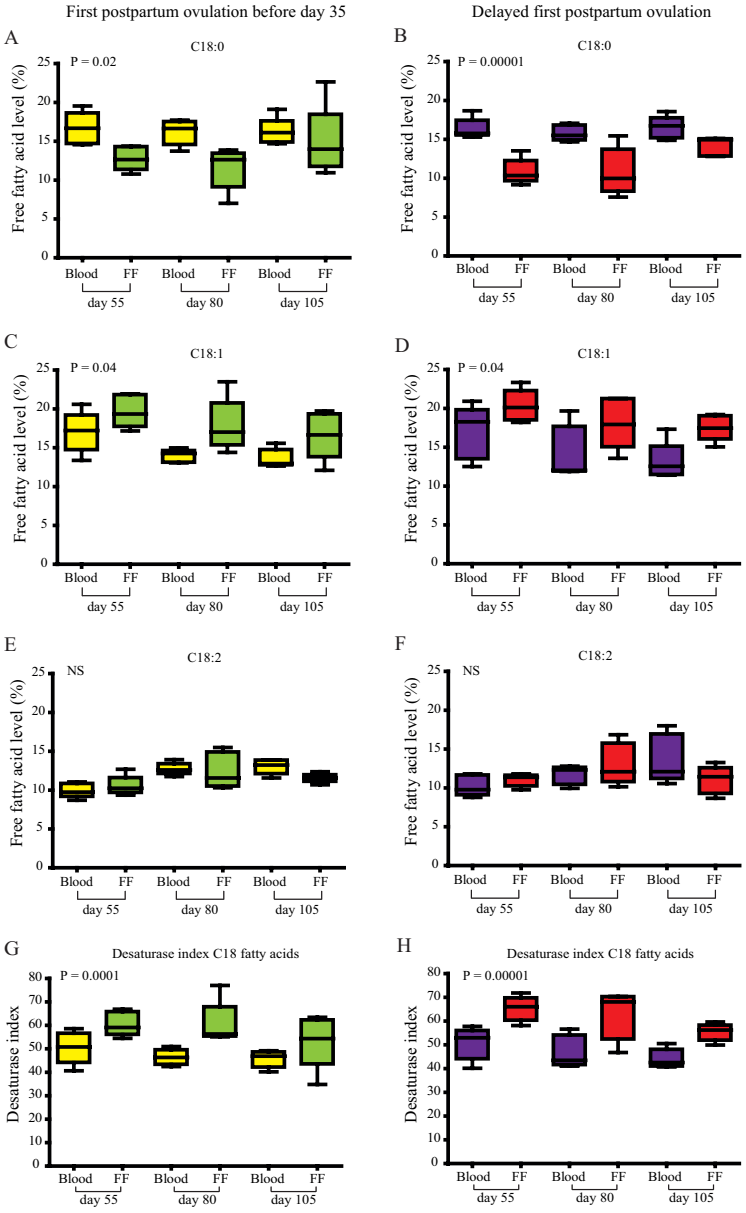

Figure 5. Box and whisker plots for the relative amounts of C18 free fatty acids in blood and follicular fluid. The relative values of free fatty acids in blood (yellow) and follicular fluid (green) of normally ovulating cows (A, C and E) compared to blood (purple) and follicular fluid (red) from cows with a delayed first postpartum ovulation (B, D and F); saturated C18:0 (A-B), mono-unsaturated C18:1 (C-D) and poly-unsaturated linoleic acid (E-F). The desaturation index of C18 free fatty acid (C18:1/ C18:1 + C18:0 * 100) in blood and follicular fluid is shown for early (G) and delayed ovulators (H). The P values represent an overall significant difference between blood and follicular fluid. NS = non-significant.

Free fatty acid levels and molecular species composition of blood differs from follicular fluid

Interestingly, both in cows with a normal and those with a delayed first postpartum ovulation, a marked difference in the relative concentrations and most prominent free fatty acid molecular species (≥ 5% of total free fatty acid) was noted between blood and follicular fluid for the 18 carbon atom chain length fatty acid species (Fig. 5) and the 16 carbon atom length fatty acid species (Fig. 6).

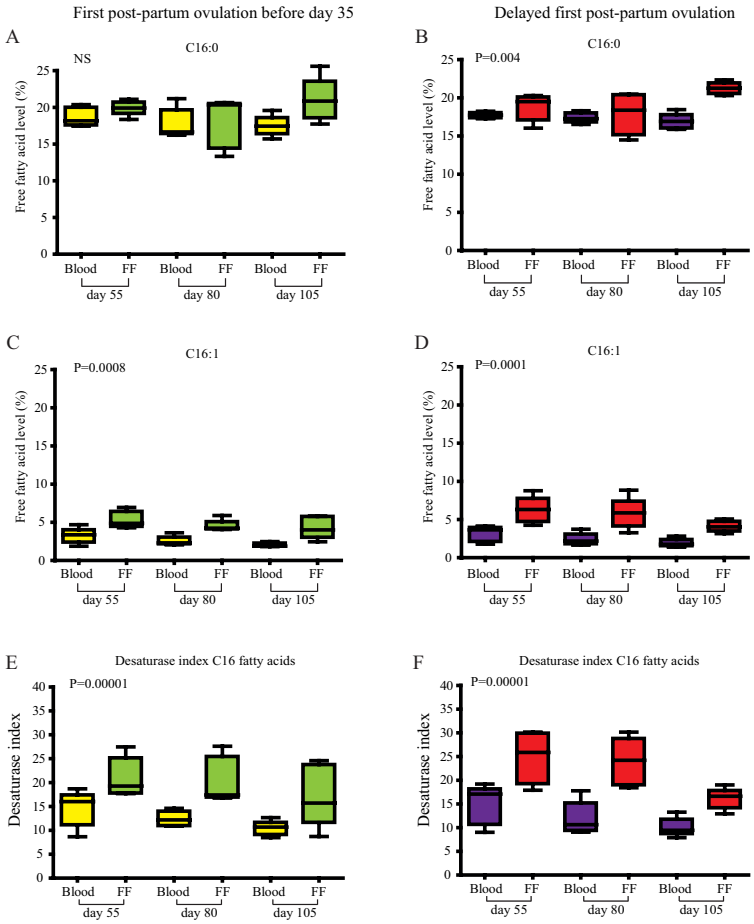

Figure 6. Box and whisker plots for the relative amounts of C16 free fatty acids in blood and follicular fluid. The relative values of free fatty acids in blood (yellow) and follicular fluid (green) of normally ovulating cows (A and C) compared to blood (purple) and follicular fluid (red) of cows with a delayed first postpartum ovulation (B and D); saturated palmitic acid (C16:0, A-B) and mono-unsaturated C16:1 (C-D). The desaturation index of C16 free fatty acid (C16:1/ C16:1 + C16:0 * 100) in blood and follicular fluid is shown for normal (E) and delayed ovulators (F). The P values represent an overall significant difference between blood and follicular fluid. NS = non-significant.

Stearic acid (C18:0) concentrations in follicular fluid were lower than in blood in both normal (P=0.02; Fig. 5A) and delayed ovulating cows (P=0.00001; Fig. 5B). By contrast, a significantly higher concentration of oleic (C18:1) and palmitoleic acid (C16:1) was detected in follicular fluid than in blood in both normal (P=0.04; Fig. 5C and P=0.0008; Fig. 6C) and delayed ovulating cows (P=0.04; Fig. 5D and P=0.0001; Fig. 6D). There was a concomitant and significant increase in the desaturation index for C18 (Fig. 5G,H) and C16 free fatty acid molecular species (Fig. 6E,F) in follicular fluid when compared to the corresponding blood samples.

Principle component analysis reveals distinct free fatty acid composition of follicular fluid and blood

A principal component analysis (PCA) was performed on the free fatty acid composition in corresponding blood and follicular fluid samples. The PCA revealed distinct clustering of blood and follicular fluid samples, which further indicated the differences in composition between blood and follicular fluid (Fig. 7). Interestingly together with the previously noted differences in C18 free fatty acid between follicular fluid and blood, less abundant free fatty acid molecular species such as poly-unsaturated C20 and C24 free fatty acid as well as uneven carbon atom chain length (C17) free fatty acid were shown to be valuable trace species for predicting differences between follicular fluid and blood. Of special interest was that the differences between follicular fluid and corresponding blood samples were time independent and remained unchanged over the 55, 80 and 105 days postpartum sampling points. Again no differences were found between cows with a normal versus a delayed first postpartum ovulation.

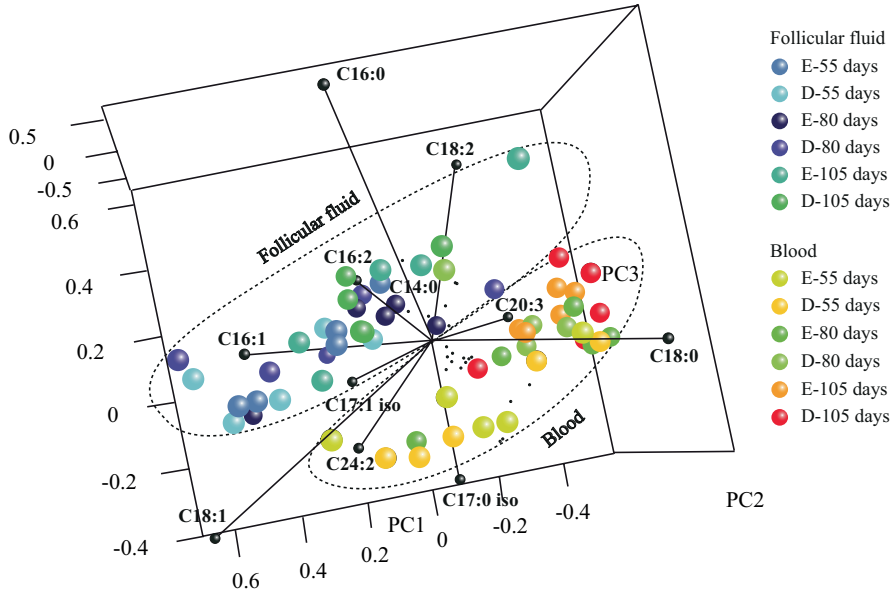

Figure 7. Principal component analysis revealed consistently distinct free fatty acid compositions in blood and follicular fluid, as shown by the different clustering in the score plot of blood and follicular fluid samples from cows with an early (E) and delayed (D) first post-partum ovulation. The major contributors to the distinct free fatty acid compositions were saturated C18:0 and poly-unsaturated linoleic acid (C18:2), as they had coordinates with the highest values in the loading plot. Fatty acids with PCA Loading values outside the 75% quartile range were plotted as black spheres, fatty acids within this range were plotted as small gray spheres.

DISCUSSION

Data from this study demonstrates that cows with a delayed first postpartum ovulation have significantly higher total blood free fatty acid concentrations during the early postpartum period than cows with an earlier (< day 35) first postpartum ovulation. Early resumption of ovarian activity and an early first postpartum ovulation have generally been considered useful indicators for likely fertility of the cow in the subsequent period, with an ovulation after day 35 associated with reduced fertility in the postpartum period [25, 32, 33]. The extent to which free fatty acid concentrations are elevated in the postpartum period is related to the degree of lipid mobilization from body fat reserves, and thus to the severity of the NEB. In cows with a delayed ovulation, the free fatty acid levels remained > 500 μM until the second week postpartum, whereas in the group with a pre-day 35 first ovulation the concentrations dropped below 500 μM in the first week. Interestingly, in both groups dominant follicles developed to a pre-ovulatory size before day 35 postpartum, the difference being that

they failed to ovulate in the delayed ovulation group. The 3 potential fates of a dominant follicle are ovulation, atresia (regression) or cyst formation [34]. In the current study, most of the dominant follicles in the delayed ovulation group became atretic in the early postpartum period. However, two out of the five cows in the delayed ovulation group developed a follicle larger than those ovulated in the other group. The progress to ovulation of a dominant follicle is presumed to depend on the combination of production of 17β-oestradiol to above a certain threshold, which induces an ovulatory LH surge, and development of LH responsiveness by the follicle. Indeed, cows with non-ovulatory follicles during the first postpartum follicular wave appear to have lower blood 17β-oestradiol concentrations than cows with ovulatory follicles, even though the diameter of the follicles is comparable [8]. In line with our data, cows with non-ovulatory first wave follicles show a longer interval to the day of the energy balance nadir [8, 9]. It may be relevant that IGF-I concentrations are positively related to 17β-oestradiol in the blood [8] and the number of LH-binding sites on theca cells [35]. IGF-I concentrations are depressed during NEB, and it is therefore possible that reduced IGF-I contributes to the observed delay in ovulation in cows with a more severe NEB.

In contrast to the higher early postpartum free fatty acid concentrations in the circulation of cows with a delayed ovulation, the free fatty acid concentrations and relative abundance in follicular fluid and blood did not differ between normal and delayed ovulating cows at days 55, 80 and 105 postpartum.

The development of a follicle from an early pre-antral to the peri-ovulatory stage has been estimated to take 60-80 days [4]. Therefore, follicles that assume dominance around the preferred time of insemination, which is from around day 60 postpartum assuming a desired inter-calving interval of around 12-13 months [24], will have experienced NEB during the early stages of follicular development. Britt [4] hypothesized that oocytes from dominant follicles exposed to the NEB during early development may suffer from impaired developmental competence, and that this could form the missing link between more severe NEB and reduced fertility. Oocytes present in early stage recruited follicles may even be more susceptible to the effects of NEB because they have only one or two layers of granulosa cells to isolate them from the blood containing the elevated free fatty acid concentrations [36]. In this respect, at around day 80 postpartum oocytes from first and second parity cows had a lower quality than those retrieved earlier in the postpartum period, which appears to support Britt's hypothesis [3]. Previous in vitro studies have also indicated that elevated free fatty acid levels can have a detrimental impact on both theca and granulosa

cells [18-20], i.e. the cells that line the follicle, as well as on the oocyte itself [21-23]. This further indicates that elevated free fatty acid concentrations during the early postpartum period have the potential to negatively influence the follicle and the oocyte.

In this study we found no indication that early postpartum circulating free fatty acid concentrations were directly reflected in the free fatty acid composition of follicular fluid recovered from dominant follicles at potential insemination times. On the other hand, marked differences in free fatty acid compositions between blood and corresponding follicular fluid samples were evident in both groups of cows, independent of the time postpartum. The distinct free fatty acid composition of blood and follicular fluid indicates the presence of an intact blood-follicle barrier, composed of the capillary endothelium, sub-endothelial basal lamina, theca cells, follicular basement membrane and granulosa cell layer, separating follicular fluid from the free fatty acids present in the circulation [37-39]. Follicular fluid has a relative abundance of mono-unsaturated C18:1 and paucity of saturated C18:0. Therefore, final follicular growth and oocyte maturation presumably occurred under normal free fatty acid conditions in both the normal and delayed ovulating cows [23].

The different free fatty acid composition of blood and follicular fluid may derive from selective transport of free fatty acids through the blood-follicle barrier, or subsequent intra-follicular metabolism or storage in the cells that line the follicle. For instance, follicular cells may, by means of the enzyme stearoyl-CoA-desaturase (SCD), convert saturated free fatty acid into mono-unsaturated free fatty acid by dehydrogenation at the Δ^9carbon chain position of C18 and C16 fatty acids. SCD has also been identified in granulosa and cumulus cells of the rat [40] and may contribute to the distinct composition of free fatty acids in follicular fluid compared to blood.

In conclusion, an overall elevation in circulating free fatty acid concentrations during the early postpartum period is associated with delayed ovulation. However, the free fatty acid composition of the follicular fluid of dominant follicles, and in the blood at days 55, 80 and 105 postpartum, appears to be similar in cows with delayed ovulation and those that ovulate before day 35. There are however marked differences in the free fatty acid composition of follicular fluid and blood, which indicates a functional blood follicle barrier. Future research should investigate the impact of the early postpartum elevation in free fatty acid concentrations on the competence of the oocytes in follicles that assume dominance in the preferred time-window for insemination.

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REFERENCES

1. Diskin MG, Murphy JJ, Sreenan JM. Embryo survival in dairy cows managed under pastoral conditions. Anim Reprod Sci 2006; 96:297-311.
2. Sartori R, Sartor-Bergfelt R, Mertens SA, Guenther JN, Parrish JJ, Wiltbank MC. Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. J Dairy Sci 2002; 85:2803-2812.
3. Walters AH, Bailey TL, Pearson RE, Gwazdauskas FC. Parity-related changes in bovine follicle and oocyte populations, oocyte quality, and hormones to 90 days postpartum. J Dairy Sci 2002; 85:824-832.
4. Britt JH. Impacts of early postpartum metabolism on follicular development and fertility. Bov Proc 1992; 24:39-43.
5. Butler WR. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livest Prod Sci 2003; 83:211-218.
6. van Knegsel AT, van den Brand H, Dijkstra J, Tamminga S, Kemp B. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. Reprod Nutr Dev 2005; 45:665-688.
7. Kawashima C, Matsui M, Shimizu T, Kida K, Miyamoto A. Nutritional factors that regulate ovulation of the dominant follicle during the first follicular wave postpartum in high-producing dairy cows. J Reprod Dev 2012; 58:10-16.
8. Beam SW, Butler WR. Energy balance, metabolic hormones, and early postpartum follicular development in dairy cows fed prilled lipid. J Dairy Sci 1998; 81:121-131.
9. Butler WR, Smith RD. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. J Dairy Sci 1989; 72:767-783.
10. Contreras GA, Sordillo LM. Lipid mobilization and inflammatory responses during the transition period of dairy cows. Comp Immunol Microbiol Infect Dis 2011; 34:281-289.
11. Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. Diabetes 2001; 50:1771-1777.
12. Coll T, Eyre E, Rodriguez-Calvo R, Palomer X, Sanchez RM, Merlos M, Laguna JC, Vazquez-Carrera M. Oleate reverses palmitate-induced insulin resistance and inflammation in skeletal muscle cells. J Biol Chem 2008; 283:11107-11116.
13. Henique C, Mansouri A, Fumey G, Lenoir V, Girard J, Bouillaud F, Prip-Buus C, Cohen I. Increased mitochondrial fatty acid oxidation is sufficient to protect skeletal muscle cells from palmitate-induced apoptosis. J Biol Chem 2010; 285:36818-36827.
14. Listenberger LL, Han X, Lewis SE, Cases S, Farese RV,Jr, Ory DS, Schaffer JE. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc Natl Acad Sci U S A 2003; 100:3077-3082.
15. Listenberger LL, Ory DS, Schaffer JE. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. J Biol Chem 2001; 276:14890-14895.
16. Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, Donath MY. Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. Diabetes 2001; 50:69-76.
17. Mishra R, Simonson MS. Saturated free fatty acids and apoptosis in microvascular mesangial cells: palmitate activates pro-apoptotic signaling involving caspase 9 and mitochondrial release of endonuclease G. Cardiovasc Diabetol 2005; 4:2.

18. Mu YM, Yanase T, Nishi Y, Tanaka A, Saito M, Jin CH, Mukasa C, Okabe T, Nomura M, Goto K, Nawata H. Saturated FFAs, palmitic acid and stearic acid, induce apoptosis in human granulosa cells. Endocrinology 2001; 142:3590-3597.
19. Vanholder T, Leroy JL, Soom AV, Opsomer G, Maes D, Coryn M, de Kruif A. Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation in vitro. Anim Reprod Sci 2005; 87:33-44.
20. Vanholder T, Leroy JLMR, Van Soom A, Maes D, Coryn M, Fiers T, de Kruif A, Opsomer G. Effect of non-esterified fatty acids on bovine theca cell steroidogenesis and proliferation in vitro. Anim Reprod Sci 2006; 92:51-63.
21. Leroy JL, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G, Van Soom A. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. Reproduction 2005; 130:485-495.
22. Wu LL, Russell DL, Norman RJ, Robker RL. Endoplasmic Reticulum (ER) Stress in Cumulus-Oocyte Complexes Impairs Pentraxin-3 Secretion, Mitochondrial Membrane Potential (ΔΨm), and Embryo Development. Mol Endocrinol 2012; 26:562-573.
23. Aardema H, Vos PL, Lolicato F, Roelen BA, Knijn HM, Vaandrager AB, Helms JB, Gadella BM. Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. Biol Reprod 2011; 85:62-69.
24. Stevenson JS. Clinical reproductive physiology of the cow. In: Youngquist RS and Threlfall WR (eds.), Current therapy in large animal theriogenology, 2nd ed. Philadelphia: 2007: 286.
25. Gautam G, Nakao T, Yamada K, Yoshida C. Defining delayed resumption of ovarian activity postpartum and its impact on subsequent reproductive performance in Holstein cows. Theriogenology 2010; 73:180-189.
26. Pieterse MC, Vos PL, Kruip TA, Wurth YA, van Beneden TH, Willemse AH, Taverne MA. Transvaginal ultrasound guided follicular aspiration of bovine oocytes. Theriogenology 1991; 35:857-862.
27. Aardema H, Lolicato F, van de Lest CH, Brouwers JF, Vaandrager AB, van Tol HT, Roelen BA, Vos PL, Helms JB, Gadella BM. Bovine Cumulus Cells Protect Maturing Oocytes from Increased Fatty Acid Levels by Massive Intracellular Lipid Storage. Biol Reprod 2013; 88:164.
28. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; 37:911-917.
29. Kates M. Techniques of Lipidology: Isolation, Analysis and Identification of Lipids. In: Burdon RH and Van Knippenberg PH (eds.), Laboratory Techniques in Biochemistry and Molecular Biology, 2nd ed. Elsevier Amsterdam; 1986: 125.
30. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal Chem 2006; 78:779-787.
31. Pinheiro J, Bates D, DebRoy S, Sarkar D, the R Development Core Team. Linear and Nonlinear Mixed Effects Models. 2011; R package version 3.1-101:
32. Galvao KN, Frajblat M, Butler WR, Brittin SB, Guard CL, Gilbert RO. Effect of early postpartum ovulation on fertility in dairy cows. Reprod Domest Anim 2010; 45:e207-11.
33. Staples CR, Thatcher WW, Clark JH. Relationship between ovarian activity and energy status during the early postpartum period of high producing dairy cows. J Dairy Sci 1990; 73:938-947.

34. Peter AT, Vos PL, Ambrose DJ. Postpartum anestrus in dairy cattle. Theriogenology 2009; 71:1333-1342.
35. Stewart RE, Spicer LJ, Hamilton TD, Keefer BE. Effects of insulin-like growth factor I and insulin on proliferation and on basal and luteinizing hormone-induced steroidogenesis of bovine thecal cells: involvement of glucose and receptors for insulin-like growth factor I and luteinizing hormone. J Anim Sci 1995; 73:3719-3731.
36. Fair T, Hulshof SC, Hyttel P, Greve T, Boland M. Oocyte ultrastructure in bovine primordial to early tertiary follicles. Anat Embryol (Berl) 1997; 195:327-336.
37. Hennet ML, Combelles CM. The antral follicle: a microenvironment for oocyte differentiation. Int J Dev Biol 2012; 56:819-831.
38. Hess KA, Chen L, Larsen WJ. The ovarian blood follicle barrier is both charge- and size-selective in mice. Biol Reprod 1998; 58:705-711.
39. Shalgi R, Kraicer P, Rimon A, Pinto M, Soferman N. Proteins of human follicular fluid: the blood-follicle barrier. Fertil Steril 1973; 24:429-434.
40. Moreau C, Froment P, Tosca L, Moreau V, Dupont J. Expression and regulation of the SCD2 desaturase in the rat ovary. Biol Reprod 2006; 74:75-87.

Chapter 6

Summarizing discussion

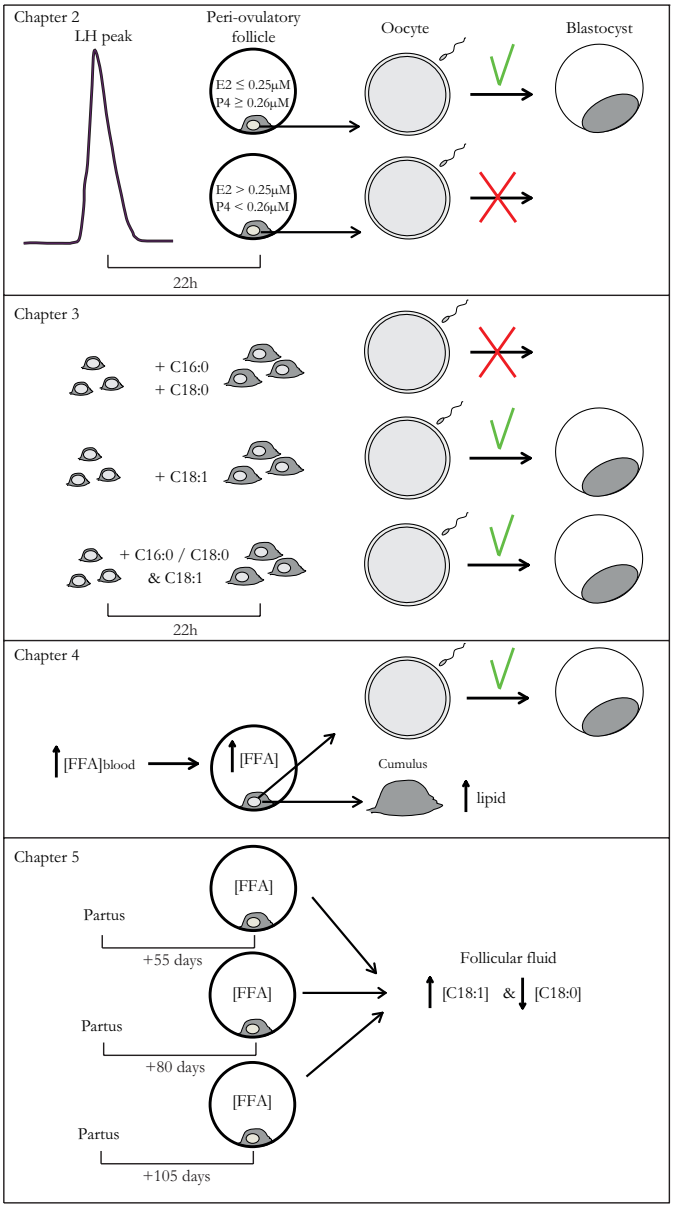

Figure 1. A summary of the major findings of this thesis per chapter. In Chapter 2 it was shown that only oocytes that derived from peri-ovulatory follicles with a 17β-estradiol ≤ 0.25 µM and progesterone ≥ 0.26 µM developed into a blastocyst. Chapter 3 demonstrated a dose-dependent negative impact of saturated C16:0 and C18:0 on the developmental competence of the oocyte, in contrast to the harmless impact of mono-unsaturated C18:1. Furthermore, C18:1 prevented the negative impact of saturated FFA on the oocyte. Chapter 4 showed that oocytes in peri-ovulatory follicles appear to be protected against elevated FFA in blood by the combination of a relatively high level of C18:1 in the follicular fluid and the surrounding cumulus cells that massively accumulated the fatty acids in intracellular lipid droplets. Chapter 5 showed that the FFA composition in follicular fluid at different postpartum insemination moments was not different and contained a relatively high C18:1 and low C18:0 concentration in comparison to blood. E2 = 17β-estradiol, P4 = progesterone and FFA = free fatty acid.

During the last four decades the fertility of high-producing dairy cows has declined dramatically [1-6]. This decline in fertility has been related to negative energy balance (NEB) in the early post-partum period of cows, characterized by strongly elevated free fatty acid concentrations in the blood. While the association of a metabolic condition characterized by elevated free fatty acid concentrations and reduced fertility is not restricted to the cow, a similar effect has been observed in women [7-16], the effects of elevated levels of free fatty acids on female gonadal function and gamete quality are largely unknown. The aim of this thesis was to advance our understanding of the impact of elevated free fatty acid concentrations on fertility, with a particular focus on the maturing oocyte and its post-fertilization developmental competence. Figure 1 presents the major findings of this thesis in a schematic picture.

The selection of matured follicles after super-stimulation treatment

In this thesis, a timed FSH ovarian stimulation protocol was used to increase the number of presumptive pre-ovulatory follicles in the mono-ovulatory cow. However, a potential disadvantage of ovarian stimulation is that, as well as the stimulation of competent follicles, the gonadotrophins also partly rescue and stimulate "non-competent follicles" [17, 18]. In **Chapter 2** we demonstrated that hormone concentrations in the follicular fluid of FSH stimulated follicles at the end of the maturation phase can be used as a predictor of the post-fertilization developmental competence of oocytes recovered after ovarian stimulation. Specifically, oocytes that originated from follicles with 17β-estradiol concentrations ≤ 0.25 μM and progesterone concentrations ≥ 0.26 μM were fully competent and able to develop into a blastocyst after fertilization. The observed < 1 ratio of 17β-estradiol to progesterone in follicular fluid from competent follicles of super-stimulated heifers is in agreement with the hormonal environment within the mature pre-ovulatory follicle of non-stimulated cows near the time of ovulation [19]. The LH peak marks the switch from 17β-estradiol dominance at the start of maturation to progesterone dominance at the end of maturation, and results from the stimulation of the synthesis of progesterone by luteinized theca and granulosa cells [19, 20]. Indeed the concentrations of 17β-estradiol and progesterone in follicular fluid can be used to select mature follicles containing a competent oocyte. Progesterone appears to have an impact on the nuclear maturation of the oocyte [17, 21]. The inhibition of progesterone synthesis with trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase) in cumulus cells during *in vitro* maturation has been shown to impair post-fertilization developmental capacity of the oocyte, although this can be recovered

by addition of progesterone to the maturation medium [22]. These observations emphasize the importance of appropriate hormone concentrations for the cumulus-oocyte-complex (COC) during final maturation. The predictive value of hormone concentrations in follicular fluid is of particular interest for human *in vitro* fertilization (IVF) where follicles stimulated to develop by gonadotrophin treatment are punctured, in contrast to commercial bovine IVF in which immature follicles are aspirated to obtain oocytes for *in vitro* fertilization and embryo production. The potential to determine the developmental competence of the retrieved oocytes using a non-invasive, cost-effective method may be of considerable value for human IVF [23]. Rapid assay of follicular fluid steroid hormone concentrations may therefore be a useful adjunct to human IVF, especially as it does not require any extra samples since it can be performed on a by-product of oocyte recovery, the follicular fluid.

Extracellular fatty acids are incorporated into the oocyte

Fatty acids are essential nutrients and building blocks for all cells including the oocyte and the cells of the early embryo [24-33]. The importance of fatty acids for the developing oocyte and early embryo is described in **Chapter 1**. However, the origin of the fatty acids in oocytes, which are mainly present in the form of triacylglycerol (TAG) stored in lipid droplets [29, 34, 35], has not been clear. Lipid droplets are present in oocytes from an early stage of development, as early as the primordial follicle stage, and their numbers gradually increase during the successive follicular developmental stages [36]. Fatty acids in cells can be either synthesized *de novo* or incorporated from the extracellular environment, and it has been suggested that oocytes express enzymes required for *de novo* fatty acid synthesis [37-39]. On the other hand, because oocytes are surrounded by a glycoprotein layer, the zona pellucida, incorporation of extracellular fatty acids may be inhibited. However, data presented in this thesis demonstrate that oocytes can incorporate free fatty acids from their environment, despite the presence of the zona pellucida (**Chapter 3**). Oocytes with (**Chapter 3**) or without a surrounding cumulus complex were both able to incorporate free fatty acids, where denuded oocytes do so initially at a higher rate (Aardema et al., unpublished data). In this respect, cumulus cells appear to play a pivotal role in the uptake of fatty acids and, in particular, seem to restrict and delay the uptake of extracellular free fatty acids into the oocyte (**Chapter 4** and Aardema et al., unpublished data). Fatty acids are usually transported across a cell's phospholipid bilayer either actively or passively [40]. However, cumulus cells have gap junctional connections to the oocyte via cellular protrusions

through the zona pellucida, and are thus able to transfer small metabolites and, presumably, fatty acids directly to the oocyte [41, 42]. The uptake of free fatty acids by the oocyte implies that extracellular fatty acids may influence an oocyte's metabolism and maturation and thus have the ability to (de)regulate its post-fertilization developmental competence. Interestingly, the fatty acid composition of oocytes from COCs appears to differ between COCs of high and those of moderate and low quality [29]. While palmitic acid (C16:0) is the most abundant fatty acid type in oocytes from both types of COCs, the second most abundant fatty acid in oocytes of high quality COCs is oleic acid (C18:1) as compared to stearic acid (C18:0) in COCs of moderate or low quality [29]. This suggests that exposure to C18:1 and, presumably, uptake of C18:1 by the oocyte may improve oocyte quality. Furthermore, external fatty acids can also be incorporated into the phospholipid fraction of the oocyte (**Chapter 3**) which may affect membrane function and, for example, the freezeability of the oocyte and future embryo. Indeed, exposure of maturing COCs to either saturated C16:0 or C18:0 reduced the tolerance of embryos to cryopreservation, whereas exposure to C18:1 did not affect cryopreservability [43]. Since both human embryos and commercially produced bovine embryos are frequently cryopreserved for subsequent embryo transfer, optimization of the maturation conditions with free fatty acids could increase the proportion of embryos able to survive cryopreservation and may therefore have broad consequences for IVF.

Follicular fluid appears to have a relatively high level of oleic acid compared to blood

Elevated levels of free fatty acids in the blood are reflected in the follicular fluid and thus in the direct environment of the oocyte (**Chapter 4**). The most prominent free fatty acids in blood and follicular fluid were C16:0, C18:0 and C18:1 (**Chapters 4** and **5)**. These findings correspond with data derived from cows during the post-partum NEB period. Indeed, bovine adipose tissue, from where the free fatty acids are mobilized during periods of inadequate energy intake is dominated by the same three fatty acid types [44, 45]. Nevertheless, the precise free fatty acid compositions of blood and follicular fluid appear to be distinct (**Chapter 4** and **5)**. In cows with elevated levels of circulating free fatty acids (**Chapter 4**), including post-partum cows (**Chapter 5**), a relatively higher level of C18:1 and a lower level of saturated free fatty acids are detected in follicular fluid than in blood. By contrast, Bender et al. (2010) found that linoleic acid (C18:2) was the most prominent fatty acid in follicular fluid [46]. The different outcome of these studies may be due to the different way in which

fatty acid analysis was performed in the study of Bender et al. (2010; [46]). Bender et al. (2010; [46]) performed a total fatty acid analysis rather than the analysis of the isolated free fatty acid fraction performed in the work reported in this thesis. The fatty acids reported by Bender et al. (2010) thus include the fatty acids that were packed in lipoproteins [46]. Since metabolic stress affects the free fatty acid fraction (**Chapter 4**) and this appears to be the fraction to which oocytes (**Chapter 3**) and embryos respond [47, 48], we preferred to concentrate analysis on the free fatty acid fraction rather than the total fatty acids present in follicular fluid.

Lipid accumulation in cumulus cells exposed to elevated free fatty acid levels

Elevated free fatty acid concentrations in follicular fluid resulted in a massive accumulation of lipids in cumulus cells during maturation both *in vivo* and *in vitro* (**Chapter 4**). Remarkably, the lipid composition of the oocytes did not change after exposure to elevated free fatty acid concentrations (**Chapter 4**) whereas oocytes appeared to be able to rapidly incorporate free fatty acids from the surrounding fluid (**Chapter 3)**. Nevertheless, the unaffected total amount of fatty acids in oocytes after exposure to elevated free fatty acid levels (**Chapter 4**) does not exclude a potentially present exchange of fatty acids between the environment and the oocyte (**Chapter 3**).

A distinct effect of saturated and mono-unsaturated free fatty acids on the oocyte

The different effects of saturated versus mono-unsaturated free fatty acids on the maturing oocyte were marked (**Chapter 3**). Saturated C16:0 and C18:0 had a dose-dependent negative impact on the developmental competence of the oocyte. This was in sharp contrast to mono-unsaturated C18:1, which appeared to be harmless to the oocyte, even at high concentrations. Interestingly, C18:1 completely prevented an adverse effect of C16:0 and C18:0 on the oocytes and rescued the developmental competence of the oocyte (**Chapter 3**). Based on these *in vitro* findings (**Chapter 3**), a follicular fluid composition with a relatively high C18:1 concentration (**Chapter 4** and **5)** is proposed to be the most compatible to successfully maturing oocytes. Since C18:1 appears to protect the oocyte against the lipotoxic impact of saturated free fatty acids.

Lipotoxic effects of saturated free fatty acids

The negative effects of saturated free fatty acids on COCs have been suggested to include an endoplasmic reticulum (ER) stress response and apoptosis in cumulus cells and mitochondrial membrane depolarization in the oocyte, which are all signs of lipotoxic stress [45, 49]. Interestingly, the developmental

competence of fertilized oocytes could be completely restored after inhibition of the, by C16:0, induced ER stress with the ER stress inhibitor salubrinal [49]. The lipotoxic response of COCs exposed to saturated free fatty acids *in vitro* is largely comparable to the reaction of mouse oocytes to *in vivo* conditions in obese animals, which also includes the appearance of ER stress markers in the cumulus cells, mitochondrial membrane depolarization in the oocytes and decreased fertilization rates [15, 16]. Obese women also show signs of lipotoxic stress including, for example, increased Ca^{2+} levels in the follicular fluid [15]. The similarity in responses to elevated environmental C16:0 levels and obesity indicate that elevated concentrations of, in particular saturated, free fatty acids may play a significant role in the reported impaired fertility of obese women [8, 15, 16, 49]. This strongly suggests that more information on the free fatty acid conditions in follicular fluid during obesity should be generated. Since C18:1 appears to be able to compensate for the toxic impact of saturated free fatty acids (**Chapter 3**), intervention in a way that elevates intra-follicular C18:1 should be considered as a potential therapy.

Intervention with dietary fatty acids

A major dietary source of C18:1 is olive oil. C18:1 can be taken up by the intestine, packed into chylomicrons, transported via the circulation and stored in adipose tissue for later release in the form of free fatty acids. Increased dietary intake of olive oil may result in a relatively high oleic acid concentration in the free fatty acid fraction and, since circulating free fatty acid concentrations are reflected in follicular fluid, also in the fluid that surrounds the oocyte. Indeed, a positive effect on the pregnancy rate has been reported for women on Mediterranean diets, which are traditionally rich in olive oil, during the preconception period [50]. This indicates a potential positive impact of dietary olive oil intake on the developmental competence of the oocyte and early embryo. Unfortunately, intervention by altering dietary fatty acid intake is more complex in ruminants than non-ruminants. After entry of fatty acids into the rumen, bacteria hydrogenate unsaturated fatty acids to saturated fatty acids [51]. To overcome this phenomenon, fatty acids can be packed into calcium salts that protect against ruminal biohydrogenation [52]. Several such conjugated poly-unsaturated fatty acids are commercially available, including linoleic (C18:2) and linolenic acid (C18:3). Dietary provision of these fatty acids results in elevated levels of the "encapsulated fatty acids" in the blood and follicular fluid of ruminants [48, 53]. All of which suggests that dietary intervention may be a worthwhile strategy to attempt to improve fertility in both ruminants and non-ruminants.

Developmental competence of oocytes is not impaired by elevated levels of free fatty acids

Interestingly, elevated levels of free fatty acids in the follicular fluid did not impair the capacity of an oocyte to develop into a blastocyst (**Chapter 4**). By contrast, when oocytes were cultured without a cumulus cell layer during only the last six hours of maturation the developmental competence tended to be impaired by exposure to a mix of free fatty acids at levels consistent with physiological elevation (**Chapter 4**). The findings of **Chapters 3** and **4** suggest that the combination of C18:1 in follicular fluid and the cumulus cells surrounding the oocyte help protect the oocyte against lipotoxicity, their massive accumulation of fatty acids appears to restrict active or passive transport of free fatty acids towards the oocyte and may therefore prevent negative effects of elevated free fatty acids in follicular fluid. The cumulus cells also appear to protect the oocyte against reactive-oxygen-species [54]. The importance of cumulus cells to nurture the oocyte and allow proper nuclear maturation and subsequent fertilization have been well established [42, 55]. Apart from this supportive role during oocyte development, data from this thesis indicate that cumulus cells also appear to be crucial for the protection of the oocyte against potentially toxic free fatty acids.

Exposure to fatty acids complexed to albumin versus fatty acids solubilized in ethanol

In this thesis, it was demonstrated that C18:1 can reverse the detrimental effects of C16:0 and C18:0 on post-fertilization oocyte developmental competence (**Chapter 3**). By contrast, Van Hoeck et al. (2011; [56]) reported a reduced post-fertilization developmental competence of oocytes after exposure to similar free fatty acid concentrations and combinations (conform **Chapter 4**). This discrepancy is probably due to the method by which fatty acids were added to the maturation medium. Van Hoeck et al. (2011; [56]) injected stocks of ethanol-solubilized free fatty acids into the incubation bufferVan Hoeck et al. 2011). We took an, in our opinion, more physiological approach in which fatty acids were complexed with fatty- acid-free-albumin before being introduced to the maturation medium for COCs. The expected fatty acid micelle formation after ethanol injection into the maturation buffer, as well as the high level of ethanol (0.5%) may have resulted in the impaired developmental competence [56] that we did not observe when using free fatty acid-albumin complexes (**Chapter 4**).

Lipotoxic stress effects of saturated free fatty acids can be prevented by oleic acid

The protective effect of mono-unsaturated fatty acids against the by saturated fatty acids induced lipotoxicity on the oocyte (this thesis has also been observed in several somatic cell types [57-63]. C18:1 appears to support the storage of saturated fatty acids in lipid droplets and in so doing keeps these fatty acids away from apoptotic pathways [57, 58, 61-63]. Saturated fatty acids can increase concentrations of the toxic lipid-intermediates DAG and ceramides and can thereby down-regulate the synthesis of TAG from DAG by diacyl-glycerol acyltransferase 2 (DGAT 2) [57, 61, 63]. Co- or pre-incubation with oleic acid can increase the expression of DGAT 2 and can channel the saturated fatty acids into the TAG fraction, thereby increasing the amount of TAGs while reducing the amounts of ceramide and DAG [57, 61, 63]. TAG accumulation is cyto-protective because it involves sequestering potentially toxic fatty acids into lipid droplets. A second potential protective mechanism of oleic acid is the stimulation of fatty acid breakdown, which has been observed in skeletal muscle cells, by inducing an increase in the expression of carnitine-palmitoyl-transferase I (CPT-1), the rate limiting enzyme in fatty acid breakdown [58, 63]. The lipid accumulation in cumulus cells after exposure to elevated free fatty acid levels also appears to protect the oocyte against potential negative effects of elevated free fatty acid concentrations (**Chapter 4**). Furthermore, *in vitro* exposure of maturing oocytes to C18:1 prevented lipid depletion induced by saturated fatty acids and restored the post-fertilization developmental competence of the oocytes. These results indicate that diversion of saturated fatty acids to lipid droplets by C18:1 facilitated normal development (**Chapter 3**).

SCD activity in the follicle may be responsible for high oleic acid concentrations in follicular fluid

The lower levels of C18:0 and concomitant higher levels of C18:1 in follicular fluid were related to a significantly higher index in that follicular fluid of stearoyl-CoA desaturase (SCD) activity, the enzyme that transforms C18:0 into C18:1 by the introduction of a cis-double bond between the 9^{th} and 10^{th} carbon atoms (**Chapter 5)**. This indicates that the follicle may indeed actively convert C18:0 to C18:1. Both gene and protein expression for SCD have been detected in the rat ovary and it appears that SCD is highly expressed in both granulosa and cumulus cells, whereas there is only low expression in theca cells and no gene expression in oocytes [64]. Furthermore, gene expression for SCD in bovine cumulus cells was up-regulated during maturation (Aardema et al., unpublished data). SCD expression appears to be highly related to the fatty acid composition

of milk and in particular with the mono-unsaturated fatty acids present in milk [65-67]. The free fatty acid composition of follicular fluid may also be related to the SCD expression in the dominant follicle of cows. Future research should investigate whether SCD and free fatty acid compositions of follicular fluid are related to the fertility of individual cows. A relationship between SCD and fertility would be of particular interest because the SCD activity varies among individual cows [67, 68]. Furthermore, desaturase activity, and thus the ability to convert saturated fatty acids into mono-unsaturated fatty acids by SCD, appears to be heritable [67-70]. This indicates that selection for desaturase indices, and thus SCD activity, may be valuable in future breeding programs.

FUTURE ASPECTS

1. The impact of free fatty acids on early stage follicles

In this thesis the impact of elevated free fatty acid concentrations on oocyte developmental competence during final maturation was investigated. However, the growth phase of the follicle, from the primordial stage until the pre-ovulatory stage, takes around 100 days in the cow [5], and during all the different stages of development the oocyte may be exposed to harmful elevated free fatty acid concentrations. Initially, in the early follicular phase, the follicle contains only a single layer of granulosa cells surrounding the oocyte but, during subsequent growth phases, the follicle develops into a structure with several cell layers [36]. It follows that oocytes may be more prone to elevated levels of circulating free fatty acids during early follicle development. This is of particular interest because the NEB in dairy cows takes place during the early weeks after calving, when the follicles that may become dominant at 60-80 days post-partum have been recruited and have started their development. Exposure of these oocytes in pre-antral follicles to toxic reagents may lead to oocytes of inferior quality at the time of fertilization. Indeed, Walters et al. [3] demonstrated that the quality of oocytes, that started their development during the NEB period, was reduced at around 70 days post-partum. For these reasons, future research should investigate the impact of elevated free fatty acid concentrations on oocytes in early stage follicles and follow their subsequent development until ovulation. Furthermore, if evidence emerges of damage to early developing oocytes by increased concentrations and the increased saturation of free fatty acids in the blood, post-partum intervention strategies based around oleic acid supplementation should be considered and their utility investigated.

2. The impact of free fatty acids on the early embryo and its direct environment

Elevated free fatty acid levels may also affect the early embryo in the oviduct and uterus. Pre-implantation embryos are able to take up free fatty acids and appear to be sensitive to the effects of free fatty acids [71-75]. Exposure of mouse and rat embryos to the saturated palmitic acid *in vitro* reduced the percentage of blastocysts that were formed, whereas oleic acid alone or in combination with palmitic acid promoted embryo development [76, 77]. This suggests that elevated levels of free fatty acids in the oviduct and uterus could impair embryo development. However, information on the fatty acid composition in the oviduct and uterus is scarce [78, 79] and information on the impact of elevated free fatty acid levels in the blood on the free fatty acid concentrations in the oviduct and uterus is lacking. Future research should focus on establishing the free fatty acid environments that an embryo encounters during its transfer from the oviduct to the uterus. This is of particular interest because the embryonic genome is demethylated and remethylated between fertilization and the blastocyst stage. This process of embryonic genome de- and remethylation can be influenced by the environment and can in turn result in altered gene expression patterns [80]. These epigenetic changes can result in a lifelong impact on the offspring. A well-described example, in this respect, is the Dutch 'hunger winter', during which many Dutch people suffered severe malnutrition. Children from women that were in their early stages of pregnancy during the hunger winter subsequently transpired to have a higher risk of circulatory and metabolic diseases, like type II diabetes, during adulthood [81-83]. Similarly, a high concentration of fetal-calf-serum during *in vitro* culture of cattle embryos results in an increased size of the offspring at birth, a condition known as 'large offspring syndrome' [84-86]. This large offspring syndrome is caused by epigenetic changes in the imprinted genes that occurred during the *in vitro* embryo culture period. Furthermore, in a recent study mouse embryos that were only exposed to palmitic acid during early embryonic development showed a reduced size at birth, followed by catch-up growth postnatally [87]. Free fatty acid exposure during early embryonic development may thus be considered as a potential epigenetic effector. The *in vitro* bovine culture system is an ideal environment to investigate the "epigenetic" impact of free fatty acids on the oocyte and early embryo in a standardized setting. The "epigenetic" impact of free fatty acids on the embryo can be investigated after culture by analysis of DNA methylation and potential histone modifications. The cow is a good animal model to investigate the impact of potential epigenetic factors on human embryos because both species are mono-ovulatory and have a comparable estrous cycle and maturation length. Furthermore oocytes from the

ovaries of slaughtered cows are readily available, in stark contrast to the limited availability of human material and ethical concerns of working on early human embryos. In addition, the use of slaughterhouse material significantly reduces the use of experimental animals, like mice.

3. How are fatty acids incorporated into cumulus cells and the oocyte during maturation?

The results presented in this thesis clearly indicate a role for the cumulus cells in the transfer of free fatty acids to the oocyte, with reduced uptake by oocytes surrounded by cumulus cells. However, it is not currently known how free fatty acid transport into the oocyte is regulated. The cumulus cells appear to present a functional barrier between the free fatty acids in the follicular fluid and those in the oocyte. In this respect, it has been suggested that cumulus cell expansion functions as a molecular sieve that restricts the transfer of potentially toxic metabolites to the oocyte during final maturation [88]. For this reason, it is of particular interest to investigate whether reduced cumulus cell expansion by the COC, which appears to be related to reduced developmental competence of the oocyte (**Chapter 2**), corresponds to a reduced "molecular sieve function" of the cumulus cells and increased exposure of oocytes to potentially toxic free fatty acids. Future research should aim to establish how extracellular fatty acids are taken up by the oocyte.

4. Human relevance

The negative impact of elevated levels of saturated fatty acids on the oocyte is also of great interest for human gamete function and embryonic development. Particularly given that obesity has become a serious metabolic problem in developed countries and is directly associated with elevated levels of free fatty acids in the blood [89]. Furthermore, metabolic conditions characterized by elevated free fatty acid levels have been associated with impaired fertility not only in cows, but also in women [7-16]. The cow appears to be a good model to study the period from the primordial follicle stage until the entrance of the fertilized embryo into the uterus, as follicular growth and the time from fertilization until the blastocyst stage are of comparable length in man and cow; this is in marked contrast to the extensively used mouse model [5, 90-93]. The impact of metabolic conditions can be investigated both *in vitro* and *in vivo* by using obese cows, with a high body-condition-score, as an animal model for the human situation.

OVERALL CONCLUSION

Elevated concentrations of saturated free fatty acids in the follicular fluid of the maturing COC can seriously impair oocyte maturation; C16:0 and C18:0 fatty acids dose-dependently decrease the post-fertilization developmental competence of maturing oocytes. However, the adverse effects of these saturated free fatty acids can be completely prevented by C18:1. These findings strongly indicate that the free fatty acid composition, rather than simply the total amount of free fatty acids present, determines the effect on the oocyte. This is also of interest for the human situation since obesity, which is also characterized by elevated circulating free fatty acid concentrations, results in lipotoxic responses presumably provoked by saturated free fatty acids. Interestingly, C18:1 is a relatively abundant fatty acid in the follicular fluid of metabolically stressed and post-partum cows. The combination of the relatively high C18:1 concentration and massively increased storage of fatty acids in the cumulus cells appears to protect the oocyte against potential harm from elevated saturated free fatty acid concentrations in follicular fluid. The elevated levels of free fatty acids did not impair the post-fertilization developmental competence of oocytes from maturing COCs in peri-ovulatory follicles. However, elevated free fatty acid levels may pose more of a threat to early stage follicles which have a less extensive granulosa and cumulus investment to protect them and are likely to be exposed to significant elevations in blood free fatty acid concentrations. The impact of free fatty acids on these early stage follicles certainly needs further investigation. Dietary intervention with C18:1, both in women and cows, is a strategy that should be investigated with respect to its ability to prevent the potential negative effects of saturated free fatty acids on the oocyte.

REFERENCES

1. Diskin MG, Murphy JJ, Sreenan JM. Embryo survival in dairy cows managed under pastoral conditions. Anim Reprod Sci 2006; 96:297-311.
2. Sartori R, Sartor-Bergfelt R, Mertens SA, Guenther JN, Parrish JJ, Wiltbank MC. Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. J Dairy Sci 2002; 85:2803-2812.
3. Walters AH, Bailey TL, Pearson RE, Gwazdauskas FC. Parity-related changes in bovine follicle and oocyte populations, oocyte quality, and hormones to 90 days postpartum. J Dairy Sci 2002; 85:824-832.
4. van Knegsel AT, van den Brand H, Dijkstra J, Tamminga S, Kemp B. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. Reprod Nutr Dev 2005; 45:665-688.
5. Britt JH. Impacts of early postpartum metabolism on follicular development and fertility. Bov Proc 1992; 24:39-43.
6. Butler WR. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livest Prod Sci 2003; 83:211-218.
7. Jungheim ES, Macones GA, Odem RR, Patterson BW, Lanzendorf SE, Ratts VS, Moley KH. Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during in vitro fertilization. Fertil Steril 2011; 95:1970-1974.
8. Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE, Moley KH. Diet-induced obesity model: abnormal oocytes and persistent growth abnormalities in the offspring. Endocrinology 2010; 151:4039-4046.
9. Metwally M, Saravelos SH, Ledger WL, Li TC. Body mass index and risk of miscarriage in women with recurrent miscarriage. Fertil Steril 2010; 94:290-295.
10. Jorritsma R, Wensing T, Kruip TA, Vos PL, Noordhuizen JP. Metabolic changes in early lactation and impaired reproductive performance in dairy cows. Vet Res 2003; 34:11-26.
11. Gesink Law DC, Maclehose RF, Longnecker MP. Obesity and time to pregnancy. Human Reproduction 2007; 22:414-420.
12. Rich-Edwards JW, Spiegelman D, Garland M, Hertzmark E, Hunter DJ, Colditz GA, Willett WC, Wand H, Manson JE. Physical activity, body mass index, and ovulatory disorder infertility. Epidemiology 2002; 13:184-190.
13. Kawashima C, Sakaguchi M, Suzuki T, Sasamoto Y, Takahashi Y, Matsui M, Miyamoto A. Metabolic profiles in ovulatory and anovulatory primiparous dairy cows during the first follicular wave postpartum. J Reprod Dev 2007; 53:113-120.
14. Robker RL, Wu LL, Yang X. Inflammatory pathways linking obesity and ovarian dysfunction. J Reprod Immunol 2011; 88:142-148.
15. Wu LL, Dunning KR, Yang X, Russell DL, Lane M, Norman RJ, Robker RL. High-fat diet causes lipotoxicity responses in cumulus-oocyte complexes and decreased fertilization rates. Endocrinology 2010; 151:5438-5445.
16. Yang X, Wu LL, Chura LR, Liang X, Lane M, Norman RJ, Robker RL. Exposure to lipid-rich follicular fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus-oocyte complexes. Fertil Steril 2012; 97:1438-5.
17. de Loos FA, Bevers MM, Dieleman SJ, Kruip TA. Follicular and oocyte maturation in cows treated for superovulation. Theriogenology 1991; 35:537-546.

18. Callesen H, Greve T, Hyttel P. Preovulatory endocrinology and oocyte maturation in superovulated cattle. Theriogenology 1986; 25:71-86.
19. Dieleman SJ, Bevers MM, Poortman J, van Tol HT. Steroid and pituitary hormone concentrations in the fluid of preovulatory bovine follicles relative to the peak of LH in the peripheral blood. J Reprod Fertil 1983; 69:641-649.
20. Komar CM, Berndtson AK, Evans AC, Fortune JE. Decline in circulating estradiol during the periovulatory period is correlated with decreases in estradiol and androgen, and in messenger RNA for p450 aromatase and p450 17alpha-hydroxylase, in bovine preovulatory follicles. Biol Reprod 2001; 64:1797-1805.
21. Siqueira LC, Barreta MH, Gasperin B, Bohrer R, Santos JT, Buratini J,Jr, Oliveira JF, Goncalves PB. Angiotensin II, progesterone, and prostaglandins are sequential steps in the pathway to bovine oocyte nuclear maturation. Theriogenology 2012; 77:1779-1787.
22. Aparicio IM, Garcia-Herreros M, O'Shea LC, Hensey C, Lonergan P, Fair T. Expression, regulation, and function of progesterone receptors in bovine cumulus oocyte complexes during in vitro maturation. Biol Reprod 2011; 84:910-921.
23. Wallace M, Cottell E, Gibney MJ, McAuliffe FM, Wingfield M, Brennan L. An investigation into the relationship between the metabolic profile of follicular fluid, oocyte developmental potential, and implantation outcome. Fertil Steril 2012; 97:1078-84.
24. Kane MT. Minimal nutrient requirements for culture of one-cell rabbit embryos. Biol Reprod 1987; 37:775-778.
25. Ferguson EM, Leese HJ. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. Mol Reprod Dev 2006; 73:1195-1201.
26. Ferguson EM, Leese HJ. Triglyceride content of bovine oocytes and early embryos. J Reprod Fertil 1999; 116:373-378.
27. Abe H, Yamashita S, Itoh T, Satoh T, Hoshi H. Ultrastructure of bovine embryos developed from in vitro-matured and -fertilized oocytes: comparative morphological evaluation of embryos cultured either in serum-free medium or in serum-supplemented medium. Mol Reprod Dev 1999; 53:325-335.
28. Sturmey RG, Leese HJ. Energy metabolism in pig oocytes and early embryos. Reproduction 2003; 126:197-204.
29. Kim JY, Kinoshita M, Ohnishi M, Fukui Y. Lipid and fatty acid analysis of fresh and frozen-thawed immature and in vitro matured bovine oocytes. Reproduction 2001; 122:131-138.
30. Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ, Robker RL. Beta-Oxidation Is Essential for Mouse Oocyte Developmental Competence and Early Embryo Development. Biol Reprod 2010; 83:909-918.
31. Downs SM, Mosey JL, Klinger J. Fatty acid oxidation and meiotic resumption in mouse oocytes. Mol Reprod Dev 2009; 76:844-853.
32. Paczkowski M, Silva E, Schoolcraft WB, Krisher RL. Comparative importance of Fatty Acid Beta-oxidation to nuclear maturation, gene expression, and glucose metabolism in mouse, bovine, and porcine cumulus oocyte complexes. Biol Reprod 2013; 88:111.
33. Sutton-McDowall ML, Feil D, Robker RL, Thompson JG, Dunning KR. Utilization of endogenous fatty acid stores for energy production in bovine preimplantation embryos. Theriogenology 2012; 77:1632-1641.

34. Homa ST, Racowsky C, McGaughey RW. Lipid analysis of immature pig oocytes. J Reprod Fertil 1986; 77:425-434.
35. McEvoy TG, Coull GD, Broadbent PJ, Hutchinson JS, Speake BK. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. J Reprod Fertil 2000; 118:163-170.
36. Fair T, Hulshof SC, Hyttel P, Greve T, Boland M. Oocyte ultrastructure in bovine primordial to early tertiary follicles. Anat Embryol (Berl) 1997; 195:327-336.
37. Auclair S, Uzbekov R, Elis S, Sanchez L, Kireev I, Lardic L, Dalbies-Tran R, Uzbekova S. Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes. Am J Physiol Endocrinol Metab 2013; 304:599-613.
38. Cetica P, Pintos L, Dalvit G, Beconi M. Involvement of enzymes of amino acid metabolism and tricarboxylic acid cycle in bovine oocyte maturation in vitro. Reproduction 2003; 126:753-763.
39. Cetica P, Pintos L, Dalvit G, Beconi M. Activity of key enzymes involved in glucose and triglyceride catabolism during bovine oocyte maturation in vitro. Reproduction 2002; 124:675-681.
40. Glatz JFC, Luiken JJFP, Bonen A. Membrane Fatty Acid Transporters as Regulators of Lipid Metabolism: Implications for Metabolic Disease. Physiological Reviews 2010; 90:367-417.
41. de Loos F, Kastrop P, Van Maurik P, Van Beneden TH, Kruip TA. Heterologous cell contacts and metabolic coupling in bovine cumulus oocyte complexes. Mol Reprod Dev 1991; 28:255-259.
42. Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum Reprod Update 2008; 14:159-177.
43. Shehab-El-Deen MA, Leroy JL, Maes D, Van Soom A. Cryotolerance of bovine blastocysts is affected by oocyte maturation in media containing palmitic or stearic acid. Reprod Domest Anim 2009; 44:140-142.
44. Rukkwamsuk T, Geelen MJ, Kruip TA, Wensing T. Interrelation of fatty acid composition in adipose tissue, serum, and liver of dairy cows during the development of fatty liver postpartum. J Dairy Sci 2000; 83:52-59.
45. Leroy JL, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G, Van Soom A. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. Reproduction 2005; 130:485-495.
46. Bender K, Walsh S, Evans AC, Fair T, Brennan L. Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. Reproduction 2010; 139:1047-1055.
47. Hughes J, Kwong WY, Li D, Salter AM, Lea RG, Sinclair KD. Effects of omega-3 and -6 polyunsaturated fatty acids on ovine follicular cell steroidogenesis, embryo development and molecular markers of fatty acid metabolism. Reproduction 2011; 141:105-118.
48. Wonnacott KE, Kwong WY, Hughes J, Salter AM, Lea RG, Garnsworthy PC, Sinclair KD. Dietary omega-3 and -6 polyunsaturated fatty acids affect the composition and development of sheep granulosa cells, oocytes and embryos. Reproduction 2010; 139:57-69.
49. Wu LL, Russell DL, Norman RJ, Robker RL. Endoplasmic Reticulum (ER) Stress in Cumulus-Oocyte Complexes Impairs Pentraxin-3 Secretion, Mitochondrial Membrane Potential (ΔΨm), and Embryo Development. Mol Endocrinol 2012; 26:562-573.

50. Vujkovic M, de Vries JH, Lindemans J, Macklon NS, van der Spek PJ, Steegers EAP, Steegers-Theunissen RPM. The preconception Mediterranean dietary pattern in couples undergoing in vitro fertilization/intracytoplasmic sperm injection treatment increases the chance of pregnancy. Fertil Steril 2010; 94:2096-2101.
51. Nafikov RA, Beitz DC. Carbohydrate and lipid metabolism in farm animals. J Nutr 2007; 137:702-705.
52. Jenkins TC, Palmquist DL. Effect of fatty acids or calcium soaps on rumen and total nutrient digestibility of dairy rations. J Dairy Sci 1984; 67:978-986.
53. Zachut M, Arieli A, Moallem U. Incorporation of dietary n-3 fatty acids into ovarian compartments in dairy cows and the effects on hormonal and behavioral patterns around estrus. Reproduction 2011; 141:833-840.
54. Fatehi AN, Roelen BA, Colenbrander B, Schoevers EJ, Gadella BM, Beverst MM, van den Hurk R. Presence of cumulus cells during in vitro fertilization protects the bovine oocyte against oxidative stress and improves first cleavage but does not affect further development. Zygote 2005; 13:177-185.
55. Albertini DF, Combelles CM, Benecchi E, Carabatsos MJ. Cellular basis for paracrine regulation of ovarian follicle development. Reproduction 2001; 121:647-653.
56. Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PE, Leroy JL. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. PLoS One 2011; 6:e23183.
57. Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. Diabetes 2001; 50:1771-1777.
58. Henique C, Mansouri A, Fumey G, Lenoir V, Girard J, Bouillaud F, Prip-Buus C, Cohen I. Increased mitochondrial fatty acid oxidation is sufficient to protect skeletal muscle cells from palmitate-induced apoptosis. J Biol Chem 2010; 285:36818-36827.
59. Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, Donath MY. Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. Diabetes 2001; 50:69-76.
60. Maedler K, Oberholzer J, Bucher P, Spinas GA, Donath MY. Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. Diabetes 2003; 52:726-733.
61. Listenberger LL, Han X, Lewis SE, Cases S, Farese RV,Jr, Ory DS, Schaffer JE. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc Natl Acad Sci U S A 2003; 100:3077-3082.
62. Listenberger LL, Ory DS, Schaffer JE. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. J Biol Chem 2001; 276:14890-14895.
63. Coll T, Eyre E, Rodriguez-Calvo R, Palomer X, Sanchez RM, Merlos M, Laguna JC, Vazquez-Carrera M. Oleate reverses palmitate-induced insulin resistance and inflammation in skeletal muscle cells. J Biol Chem 2008; 283:11107-11116.
64. Moreau C, Froment P, Tosca L, Moreau V, Dupont J. Expression and regulation of the SCD2 desaturase in the rat ovary. Biol Reprod 2006; 74:75-87.
65. Rincon G, Islas-Trejo A, Castillo AR, Bauman DE, German BJ, Medrano JF. Polymorphisms in genes in the SREBP1 signalling pathway and SCD are associated with milk fatty acid composition in Holstein cattle. J Dairy Res 2012; 79:66-75.

66. Bouwman AC, Bovenhuis H, Visker MH, van Arendonk JA. Genome-wide association of milk fatty acids in Dutch dairy cattle. BMC Genet 2011; 12:43-2156-12-43.

67. Garnsworthy PC, Feng S, Lock AL, Royal MD. Short communication: heritability of milk fatty acid composition and stearoyl-CoA desaturase indices in dairy cows. J Dairy Sci 2010; 93:1743-1748.

68. Soyeurt H, Dehareng F, Mayeres P, Bertozzi C, Gengler N. Variation of Delta 9-desaturase activity in dairy cattle. J Dairy Sci 2008; 91:3211-3224.

69. Bastin C, Soyeurt H, Gengler N. Genetic parameters of milk production traits and fatty acid contents in milk for Holstein cows in parity 1-3. J Anim Breed Genet 2013; 130:118-127.

70. Soyeurt H, Dardenne P, Dehareng F, Bastin C, Gengler N. Genetic parameters of saturated and monounsaturated fatty acid content and the ratio of saturated to unsaturated fatty acids in bovine milk. J Dairy Sci 2008; 91:3611-3626.

71. Haggarty P, Wood M, Ferguson E, Hoad G, Srikantharajah A, Milne E, Hamilton M, Bhattacharya S. Fatty acid metabolism in human preimplantation embryos. Hum Reprod 2006; 21:766-773.

72. Khandoker MAMY, Tsujii H. The metabolism of exogenous fatty acid by preimplantation rat embryos. Asian-Aus J Anim Sci 1999a; 12:1181-1187.

73. Flynn TJ, Hillman N. The metabolism of exogenous fatty acids by preimplantation mouse embryos developing in vitro. J Embryol Exp Morphol 1980; 56:157-168.

74. Wang G, Tsujii H. Incorporation and Distribution of Both ^{3}H-Palmitic and ^{14}C-Oleic Acids in Lipids by Preimplantation Mouse Embryos. J Mamm Ova Res 1998; 15:185-5.

75. Khandoker MAMY, Nishioka M, Tsujii H. Effect of BSA binding fatty acids on mouse and rat embryo development. J Mamm Ova Res 1995; 12:113-5.

76. Khandoker MAMY, Tsujii H. Effect of exogenous fatty acids on in vitro development of rat embryos. Asian-Aus J Anim Sci 1999b; 12:169-173.

77. Quinn P, Whittingham DG. Effect of fatty acids on fertilization and development of mouse embryos in vitro. J Androl 1982; 3:660-666.

78. Khandoker MAMY, Tsujii H, Karasawa D. Fatty acid compositions of oocytes, follicular, oviductal and uterine fluids of pig and cow Asian-Aus J Anim Sci 1997; 10:523-4.

79. Khandoker MAMY, Tsujii H, Karasawa D. Fatty acid analysis of oocytes, oviductal and uterine fluids of rabbit Anim Sci Technol 1996; 67:549-4.

80. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. Science 2001; 293:1089-1093.

81. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, Slagboom PE, Lumey LH. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A 2008; 105:17046-17049.

82. Roseboom TJ, Painter RC, van Abeelen AF, Veenendaal MV, de Rooij SR. Hungry in the womb: what are the consequences? Lessons from the Dutch famine. Maturitas 2011; 70:141-145.

83. Tobi EW, Lumey LH, Talens RP, Kremer D, Putter H, Stein AD, Slagboom PE, Heijmans BT. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. Hum Mol Genet 2009; 18:4046-4053.

84. Hori N, Nagai M, Hirayama M, Hirai T, Matsuda K, Hayashi M, Tanaka T, Ozawa T, Horike S. Aberrant CpG methylation of the imprinting control region KvDMR1 detected in assisted reproductive technology-produced calves and pathogenesis of large offspring syndrome. Anim Reprod Sci 2010; 122:303-312.

85. Young L, Sinclair K, Wilmut I. Large offspring syndrome in cattle and sheep. Rev Reprod 1998; 3:155-163.
86. Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. Biology of Reproduction 1995; 53:1385-1391.
87. Jungheim ES, Louden ED, Chi MM, Frolova AI, Riley JK, Moley KH. Preimplantation exposure of mouse embryos to palmitic acid results in fetal growth restriction followed by catch-up growth in the offspring. Biol Reprod 2011; 85:678-683.
88. Dunning KR, Watson LN, Sharkey DJ, Brown HM, Norman RJ, Thompson JG, Robker RL, Russell DL. Molecular filtration properties of the mouse expanded cumulus matrix: controlled supply of metabolites and extracellular signals to cumulus cells and the oocyte. Biol Reprod 2012; 87:89.
89. Holte J, Bergh T, Berne C, Lithell H. Serum lipoprotein lipid profile in women with the polycystic ovary syndrome: relation to anthropometric, endocrine and metabolic variables. Clin Endocrinol (Oxf) 1994; 41:463-471.
90. Gougeon A. Ovarian follicular growth in humans: ovarian ageing and population of growing follicles. Maturitas 1998; 30:137-142.
91. Oakberg EF, Tyrrell PD. Labeling the Zona Pellucida of the Mouse Oocyte. Biology of Reproduction 1975; 12:477-482.
92. Cockburn K, Rossant J. Making the blastocyst: lessons from the mouse. J Clin Invest 2010; 120:995-1003.
93. Spencer TE, Johnson GA, Bazer FW, Burghardt RC. Implantation mechanisms: insights from the sheep. Reproduction 2004; 128:657-668.

Chapter 7

Nederlandse samenvatting

Tijdens de laatste decennia is de vruchtbaarheid van de hoogproductieve melkkoe drastisch afgenomen. Deze daling van de vruchtbaarheid wordt in verband gebracht met de toegenomen negatieve energiebalans, net na afkalven, als gevolg van de sterk gestegen melkproductie. De hoge melkproductie leidt tot een groot verlies van energie via de melk die op dat moment onvoldoende gecompenseerd kan worden met energie vanuit de voeding. De hierdoor verlaagde glucose concentraties en als gevolg daarvan verlaagde insuline concentraties in het bloed leiden tot het mobiliseren van vrije vetzuren vanuit vetweefsel en tot de voor een negatieve energiebalans kenmerkende verhoogde vrije vetzuurconcentraties in het bloed leiden. De vrije vetzuren zijn voor cellen een alternatieve energiebron tijdens energieschaarste, maar verhoogde vrije vetzuurconcentraties van met name verzadigde vetzuren kunnen schadelijk zijn voor veel verschillende typen cellen. Mogelijk vormen deze verhoogde concentraties van vrije vetzuren de link tussen de negatieve energiebalans en de verlaagde vruchtbaarheid. Het doel van dit proefschrift was om het effect van verhoogde vrije vetzuurconcentraties op de vruchtbaarheid en in het bijzonder op de ontwikkelingscapaciteit van de rijpende eicel te onderzoeken. De hier beschreven uitkomsten zijn ook potentieel interessant voor het humane voortplantingsonderzoek omdat metabole aandoeningen met verhoogde concentraties van vrije vetzuren, zoals obesitas en anorexia, ook bij vrouwen geassocieerd zijn met een verminderde vruchtbaarheid.

De eicel heeft een lange weg te gaan voor uiteindelijke ovulatie kan optreden

Eicellen bevinden zich in follikels in de ovaria van het vrouwelijk dier en vanaf de puberteit treedt er iedere reproductie cyclus een eisprong op van 1 of meerdere follikels. Gedurende het reproductieve leven van het vrouwelijke dier start in cycli de ontwikkeling van follikeltjes, geïnitieerd door het hormoon FSH, waarbij er slechts enkelen uiteindelijk zullen ovuleren. Gedurende dit proces kan een follikel zich ontwikkelen van een enkele cellaag met daarbinnen de eicel, de primordiale follikel, tot een compleet ontwikkelde follikel. Deze zogenaamde pre-ovulatoire follikel bestaat uiteen buitenlaag van theca en granulosa cellen met daarbinnen een met follikelvloeistof gevulde holte en binnenin de eicel die samen met de omringende laag cumuluscellen het cumulus-eicel-complex (COC) vormt. De hele groeiperiode van primordiaal naar pre-ovulatoire follikel beslaat ongeveer 60-80 dagen in de koe en maar een enkeling redt het tot het dominante stadium en kan uiteindelijk ovuleren, want standaard ovuleert er iedere cyclus van gemiddeld 21 dagen maar één follikel in de koe. Tijdens de gehele periode van follikel en eicel ontwikkeling kunnen vrije vetzuren een effect hebben op de

eicel, maar in dit proefschrift is de impact van vrije vetzuren tijdens de finale maturatie van de eicel onderzocht. De finale periode van rijping, gedurende de laatste 22 uur voor ovulatie, is een cruciale periode voor de eicel waarin een cytoplasmatische- en een kernmaturatie op moet treden die de eicel geschikt maakt voor bevruchting. In het vrouwelijke dier wordt deze periode geïnitieerd door de LH piek, maar deze laatste 22 uur kunnen ook in het lab, *in vitro*, dus buiten het vrouwelijke dier plaatsvinden en na maturatie kunnen de eicellen *in vitro* bevrucht (IVF) en gekweekt worden tot het embryonale stadium. In dit proefschrift is de *in vitro* maturatie, fertilisatie en kweek gebruikt als aanvulling op de *in vivo* experimenten, om zeer gericht te kunnen kijken naar de effecten van vrije vetzuren tijdens de eicel.

Extracellulaire vetzuren worden ingebouwd in de eicel

Vetzuren zijn in de eerste plaats essentieel voor cellen als energiebron en kunnen als bouwstof dienen voor onder andere celmembranen. Het belang van vetzuren voor de ontwikkeling van de eicel en het embryo is beschreven in **Hoofdstuk 1**. Onbekend is echter waar de vetzuren, die hoofdzakelijk liggen opgeslagen als triacylglycerol in vetdruppels in de eicel, vandaan komen. Vetdruppels zijn aanwezig in de eicel vanaf een vroeg stadium in de ontwikkeling, vanaf het primordiale follikelstadium, en nemen geleidelijk toe in aantal tijdens de opeenvolgende follikelgroeistadia. De meeste cellen in het lichaam zijn in staat om zelf vetzuren te synthetiseren en een eerdere studie liet zien dat eicellen ook de enzymen bevatten om vetzuren te synthetiseren. Of eicellen in staat zijn om, net als veel andere cellen, vetzuren op te nemen vanuit de extracellulaire omgeving was onbekend. Doordat de eicel wordt omringd door een laag van glycoproteïnen, de zona pellucida, zou de opname van vetzuren vanuit de omgeving van de eicel bemoeilijkt kunnen worden. De *in vitro* bevindingen in **Hoofdstuk 3** tonen aan dat eicellen in staat zijn om vrije vetzuren vanuit het milieu op te nemen. De opname van vetzuren door de eicel impliceert dat extracellulaire vetzuren de eicel kunnen beïnvloeden en in potentie een effect kunnen hebben op de ontwikkelingscapaciteit van de eicel. De mogelijkheid tot opname van vrije vetzuren door de eicel bleek onafhankelijk van de aanwezigheid van een laag cumuluscellen, maar eicellen zonder omringende laag van cumuluscellen namen de vetzuren initieel met een hogere snelheid op (Aardema et al., ongepubliceerde data). Dit lijkt erop te wijzen dat cumuluscellen naast de cruciale al bekende ondersteunende functies tijdens de eicelrijping ook een rol spelen in de opname van vetzuren door de eicel en mogelijk de opname van vetzuren door de eicel kunnen beperken, dan wel vertragen (**Hoofdstuk 4** en Aardema et al., ongepubliceerde data).

Een experimenteel model om metabole stress en meer follikelgroei per koe te induceren

In **Hoofdstuk 4** is een gestandaardiseerd experimenteel model gebruikt om metabole stress te induceren in koeien. Metabole stress zoals een negatieve energie balans beïnvloedt primair de vrije vetzuurfractie die vanuit vetweefsel wordt gemobiliseerd, dit is dan ook de fractie die geanalyseerd is in dit proefschrift (**Hoofdstuk 4 en 5**).

De koe ovuleert gemiddeld maar één eicel per 21 dagen wat zou betekenen dat veel proefdieren nodig zouden zijn om een effect op de eicel in het dominante follikel stadium te kunnen onderzoeken. Gelukkig kan het aantal dominante follikels per koe vergroot worden door middel van een hormonale stimulatie van de eierstokken. Een groter aantal dominante follikels leidt tot een groter aantal ovulaties van eicellen die na bevruchting tot een embryo kunnen uitgroeien. In **Hoofdstuk 4** hebben we hormonale superstimulatie toegepast om het aantal dominante follikels per dier en dus de hoeveelheid onderzoeksmateriaal per dier te verhogen. Een belangrijk nadeel van superstimulatie is echter dat ook stimulatie van niet-competente follikels kan optreden. In **Hoofdstuk 2** is aangetoond dat de competente follikels op basis van hormoonconcentraties in de follikelvloeistof kunnen worden onderscheiden van niet-competente follikels van gesuperstimuleerde koeien. In de ongestimuleerde koe vindt er in de pre-ovulatoire follikel in de laatste 22 uur voor ovulatie een hormonale omschakeling plaats van een oestrogeen-gedomineerde omgeving rondom de LH piek naar een progesteron-gedomineerde omgeving vlak voor de ovulatie. Bij de gestimuleerde koeien in **Hoofdstuk 2** bleken alleen eicellen die afkomstig waren van follikels met een 17β-oestradiol concentratie ≤ 0.25 μM en progesteron concentratie ≥ 0.26 μM aan het eind van de maturatie in staat om zich tot een embryo in het blastocyst stadium te ontwikkelen. Overeenkomstig met de niet gestimuleerde koeien werd de follikelvloeistof van competente follikels bij gestimuleerde koeien ook door progesteron gedomineerd vlak voor ovulatie. Deze uitkomsten geven aan dat hormonale veranderingen in de follikel noodzakelijk zijn voor een optimaal verloop van de finale rijping van de eicel en dat hormoonconcentraties in follikelvloeistof gebruikt kunnen worden voor de selectie van competente eicellen na superstimulatie. In **Hoofdstuk 4** hebben we de hormoonconcentraties van 17β-oestradiol en progesteron in follikelvloeistof gebruikt om de competente follikels te selecteren van de totale groepvan follikels.

Follikelvloeistof bevat een relatief hoge concentratie oleaat in vergelijking met bloed

Het experimentele model om metabole stress te induceren zorgde voor een stijging van het gehalte aan vrije vetzuren in bloed (**Hoofdstuk 4**) die vergelijkbaar was met de stijging van de vrije vetzuurconcentratie die optreedt in de hoogproductieve koe net na afkalven (**Hoofdstuk 5**). De stijging van de vrije vetzuurconcentraties in bloed werd gereflecteerd in de follikelvloeistof en zorgde voor een stijging van de concentratie van vrije vetzuren in de dominante follikel, dus in de directe omgeving van de eicel (**Hoofdstuk 4**). De sterk gestegen vrije vetzuur concentratie in bloed net na afkalven bleek geen effect te hebben op de vrije vetzuursamenstelling van dominante follikels rond de periode waarop koeien in de reguliere melkveehouderij worden geïnsemineerd, ondanks de blootstelling aan hoge concentraties vrije vetzuren tijdens de vroege ontwikkelingsstadia van deze follikels (**Hoofdstuk 5**). Zowel in bloed als in de follikelvloeistof waren de meest prominente vrije vetzuren de verzadigde vetzuren palmitaat (C16:0) en stearaat (C18:0) en het enkelvoudig onverzadigde vetzuur oleaat (C18:1) (**Hoofdstuk 4 en 5**). In de koe zijn dit ook de meest voorkomende vetzuren die liggen opgeslagen in het vetweefsel waarvan uit de vetzuren worden vrijgemaakt. Opvallend was dat follikelvloeistof relatief minder verzadigde en meer enkelvoudig onverzadigde vetzuren bevatte dan bloed, hetgeen met name veroorzaakt werd door een lager gehalte van C18:0 en hoger gehalte van C18:1 in follikelvloeistof (**Hoofdstuk 4 en 5**).

Vetstapeling in cumulus cellen na blootstelling aan verhoogde vrije vetzuurconcentraties

Blootstelling aan de sterk gestegen vrije vetzuurconcentraties in follikelvloeistof resulteerde in een massale stapeling van vetzuren in de cumuluscellen die de eicel omringen (**Hoofdstuk 4**). Dit effect werd zowel in de koe, dus *in vivo*, als ook tijdens de maturatie buiten de koe, *in vitro*, waargenomen. Deze blootstelling aan verhoogde vrije vetzuurconcentraties leidde niet tot een verandering van de vetzuursamenstelling in de eicellen (**Hoofdstuk 4**). Dat is opmerkelijk omdat is aangetoond dat eicellen vrije vetzuren op kunnen nemen vanuit hun omgeving (**Hoofdstuk 3**).

Verzadigde en enkelvoudig onverzadigde vrije vetzuren hebben een verschillend effect op de eicel

De ontwikkelingscompetentie van eicellen die tijdens de *in vitro* rijping werden blootgesteld aan verhoogde concentraties van vrije vetzuren bleek sterk afhankelijk

te zijn van het type vetzuur waaraan ze werden blootgesteld (**Hoofdstuk 3**). De verzadigde vrije vetzuren C16:0 en C18:0 hadden een dosis-afhankelijk negatief effect op de ontwikkelingscompetentie van de eicel, de blootstelling aan deze verzadigde vrije vetzuren tijdens de rijping van eicellen verlaagde de kans op ontwikkeling van een embryo. Het enkelvoudig onverzadigde vetzuur C18:1 was in tegenstelling tot de verzadigde vetzuren niet schadelijk, ook niet bij een hoge concentratie. Zeer interessant was de bevinding dat wanneer eicellen gelijktijdig werden blootgesteld aan een verzadigd vetzuur en aan C18:1, het C18:1 in staat bleek om de negatieve effecten van verzadigd C16:0 en C18:0 op de eicel volledig te voorkomen en de ontwikkelingscompetentie van de eicel intact bleef (**Hoofdstuk 3**). Deze *in vitro* bevindingen tonen aan dat de relatief hoge C18:1 concentratie in follikelvloeistof (**Hoofdstuk 4 en 5**) gunstig lijkt te zijn voor de rijpende eicel, omdat C18:1 de eicel kan beschermen tegen de potentieel schadelijke impact van verzadigde vrije vetzuren.

De ontwikkelingscompetentie van eicellen is niet verminderd na blootstelling aan verhoogde concentraties van vrije vetzuren

Interessant was dat de sterk verhoogde concentratie van vrije vetzuren in follikelvloeistof van metabool gestresste dieren geen nadelig effect had op de ontwikkelingscompetentie van de eicel om zich tot een embryo te ontwikkelen (**Hoofdstuk 4**). Ook bij nabootsing van de *in vivo* follikelcondities van vrije vetzuren tijdens de *in vitro* maturatie van eicellen leidde dit niet tot een nadelig effect op de eicel. Terwijl eicellen die zonder cumuluslaag aan een fysiologisch verhoogde concentratie van vrije vetzuren werden blootgesteld tijdens de laatste 6u van de maturatie, van de totale *in vitro* maturatieperiode van 22 uur, een tendens lieten zien voor een verminderde ontwikkelingscompetentie (**Hoofdstuk 4**). De bevindingen van **Hoofdstuk 3** en **4** suggereren dat de combinatie van een relatief hoge concentratie C18:1 in follikelvloeistof en de cumuluslaag om de eicel heen de eicel hebben beschermd tegen schade door verhoogde concentraties van vrije vetzuren. Uit studies uitgevoerd met andere celtypen is bekend dat C18:1 de stapeling van vetzuren in intracellulaire vetdruppels stimuleert en dit een manier is om een cel te beschermen tegen potentieel schadelijke vetzuren. De relatief hoge concentratie C18:1 in follikelvloeistof in combinatie met de massale vetstapeling in de cumuluscellen lijken de eicel actief te hebben beschermd tegen de verhoogde concentraties van potentieel toxische verzadigde vrije vetzuren in de follikelvloeistof.

CONCLUSIE

Verhoogde concentraties van de verzadigde vrije vetzuren C16:0 en C18:0 hebben een dosis-afhankelijk negatief effect op de ontwikkelingscompetentie van rijpende eicellen. Deze nadelige effecten worden volledig teniet gedaan door gelijktijdige blootstelling aan het enkelvoudig onverzadigde vetzuur C18:1. Deze bevindingen tonen aan dat de samenstelling van de vrije vetzuren in follikelvloeistof, en niet per se de totale concentratie van vrije vetzuren, bepalend zijn voor het uiteindelijke effect op de competentie van de eicel. Verhoogde concentraties van vrije vetzuren in het bloed worden gereflecteerd in de follikelvloeistof, maar de combinatie van een relatief hoge C18:1 concentratie in de follikelvloeistof en de massale vetstapeling in de cumuluscellen lijken een nadelig effect op de eicel te voorkomen. Op basis van de hier beschreven studies is er geen aanwijzing voor een nadelig effect van een stijging van de vrije vetzuurconcentraties op de eicel gedurende de finale rijping, mits C18:1 in voldoende mate aanwezig is. Mogelijk kan de link tussen de negatieve energiebalans en de verminderde vruchtbaarheid worden verklaard door een vroeg na afkalven optredend negatief effect van verhoogde concentraties van vrije vetzuren op de eicel, wanneer de eicel zich in een vroeg stadium van de folliculaire ontwikkeling bevindt, wat zou kunnen resulteren in een verminderde kwaliteit van de eicel op moment van inseminatie maanden later. Toekomstig onderzoek zou zich daarom moeten richten op de mogelijke impact van verhoogde concentraties van vrije vetzuren in bloed op de kwaliteit van de follikel en de eicel tijdens een eerder ontwikkelingsstadium van de follikelgroei, op een moment waarop de eicel wordt omgeven door minder cellagen en er een meer rechtstreekse blootstelling is aan de meer verzadigde vetzuursamenstelling in bloed.

Dankwoord

Curriculum Vitae

List of awards and publications

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Hilde Aardema was born on June 4th 1980 in De Bilt, the Netherlands. In 1998 she finished her secondary education 'VWO' at the 'Meander College' in Zwolle. In 1999 she started the study Veterinary Medicine at Utrecht University and graduated as a DVM in 2005. During her study she performed a research internship on estrus behavior in cows at Teagasc Moorepark in Fermoy, Ireland in 2003. She worked as a veterinarian at the veterinary practice 'DAP van Waard tot Klif' in Koudum, the Netherlands in 2006. In 2007 she started a PhD track and residency in Reproduction at the Department of Farm Animal Health at the Faculty of Veterinary Medicine, Utrecht University. Since February 2014 she is the Head of the Farm Animal Health Clinic at the Faculty of Veterinary Medicine, Utrecht University.

Hilde Aardema werd geboren op 4 juni 1980 in De Bilt. In 1998 behaalde ze haar VWO-diploma aan het Meander College te Zwolle. In 1999 begon zij haar studie Diergeneeskunde aan de Universiteit Utrecht en in 2005 studeerde ze af als dierenarts. Tijdens de studie verrichte ze onderzoek naar oestrusgedrag van koeien gedurende een onderzoekstage bij Teagasc Moorepark in Fermoy, Ierland in 2003. Ze was in 2006 werkzaam als rundvee dierenarts in dierenartsenpraktijk 'DAP van Waard tot Klif' te Koudum. In 2007 begon ze een promotie-en specialisatietraject in reproductie bij het Departement Gezondheidszorg Landbouwhuisdieren van de Faculteit Diergeneeskunde, Universiteit Utrecht, waarvan dit proefschrift het resultaat is. Vanaf februari 2014 is zij werkzaam als Chef de Clinique bij het Departement Gezondheidszorg Landbouwhuisdieren.

PEER REVIEWED PUBLICATIONS

- **H. Aardema**, P.L.A.M. Vos, F. Lolicato, B.A.J. Roelen, H.M. Knijn, A.B. Vaandrager, J.B. Helms and B.M. Gadella. Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. Biology of Reproduction 2011; **85** (1): 62-69.

- **H. Aardema**, F. Lolicato, C.H.A. van de Lest, J.F. Brouwers, A.B. Vaandrager, H.T.A. van Tol, B.A.J. Roelen, P.L.A.M. Vos, J. B. Helms and B.M. Gadella. Bovine cumulus cells protect maturing oocytes from increased fatty acid levels by massive intracellular lipid storage. Biology of Reproduction 2013; **88** (6): 164, 1-15.

- **H. Aardema**, B.A.J. Roelen, H.T.A. van Tol, C.H.Y. Oei, B.M. Gadella and P.L.A.M. Vos. Follicular 17β-estradiol and progesterone concentrations and degree of cumulus cell expansion as predictors of in vivo-matured oocyte developmental competence in super-stimulated heifers. Theriogenology 2013; **80** (6): 576-83.

AWARDS

Tebu-bio	Travel Grant Winning Abstract	2009
Veterinary Science Day	Winner Presentation Prize	2010
Vereniging voor Fertiliteitsstudie	Winner Student Competition	2011
European Embryo Transfer Association	Winner Student Competition	2012
International Embryo Transfer Society	Runner up Student Competition	2013

ORAL CONTRIBUTIONS TO SCIENTIFIC CONFERENCES

- **H Aardema**, HM Knijn, PLAM Vos and BM Gadella. 2008. In vitro maturatie van bovine eicellen in aanwezigheid van verzadigde vrije vetzuren heeft een remmende werking op vroeg embryonale ontwikkeling. Vereniging voor Fertiliteitsstudie, Leiden.

- **H. Aardema**. 2010. The impact of free fatty acids on oocyte quality. WIAS seminar, Wageningen, The Netherlands.

- **H. Aardema**, P.L.A.M. Vos, B.A.J. Roelen, J.B. Helms, B.M. Gadella. 2010. Het onverzadigde vetzuur oleaat gaat schadelijke effecten van verzadigd palmitaat tijdens rijping rundereicellen tegen. Vereniging voor Fertiliteitsstudie, Nijmegen, The Netherlands.

- **H. Aardema**. 2010. Het effect van vrije vetzuren op de kwaliteit van de eicel. KNMvD, Vakgroep Gezondheidszorg Herkauwer, Utrecht, The Netherlands.

- **H. Aardema**, P.L.A.M. Vos, B.A.J. Roelen, J.B. Helms, B.M. Gadella. 2010. Saturated free fatty acid during maturation of bovine oocytes impairs early embryonic development. 8th International Ruminant Reproduction Symposium, Anchorage, Alaska, USA.

- **H. Aardema**. 2011. The impact of NEFAs on oocyte quality. KNMvD, Vakgroep Gezondheidszorg Herkauwer, Arnhem, The Netherlands.

- **H. Aardema**, P.L.A.M. Vos, B.A.J. Roelen and B.M. Gadella. 2011. Metabole stress verhoogt vrije vetzuurconcentraties in de follikel. Vereniging voor Fertiliteitsstudie, Utrecht, The Netherlands.

- **H. Aardema**, P.L.A.M. Vos, B.A.J. Roelen and B.M. Gadella. 2012. Metabole stress verhoogt vrije vetzuurconcentraties in de follikel. Belgian Society for Reproductive Medicine, Elewijt, Belgium.

- **H. Aardema**, B.A.J. Roelen, H.T.A. van Tol, B.M. Gadella and P.L.A.M. Vos. 2012. Follicle size is not related to the concentrations of estradiol and progesterone in bovine follicles after superstimulation. European Embryo Transfer Association, St Malo, France.

- **H. Aardema**, F. Lolicato, B.A.J. Roelen, P.L.A.M. Vos, J.B. Helms and B.M. Gadella. 2013. Cumulus cells protect oocytes against potential lipotoxicity from elevated free fatty acid concentrations in follicular fluid. International Embryo Transfer Society, Hannover, Germany.

- **H. Aardema**, F. Lolicato, H.T.A. van Tol, B.A.J. Roelen, P.L.A.M. Vos, J.B. Helms and B.M. Gadella. 2013. Nano-lipidomics reveals a major role of oleic acid and cumulus cells in protecting oocytes against lipotoxic stress, Society for the Study of Reproduction, Montreal, Canada.