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Research Article

Bovine embryo elongation is altered due to maternal fatty acid supplementation

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Abstract

The pre-implantation period is prone to embryonic losses in bovine. Embryo–maternal communication is crucial to support embryo development. Thereby, factors of the uterine fluid (UF) are of specific importance. The maternal diet can affect the UF composition. Since omega 3 fatty acids (omega 3 FA) are considered to be beneficial for reproduction, we investigated if dietary omega 3 FA affected factors in the UF related to embryo elongation. Angus heifers (n = 37) were supplemented with either 450 g of rumen-protected fish oil (omega 3 FA) or sunflower oil (omega 6 FA) for a period of 8 weeks. Following cycle synchronization and artificial insemination, the uteri were flushed post mortem to recover the embryos on day 15 of pregnancy. The UF and tissue samples of endometrium and corpus luteum (CL) were collected. Strikingly, the embryo elongation in the omega 3 group was enhanced compared to the omega 6 group. No differences were observed in uterine prostaglandins, even though the endometrial concentration of their precursor arachidonic acid was reduced in omega 3 compared to omega 6 heifers. The dietary FA neither led to differential expression of target genes in endometrium nor CL nor to a differential abundance of low-density lipoprotein cholesterol, cortisol or amino acids in the UF. Interestingly, the omega 3 group displayed a higher plasma progesterone concentration during luteal growth than the omega 6 group, possibly promoting embryo elongation. Further research should include an ovarian perspective to understand the functional link between dietary omega 3 FA and reproductive outcome.

Summary Sentence

The pre-implantation embryo elongation in bovine is affected by dietary omega 3/6 fatty acids likely due to an effect on the ovary rather than a change of the uterine secretome.

Key words: omega 3 fatty acids, omega 6 fatty acids, progesterone, prostaglandins, uterine fluid, endometrium.

Introduction

Early embryonic losses in the pre-implantation phase are commonly observed in cattle. Even though the fertilization rate averages 90%, the actual calving rate reaches only 55% [1]. In high-producing dairy cows, the rate of early embryonic losses is even more pronounced [2, 3]. The most critical period during which embryo losses occur in bovines is prior to implantation between day 8 and 16 of pregnancy.

As reason for this, a failure of embryo elongation and maternal recognition of pregnancy is considered [4]. For successful establishment and maintenance of pregnancy, an intense embryo–maternal communication via embryonic signals and maternal factors is crucial. In bovines, direct contact of mother and embryo is established through implantation only from day 18 on. Until then, the communication is crucially dependent on the uterine fluid (UF), a complex mixture of nutritive and regulatory factors such as amino acids, sugars, fatty acids (FA), enzymes and other proteins, growth factors and hormones (reviewed by Filant and Spencer [5]). The UF comprises secretions by both the endometrium and the embryo as well as molecules that are transudated from the blood. It may be affected by the maternal diet [6].

Dietary supplementation with fat in general and specifically with ω3 FA has been discussed to improve bovine fertility [7]. However, the study outcomes are inconsistent. Some studies showed an increased conception or pregnancy rate or reduced pregnancy losses following dietary polyunsaturated FA (PUFA) supplementation compared to supplementation with saturated FA (SFA) [8, 9]. In contrast, other authors [10–12] found no effect of dietary FA on the pregnancy rate. In a study by Burke et al. [13], dietary ω3 FA increased the pregnancy rate in only one of two investigated farms where overall fertility was initially poor. Supplementing beef cows with linoleic acid (LA) reduced pregnancy rates [14].

Before implantation, the bovine trophoblast undergoes rapid elongation, starting around day 15 of pregnancy. At this developmental stage, the trophoblast mononuclear cells synthesize large amounts of interferon tau (IFNT), the signal for maternal recognition of pregnancy [15, 16]. IFNT induces the endometrial gene expression of interferon-stimulated genes (ISGs), which are affecting the endometrium and the trophoblast to support embryo elongation and implantation [17]. IFNT also prevents the pulsatile release of endometrium-derived prostaglandin (PG) $F_{2\alpha}$ and thus luteolysis, whereby the progesterone (P4) production of the corpus luteum (CL) is maintained [15].

Starting shortly after ovulation, luteal P4 affects the endometrium by alterating gene expression and secretion of embryotropic factors [18–20]. The administration of P4 during days 1–4 or days 3–16 of pregnancy caused an increased elongation of the embryo on days 13–16 of pregnancy in beef heifers [18, 21] and was associated with the induction of gene expression changes in the endometrium [22, 23]. Factors secreted by both the embryo (e.g. IFNT, PG, and cortisol) and the endometrium (e.g. PG and cortisol) are then assumed to further modulate the endometrial gene expression [24, 25] and lead to an ongoing modification of the UF [24–27].

Prostaglandins are local signaling molecules that exhibit a broad spectrum of functions. The main series-2 PG precursor is the ω6 FA arachidonic acid (AA; C20:4) that is incorporated into the phospholipid bilayer of cell membranes. After liberation from the cell membrane by phospholipase A2 (PLA2), prostaglandin-endoperoxide synthase 2 (PTGS2; previously known as prostaglandin G/H synthase and cyclooxygenase), the key enzyme of PG synthesis, converts AA to PGH_2 which is the common precursor for all proinflammatory downstream series-2 PG, namely $PGF_{2\alpha}$, PGE_2, PGD_2, PGI_2, and thromboxane A_2 (TXA_2). Increasing endometrial PTGS2 expression during diestrus induces the synthesis and release of luteolytic $PGF_{2\alpha}$ pulses [28–30]. In addition, PG in the uterine lumen promote embryonic development and elongation [29, 31, 32]. As both the endometrium and the embryo synthesize PG [28, 33–35], the concentration of uterine luminal PG is higher during pregnancy than during the estrous cycle [31, 36]. Even though luteolytic $PGF_{2\alpha}$ pulses are absent during pregnancy, constant levels of PGE_2 have been detected in the bovine caudal vena cava [37]. An increased PGE_2 production during early pregnancy is induced by IFNT through the upregulation of endometrial PTGS2 [38–40]. PGE_2 has furthermore been shown to promote luteal maintenance and subsequent embryo elongation and implantation [38].

A modification of the PG synthesis may be at least in part involved in the improved fertility observed following a dietary FA supply. The utilization of the ω3 FA eicosapentaenoic acid (EPA; C20:5) as a substrate for PTGS2 results in the formation of series-3 PG. Series-3 PG are much less bioactive than the AA-derived series-2 PG and do not seem to be involved in luteolysis in bovines [41, 42]. The ω3 FA docosahexaenoic acid (DHA; C22:6) does not serve as a precursor for PG synthesis, but has been shown to inhibit PTGS2 activity [43]. Both ω6 and ω3 FA compete for the incorporation into cell membrane phospholipids as well as for an utilization by PTGS2. Therefore, dietary ω3 FA may reduce the formation of AA-derived series-2 PG in favor of increasing the synthesis of series-3 PG. Discussed pathways for ω3 FA-induced reduction of series-2 PG include an inhibiting effect on the synthesis and activity of Δ-6-desaturase involved in AA synthesis. Another potential pathway is the ω3 FA-induced inhibition of PTGS2 involved in PGH_2 synthesis and the replacement of cell membrane AA by ω3 FA, thereby reducing the precursor for series-2 PG production [14, 41]. PG as well as ω3 and ω6 FA are ligands for nuclear receptors such as the transcription factor family of peroxisome proliferator activated receptors (PPARs), which impact on gene expression [44–47]. An involvement of PPARs in development and reproduction has already been demonstrated [44, 48] and a supplementation with ω3 FA has been reported to alter the expression of genes involved in reproduction in the bovine endometrium [49]. Trophectodermal peroxisome proliferator activated receptor gamma (*PPARG*) signaling involved in glucose and FA uptake and metabolism has been shown to be essential for early embryo elongation and survival in sheep [50].

A number of studies have investigated the effects of dietary ω3 and ω6 FA on PG concentrations in plasma. At parturition, ω3 FA from fish oil have been shown to decrease the concentration of plasma PGF metabolites (PGFM) compared to olive oil rich in ω6 FA [51]. Contrarily, feeding diets rich in ω3 FA significantly increased PGFM plasma concentrations post partum in beef cattle [52] as well as in primiparous, but not in multiparous dairy cattle [9]. Several studies have also determined the effect of ω3 FA on oxytocin-stimulated PGFM concentrations. A supplementation of fish oil and feeding fish meal or linseed decreased the PGFM response to oxytocin stimulation in beef heifers and dairy cows compared to control groups which lacked additional dietary fat or which were supplemented with palmitic acid [41, 53–55]. However, feeding linseed in comparison to soybean meal did not affect the oxytocin-induced PGFM response [56].

We hypothesize an effect of dietary ω3 FA on embryo elongation. As the cell membrane composition and thus the content of the PG precursors ω6 and ω3 FA can vary depending on dietary available FA, we supplemented growing Angus heifers with rumen-protected fish oil rich in ω3 FA (EPA and DHA) to ensure the incorporation of FA into the body tissues. To maximize the differences in the ω6/ω3 ratio between the experimental and the control group, we supplemented the latter with rumen-protected sunflower oil rich in the ω6 FA LA, a precursor for AA. We studied embryo elongation and development and further associated parameters in the endometrium and the CL. We specifically aimed at focusing whether the composition of the UF was affected by the diet.

Materials and methods

Animals and study design

In total, 37 Angus heifers (*Bos taurus*) aged between 10 and 27 months (mean ± SEM: 19 ± 0.7) with an average body weight of 348.1 ± 6.4 kg were randomly assigned to two age- and weight-matched study groups. The animals were housed in a freestall barn with ad libitum access to water at the former ETH research station Chamau (Canton Zug, Switzerland) and fed a ration (7.54 kg/day; containing barley straw [3.98 kg], hay [0.57 kg], soy [01.02 kg], wheat [0.69 kg], molasses [0.68 kg], beta-carotene [0.09 kg], commercial mineral mix [0.04 kg] and sodium chloride [0.02 kg]) that included either 450 g of a rumen-protected sunflower oil ("Sonnenblumenöl 100528" from NUTRISWISS AG, Lyss, Switzerland) supplement rich in ω6 FA (ω6 group, n = 15 animals) or 450 g of a rumen-protected fish oil ("Marineöl Omega-3-Konzentrat 33/22" from Henry Lamotte Oils GmbH, Bremen, Germany) supplement rich in in ω3 FA (ω3 group, n = 22 animals). The detailed diet composition is presented in Supplementary Table S1 according to Wolf et al. [57]. The feeding experiment was performed in two separate runs, with 18 animals (n = 9 for both ω3 and ω6 groups) in 2014 and 19 animals (n = 13 for ω3 and n = 6 for ω6) in 2015. Management reasons prevented equal group sizes in the second run. The feeding gate in the free-stall barn was equipped with a gridlock where animals were fixed during feeding. The troughs between animals were separated by wooden barriers during the last two weeks before slaughter. Rumen protection of the supplement oils was established by mixing of the respective oil with hydrogenated rapeseed oil and subsequent spray chilling (Erbo Spraytec AG, Bützberg, Switzerland). The ω6/ω3 ratio of the diet in the ω6 group was 4:1 and in the ω3 group 1:2. The selected fish oil contained 33% EPA and 22% DHA, leading to a combined intake of EPA (37g) and DHA (25 g) of 62 g. Approximately 30 g EPA plus DHA were provided to the animals' intestinal metabolism, assuming a protection of ruminal biohydrogenation of 50%.

The animals were maintained on the respective diets for 8 weeks, leading to a similar average weight gain during the supplementation period of 70 kg. After 5 weeks on the diet, the animals were cycle synchronized by using a controlled internal drug release (Eazi-Breed CIDR 1380®, Zoetis, Zurich, Switzerland) that was removed after 8 days. During the first run, for a few animals in both groups, synchronization by two injections of $PGF_{2\alpha}$ (Estrumate®, MSD Animal Health/Intervet International GmbH, Unterschleissheim, Germany) in an interval of 11 days was applied instead of the CIDR due to management reasons. One day before CIDR removal, animals were injected intramuscularly with 2 ml Estrumate (MSD Animal Health GmbH, Luzern, Switzerland). Three days after Estrumate injection, independently from the synchronization protocol used, the animals were artificially inseminated with sperm from the same Angus bull. The day of artificial insemination (AI) was defined as day 0 of pregnancy. Blood samples were collected on days 0, 3, 6, 9, 12, and 15 from a randomly chosen subset of animals of both groups (ω3: n = 11–17; ω6: n = 6–13). Immediately after slaughter on day 15, the reproductive tract was removed and the uterus was flushed ex vivo with 10 ml PBS to obtain the embryo. The length of the embryos was determined using a stereo microscope prior to snap freezing in liquid nitrogen. Endometrial and CL tissue samples were carefully collected and likewise snap frozen. The recovered UF was centrifuged to remove cellular debris at 800 × g for 10 min. Tissues, plasma, and UF were stored at −80°C until analysis. Animals were only included in the study if an embryo was recovered (ω3: n = 18; ω6: $n = 13$). With the presence of an embryo on day 15 the respective animal was defined pregnant. During the collection, one of the embryos from the ω3 group was destroyed and thus its length could not be determined. However, this animal was included in the study as the presence of an embryo was confirmed. Because of the high prevalence of pre-implantation embryo mortality in bovine, the embryos' genomic DNA was controlled for apoptotic laddering on an agarose gel to ensure the inclusion of animals with viable embryos only. Apoptotic DNA fragmentation was not observed for any of the embryos.

The experimental protocol was approved by the Veterinary office of the Canton Zug (Switzerland) in accordance with the Swiss legislation on animal rights and welfare (Permit numbers: ZG 64/14 and ZG 71/15).

Quantification of fatty acids by gas chromatography with flame ionization detector

The FA composition of plasma and endometrial tissue was determined using FA methyl esters (FAME) prepared by transesterification with trimethylsulfonium hydroxide (TMSH). Briefly, for both plasma and endometrial tissue 1 g was extracted two times with 7 ml of chloroform/methanol. The chloroform layer was drained and evaporated. The residues were resuspended in tert-butylmethylether with TMSH added. The analysis of FAMEs was performed by gas chromatography with a flame ionization detector (GC 6890, Agilent Technologies, Waldbronn, Germany). The quantification of FA was performed using Chromeleon® 6.8 Chromatography Software (Dionex, Sunnyvale, USA) [58].

Quantification of prostanoids by liquid chromatography-tandem mass spectrometry

Prostaglandins ($PGF_{2\alpha}$, PGD_2, PGE_2, 6-keto-$PGF_{1\alpha}$) and thromboxane B_2 (TXB_2) were determined in the UF by solid-phase sample extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Extraction and analysis were performed as previously described [31]. Briefly, a stock solution with 100 μg/ml PGE_2, PGD_2, TXB_2, $PGF_{2\alpha}$, and 6-keto $PGF_{1\alpha}$ was prepared in methanol and then further diluted to obtain working standards. Bovine UF samples were prepared with 200 μl sample and 20 μl methanol were added. The prepared samples were extracted, the organic phase was removed and the residues were reconstituted with 50 μl acetonitrile/water/formic acid (20:80:0.0025, v/v, pH 4.0), centrifuged and then transferred to glass vials (Macherey-Nagel, Düren, Germany) prior to injection into the LC-MS/MS system. A Synergi Hydro-RP column (150 × 2 mm I.D., 4 μm particle size, and 80 Å pore size from Phenomenex, Aschaffenburg, Germany) was used to separate PG and TXB_2 before being determined with an API 4000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany). The quantification was performed with Analyst Software V1.4.2 (Applied Biosystems).

Analysis of cortisol in the uterine fluid

The cortisol content was determined in UF using a commercial immunoassay (Cortisol Free Saliva Kit, Demeditec, Kiel, Germany) according to the manufacturer's instructions.

Analysis of amino acids and total protein content

The analysis of amino acids was performed as described earlier [59]. Briefly, the aTRAQ Reagent Kit was used for 40 μl of UF according

to the manufacturer's instructions (Applied Biosystems) by subsequent analysis via targeted LC-MS/MS (AB SCIEX QTrap 3200 LC-MS/MS System, AB SCIEX, Framingham, MA, USA). The data were analyzed using the Analyst® 1.5 Software, and quantitative measurements of the amino acids were obtained. The total protein content in UF and plasma was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA). Because total protein content and total amino acid concentration (sum of all measured amino acids) were well correlated ($r = 0.82$, $P < 0.001$), the data are presented as mean nmol/mg total protein ± SEM.

Total RNA isolation

Total RNA was extracted from the ipsilateral intercaruncular endometrial and CL tissue using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Tissues were homogenized using the MagNA Lyser and MagNA Lyser Green Beads (Roche, Rotkreuz, Switzerland). RNA concentrations were quantified with the NanoDrop 2000 (peqLab, Erlangen, Germany). RNA integrity was monitored using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) with the Agilent RNA 6000 Nano Kit. RNA integrity numbers ranged between 8.9 and 10 for all samples. RNA aliquots were stored at −80°C until cDNA synthesis.

Reverse transcription and qPCR

Total RNA (1 μg from endometrium and 500 ng from CL, respectively) was used for cDNA synthesis with the GoScript Reverse Transcription System (Promega, Madison, USA). The reaction mix was composed as follows: 10 μl RNA in H_2O, 0.5 μl $Oligo(dT)_{15}$ primer, 0.5 μl random primer, 4 μl reaction buffer, 2.5 μl $MgCl_2$, 1 μl dNTPs, 0.5 μl RNasin, 1 μl reverse transcriptase. Incubation of the reaction mix was performed in a PCR cycler (25°C for 5 min, 42°C for 60 min, 70°C for 15 min).

Quantitative real-time PCR (qPCR) was carried out using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, USA) on a CFX384 Real-Time PCR Detection System (Bio-Rad, Munich, Germany). The cycle of quantification (Cq) values were obtained using a single threshold. The relative expression level (ΔCq) of each gene was generated by scaling the target gene Cq of each individual sample to the geometric mean of the Cqs of three reference genes (ubiquitin B (*UBB*), H3 histone family member 3A (*H3F3A*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), according to the bestkeeper method [60]. Fold-changes were calculated according to the ΔΔCt method. The sequences of commercially synthesized primers (Microsynth, Balgach, Switzerland) used are listed in Supplementary Table S2.

Plasma progesterone concentration

Plasma P4 was determined using the Immunotech RIA (Beckman Coulter, CA, USA), previously validated for bovine plasma [61]. The analytical sensitivity for P_4 was 0.03 ng/ml, intraassay CV was 7.6%, and interassay CV was 9.7%.

Quantification of LDL cholesterol

A commercial assay kit (high-density lipoprotein (HDL) and low-density lipoprotein (LDL)/very low-density lipoprotein (VLDL) Cholesterol Assay kit, abcam, Cambridge, UK) was used to measure cholesterol concentration in the LDL fractions of plasma samples collected at slaughter according to the manufacturer's instructions.

Quantification of tocopherols by high-performance liquid chromatography

Tocopherols were quantified in plasma (collected at slaughter), liver, and endometrium by a validated method described earlier [62]. Briefly, tissue samples were saponified for 30 min at 70°C in a shaking water bath. To both tissue and plasma samples butylated hydroxytoluene (BHT; 25 μL of a 1 mg/ml ethanolic solution) was added. Then all samples were extracted twice with n-Hexane. The supernatants were pooled and evaporated (Christ SpeedDry; Christ, Osterode Germany). The dried residues were resuspended and injected into a Jasco HPLC system (AS-950 Plus autosampler, PU-980 Plus pump, FP-950 Plus fluorescence detector, LG-980-02 gradient unit, and a 3-line degasser; Jasco, Groß-Umstadt, Germany). The tocopherols were separated on a Kinetex PFP column (2.6 μm, 150 × 4.6 mm; Phenomenex, Aschaffenburg, Germany) using a methanol: water (85:15, vol/vol) mobile phase. The fluorescence detector was set to an excitation wavelength of 296 nm and emission wavelength of 325 nm. Peaks were recorded and integrated using ChromPass version 1.8.6.1 (Jasco). The concentrations of tocopherols were quantified against external standard curves with authentic compounds (Sigma Aldrich, St. Louis, MO).

Statistical analysis

The statistical analysis was performed using SPSS version 22 (SPSS GmbH Software, Munich, Germany). The Shapiro–Wilk test was used to test for normal distribution of data and residuals. For comparisons between diet groups, data following a Gaussian distribution were analyzed by Student *t* test. For data not following a Gaussian distribution (as observed for embryo length), a Mann–Whitney *U* test was performed. In case of a significant effect of embryo length on dependent variables (endometrial gene expression, uterine prostaglandins, and amino acids), the "least-square ANOVA general linear models procedure" was used with diet group (ω3 or ω6) as a fixed factor and embryo length (in cm) as a covariable. Data analysis for qPCR results was performed on ΔCq values. Results are presented as means ± SEM and P values ≤ 0.05 were considered statistically significant.

Results

Rumen protected ω3 FA lowered the ω6/ω3 ratio and changed the FA pattern in plasma and endometrium

The amounts of single ω3 and ω6 FA in both plasma and endometrium differed significantly between animals supplemented with fish oil (ω3 FA) and animals supplemented with sunflower oil (ω6 FA) as presented in Table 1. In both diet groups, the percentage of AA, docosapentaenoic acid, and DHA of total ω6 and ω3 FA was higher in the endometrium compared to plasma whereas the percentages of LA, alpha-linolenic acid (ALA), and EPA were decreased in endometrium compared to plasma (all $P < 0.001$).

The total PUFA concentration in plasma was significantly increased in the ω3 group compared to the ω6 group (Table 1). This change was due to increased concentrations of total ω3 FA, whereas total ω6 FA concentration was unaffected in plasma. However, in the endometrium, the total PUFA concentration did not differ between diet groups. This was caused by a significant increase in total ω3 FA and a simultaneous significant decrease in total ω6 FA. Saturated FA and monounsaturated FA (MUFA) were not affected by diet neither in plasma nor in endometrium.

Table 1. Concentration and percentage of selected ω3 and ω6 FA to total ω3 and ω6 FA in plasma and endometrium of Angus heifers.

		Plasma					Endometrium				
		μg/g		*P* value	% of total ω6 and ω3 FA		μg/g		*P* value	% of total ω6 and ω3 FA	
		ω6 FA diet	ω3 FA diet		ω6 FA diet	ω3 FA diet	ω6 FA diet	ω3 FA diet		ω6 FA diet	ω3 FA diet
SFA		596 ± 37	675 ± 33	0.1			1616 ± 117	1489 ± 105	0.4		
MUFA		304 ± 15	264 ± 12	0.06			1266 ± 106	1030 ± 65	0.1		
PUFA		749 ± 42	916 ± 47	0.02			1449 ± 105	1352 ± 103	0.5		
Linoleic acid		513 ± 30	458 ± 22	0.2	68	50	338 ± 25	315 ± 24	0.3	23	23
gamma-Linolenic acid		19.8 ± 1.5	5.3 ± 0.5	<0.001	3	1	17.0 ± 1.8	10.2 ± 1.1	0.002	1	1
Eicosadienoic acid	ω6	0.8 ± 0.1	1.7 ± 0.1	0.005	-	-	18.8 ± 3.0	15.1 ± 1.6	0.1	1	1
Eicosatrienoic 8c,11c, 14c acid		29.6 ± 1.9	13.3 ± 0.7	<0.001	4	2	117 ± 10	80.7 ± 7.4	0.006	8	6
Arachidonic acid		51.6 ± 2.7	59.3 ± 3.6	0.4	7	6	584 ± 45	388 ± 22	<0.001	40	29
ω6 FA (total)		614 ± 35	536 ± 26	0.09	82	59	1075 ± 80	810 ± 53	0.009	73*	60
Linolenic acid		69.0 ± 4.7	117 ± 6	<0.001	9	13	12.2 ± 4.9	17.8 ± 1.9	0.1	1	1
Eicosapentaenoic acid		34.7 ± 3.2	187 ± 18	<0.001	5	20	24.6 ± 3.1	101 ± 13	<0.001	2	7
Docosapentaenoic acid	ω3	20.8 ± 1.2	30.8 ± 1.4	<0.001	3	3	116 ± 8	158 ± 14	0.2	8	12
Docosahexaenoic acid		10.7 ± 0.9	43.7 ± 2.6	<0.001	1	5	221 ± 19	266 ± 23	0.2	15	20
ω3 FA (total)		135 ± 9	378 ± 26	<0.001	18	41	376 ± 29	545 ± 50	0.045	26	40
ω6/ω3		4.7 ± 0.2	1.5 ± 0.1	<0.001			2.8 ± 0.1	1.6 ± 0.1	<0.001		

Heifers in the ω3 group have significantly more ω3 FA in plasma and endometrium than ω6 heifers. This was caused by an increase in all ω3 FA in plasma whereas in endometrium it caused by solely a higher EPA concentration. Conversely, heifers in the ω6 group display a higher concentration of AA in endometrium. Values are given as means ± sem.

*Rounding error.

The supplementation with rumen-protected ω3 FA significantly decreased the ω6/ω3 ratio in plasma as well as in endometrium compared to supplementation with rumen-protected ω6 FA (Table 1). This change in the ω6/ω3 ratio in plasma was caused by significant increases of all detectable ω3 FA including EPA and DHA in the ω3 group compared to the ω6 group. The amount of AA in plasma did not significantly differ between the two groups. In the endometrium, dietary ω3 FA significantly decreased the amount of AA and significantly increased the amount of EPA compared to dietary ω6 FA. Endometrial DHA did not differ between groups.

Day 15 preimplantation embryos differed in length due to supplementation with rumen protected ω3 and ω6 FA

The embryo length varied greatly within and between the experimental groups (Figure 1), namely from 0.2 to 20.3 cm in the ω3 group, but only from 1.4 to 7.7 cm in the ω6 group. On average, embryos from ω3 FA supplemented animals were significantly longer than embryos in the ω6 group (median ± SEM: ω6 group: 2.5 ± 0.5 cm; ω3 group: 6.4 ± 1.3 cm; $P = 0.043$). The pregnancy rate did not differ between treatment groups and was 82% in the ω3 and 87% in ω6 group, respectively.

Different concentrations of AA and EPA in the endometrium caused by FA supplementation did not affect uterine prostaglandin concentrations

The concentrations of different series-2 PG and their metabolites (PGE_2, PGD_2, TXB_2, $PGF_{2\alpha}$, PGFM, and 6-keto-$PGF_{1\alpha}$) in the UF did not differ between groups (Table 2). $PGF_{2\alpha}$ and the PGI_2 metabolite 6-keto-$PGF_{1\alpha}$ were the most abundant prostaglandines followed

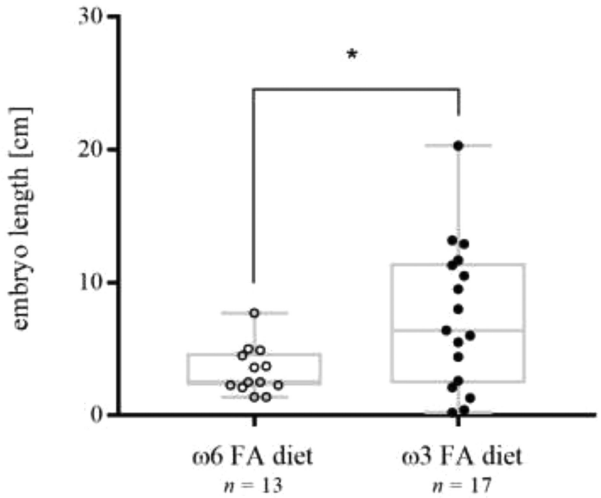

Figure 1. Supplementation with ω3 FA compared to ω6 FA led to significantly longer embryos on day 15 of pregnancy in Angus heifers (*$P \leq 0.05$).

Table 2. Uterine concentrations of prostaglandins (ng/ml uterine fluid; means ± SEM).

	ω6 FA diet	ω3 FA diet	*P*-value
$PGF_{2\alpha}$	17.5 ± 7.0	31.5 ± 5.8	0.9
PGE_2	0.6 ± 0.2	1.0 ± 0.2	0.4
6-keto-$PGF_{1\alpha}$	12.6 ± 3.4	15.2 ± 2.8	0.6
PGD_2	0.2 ± 0.1	0.2 ± 0.03	0.5
TXB_2	0.2 ± 0.1	0.1 ± 0.04	0.4
PGFM	1.5 ± 0.2	1.3 ± 0.2	0.4

by PGFM and PGE_2. PGD_2 and TXB_2 were present in similar concentrations and represented the least prominent PG in the UF.

Dietary FA affected intrauterine alanine concentration but did neither impact on other amino acids nor intrauterine cortisol

With the exception of a significantly higher concentration of alanine in the $\omega6$ compared to the $\omega3$ group, no differences were observed in amino acid concentrations in the UF of day 15 pregnant heifers (Supplementary Table S3). The cortisol concentration in the UF did not significantly differ between groups ($\omega6$ group: 0.59 ± 0.1 ng/ml; $\omega3$ group: 0.58 ± 0.1 ng/ml).

The different concentrations of AA and EPA in the endometrium had only a minor impact on endometrial gene expression

The endometrial expression of a broad range of genes, which were selected due to their respective involvement in embryo elongation and metabolism as well embryo–maternal communication, was widely unaffected by the dietary FA supplementation. Out of the genes under investigation, only perilipin 2 (*PLIN2*; previously known as adipose differentiation-related protein (*ADFP*)), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma (*YWHAG*), and sperm associated antigen 9 (*SPAG9*) were differently regulated in the endometrium of pregnant $\omega3$ supplemented heifers compared to pregnant $\omega6$ supplemented heifers (Table 3). The insulin receptor (*INSR*) transcript abundance showed a trend ($P = 0.058$).

Plasma progesterone was affected by dietary FA

In both groups, the concentration of plasma P4 increased from day 3 to day 15 (Figure 2). Animals from the $\omega3$ group had higher P4 concentrations compared to animals from the $\omega6$ group. The average higher concentration of P4 in the $\omega3$ group was due to the animals from which embryos >8 cm in length were recovered.

Luteal gene expression of enzymes involved in P4 synthesis did not differ on day 15 between diet groups

The luteal gene expression of neither hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*HSD3B1*), steroidogenic acute regulatory protein (*STAR*), prostaglandin F receptor (*PTGFR*), aldo-keto reductase family 1 member C3 (*AKR1C3*; previously known as hydroxysteroid (17-beta) dehydrogenase 5 (HSD17B5)), caspase 3 (*CASP3*), caspase 8 (*CASP8*) or *PPARG* differed between diet groups (Table 4).

Dietary FA neither impacted on plasma LDL nor on plasma nor endometrial α- and γ-tocopherol concentrations

The LDL cholesterol concentration in plasma did not differ between the two diet groups ($\omega6$ group: 0.24 ± 0.03 mmol/l; $\omega3$ group: 0.23 ± 0.03 mmol/l) on day 15 of pregnancy. Despite liver concentrations of α- and γ-tocopherol being significantly lower in the $\omega3$ group compared to the $\omega6$ group, neither plasma nor endometrial concentrations of α- and γ-tocopherol differed between groups, respectively (Supplementary Table S4).

Table 3. Endometrial relative gene expression (means ± SEM) and mean fold-changes of pregnant heifers as determined by qPCR.

	ΔCt $\omega6$ FA diet (n = 13)	ΔCt $\omega3$ FA diet (n = 18)	Mean fold-change ($\omega3$ vs $\omega6$)	*P*-value
IFNt-stimulated genes				
ISG15	−1.19 ± 0.13	−1.05 ± 0.27	1.1	0.4
OAS1	−0.39 ± 0.13	−0.30 ± 0.17	1.1	0.7
MX1	0.25 ± 0.10	0.34 ± 0.18	1.1	0.9
Antioxidant defense				
GPX1	−2.09 ± 0.18	−2.15 ± 0.09	1.0	0.6
GPX4	−3.72 ± 0.14	−3.71 ± 0.09	1.0	0.6
SOD1	−3.00 ± 0.48	−3.66 ± 0.75	0.6	0.8
SOD2	−5.39 ± 0.94	−5.35 ± 0.86	1.0	0.8
NQO1	−9.85 ± 0.31	−10.25 ± 0.21	0.8	0.4
GSTA2	−7.65 ± 0.86	−7.02 ± 0.17	1.6	0.7
GSR	−4.44 ± 0.10	−4.42 ± 0.10	1.0	0.6
GSS	−4.44 ± 0.62	−3.67 ± 0.19	1.7	0.7
CAT	−4.35 ± 0.13	−4.52 ± 0.23	0.9	0.6
MT1	−8.33 ± 1.32	−7.44 ± 0.77	1.9	0.7
MT2	−7.86 ± 0.68	−7.18 ± 0.28	1.6	0.7
NFE2L2	−2.85 ± 0.12	−4.14 ± 0.87	0.4	0.3
Amino acid transport and metabolism				
SLC1A1	−1.08 ± 0.15	−1.19 ± 0.13	0.9	0.7
SLC1A5	−5.94 ± 0.21	−5.81 ± 0.23	1.1	0.3
SLC6A6	−5.86 ± 0.14	−5.98 ± 0.12	0.9	0.2
SLC7A1	−4.95 ± 0.10	−4.93 ± 0.11	1.0	0.2
SLC7A5	−8.33 ± 0.15	−8.29 ± 0.19	1.0	0.8
SLC7A8	−5.81 ± 0.30	−5.67 ± 0.27	1.1	0.9
SLC15A1	−5.99 ± 0.19	−6.00 ± 0.16	1.0	0.4
SLC15A2	−6.20 ± 0.11	−6.05 ± 0.13	1.1	0.09
SLC15A3	−6.10 ± 0.17	−5.98 ± 0.23	1.1	0.5
GTP	−6.78 ± 0.13	−6.90 ± 0.15	0.9	0.5
HDC	−10.37 ± 0.50	−10.29 ± 0.52	1.1	0.8
IGF signaling				
IGF1	−5.59 ± 0.39	−5.32 ± 0.30	1.2	0.8
IGF1R	−3.53 ± 0.14	−3.39 ± 0.16	1.1	0.3
IGF2	−4.82 ± 0.27	−4.10 ± 0.21	1.7	0.2
IGF2R	−5.66 ± 0.23	−5.40 ± 0.24	1.2	0.7
INSR	−5.00 ± 0.06	−4.89 ± 0.07	1.1	0.06
PPAR signaling				
PPARA	−4.10 ± 0.09	−4.18 ± 0.07	0.9	0.2
PPARD	−5.47 ± 0.15	−5.38 ± 0.11	1.1	0.8
PPARG	−9.97 ± 0.29	−9.72 ± 0.20	1.2	0.6
Prostaglandin signaling				
PLA2	−5.80 ± 0.12	−5.80 ± 0.07	1.0	0.5
PTGS2	−1.81 ± 0.20	−1.99 ± 0.15	0.9	0.4
PTGER2	−5.38 ± 0.11	−5.74 ± 0.41	0.8	0.6
PTGER4	−7.21 ± 0.19	−7.11 ± 0.14	1.1	0.9
PTGIS	−7.76 ± 1.04	−7.33 ± 0.72	1.4	0.7
PTGIR	−9.62 ± 0.21	−9.48 ± 0.29	1.1	0.8
HPGD	−2.04 ± 0.38	−1.66 ± 0.15	1.3	0.8
SLCO2A1	−7.65 ± 0.13	−7.89 ± 0.24	0.9	0.1
EDN1	−7.44 ± 0.26	−7.54 ± 0.24	0.9	0.8
Lipid and cholesterol metabolism and transport				
LPL	−9.37 ± 0.15	−9.26 ± 0.16	1.1	0.9
FABP3	−2.84 ± 0.27	−2.55 ± 0.31	1.2	0.1
STAR	−7.95 ± 0.19	−8.11 ± 0.21	0.9	0.3
PLIN2	**−3.67 ± 0.17**	**−3.97 ± 0.14**	**0.8**	**0.04**
Immunoregulation				
CPN1	−5.55 ± 0.23	−5.90 ± 0.18	0.8	0.9
TCF7	−5.44 ± 0.11	−5.47 ± 0.07	1.0	0.4

Table 3 *–continued*

	ΔCt ω6 FA diet (n = 13)	ΔCt ω3 FA diet (n = 18)	Mean fold-change (ω3 vs ω6)	*P*-value
Wnt signaling				
SFRP2	−4.57 ± 0.35	−4.54 ± 0.26	1.0	0.9
WNT11	−6.00 ± 0.16	−5.97 ± 0.12	1.0	0.5
Signal transduction				
YWHAG	**−1.44 ± 0.07**	**−1.50 ± 0.06**	**1.0**	**0.04**
SPAG9	**−2.61 ± 0.06**	**−2.72 ± 0.05**	**0.9**	**0.03**

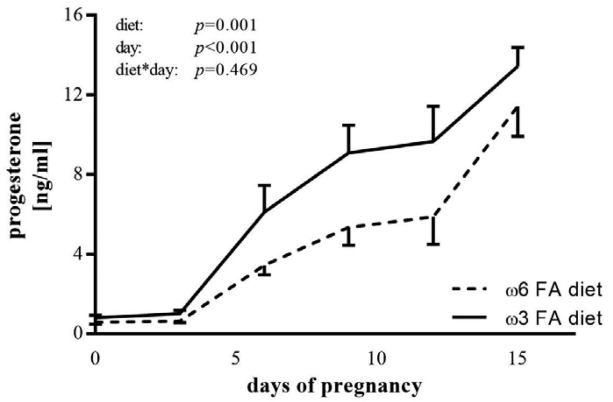

Figure 2. Plasma progesterone concentrations were higher in ω3 FA supplemented heifers than in ω6 FA supplemented heifers. Values are given as means ± SEM.

Table 4. Luteal relative gene expression (mean ± SEM) and mean fold-changes of pregnant heifers as measured via qPCR.

	ΔCt ω6 FA diet (n = 6)	ΔCt ω3 FA diet (n = 12)	Mean fold-change (ω3 vs ω6)	*P*-value
HSD3B1	2.80 ± 0.16	2.73 ± 0.13	0.95	0.8
STAR	2.43 ± 0.28	2.58 ± 0.14	1.11	0.6
PTGFR	0.57 ± 0.11	0.73 ± 0.13	1.12	0.4
AKR1C3	−4.13 ± 0.08	−4.07 ± 0.05	1.04	0.5
CASP8	−5.45 ± 0.10	−5.39 ± 0.08	1.04	0.7
CASP3	−11.88 ± 0.24	−11.97 ± 0.06	0.94	0.6
PPARG	−8.22 ± 0.28	−8.50 ± 0.11	0.82	0.3

Discussion

The present study is the first to report differences in embryo elongation in heifers supplemented with ω3 FA-rich fish oil and ω6 FA-rich sunflower oil, respectively. The main findings were that (1) day 15 embryos were significantly longer in ω3 compared to ω6 fed animals; (2) the concentration of AA was significantly lower, while the concentration of EPA was significantly higher and DHA was unaffected in the endometrium of ω3 compared to ω6 fed animals; (3) the concentrations of uterine prostaglandins did not differ between diet groups despite significantly different endometrial amounts of their precursor AA; and (4) increased plasma P4 concentration in ω3 compared to ω6 fed animals during luteal growth was associated with filamentous embryos present on day 15 of pregnancy.

There is no general agreement on how to categorize embryos according to their length, specifically at the beginning of elongation. The classification of embryos differs between published studies [63–67]. Bovine embryos are suggested to have an ovoid (day 12), a tubular (day 14), or a filamentous (day 16–18) shape, and the term "elongation" is generally applied to embryos with an ovoid to slightly tubular shape [63, 68]. The day 15 embryos of the present study showed a great variance in length from the ovoid to the filamentous forms. The embryos recovered from ω3 FA supplemented animals were not only significantly longer than those recovered from ω6 FA supplemented animals, but also the variance in embryo length was greater in the ω3 group than in the ω6 group. Staples et al. [69] reported bovine embryo lengths on day 15 of pregnancy ranging from 0.6 to 17 cm, whereas Betteridge et al. [70] found day 15 embryo lengths ranging between 0.3 and 4 cm. Following superovulation, an enormous range of variation in embryo length (e.g. 0.4–4 cm on day 14) was observed even within a single donor [70]. Therefore, it remains difficult to state with certainty whether ω3 FA promoted or ω6 FA delayed embryo elongation in comparison to the respective other supplementation, or both.

Dietary PUFA normally undergo partial biohydrogenation by ruminal microorganisms. Our FA supplements were coated with hydrogenated rapeseed oil for rumen protection. We chose heifers during their postpubertal growing phase to maximize the incorporation of dietary PUFA into the body lipid pools. The plasma and the endometrial FA profiles of our heifers confirm the successful rumen protection of ω3 FA. The higher EPA and DHA concentration in plasma of ω3 compared to ω6 supplemented animals is in accordance with the literature, where a stronger increase in EPA compared to DHA concentration as well as an increase in total ω3 PUFA lacking a difference in total ω6 PUFA has been shown [71, 72]. Increased plasma levels of supplemented ω3 FA led to an increased endometrial EPA concentration compared to the supplementation with ω6 FA. We observed an incorporation of dietary ω3 FA into endometrial tissue. This is in line with previously published studies [55, 71, 72], leading to a decreased ω6/ω3 ratio [55, 72, 73]. The latter has also been observed for luteal tissue [72].

During early embryonic development, PUFA play an important role by affecting membrane fluidity and permeability. They also serve as essential precursors for signaling molecules such as PG. Childs et al. [55] did not find any difference in plasma AA concentrations after 50 days of ω3 FA supplementation compared to an unsupplemented control group. This is in accordance with our findings, though we compared to a supplementation with ω6 FA. The supplementation with ω3 FA increased EPA almost threefold and decreased AA concentration in the UF of beef heifers compared to an ω6 supplementation, leading to significantly different ω6/ω3 ratios in the UF at day 7 of pregnancy [54]. Even though we did not analyze the FA in the UF, we have observed similar changes in EPA and AA in the endometrium. Consequently, we assume that our rumen-protected ω3 and ω6 FA supplementation has most likely resulted in a similar FA profile in the UF. Interestingly, Childs et al. [54] did not detect differences in DHA concentrations in the UF when comparing ω3 and ω6 supplemented animals. Accordingly, despite significant differences in DHA concentrations in plasma, we neither observed differing DHA concentrations in the endometrium of the two diet groups. Furthermore, the concentration of EPA was lower in endometrium compared to plasma, although endometrial AA and DHA concentrations were higher than those in plasma. Thus, we assume that AA and DHA have preferentially been incorporated into the endometrium. These results point toward a selective uptake of specific FA into the endometrium and the UF, respectively. We hypothesize that this regulatory mechanism might be due to specific needs of the developing embryo.

It has been hypothesized that ω3 FA may act synergistically with IFNT to inhibit endometrial production of $PGF_{2\alpha}$ and thus luteolysis [74]. In our study, we could not observe any diet-induced differences in endometrial gene expression of enzymes involved in PG synthesis. Moreover, with regard to the significantly decreased endometrial availability of AA in ω3 compared to ω6 FA supplemented animals, we hypothesized to find decreased series-2 PG in the UF. Surprisingly, this was not case. In several animal cell culture systems including bovine endometrial cells, incubation with ω3 FA decreased $PGF_{2\alpha}$ production and secretion [74–77]. In addition, the PG-reducing effect of ω3 FA in the bovine reproductive tract has repeatedly been reported, especially in cyclic animals [78]. However, this was assessed by determining circulating PGFM. Our data show that irrespective of decreased AA, the local intrauterine series-2 PG concentrations during preimplantation development remained unaffected. We doubt whether the AA shift induced by a maternal diet can be pronounced enough at all to impact on the intrauterine hormonal signaling of series-2 PG. Most studies that report an effect of PUFA supplementation on PG concentrations were performed with cyclic animals. Therefore, we cannot rule out an effect of dietary ω3 and ω6 PUFA on PG concentrations in nonpregnant animals.

Increasing plasma P4 concentrations might present a mechanism by which ω3 FA increase pregnancy rates in cows [79]. Intervention studies showed that higher P4 concentrations during the early stage of pregnancy were associated with a greater embryo elongation [18, 20–22, 80–82]. The importance of the P4 regulatory effect has been demonstrated in synchronous versus asynchronous embryo transfer [83, 84]. However, dietary ω3 and ω6 FA effects on plasma P4 concentrations led to contradictory results. In lactating Holstein cows, the supplementation of ω3 FA slightly increased mean P4 concentrations in some studies [85, 86], but lacked to show an effect in others [85, 87, 88]. Robinson et al. [89] observed lower plasma P4 concentrations when dairy cows were supplemented with FA. However, the effect was irrespective of a ω3 or ω6 FA supplementation. The incubation of bovine luteal cells with ω3 FA decreased P4 synthesis [90], whereas ω6 FA increased P4 in bovine CL cells [91].

Dietary ω3 FA are suggested to prevent the regression of the CL by decreased endometrial $PGF_{2\alpha}$ released into the circulation (indicated by reduced PGFM), which may result in a prolonged P4 secretion [13, 92]. Indeed, a fish meal supplementation to lactating animals resulted in P4 concentrations >1 ng/ml 2 days after $PGF_{2\alpha}$ injection. This led to the assumption of an inhibitory effect of ω3 FA on $PGF_{2\alpha}$-induced CL regression [13]. Dietary supplementation with flaxseed containing ALA led to higher P4 values on the day of AI in lactating Holstein cows compared to sunflower seed, also suggesting a delay in luteolysis [87]. In our experiment, we did not determine the timing of luteolysis and ovulation. If ω3 FA supplementation had caused a delay in luteolysis in our animals as described in the literature [13, 87], a delayed ovulation would be expected to result in less elongated embryos because of a relative younger embryonic age. However, we observed the opposite, thereby rather strengthening the hypothesis of promoted embryo elongation via ω3 FA supplementation. In our data, the heterogeneity in P4 was higher in the ω3 than in the ω6 group. Those heifers carrying an elongated embryo on day 15 had higher P4 from day 6 onwards.

The supplementation with dietary ω3 FA has been associated with reduced plasma cholesterol compared to dietary ω6 FA [89]. While our data indicate the lack of change in plasma cholesterol, we found higher plasma P4 concentrations in the ω3 FA supplemented animals compared to the ω6 FA supplemented animals during luteal growth. However, this appeared due to those animals in the ω3 group where a filamentous embryo was present. Clearly, the rise in P4 of these latter ones could have caused the enhanced embryo elongation. However, it is unclear why only some animals responded to the ω3 treatment with an increase in P4 while others of the same treatment group did not. A direct effect of the dietary ω3 FA on P4 synthesis and luteal function will thus need further substantiation.

We further analyzed whether the supplementation with ω3 compared to ω6 FA altered the response of the endometrium to P4. Specifically, gene expression changes via FA-sensitive transcription factors were of interest. In general, ω3 FA seem to be more effective in modifying gene expression than ω6 FA or SFA and increased EPA plasma levels are associated with increased *PPAR* gene expression [93, 94]. Interestingly, the endometrial gene expression of *PPAR*s did not differ in the present study. Waters et al. [49] demonstrated that feeding ω3 FA led to differential gene expression of a variety of genes in the endometrium. We could confirm only some of these reported gene expression changes in our study. However, the control group in Waters et al. [49] received palmitic acid and not ω6 FA as reference.

With the exception of alanine, neither the concentration of amino acids in the UF, the expression of amino acid transporters, the presence of endometrial α- and gamma-tocopherol, or uterine cortisol was affected by the diet. Surprisingly, even the gene expression of ISGs did not significantly differ between animals with embryos of different lengths. Because day 7 embryos already induce endometrial ISG15 ubiquitin-like modifier (*ISG15*; previously known as interferon, alpha-inducible protein (clone IFI-15K) (*G1P2*)), 2′-5′-oligoadenylate synthetase 1 (*OAS1*), and MX dynamin like GTPase 1 (*MX1*) gene expression [95], we assume that embryos may have induced the respective gene expression to a maximum already at an earlier stage of elongation. This would explain the lack of an observable difference. Taken together, endometrial gene expression differences in the present study in general were very scarce, even though the endometrial concentration of ω3 and ω6 FA was significantly altered. It remains to be analyzed to which extent gene expression and PG concentration differed before day 15 of pregnancy and may hereby had affected embryo elongation.

Despite a significant difference in embryo length between the ω3 and ω6 FA groups, we cannot identify with certainty a mechanism by which ω3 and ω6 FA differentially affected embryo elongation. Our study rules out (1) a direct effect of ω3 FA altering endometrial embryotropic series-2 PG as well as (2) a direct or indirect effect of ω3 FA altering uterine amino acids, α- and γ-tocopherol, or cortisol furthering embryo elongation. In the present study, we neither investigated possible effects of dietary FA on uterine series-3 PG nor FA effects on the ovaries and thus follicles and oocytes. The latter remains a specifically interesting target because dietary ω3 and ω6 FA have been shown to affect follicular development and oocyte competency [96–98]. Therefore, further research is needed to understand the functional link between dietary ω3 FA and reproductive outcome.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Table S1 Proximate (mean of pooled diet samples) and fatty acid composition of diets (n = 3) adapted from [57]. Values are given as means ± standard deviation.
Supplementary Table S2 Sequences of primers.

Supplementary Table S3 Amino acid concentrations in the uterine fluid. Values are given as means ± SEM.

Supplementary Table S4 Plasma, liver and endometrial concentrations of alpha- and gamma-tocopherol. Values are given as means ± SEM.

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