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Bovine Cumulus Cells Protect Maturing Oocytes from Increased Fatty Acid Levels by Massive Intracellular Lipid Storage[1]

Hilde Aardema,[3] Francesca Lolicato,[4] Chris H.A. van de Lest,[4] Jos F. Brouwers,[4] Arie B. Vaandrager,[4] Helena T.A. van Tol,[3] Bernard A.J. Roelen,[3,5] Peter L.A.M. Vos,[3] J. Bernd Helms,[4] and Bart M. Gadella,[2,3,4]

[3]*Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*
[4]*Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*
[5]*Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*

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.

cows, embryo culture, in vitro fertilization, in vitro maturation, IVF, IVM, oocyte-follicle interactions, ruminants

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 monounsaturated 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

[1]This work was funded by Pfizer Animal Health.
[2]Correspondence: E-mail: b.m.gadella@uu.nl

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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 lnonactating 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.

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TABLE 1. Details of primers used for qPCR.

Code	Gene	Primer sequence (5′ → 3′)	Position	Annealing Temp (°C)	GenBank accession no.
PLAC8	Placenta-specific 8	GACTGGCAGACTGGCATCTT	210–229	60	NM_001025325.2
		CTCATGGCGACACTTGATCC	330–349		
PTGS2	Prostaglandin-endoperoxide synthase 2	TGGCTACGGGAACACAACA	980–998	65	NM_174445.2
		TGATACTTTCTCTACTGCGACTGG	1388–1411		
CTSB	Cathepsin B	GCAACTCCTGGAACACTGAT	890–909	63	NM_174031.2
		ACTGATGAGTGCACGGCATT	984-1003		
H2AFZ	H_2A histone family, member Z	AGGACGACTAGCCATGGACGTGTG	162–185	60	NM_174809.2
		CCACCACCAGCAATTGTAGCCTTG	347–370		
PGK1	Phosphoglycerokinase	CTGGACAAGCTGGATGTGAA	72–91	61	BT021601.1
		AACAGCAGCCTTGATCCTCT	160–179		

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 semi-automated 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. At 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

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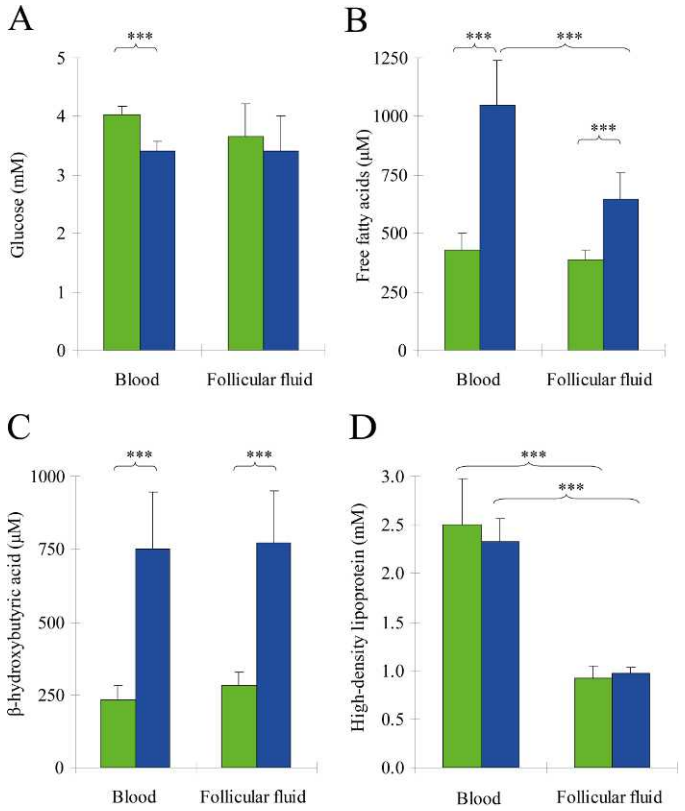


FIG. 1. Short-term fasting alters metabolic profiles in blood and follicular fluid. The short-term fasting period resulted in a decreased concentration of glucose (**A**) and an increase of the concentrations of free fatty acids (**B**) and β-hydroxybutyric acid (**C**) in the blood of the experimental 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 (***$P \leq 0.001$).

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.

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,

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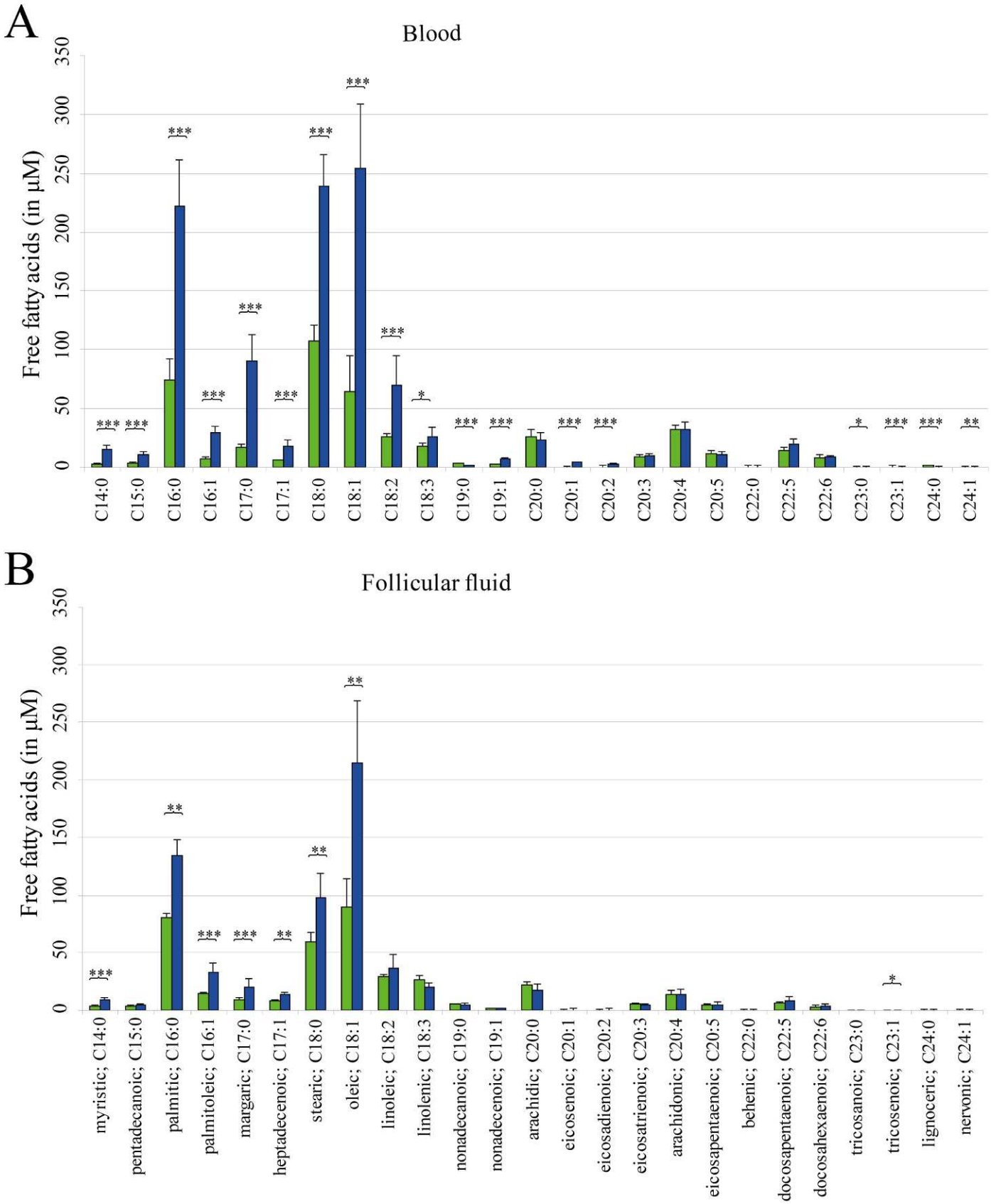


FIG. 2. C18:1 and C16:0 and C18:0 dominate blood and follicular fluid. Short-term fasting (blue bars) resulted in a massive increase in the levels of free fatty acids with a carbon length of 14–18 both in the blood (**A**) and in follicular fluid (**B**) in comparison to the control condition (green 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 ***$P \leq 0.001$).

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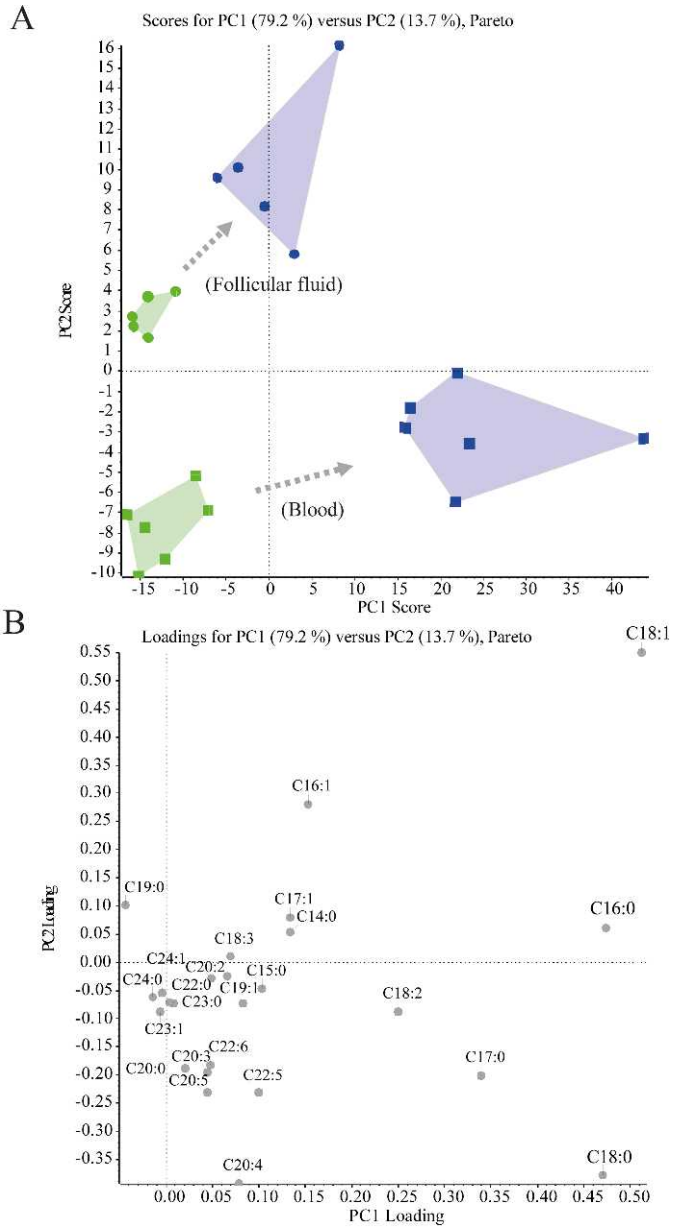


FIG. 3. Distinct free fatty acid compositions in blood and follicular fluid of control and fasted animals. The principal component analysis revealed characteristic free fatty acid compositions between the control (green) and experimental group (blue) in blood (squares) and follicular fluid (circles) 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.

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.

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). 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.

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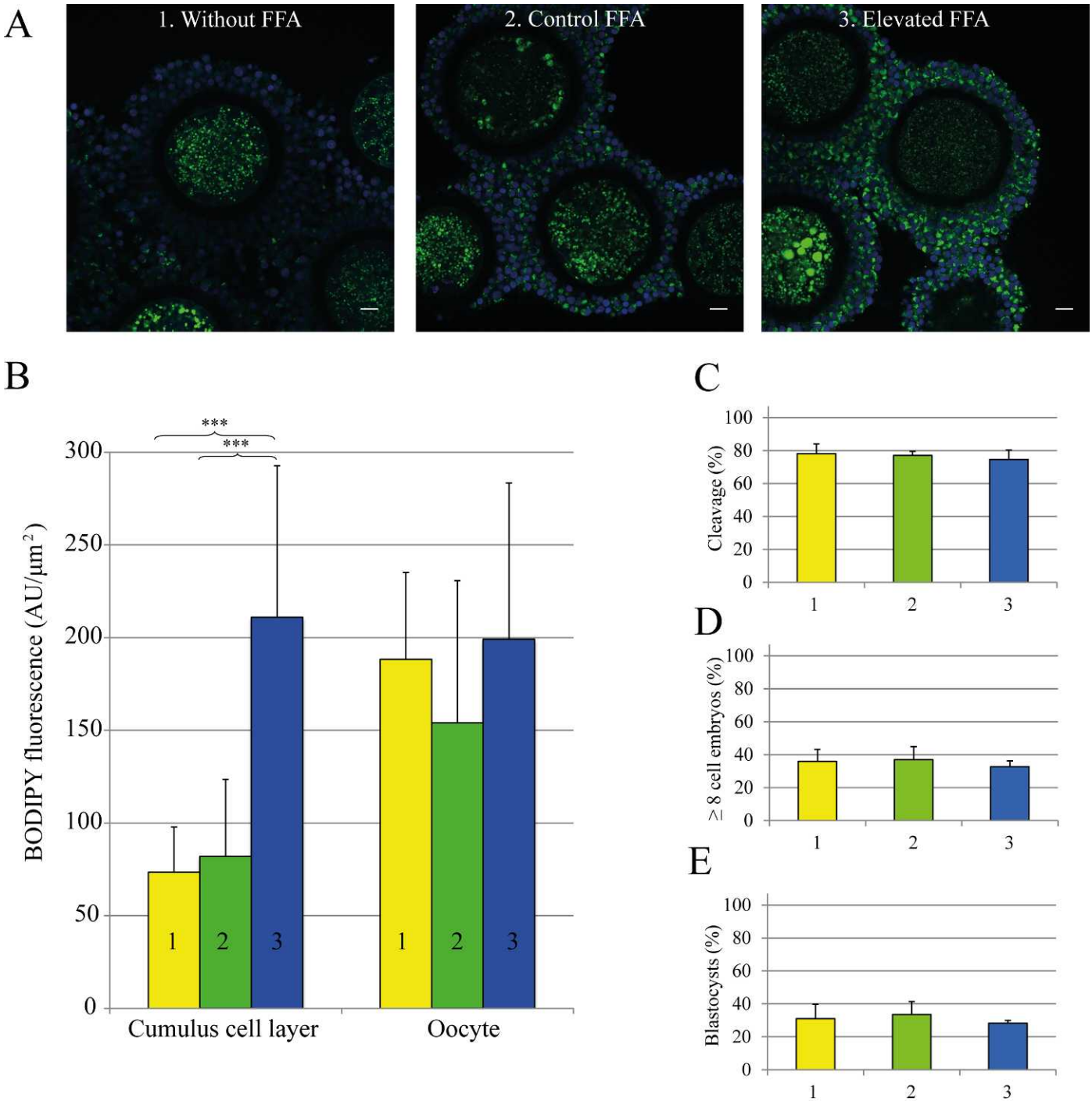


FIG. 4. Exposure to the most abundant free fatty acids in follicular fluid does not impair oocyte developmental competence. In vitro maturation with medium without FFA (1), with control FFA levels; 80 µM C16:0, 70 µM C18:0, 100 µM C18:1 (2) or with elevated FFA levels; 150 µM C16:0, 100 µM C18:0, 200 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.

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

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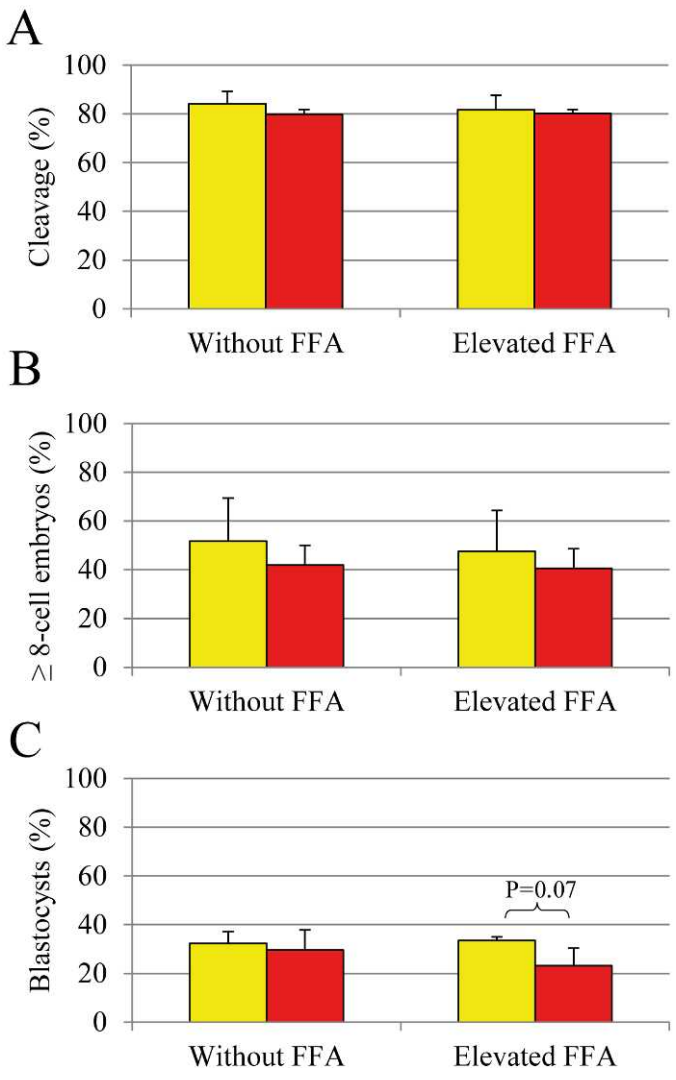


FIG. 5. Cumulus cell removal of COCs and FFA exposure during the last 6 h of maturation. Removal of cumulus cells (red bars, DOs) from COCs did not affect the developmental competence of the DOs compared to intact COCs (yellow bars). The culture conditions are defined in Figure 4. Developmental competence 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.

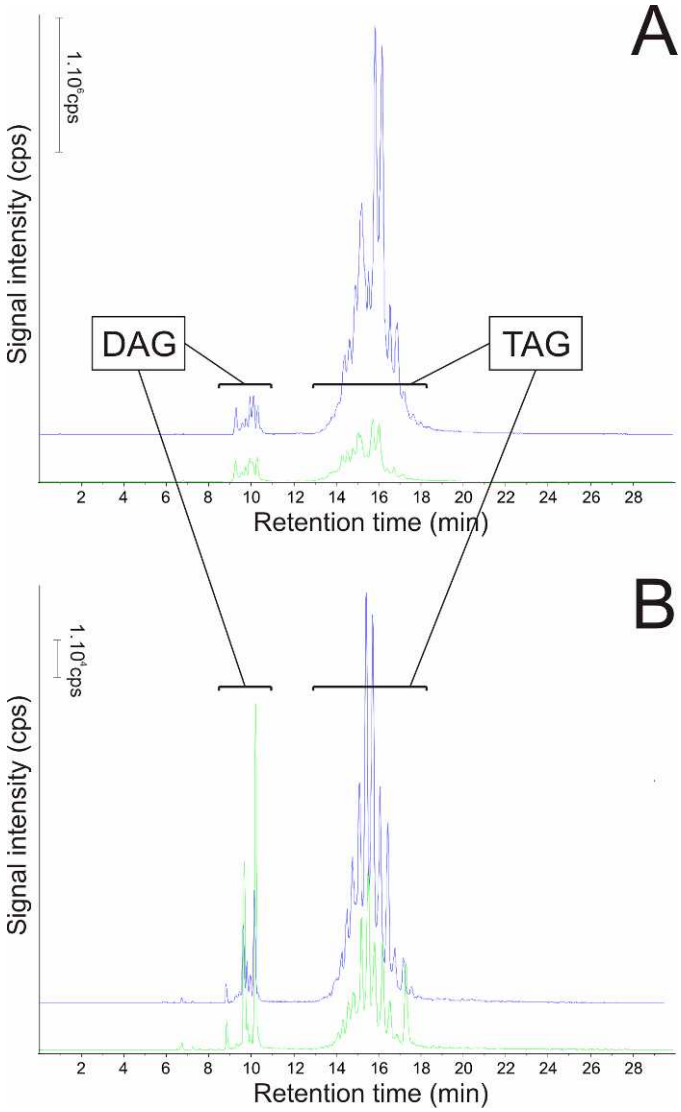


FIG. 6. Intensity HPLC chromatograms of ionized neutral lipids in cumulus cells and in oocytes measured by mass spectrometry. The intensity of ionized neutral lipids from in vivo matured cumulus cells (**A**) and oocytes (**B**) were measured after control (green line) or experimental (blue line) conditions. 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.

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

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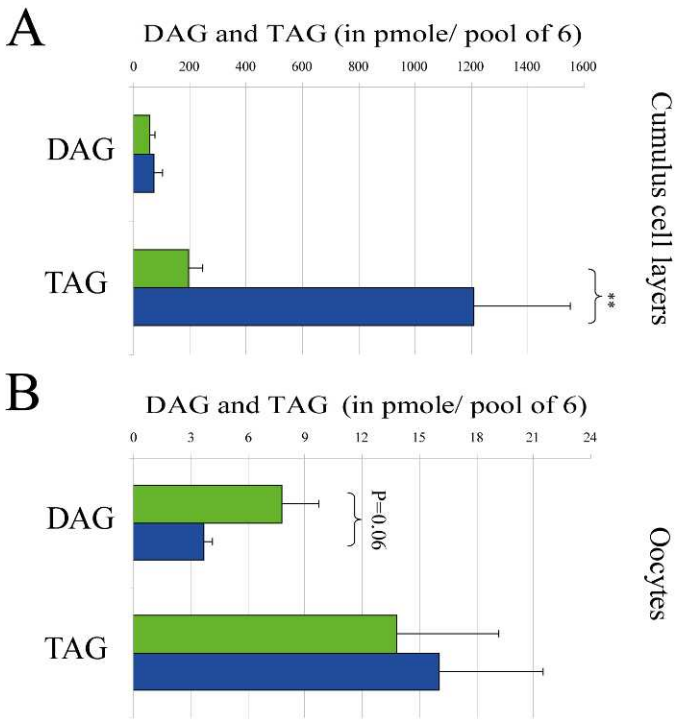


FIG. 7. Elevated free fatty acid concentrations induce massive lipid accumulation in cumulus cells and minor lipid alterations in oocytes. The cumulus cells (pool of cumulus cells from 6 oocytes per sample) that surrounded the oocytes during exposure to short-term fasting (blue bars) showed a massive 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**.

of the oocytes as determined by the cleavage and blastocyst rate after fertilization was not altered under these three conditions (Fig. 4, C–E). 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.

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). 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.

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.

Short-Term Elevated Free Fatty Acid Levels in Follicular Fluid During Maturation of Oocytes Does 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.

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.

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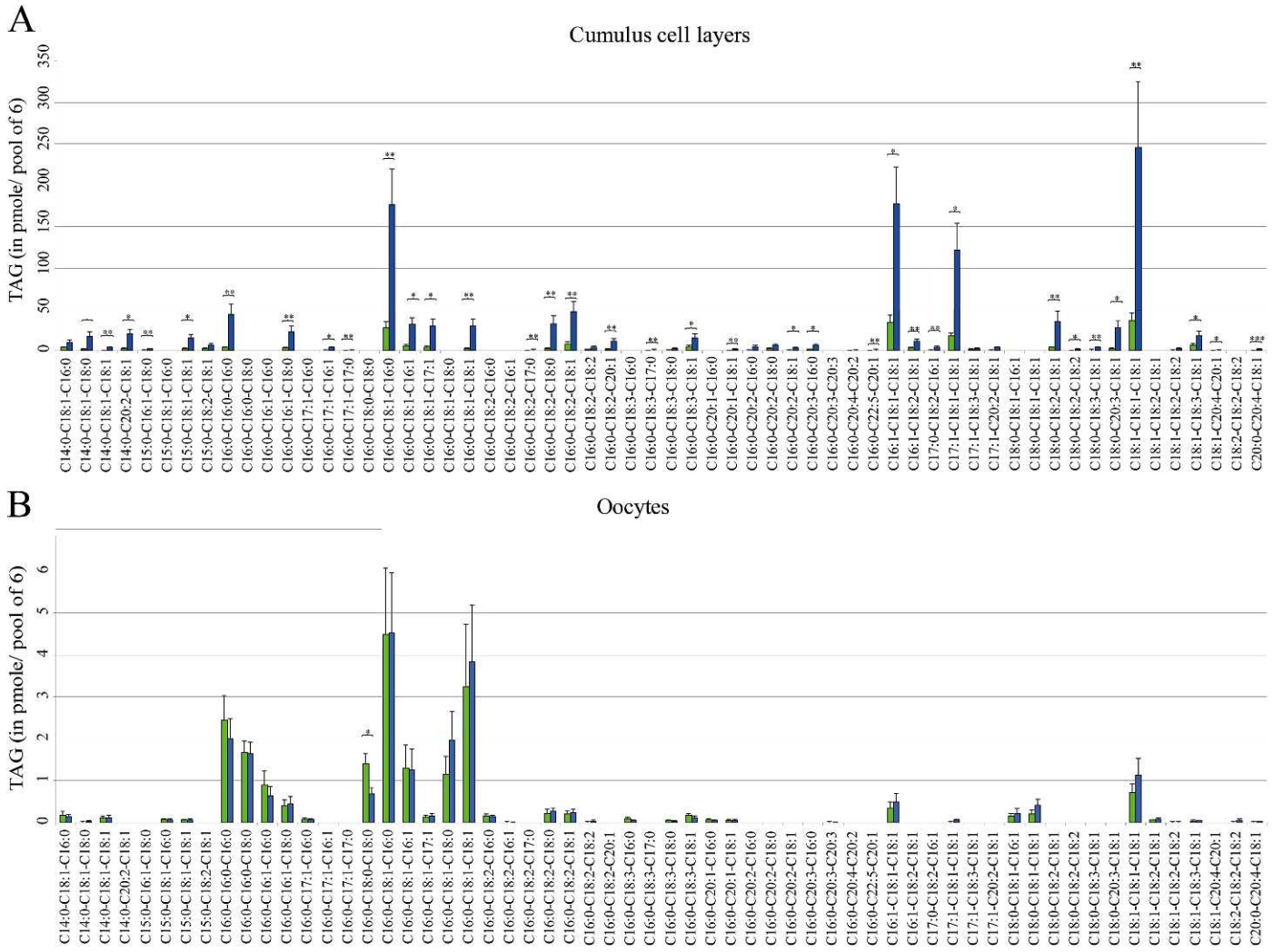


FIG. 8. The level of TAG species with C18:1 massively increases in cumulus cells exposed to elevated levels of free fatty acids in follicular fluid. Especially the concentrations of TAG species in cumulus cells (pool of cumulus cells originating from 6 COCs) with one or more acylations with C18:1 heavily 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**.

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

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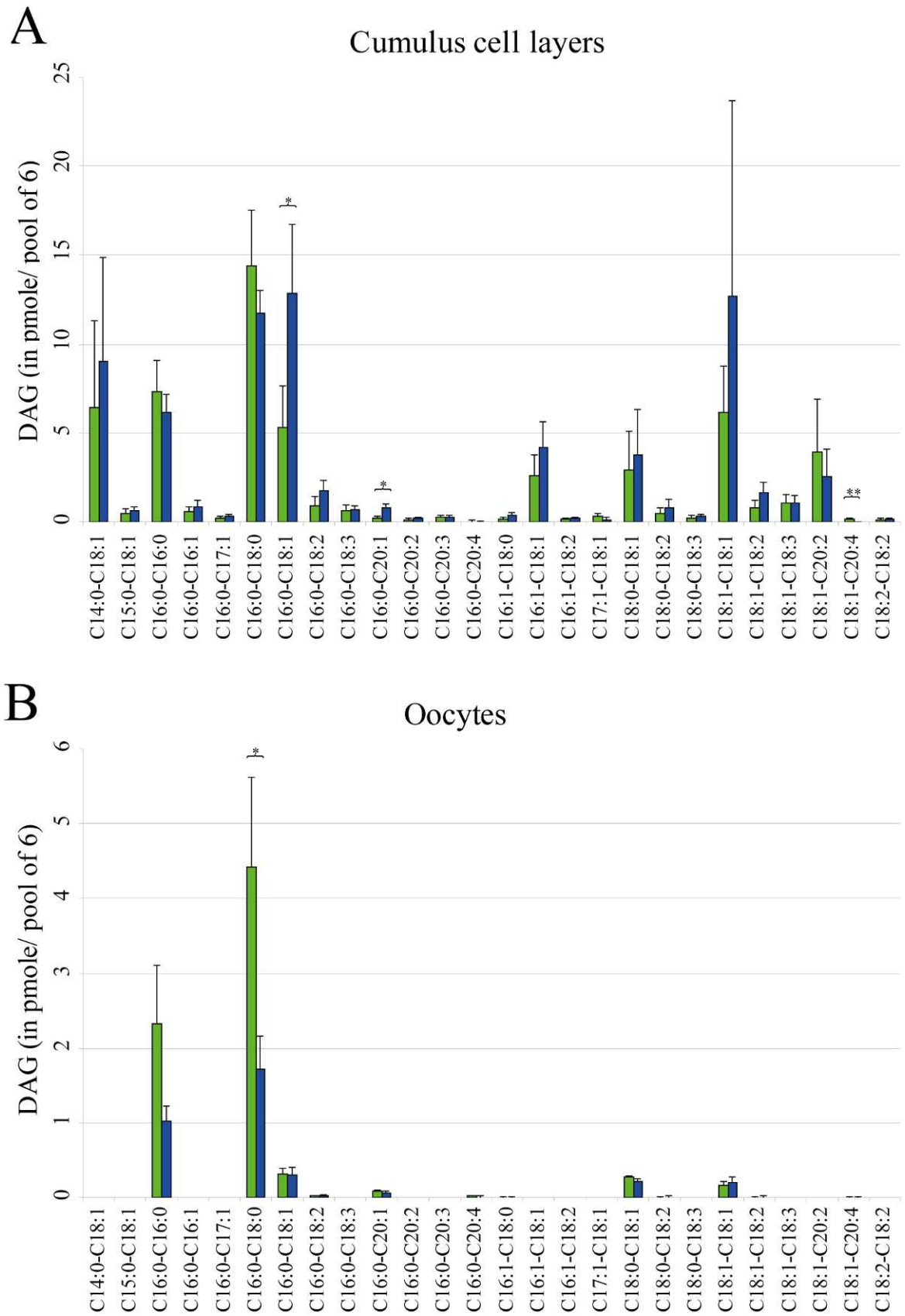


FIG. 9. The levels of DAG species in cumulus cells and oocytes. The concentrations of DAG species of cumulus cells (pool of cumulus cells originating from 6 COCs) were unaltered upon exposure to short-term fasting (blue bars) in comparison to cumulus cells of the control group (green) (**A**). In contrast 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**.

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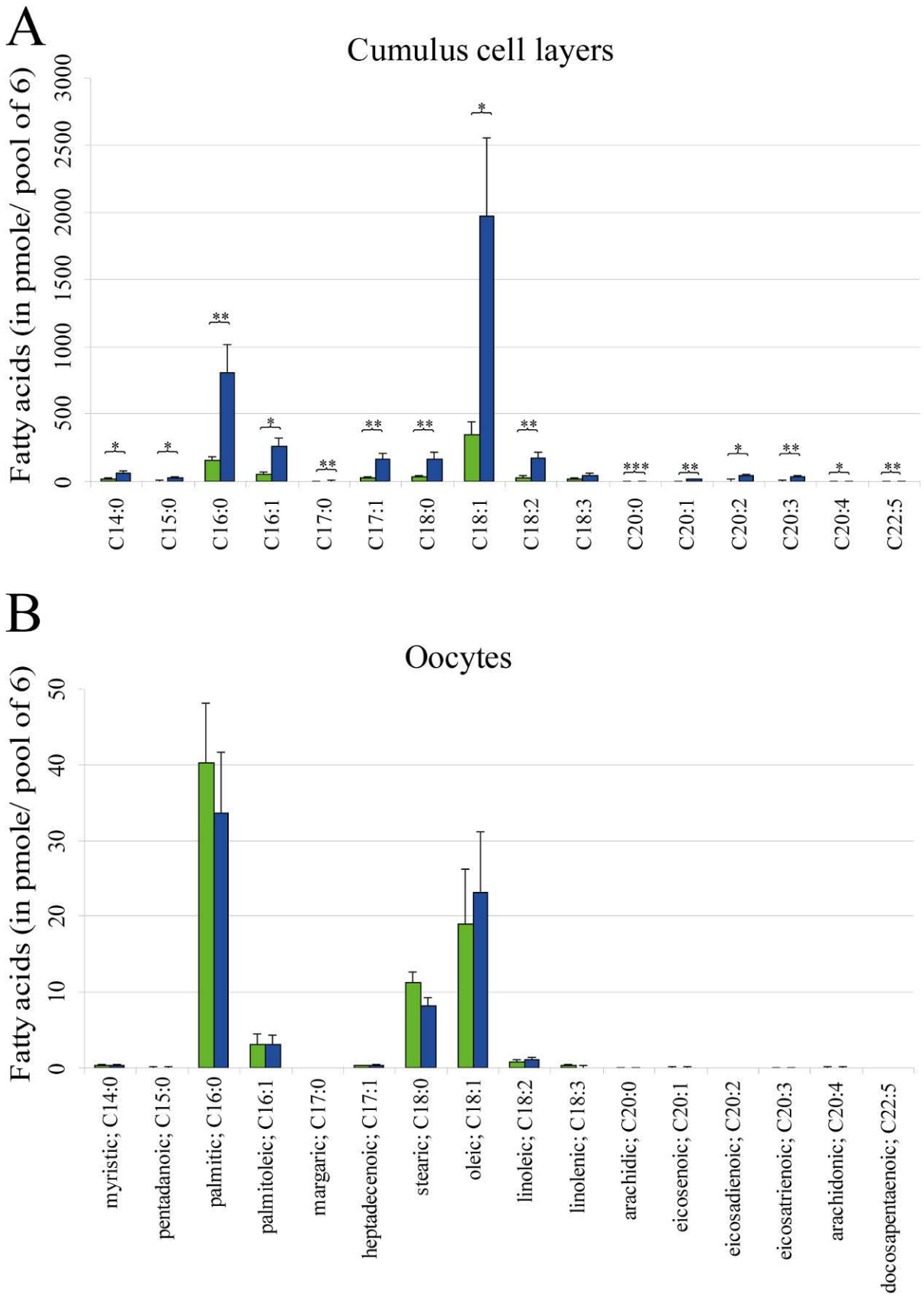


FIG. 10. The total amount of individual fatty acids present in DAG and TAG. Almost all the different fatty acids present in the lipid of cumulus cells (pool of cumulus cells 6 originating from 6 COCs) significantly increased upon exposure to short-term fasting (blue bars) compared with cumulus cells 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**.

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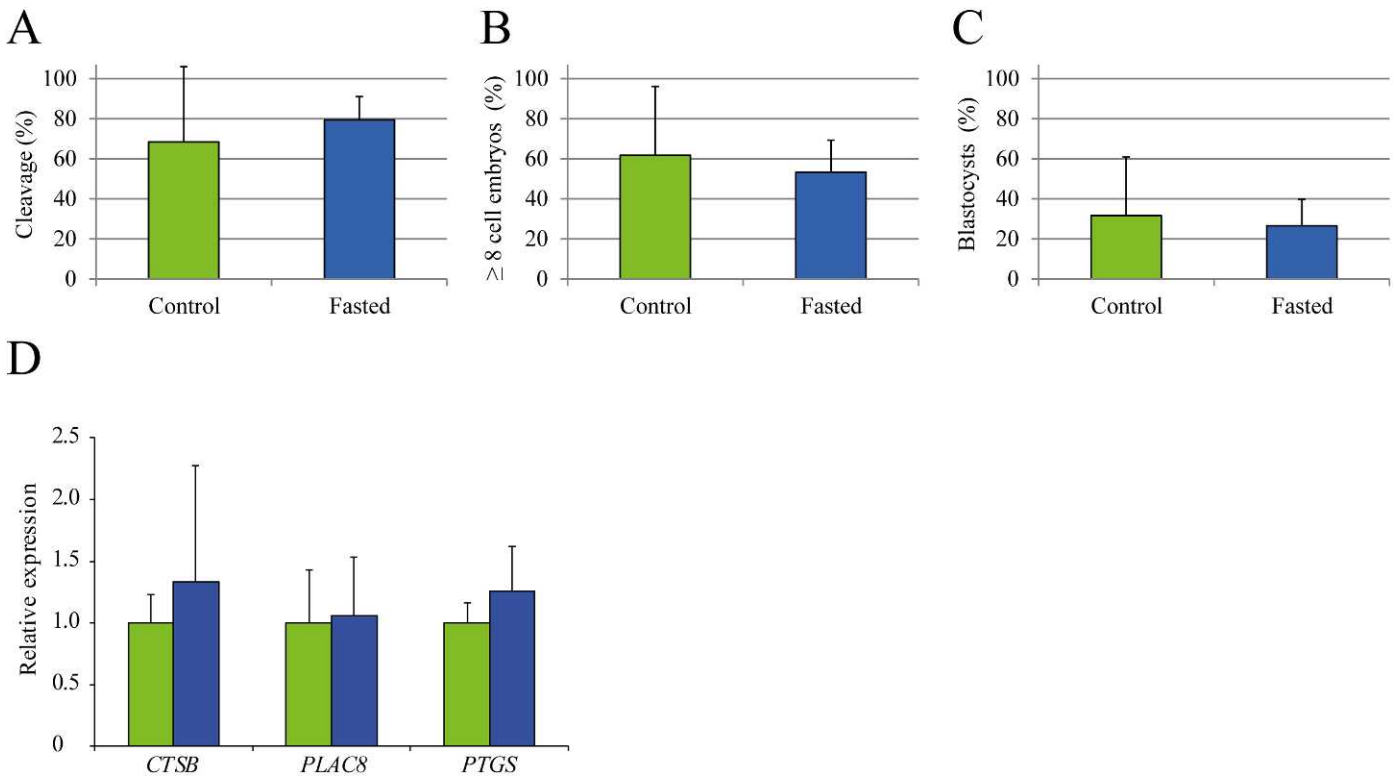


FIG. 11. Short-term fasting during final oocyte maturation does not impair the postfertilization developmental competence of the oocytes. The developmental competence of in vivo matured COCs originating from control (green bars) and fasted (blue bars) cows was measured by determining the cleavage (**A**), ≥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.

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

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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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