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Original Research Article

Periparturient oleic acid-rich fat supplementation affects the lipid profile in blood and results in an increased oocyte yield in postpartum dairy cows

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ABSTRACT

In high-producing dairy cows periparturient negative energy balance (NEB) triggers body fat mobilization, resulting in elevated blood levels of non-esterified fatty acids (NEFAs). Blood is dominated by the saturated fatty acids (SFA) palmitic (C16:0) and stearic acid (C18:0), which are associated with a negative effect on oocyte developmental competence. In contrast, the monounsaturated fatty acid (MUFA) oleic acid is harmless and is able to counteract the negative effect of saturated NEFAs on in vitro maturing oocytes. Since preantral follicles lack oleic acid-rich follicular fluid, we hypothesized that preantral follicles and oocytes may benefit from oleic acid-rich fat supplementation during NEB.

Eight-month pregnant Holstein Friesian heifers were randomly divided in two groups to receive a standard, palmitic acid-rich (CTR, n = 5), or rumen-protected oleic acid-rich (UNSAT, n = 6), periparturient fat supplementation until 4 weeks post-calving. NEFA, β-Hydroxybutyric acid and haptoglobin profiles in blood were monitored, and cumulus-oocyte-complexes (COCs) were via transvaginal ovum pick-up (OPU) collected at 8, 12, and 16 weeks postpartum for in vitro maturation, fertilization (day 0), and culture until day 8.

Oleic acid supplementation increased C18:1 and reduced C16:0 levels in blood in comparison to CTR, during the peripartum period. Interestingly, the UNSAT group exhibited a 1.6-times higher oocyte yield in comparison to the CTR, but no difference in oocyte developmental competence between the groups.

These findings suggest that peripartum oleic acid supplementation supports follicles and oocytes during NEB. Potential long-term benefits of oleic acid on fertility in dairy cows, in a higher number of animals, warrant further investigation.

1. Introduction

Negative energy balance (NEB) is common in high-producing dairy cows during the early postpartum period and has been linked to reduced fertility performance [1–5]. During NEB, the cow mobilizes body fat reserves, as an alternative energy source to meet the temporary energy deficit in the first weeks of lactation, leading to increased circulating concentrations of non-esterified fatty acids (NEFAs) [6–8]. Excessive NEFA concentrations have been associated with detrimental effects on cow health, including impaired immune function, increased risk of metabolic disorders (e.g., ketosis), and consequently, compromised reproductive performance, including decreased oocyte developmental competence [7–11]. The three most prominent NEFAs found in blood and follicular fluid of dairy cows are saturated palmitic and stearic acid (SFA), and the mono-unsaturated fatty acid (MUFA) oleic acid, which are indeed the most prominent fatty acids present in adipose tissue of cows [7,9,12,13]. Former studies demonstrated that there is a relatively high level of oleic acid present in follicular fluid, in comparison to the blood that is dominated by saturated NEFAs, independent of the postpartum timing [9,12,13]. In vitro studies demonstrated a negative impact of saturated NEFAs on oocyte developmental competence that could be counteracted by MUFA oleic acid [11,12]. Elevated levels of the MUFA oleic acid facilitate the safe storage of (saturated) fatty acids in lipid droplets of cells, also demonstrated by a significant increase of lipid accumulation in cumulus cells after exposure to metabolic stress conditions [12,14,15]. Furthermore, the cumulus cells that surround the

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oocyte are able to convert saturated fatty acids into MUFAs via enzyme activity of the highly abundant stearoyl-CoA desaturase-1 (SCD1) and are able to protect the oocyte against elevated levels of NEFAs [14].

Britt already hypothesized in 1992 that there might be a negative carry-over effect on oocytes that are developing in follicles during the NEB, long before final maturation and ovulation, which may hamper oocyte developmental competence [1]. The follicular growth phase, from primordial until preovulatory follicle has been estimated to be over 120 days for cows [16]. This indicates that a successful conception and pregnancy, until at least 120 days postpartum, depends on presumptive oocytes that have fully experienced the potential negative effects of the postpartum NEB during earlier phases of development. To this end, oocytes ovulated from preovulatory follicles at the time of the first postpartum AI may be hampered by the NEB and could have a reduced competence to develop into a good quality embryo, potentially resulting in a higher incidence of pregnancy failure and thus reduced fertility. It has indeed been shown that cows that suffered from a more severe NEB in the early weeks postpartum have reduced embryo quality, and hence reduced pregnancy rates following artificial insemination at 8–9 weeks postpartum [17]. In fact, primary and secondary oocytes from preantral stage follicles lack protection from surrounding cumulus cells and the intrafollicular levels of oleic acid. Therefore, oocytes from early follicular stages may be more directly exposed to saturated NEFAs in blood and may be more prone to the detrimental effects of NEB.

Optimizing nutrition is crucial for enhancing dairy cows' metabolic health and reproductive performance during the transition period. Supplementing omega-6 polyunsaturated fatty acids (PUFAs) around calving, such as conjugated linoleic acid, has been shown to improve follicular development, embryo quality, and pregnancy rate [18–20]. During phases of late gestation and early lactation, fat supplementation with omega-3 fatty acids, a mixture of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), or α-linolenic acid, has been related to reduced uterine prostaglandin synthesis, an anti-inflammatory response, improved embryo quality, and reduced pregnancy losses [21–23]. The supplementation of the omega-3 fatty acid flaxseed, rich in α-linoleic acid, to 256 days pregnant cows, from prepartum until 100 days in lactation, resulted in increased levels of C18:3 in follicular fluid [24]. Also, dietary supplementation of different sources of omega-3 fatty acids from prepartum until 100 days of lactation, was associated with a higher number of follicles and a tendency for an increased number of oocytes, collected by transvaginal ovum pick-up (OPU) from day 60 postpartum, and resulted in a higher blastocyst rate in comparison to a group supplemented with saturated fatty acids [25]. The dietary fat supplementation of respectively omega-6 PUFAs around calving, has been suggested to stimulate the synthesis of prostaglandin-F2α and to improve uterine health [26], while omega-3 PUFAs reduce prostaglandin-F2α levels to support embryo survival during the breeding period [27].

However, in another study, there was no significant effect of fish oil supplementation, rich in DHA and EPA, on reproductive performance in postpartum dairy cows and only a tendency for an increased number of large-sized follicles and a decreased percentage of early embryonic death, based on progesterone values in blood, at 21 post AI [28]. The effect of dietary omega-3 fatty acids on reproduction may be in response to a reduction of the omega-6:omega-3 fatty acid ration in the diet that affects the endocannabinoid system, which via a reduced availability of arachidonic acid affects metabolism and reproduction rather than via an effect of omega-3 alone [29,30]. These findings highlight the complexity of dietary fatty acid interactions and their potential influence on reproductive outcomes in dairy cows, suggesting the need for further research to clarify their mechanisms of action.

The potential positive value claimed for the dietary implementation of PUFAs in the diets of dairy cows on reproduction has long ago been recognized [31]. However, the outcomes of dietary omega-3 and omega-6 studies are not conclusive and show sometimes positive, but also negative effects on reproduction depending on the type of PUFA that was used and may also be due to the timing and duration of the fat supplementation in relation to e.g. calving or breeding [32]. Interestingly, thus far, there has been limited attention for the potential positive effects on reproduction via dietary supplementation of MUFAs, despite the proven beneficial in vitro effects of MUFA oleic acid in counteracting the negative effects of SFA on oocyte developmental competence [11]. We hypothesized that periparturient dietary fat supplementation rich in MUFA oleic acid may protect developing oocytes against the undesired high levels of SFAs in blood during NEB, particularly at the early stages of follicular growth and development when the protection by the presence of several layers of cumulus cells and an oleic acid-rich follicular fluid is lacking. To this end, periparturient cows received an oleic acid-rich fat supplement, based on rumen-protected olive oil, or a standard fat supplement, from four weeks before parturition, before the start of the metabolic transition period and continuing during the early postpartum period with high NEFA levels in blood, until four weeks postpartum. The effect of fat supplementation on cows was measured via lipidomic analysis on blood samples taken from four weeks before until 16 weeks postpartum. To evaluate the potential 'carry-over effect of NEB' on developing oocytes, COCs were collected at 8 (50/60 days), 12 (80/90 days), and 16 weeks postpartum (120/130 days) timepoints via OPU to determine the number of follicles and COCs and to test oocyte developmental competence, based on blastocyst rates, after in vitro maturation, fertilization, and embryo culture.

2. Material and methods

The study was approved by the Dutch Ethical Animal Welfare Committee. Animal health and welfare were checked by daily observation.

2.1. Experimental design

Eleven nulliparous cows, eight Holstein-Friesian, and three Holstein-Friesian x Belgian Blue, raised at the same farm, with an average age and BCS of 24.8 months and 3.3 units, respectively, and a comparable expected calving date (±10 d) were enrolled in the study. All animals were housed in the ruminant clinic of the Department of Farm Animal Health at the Faculty of Veterinary Medicine in Utrecht, in two identical tie-barn stalls. Both stalls were equipped with rubber mattresses covered with sawdust and partitions between individual feeding places. The heifers were randomly divided into two groups based on their body condition scores (BCS): an experimental group that received an additional fat supplement rich in MUFA oleic acid (UNSAT, n = 6), and a control group that received an additional fat supplement rich in saturated palmitic acid (CTR, n = 5) from four weeks (-4 wks) prepartum before their expected calving date until 4 weeks postpartum (+4 wks). The heifers had unlimited access to the isocaloric diets following the requirements for dry-off and lactating cows (see supplemental Table) and water. From the start of lactation, milk yield was twice daily recorded until the end of the trial.

2.2. Diet

The heifers were fed with a balanced ration during the dry and lactation periods twice each day. The standard ration, which was equal for both groups, consisted of grass silage, silage maize, wheat straw, soybean, beet pulp, Royaal CP, and soy meal, fed as a total mixed ration (TMR). The two diets were isocaloric but differed in the fat source supplementation. In the experimental group (UNSAT), heifers received a fatty acid mix high in rumen-protected unsaturated oleic acid (500 g/d Monofat, calcium soap based on 100 % olive oil; Schils BV, Sittard, The Netherlands), on top of the standard ration. The control group (CTR) received a fatty acid mix, high in saturated palmitic acid, which is a standard commercially available fat supplement (380 g/d Milkpower – ForFarmers, Lochem, The Netherlands + 100 g chalk). Fatty acid mixes

Table 1
Composition of the dry period and lactation ratio given including the fat supplements. Fatty acid mixtures were supplemented from four weeks before calving (-4 wks) during the dry period, until the first four weeks of the lactation period (+4 wks). The CTR group received 380 g/d of Milk-power (ForFarmers, Lochem, The Netherlands + 100 g chalk), the UNSAT group received 500 g/d Monofat (calcium soap based on 100 % olive oil – Schils BV, Sittard, The Netherlands).

Product	Dry period (-4 until 0 wks)		Lactation (0 until + 16 wks)	
	Dry matter Kg	VEM/Kg DM	Dry matter Kg	VEM/Kg DM
Grass silage	2.20	927	6.50	927
Silage maize	1.35	1019	4.0	1019
Wheat straw	3.00	432	0.52	432
Soybean	0.18	993	2.35	993
Beet pulp	0.79	939	4.50	939
Royal CP/505075	1.52	980	1.05	980
Soy meal	0.35	1015	0.45	1015
Fatty acid mix	0.45	3000	0.45	3000
Fat supplements	**CTR**		**UNSAT**	
	Milkpower g/kg (%)		**Monofat g/kg (%)**	
Palmitic acid C16:0	799.8 (79.8 %)		90.4 (13.5 %)	
Stearic acid C18:0	32.0 (3.19 %)		21.9 (3.28 %)	
Oleic acid C18:1	120.7 (12 %)		419.7 (62.9 %)	

were added to the rations starting from - 4 weeks prepartum, based on the expected calving date, until +4 wks. The specific composition of the diets is shown in Table 1.

2.3. Body condition scores and dry matter intake

Body condition scores (BCS) were determined by the same technician at the start of the experiment (−4 wks), and at around +4, +8, and +12 weeks postpartum, according to the five-point scale (1 = an emaciated cow to 5 = an obese cow), using 0.25 increments, with a healthy cow average between 3 and 3.5; [33]. Dry matter intake (DMI) was determined daily by calculating the difference between the given amount of ration and the non-consumed, leftover product (kg). After calving, the animals were clustered in follow-up groups for blood sampling and OPU sessions based on their parturition dates (Fig. 1).

2.4. Blood sampling

Blood samples were collected from the start of the experiment at four weeks (-4 wks), two weeks (-2 wks), and one week (-1 wk) prepartum, based on the expected calving date, and at 1 week (+1 wk), 2 weeks (+2 wks), 4 weeks (+4 wks) and 6 weeks (+6 wks) postpartum, and at the times of OPU at around 8 weeks (day 50/60, OPU 1), 12 weeks (day 80/90, OPU2) and 16 weeks (120/130, OPU3) postpartum. Blood samples were taken from the jugular vein using 18-gauge disposable needles and a vacutainer system, in serum tubes for metabolic analyses. After collection, the samples were centrifuged using an Eppendorf centrifuge 5804 R at 5000 rpm for 10 min at 12 °C, and serum samples were stored at −80 °C until analysis.

2.5. Blood analysis of NEFAs, β-Hydroxybutyrate (BHBA) and haptoglobin

Serum concentrations of β-Hydroxybutyric acid (BHBA), NEFAs, and haptoglobin were measured from the start of the experiment at - 4 wks, and at +1wk, +2 wks, +4 wks, and +6wks. The choice of these time points was based on a previous study that investigated haptoglobin [34]. Samples were submitted to the veterinary laboratory of Royal GD (Deventer, The Netherlands). Serum samples were analyzed for haptoglobin, and NEFA concentrations on an UniCel DxC 600 Synchron Clinical System (Beckman Coulter). A colorimetric method was used to analyze serum concentrations of haptoglobin ("PHASE" Haptoglobin Assay; Tridelta Development Limited) and enzymatic methods were used to analyze serum concentrations of NEFA (Wako NEFA-HR(2) test kit; FUJFILM Wako Chemicals Europe GmbH). Serum samples were analyzed for BHBA concentrations on a DxC 700 AU Chemistry Analyzer (Beckman Coulter). An enzymatic method was used to analyze serum concentrations of BHBA (D-3-Hydroxybutyrate (Ranbut) reagent from Randox Laboratories Ltd.). All results were evaluated according to the quality control procedures of the laboratory (meeting NEN-EN-ISO 9001:2015 requirements).

2.6. Lipidomics analysis

Lipidomic analysis to determine the lipid profiles of blood samples was performed, according to Aardema et al., 2013 ([12]) on serum collected at −4 wks, −2 wks, +2 wks, +6 wks, +8 wks (OPU 1), +12 wks (OPU2) and +16 wks (OPU3). In short, 10 μL of serum were mixed with 10 μL of SPLASH-II stable-isotope labeled internal standards (Merck, Darmstadt, Germany) and lipids were extracted with chloroform/methanol according to Bligh and Dyer [35]. The equivalent of 1 μL serum was analyzed by LCMS on a Sciex X500R QToF instrument as described previously [36]. Data were analyzed by MSDial v4.3 (reference Hiroshi Tsagawa MSDial) and statistical analysis was performed in R (R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.).

2.7. Follicle puncture and cumulus-oocyte-complexes collection via OPU

To evaluate the effect of fat supplementation on oocyte

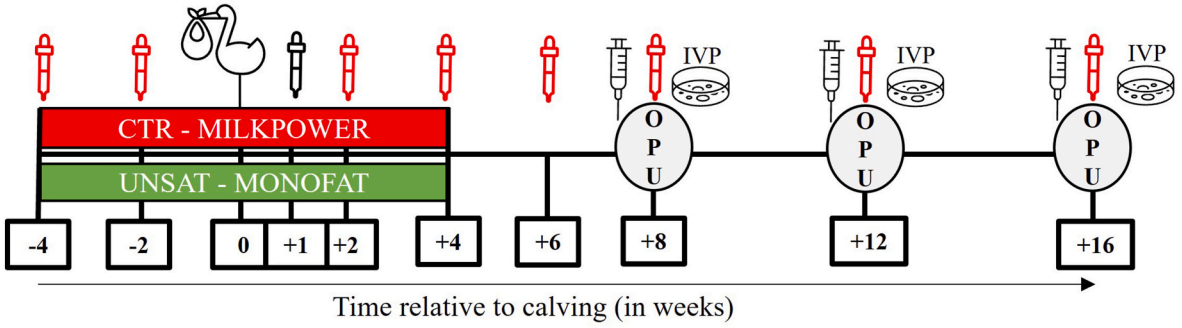

Fig. 1. *Experimental design.* Cows in the control group (CTR) received a standard fat supplement (Milkpower®) and cows of the experimental group (UNSAT) received a fat supplement rich in oleic acid (Monofat®) from four weeks before (−4) until four weeks postpartum (+4 wks). Blood sampling to check the metabolic condition of cows was performed at weeks −4 before calving (0), and +1, +2, +4, +6 postpartum and for lipidomic analysis at weeks −4, −2, +2, +4, +6, +8 (OPU 1), +12 (OPU 2) and +16 (OPU 3). Five days before the moment of transvaginal ovum pick up (OPU) cows received an injection of GnRH. Each OPU moment consisted of 2 separate sessions within a 5-day interval. Collection of COCs was followed by in vitro maturation (day −1), fertilization, and embryo culture until day 8 (IVP).

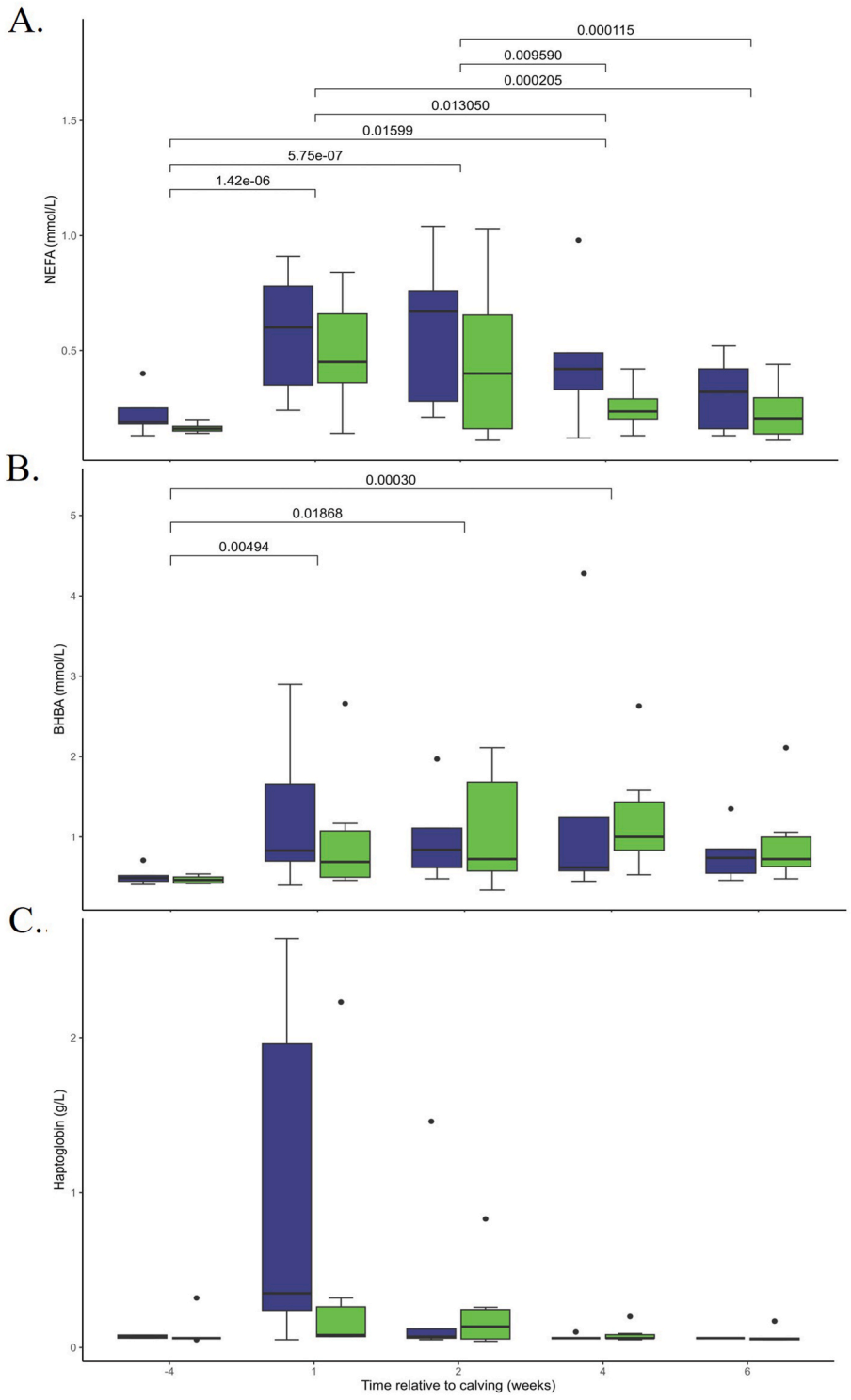

Figure 2.

Fig. 2. *Peripartum blood values of NEFA, BHBA and haptoglobin in cows of the control and experimental group.* Boxplots of the control (CTR, blue) and experimental (UNSAT, green) group of NEFA (A) and Beta-hydroxybutyric acid (BHBA; B) in blood to monitor the metabolic condition of cows, and haptoglobin (C) to measure the acute phase response during the peripartum period from four weeks before (−4) until six weeks (+6) postcalving. Significant differences have been indicated in the figure between the CTR and UNSAT groups at the time points. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

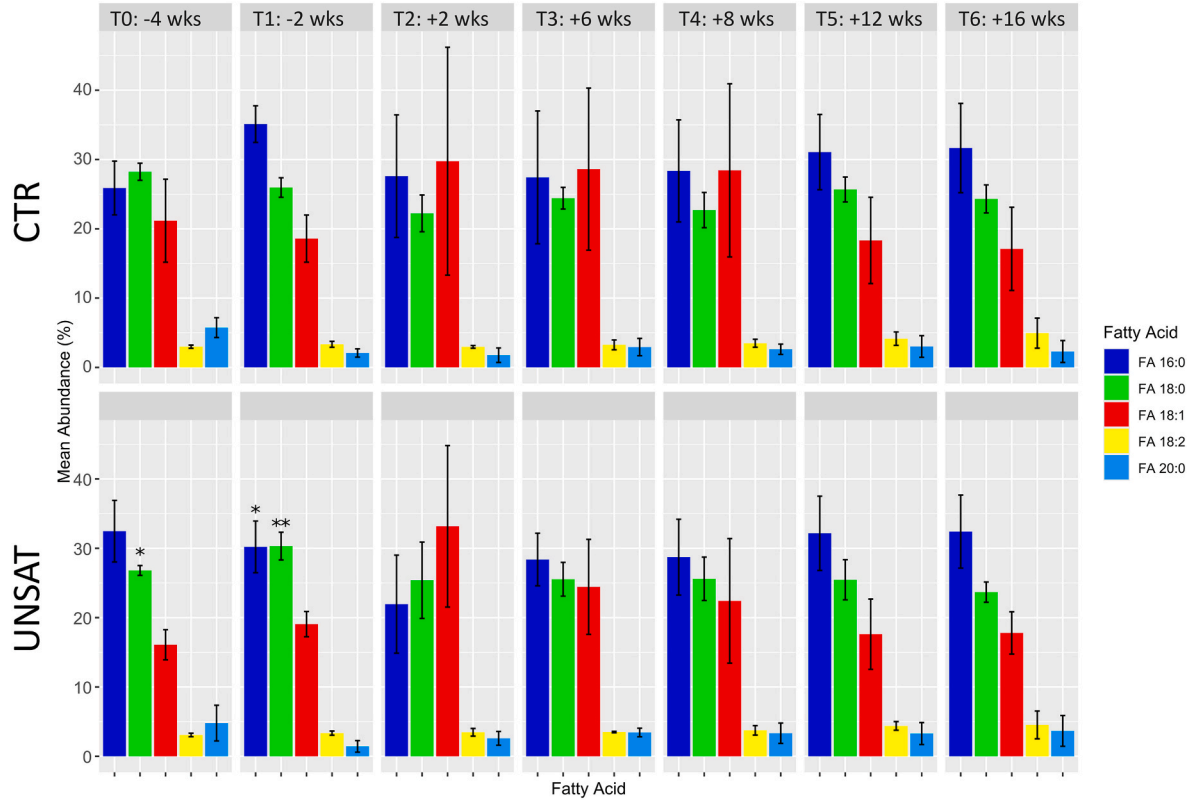

Fig. 3. Fatty acid profile of NEFAs in blood. Bars represent the relative fatty acid composition of the NEFA fraction of the five most prominent fatty acids over time for the control (CTR) and experimental (UNSAT) group. The x-axis represents the different time points from −4 weeks (T0), −2 weeks (T1), +2 weeks (T = 2), +6 weeks (T = 3), +8 weeks (T = 4, OPU 1), +12 weeks (T = 5, OPU 2) and +16 weeks (T = 6, OPU 3). The three in blood most abundant fatty acids in the NEFA fraction, with more than 15 % of the total amount of fatty acids at each time point, where palmitic (C16:0, blue bar), stearic acid (C18:0, light green bar) and oleic acid (C18:1, red bar). The y-axis represents the average percentage (%) per fatty acid for each group. Each color corresponds to a specific fatty acid, as indicated by the legend. The fatty acids are labeled by their carbon chain length and degree of saturation. Asterisks indicate a significant difference (p = * < 0.05, ** <0.01, *** <0.001) between the CTR and UNSAT group at the time points.

developmental competence, the collection of COCs by OPU was carried out at three different time points postpartum (OPU1, OPU2, and OPU3). Before the start of each OPU session, the heifers were pretreated with 20 μg of GnRH (i.m. Receptal® - MSD Animal Health, Kenilworth, New Jersey, United States) to induce a LH peak [37], and the ovulation of a present dominant follicle and start of a new follicular wave. The OPU procedure at each defined timepoint was carried out five days after the GnRH injection and was again repeated after a five-day interval to increase the total number of collected COCs per animal for each postpartum timepoint. OPU was performed by one skilled technician (Breeding company Diamond Genetics, The Netherlands) following the ultrasound-guided transvaginal puncture procedure described by Pieterse et al., 1991 ([38]) with slight modifications. After applying an epidural anesthetic by an injection of 5 ml Procaine hydrochloride 4 % ± adrenaline (Pronestesic® - ASTfarma, Oudewater, The Netherlands), the puncture device was inserted vaginally into the fornix region and under transrectal guidance the ovaries were positioned against the tip of the device, whereafter the number of visible ovarian follicles (>3 mm) was recorded. Immature COCs were aspirated from antral follicles ranging from 3 to 10 mm in diameter. For the transvaginal puncturing of the follicles sterile 18-g disposable needles were used and changed between each animal, during the collection procedure, the catheter system was repeatedly flushed with EmXcell (IMV Technologies, L'Aigle, France), and the aspirated oocytes were temporarily stored in a 50-ml conical tube at 37 °C. After the follicle puncture, the collection tubes with aspirated follicular fluid were immediately transported to the lab and COCs were searched, and counted, before the start of in vitro maturation. Oocyte recovery rate (ORR) was determined using the following formula: ORR = (the number of recovered oocytes/the number of aspirated follicles) x 100 %.

2.8. In vitro embryo production

In vitro maturation (IVM), in vitro fertilization (IVF, day 0), and in vitro embryo culture (IVC) were performed as previously described [11]. Briefly, IVM included the following steps: aspirated COCs were washed, in four-well culture plates (Nunc A/S, Roskilde, Denmark) with maturation medium (M199 Gibco BRL, Paisley, The United Kingdom), supplemented with 26.2 mM NaHCO3, 0.02 IU/ml FSH (Sioux Biochemical Inc., Sioux Center IA, USA), 0.02IU/ml LH (Sioux Biochemical Inc.), 7.7 μg/ml cysteamine, 10 ng/ml epidermal growth factor, and 1 % (v/v) penicillin-streptomycin (Gibco BRL) and incubated at 39 °C under a humidified atmosphere of 5 % CO_2 for 23 h. After the maturation of the oocytes, IVF was performed, following the procedure described by Parrish et al. (1988, [39]) with some adjustments [11]. In short, the oocytes were washed and placed in an IVM medium on plates containing fertilization medium (Fert-TALP Sigma Aldrich GmbH, Dresden, Germany). Frozen-thawed spermatozoa (1×10^6 per ml) originating from one batch of the same bull with proven fertility were added, and the plates were incubated for 20 h. The day of IVF is marked as day 0. After gametes co-incubation, the presumptive zygotes were vortexed to remove the cumulus cells and transferred to plates with a medium of synthetic oviduct fluid and stored at 39 °C under a humidified atmosphere of 5 % CO_2 and 7 % O_2. Embryos were examined on day 5 for the number of cleavages, ranging from 2 to 8 cells, and the cleaved embryos were transferred to plates with fresh medium. On day 8 of

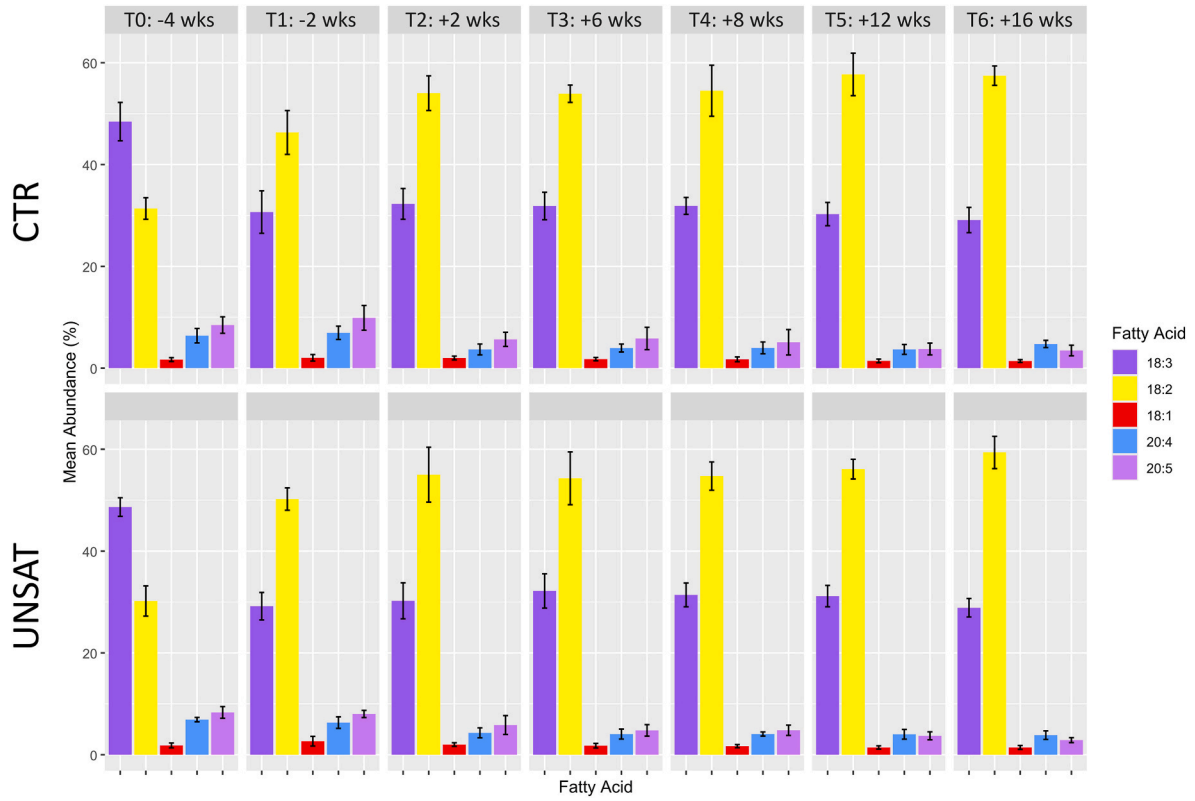

Fig. 4. *Fatty acid profile of cholesterol-esters in blood.* Bars represent the relative fatty acid composition of cholesterol-esters (CHE) of the five most prominent fatty acids over time for the control (CTR) and experimental (UNSAT) group. The x-axis represents the different time points from −4 weeks (T0), −2 weeks (T1), +2 weeks (T = 2), +6 weeks (T = 3), +8 weeks (T = 4, OPU 1), +12 weeks (T = 5, OPU 2) and +16 weeks (T = 6, OPU 3). The two in blood most abundant fatty acids in the CHE fraction, with more than 25 % of the total amount of fatty acids at each time point, where linoleic (C18:2, yellow bar) and linolenic acid (C18:3, purple bar). The y-axis represents the average percentage (%) per fatty acid for each group. Each color corresponds to a specific fatty acid, as indicated by the legend on the right side of the image. The fatty acids are labeled by their carbon chain length and degree of saturation. There were no significant differences in fatty acids esterified to cholesterol-esters between the groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

culture the embryos were assessed according to the quality and the stage: morulae, early blastocyst, blastocyst, and expanded blastocyst. The number of embryos on day 5 and blastocysts on day 8 was used to determine the developmental competence of oocytes. Blastocyst rates (BR) were determined on day 8 based on the number of after OPU recovered immature oocytes, using the following formula: BR = (the number of developed blastocysts/the number of recovered oocytes) x 100 %.

2.9. Statistical analysis

Mixed effect models were used to analyze the collected data from the OPU sessions and the blood samples. The data was imported to R (version 4.3.2) and R studio (version 2024.04.2 + 764) to be evaluated. The choice for mixed effect models was made based on the structure of the data. There are repeated measures for each cow, which are most likely related. We can account for that correlation with a mixed model. The random effect, in this case per cow, tells the model that the observations for each cow are correlated to each other, while the observations between the cows are independent. The cows themselves are also separated into two experimental groups. Mixed models are made to deal with this kind of nested data. For the blood parameters, a normal distribution was used in the model, except for haptoglobin where a t-distribution with 2 degrees of freedom was used. Either a Poisson distribution (number of aspirated follicles and number of collected oocytes) or a binomial distribution was used for the reproductive parameters conditional on the number of oocytes. Models that included random time effects or random time per cow effects failed to converge and were thus not useable. A full model using other variables, such as diet, OPU session or time a blood sample was taken, as well as the interactions between the variables was fitted. Using single-term deletion (drop1 function) and Akaike information criterion, the best fitting model was determined for the fixed effects. Estimates and 95 % profile log-likelihood confidence intervals were computed, and in the case of the reproductive parameters, exponentiated to get odds ratio's. The model assumptions were checked using randomized quantile residuals (DHARMa package in R). The covariance structure of the models is unstructured since this allows for the greatest flexibility in correlations and variances between observations. While the data was collected at roughly the same time for each cow, there is still variation in the exact point in time, as well as biological variation to account for.

3. Results

3.1. Body condition score, feed intake & milk yield

No differences in BCS were recorded between groups during the entire experimental period. The mean BCS of heifers from the CTR and UNSAT groups was respectively 3.4 ± 0.5 and 3.2 ± 0.4 at −4 wks, and 2.2 ± 0.6 and 2.5 ± 0.6 at the last BCS scoring at +12 wks. The mean loss of BCS postpartum was 1.3 ± 1.1 and 0.7 ± 1.0 for the CTR and UNSAT groups, respectively. Furthermore, no differences were detected between the CTR and UNSAT groups in the daily DMI (16.9 ± 3.3 and 17.7 ± 3.6 kg, respectively), and in the MY (27.3 ± 5.7 and 27.1 ± 6.7 kg, respectively).

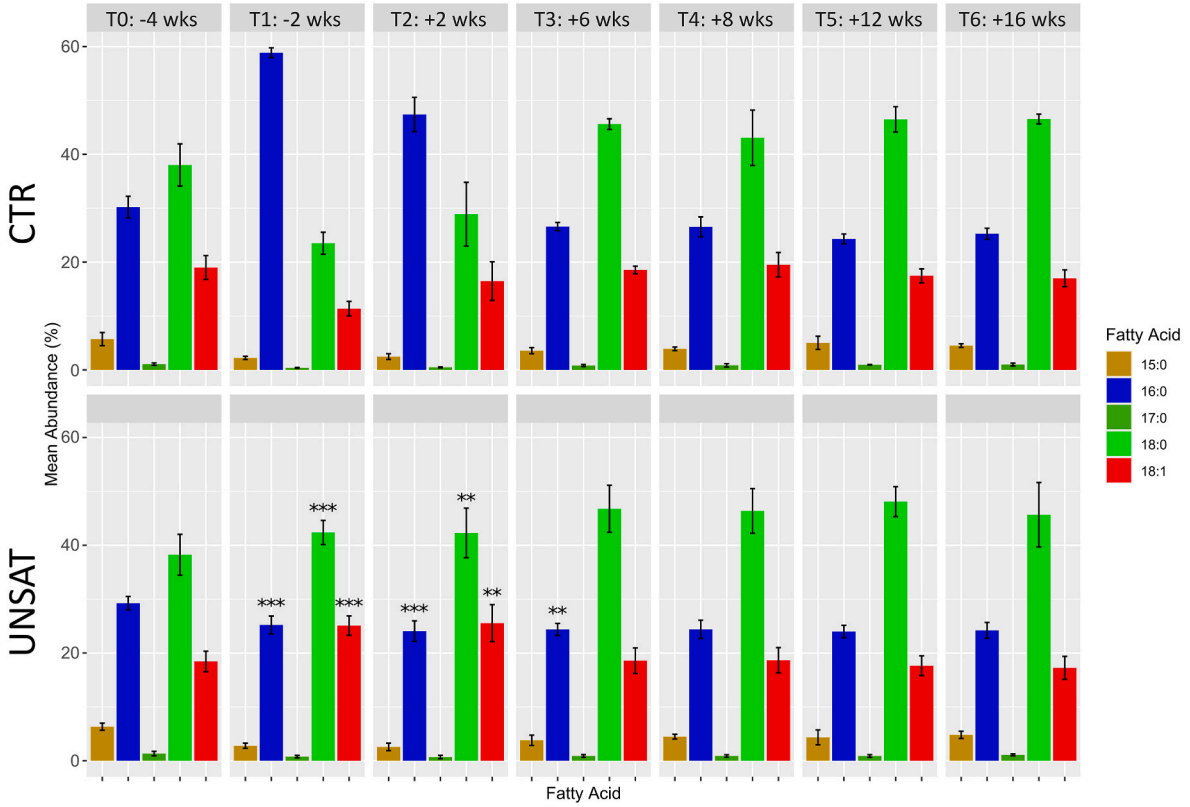

Fig. 5. *Fatty acid profile of triglyceride in blood.* Bars represent the relative fatty acid composition of triglyceride of the five most prominent fatty acids over time for the control (CTR) and experimental (UNSAT) group. The x-axis represents the different time points from −4 weeks (T0), −2 weeks (T1), +2 weeks (T = 2), +6 weeks (T = 3), +8 weeks (T = 4, OPU 1), +12 weeks (T = 5, OPU 2) and +16 weeks (T = 6, OPU 3). The three in blood most abundant fatty acids in the TAG fraction, with more than 10 % of the total amount of fatty acids at each time point, where palmitic (C16:0, blue bar), stearic acid (C18:0, light green bar) and oleic acid (C18:1, red bar). The y-axis represents the average percentage (%) per fatty acid for each group. Each color corresponds to a specific fatty acid, as indicated by the legend. The fatty acids are labeled by their carbon chain length and degree of saturation. Asterisks indicate a significant difference (p = * < 0.05, ** <0.01, *** <0.001) between the CTR and UNSAT group at the time points. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Total NEFA, BHBA, and haptoglobin concentrations

No differences in the total NEFA and BHBA concentrations were recorded between the two groups at the different time points. However, for both groups total NEFA (Fig. 2A) and BHBA (Fig. 2B) concentrations were higher ($P < 0.05$) at +1, +2, and +4 wks compared to −4 wks, with NEFAs returning to starting values at + 6 wks (Fig. 2A). At one week postpartum two cows in the CTR group, resulting in the seemingly higher haptoglobin concentration, and one cow in the UNSAT group had a higher haptoglobin value. No differences were observed in haptoglobin concentrations between the groups as well as among the different timepoints.

3.3. Fatty acid profile of NEFAs in blood

Former studies in our group demonstrated that the fatty acid composition, rather than the total amount of fatty acid, has a determining impact on oocyte quality. To this end, the fatty acid profiles of the different lipid fractions; NEFAs, cholesterol-esters and triacylglycerides, in blood were analyzed and compared between the CTR and UNSAT group. In both the CTR and UNSAT groups the most abundant NEFAs in serum were saturated palmitic (C16:0) and stearic acid (C18:0), and MUFA oleic acid (C18:1; Fig. 3). At the start of the experiment (T = 0, −4 wks) a minor, non-significant difference in the concentration of C18:0 between the CTR and UNSAT group (Fig. 3) was observed. At −2 wks (T = 1) there was a difference between the CTR and UNSAT group, with a higher C16:0 and lower C18:0 concentration in the CTR versus the UNSAT group. Overall the fatty acid profiles of NEFAs were similar among the groups, indicating that the periparturient fat supplementation had a minimal impact on the blood NEFA composition. Fig. 3

3.4. Fatty acid profile of cholesterol-esters in blood

Next, the fatty acid profile of cholesterol esters (CHEs) was analyzed. The fatty acids esterified to cholesterol were poly-unsaturated fatty acids (PUFAs), and mostly essential PUFAs. At −2 wks (T = 1) in both groups a switch was observed from CHE-C18:3 (T = 0) to CHE-C18:2, indicating a dynamic change of the fatty acid profile over time. The identified switch from linolenic acid (C18:3) towards linoleic acid (C18:2) in all animals may be in response to the dietary switch to TMR before the start of the experimental period. The fat supplementation in the CTR and UNSAT group had no effect on the CHE composition. Fig. 4.

3.5. Fatty acid profiles and individual lipid species in blood

The dietary fat supplementations of the CTR and UNSAT groups influenced the fatty acid profile of triglyceride (TAG) in serum. The cows started with a similar fatty acid profile in both groups at −4 wks (T = 0), i.e. before the start of the fat supplementation. During the fat supplementation at −2 wks and +2 wks the UNSAT group had higher concentrations of both C18:1 (+13.7 % and +9.1 %, respectively) and C18:0 (+18.9 % and +13.4 %, respectively), and lower concentrations of C16:0 (-33.6 % and – 23.3 %, respectively) in comparison to the CTR

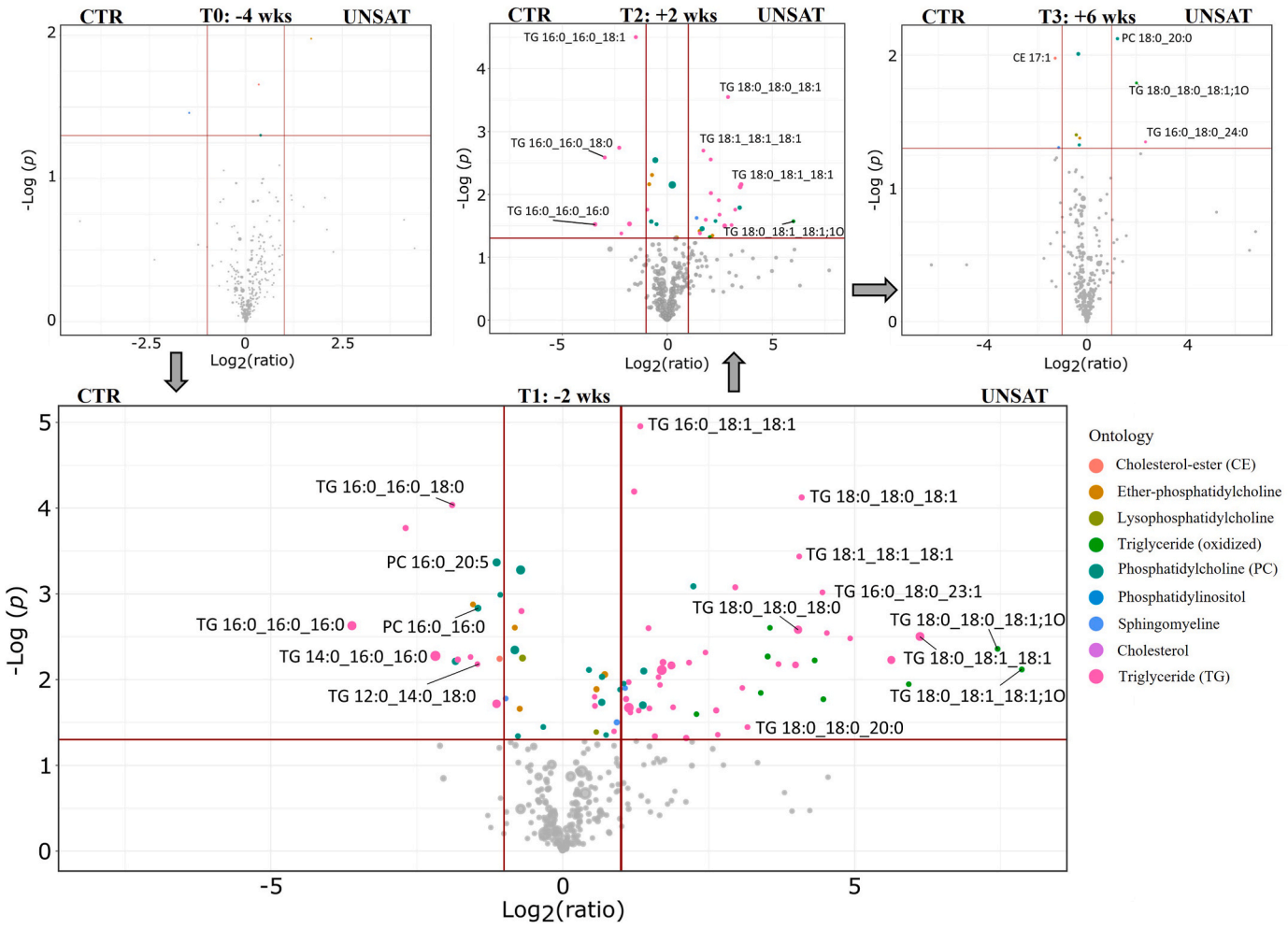

Fig. 6. *Volcano plots showing blood lipidome alterations due to oleic acid-rich fat supplementation ("UNSAT") compared to the control diet ("CTR").* The four panels represent time points from 4 weeks before calving (T0) to 6 weeks postpartum (T3), as indicated. Each dot represents a lipid species, colored by lipid class, with size proportional to its mean relative abundance. Key lipids are labeled when their ratio changes ≥2-fold (Log2(ratio) outside −1 to +1, marked by vertical lines) and significance is $p < 0.05$ (-Log10(p) > 1.3, marked by the horizontal line). Labels for triglycerides (TG) with '; 1O' indicate oxidation by one oxygen atom. Note the transient enrichment of 18:1-containing species and depletion of 16:0-containing species in the "UNSAT" group.

group (Fig. 5). At −2 wks and +2 wks the fatty acid profiles of the CTR and UNSAT groups were different. Cows in the UNSAT group had higher levels of oleic acid (C18:1, −2 wks; 25.1 ± 1.8 % and +2 wks; 25.5 ± 3.4 %) versus the CTR group (C18:1, −2 wks; 11.4 ± 1.3 % and +2 wks; 16.5 ± 3.6 %), and higher levels of stearic acid (C18:0, −2 wks; 42.4 ± 2.2 % and +2 wks; 42.3 ± 4.6 %) versus the CTR group (C18:0, −2 wks; 23.5 ± 2.0 % and +2 wks; 28.9 ± 5.9 %). Furthermore, in the UNSAT group the level of palmitic acid (C16:0) was significantly lower at −2 wks and +2 wks (25.2 ± 1.7 % and 24.1 ± 1.9 %, respectively) versus the CTR group (58.9 ± 0.9 % and 47.4 ± 3.2 %, respectively). Fig. 5

Fig. 6 illustrates the extensive and statistically significant changes in the blood lipidome following oleic acid-rich diet supplementation. These changes were most pronounced at −2 weeks (T1) and +2 weeks (T2) relative to calving. In the UNSAT group, a notable enrichment of TAG species containing C18:0 and particularly C18:1 was observed (Log2(ratio) > 0), occurring at the expense of TAGs containing 14:0 and 16:0 (Log2(ratio) < 0). Strikingly, several oxidized TAG species exhibited over a 100-fold increase (Log2(ratio) > 7), though their overall contribution to the total lipidome remains minimal. The underlying oxidation processes, while intriguing, are beyond the scope of this paper and under further investigation.

3.6. OPU and culture data

In total 1152 follicles were aspirated over the three defined OPU timepoints (i.e. 2 sessions per OPU timepoint with a 5-day interval) on +8 wks, +12 wks, and +16 wks. In total 674 immature COCs were collected and in vitro matured, fertilized (day 0), and cultured until day 8, resulting in a total number of 204 blastocysts. As shown in Table 2 and Fig. 7, the mean number of aspirated follicles was higher in the UNSAT group than in the CTR group ($p < 0.05$). The higher number of follicles in the UNSAT group resulted in a 1.61 times higher number of collected COCs from the UNSAT group. There was no difference in the oocyte developmental competence between the groups, as demonstrated by the comparable blastocyst rates, and no significant difference in the number of blastocysts per cow between the UNSAT (3.8 ± 1.0, $p = 0.16$) and CTR group (2.3 ± 0.7).

Overall, the total number of collected COCs was 1.39 times higher at the second OPU session at day 80/90 in comparison to the first OPU session, and oocyte developmental competence increased over time. The chance for an oocyte to develop into a blastocyst was overall 2.05 times higher for oocytes collected at +16 wks (OPU3) compared to those collected at + 8 wks (OPU1).

Transvaginal ovum pick-up (OPU) was performed at eight weeks (OPU 1), twelve weeks (OPU 2) and sixteen weeks (OPU 3) postpartum. The number of follicles and COCs collected via OPU are presented per

Table 2
OPU results and culture data.

OPU	Group	Follicles per cow	COCs per cow	Day 5 cleavage (in %)	Day 8 blastocyst (in %)
I	CTR	15.8 ± 7.1	7.4 ± 3.0	6.1 ± 2.1 (85.5 ± 14.4)	1.6 ± 1.4 (21.7 ± 21.1)
	UNSAT	21.9 ± 8.3	11.3 ± 5.4	9.1 ± 5.5 (73.9 ± 23.4)	2.7 ± 3.5 (19.0 ± 23.1)
II	CTR	14.1 ± 6.9	10.1 ± 5.4	8.2 ± 4.5 (83.4 ± 15.9)	2.6 ± 1.5 (29.0 ± 23.9)
	UNSAT	23.1 ± 7.5	16 ± 8.3	14.5 ± 7.8 (89.8 ± 12.4)	4.9 ± 4.9 (26.7 ± 16.5)
III	CTR	13.5 ± 2.7	6.75 ± 1.8	5.9 ± 1.7 (87.4 ± 12.3)	2.3 ± 1.0 (36.8 ± 22.6)
	UNSAT	20.5 ± 8.3	11.7 ± 5.8	9.3 ± 5.6 (78.8 ± 17.4)	3.6 ± 3.5 (27.3 ± 18.5)
Total	CTR	14.5 ± 6.0[a]	8.2 ± 4.0[A]	6.8 ± 3.2 (85.3 ± 14.0)	2.1 ± 1.4 (28.6 ± 22.6)
Total	UNSAT	21.9 ± 7.9[b]	13.1 ± 6.8[B]	11.1 ± 6.8 (81.0 ± 19.1)	3.7 ± 4.0 (24.2 ± 19.4)

cow for the control (CTR) and experimental (UNSAT) group, the average number of follicles and COCs of the three OPU sessions is presented in total. Day 5 cleavage shows the number and percentage of cleaved embryos at day 5 of embryo culture (day 0 = IVF), and day 8 shows the number and percentage of blastocysts based on the number of oocytes per cow for the CTR and UNSAT group. Different subscripts indicate a significant difference (p = <0.05) between the CTR and UNSAT group.

4. Discussion

The current study investigated whether follicle number and oocyte development in postpartum dairy cows were affected by periparturient supplementation of either oleic acid-rich or standard fat supplement, rich in palmitic acid. The current study demonstrated that periparturient oleic acid-rich supplementation resulted in higher blood levels of unsaturated oleic acid and a significantly increased oocyte yield.

The levels of NEFA and β-Hydroxybutyrate acid (BHBA) recorded at +1 wk and +2 and + 4 wks postpartum were comparable for both groups and confirmed that cows underwent NEB. In the current study the average levels of NEFA were at all times and in both groups below 0.7 mmol/L, a cutoff value that was previously associated with a higher risk for postpartum disorders [40]. The dietary supplementation with oleic acid did not affect the overall nutritional status or appetite in the cows, as indicated by similar BCS and feed intake recorded in the two groups, in agreement with previous work in dairy cattle [41].

In the current study, haptoglobin was chosen as an acute phase inflammatory marker, to evaluate the potential effect of fat supplementation on the immune response of dairy cows, since palmitic and oleic acid have been related with a pro- and anti-inflammatory response, respectively [42].

However, we did not find a difference in the inflammatory response between the groups or among different time points that were analyzed, indicating that there was no measurable inflammatory response in the cows of the current study. In a former study where cows underwent a NEB the haptoglobin levels in blood showed a subtle increase in healthy cows at day 3 postpartum, but were only significantly increased in cows a mild or severe metritis during the first week postpartum [43].

The elevated levels of, mainly saturated, NEFAs in blood associated with the NEB during the early weeks postpartum [9,12,13], may have a negative effect during the growth and development of follicles. A carry-over effect of the NEB on follicles has formerly been suggested by Britt's hypothesis [1], as the potential link between the observed reduced fertility in NEB cows. In the current study, a negative effect of the NEB on oocyte developmental competence was indeed indicated by the two times higher chance of oocytes to develop into a blastocyst when collected at +16 wks (120/130 days) postpartum, i.e. long after being exposed to the NEB, versus those collected at +8 wks (50/60 days) postpartum, independent of the group. We hypothesized that an oleic acid-rich fat supplement fed to cows in the periparturient period might be beneficial to protect growing oocytes during the phases of follicular development when they are exposed to high blood levels of, primarily, saturated NEFAs during NEB. Interestingly, the periparturient ration with oleic acid-rich fat supplementation resulted in a significantly higher number of antral follicles and hence oocytes, independent of the timing of the OPU session, in comparison to the control group that received a standard dairy cow fat supplement rich in palm oil. The 1.6 times higher yield of oocytes collected from cows fed oleic acid (UNSAT group) suggests a higher survival rate of (pre-) antral follicles and the oocytes during the NEB exposure period, though we cannot exclude an increased recruitment of follicles in the UNSAT group. The higher number of retrieved oocytes resulted in 1.6 times more blastocysts in the oleic acid-rich supplemented group, but was not significantly different between the groups (NS, p = 0.16). A former study where ewes were fed a diet supplemented with a Ca salt of fish oil rich in PUFAs also demonstrated an increased number of follicles and oocytes [44]. In another study where lactating dairy cows were fed with different sources of fatty acids, the number of oocytes collected from the treatment group that received sunflower oil, rich in oleic acid (80 %), was significantly higher compared with those fed trans oleic acid, linoleic acid, or linolenic acid [45]. In the above cited studies no beneficial effects on oocyte developmental competence were found, like in our study. In conclusion, our data indicate that oocyte yield rather than oocyte developmental competence is affected by oleic acid supplementation during NEB.

Former in vitro studies in our group demonstrated that cumulus cells are able to protect the oocyte against potential fatty acid stress by the conversion of saturated stearic acid into unsaturated oleic acid via SCD1 enzyme activity, which contributed to the safe storage of fatty acids in lipid droplets located in cumulus cells, and consequently maintained oocyte developmental competence [11,12,14,15]. The oleic acid induced intracellular lipid droplet accumulation has also been demonstrated for bovine and porcine granulosa cells, via the increased expression of genes involved in the transport of fatty acids, without affecting cell viability and the proportion of apoptotic-necrotic cells [46,47]. This observation may imply that early postpartum oocytes in preantral follicles with only one or a few layers of granulosa cells lack functional effective protection against high levels of NEFAs, and may be more prone to negative carry-over effects by the NEB. Indeed, Walters et al. [2] reported a significantly lower number of small sized antral follicles at around day 50 in comparison to day 87 postpartum, which can suggest a formerly negative impact of the NEB on preantral follicles. Taken together, this may indicate that the peripartum oleic acid-enriched supplementation resulted in a higher survival of (preantral) follicles and oocytes during earlier stages of follicular growth and development. A significantly higher level of oleic acid in the UNSAT group from −2 wks prepartum to +2 wks postpartum, in contrast to the high levels of palmitic acid in the CTR group, suggests that a balance towards unsaturated oleic acid is beneficial for both follicle and oocyte survival. During early (preantral) follicular growth stages, higher levels of oleic acid may protect the follicle and oocyte by promoting the incorporation of fatty acids in cytoplasmic triglycerides in granulosa cells, thus preventing their pro-apoptotic effects, as previously described for cumulus cells during final maturation [12,14,15]. Therefore, the higher number of follicles recorded for the UNSAT group may be due to an improved survival of periparturient recruited follicles, as a consequence of a more balanced fatty acid composition in blood. However, it

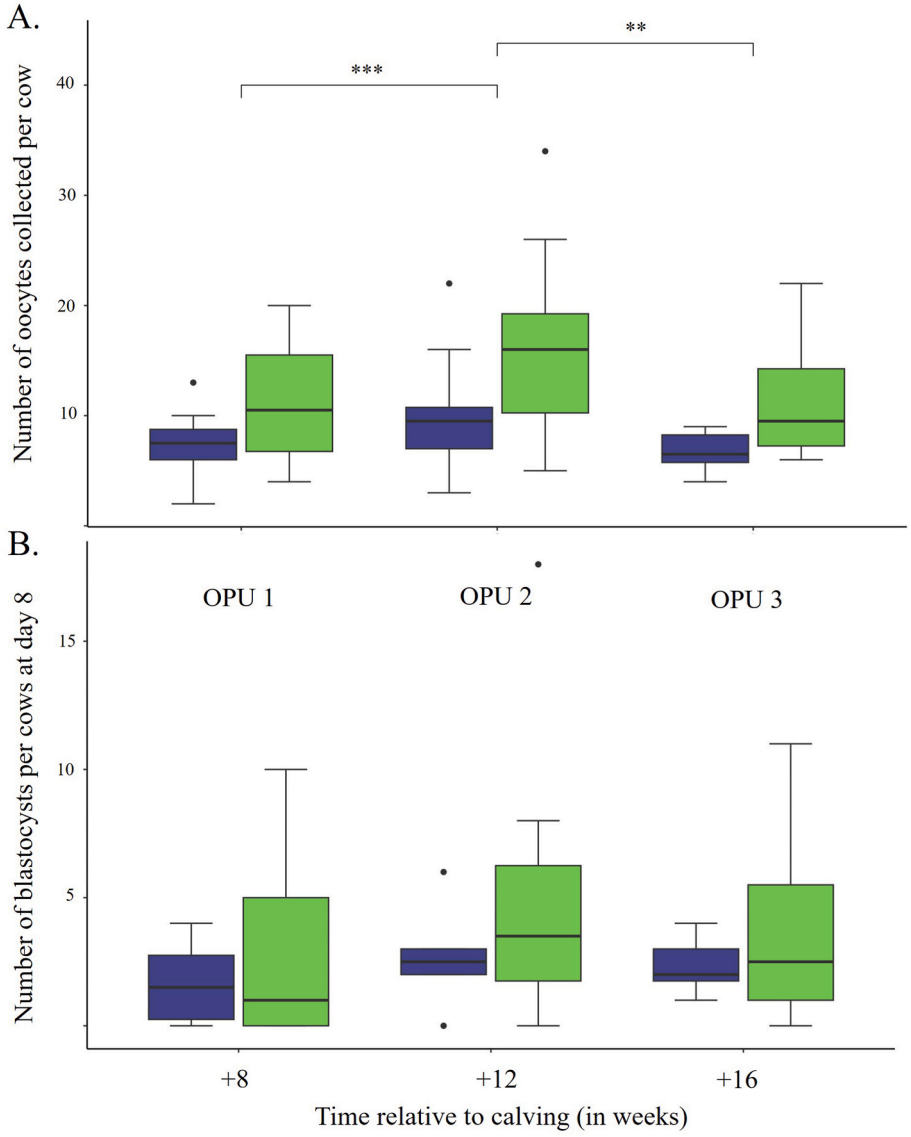

Fig. 7. *Number of oocytes and blastocysts* per *cow.* Boxplots of the control (CTR, blue) and experimental (UNSAT, green) group represent the number of collected COCs per OPU moment per cow, and the number of day 8 blastocysts that developed from the oocytes after in vitro maturation, fertilization and embryo culture. Transvaginal ovum pick-up (OPU) was performed at eight (+8), twelve (+12) and sixteen (+16) weeks postpartum. Asterisks indicate a significant difference ($p = ** < 0.01$, *** < 0.001) between the CTR and UNSAT group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

is not possible to rule out that a higher number of follicles was recruited in the UNSAT group.

It is remarkable that the UNSAT group, apart from the relatively higher oleic acid level at peripartum compared to the CTR group, showed a very stable fatty acid composition in blood over time, with comparable levels of the three most prominent fatty acids, i.e. saturated palmitic and stearic, and unsaturated oleic acid. Previous studies where omega-3 or omega-6 dietary fatty acids were supplemented to ruminants demonstrated an increase in the supplemented fatty acids in blood and follicular fluid, as well as in granulosa cells [48,49]. Wonnacott et al. reported that the fatty acid composition of both high-density and low-density lipoprotein was affected by omega-3 and omega-6 diets [48]. These findings are in line with our results of a change in the fatty acid composition of TAG and CHE, lipids that are stored in lipoproteins after dietary fat supplementation, in blood. This observation indicates that the potential effects of dietary fat sources on growing and developing follicles and in the end fertility outcomes are most likely caused by fatty acids originating from blood lipoproteins, which can be taken up by granulosa and cumulus cells via the CD36 receptor [50].

In conclusion, the present study demonstrates that dietary supplementation with oleic acid during the periparturient period appears to positively influence (pre-)antral follicle and oocyte survival in dairy cows that experience NEB. Although the metabolic parameters between groups were comparable, the periparturient supplementation of oleic acid significantly increased the number of aspirated follicles and harvested COCs, which can potentially result in more blastocysts per animal. The current findings suggest that oleic acid supplementation may positively influence follicular development during the metabolic challenges associated with NEB, highlighting the potential of dietary fatty acid supplementation as a strategy to improve reproductive performance in dairy cows during the critical metabolic transition period. The two times higher chance of oocytes to develop into a blastocyst when collected at +16 wks versus those collected at +8 wks postpartum suggests a negative carry-over effect via oocytes from follicles that develop during the NEB. The postpartum insemination successes may to this end largely depend on the timing and consequent exposure of

follicles and oocytes to the NEB. Overall, the results of the study contribute to our understanding of the complex interactions between nutrition, metabolism, and reproductive physiology in dairy cows during the transition period. However, future studies are warranted to investigate the reproductive outcomes of a larger number of animals, including conception rates and calving intervals, to fully assess the potential of oleic acid supplementation as a management strategy for improving fertility in dairy herds. A potentially higher oocyte yield is relevant for future applications, as an increased number of blastocysts produced per session results in improved ovum pick-up and genomic selection efficiency and, in turn reduced embryo production costs.

Abbreviations

AI	Artificial Insemination
BCS	Body Condition Scores
BHBA	β-Hydroxybutyric acid
CD36	CD36 receptor
CHEs	Cholesterol esters
COC	Cumulus Oocyte Complexes
CTR	Control group supplemented with a palmitic acid-rich fat supplement
DHA	Docosahexaenoic acid
DMI	Dry Matter Intake
EPA	Eicosapentaenoic acid
HDL	High-density lipoprotein
IVC	In Vitro Culture
IVF	In Vitro Fertilization
IVM	In Vitro Maturation
IVP	In Vitro Production
LDL	Low-density lipoprotein
MUFA	Mono-unsaturated fatty acid
NEB	Negative Energy Balance
NEFA	Non-Esterified Fatty Acids
OPU	Ovum Pick-Up
ORR	Oocyte Recovery Rate
PUFA	Poly-unsaturated fatty acid
SCD1	Stearoyl-CoA Desaturase-1
SFA	Saturated fatty acid
TAG	Triglyceride
TMR	Total Mixed Ration
UNSAT	Experimental group supplemented with an oleic acid-rich fat supplement

CRediT authorship contribution statement

F. Piscopo: Writing – original draft, Visualization. **B. Gasparrini:** Writing – review & editing, Writing – original draft, Visualization. **R. van Halderen:** Visualization, Formal analysis, Data curation. **J.F. Brouwers:** Formal analysis, Data curation. **J. van den Broek:** Formal analysis, Data curation. **H.T.A. van Tol:** Investigation. **P.L.A.M. Vos:** Writing – review & editing, Investigation, Conceptualization. **H. Aardema:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Investigation, Conceptualization.

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Declaration of interests

The authors declare no competing interests.

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