Supplementation with eicosapentaenoic and docosahexaenoic acids in late gestation in ewes changes adipose tissue gene expression in the ewe and growth and plasma concentration of ghrelin in the offspring[1]

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ABSTRACT: Omega-3 long chain fatty acids have a positive impact on production. When consumed during late gestation, it might have fetal programming effects on the fetus, which will have lifelong impacts on development and production. The objectives of the present study were to evaluate the effect of increasing doses of eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) in the diet of ewes in the last third of gestation on their body weight (BW), subcutaneous adipose tissue relative mRNA abundance of genes associated with adipose tissue metabolism, and growth performance and plasma metabolites and hormones of their offspring during the finishing phase. Ewes (n = 72) were blocked by BW and allotted to pens (8 per treatment) with 3 ewes per pen. Ewes were supplemented with an EPA and DHA source (Strata G113) at concentrations of 0, 1, or 2% of dry matter intake during the last 50 d of gestation. At lambing, all ewes were penned together and offered the same diet. After weaning at 60 d of age, lambs were blocked by BW and sex and fed for 56 d. All lambs were fed the same pellet diet (61.09% ground corn, 24.08% soy hulls, 11.09% soybean meal, 1.48% Ca salt of palm oil, and 2.26% mixed mineral vitamin), and were weighed every 14 d until the end of the trial. Blood samples were collected on the weight sampling days. Dry matter intake and refusals were weighed daily. Data were analyzed as a randomized complete block design with repeated measurements (SAS 9.4). Polynomial contrast (linear-L and quadratic-Q) was used for mean separation. There were no differences in ewe body condition score, milk production, milk fat, or milk protein, but there was a trend for increased (L, P = 0.06) lactose concentration, and also differences in *DGAT1* (L, P = 0.04), *Δ^5-desaturase* (Q, P = 0.06) and *Δ^6-desaturase* (Q, P = 0.07), *PPARα* (Q, P = 0.03), *ELOVL2* and *5* (Q, P < 0.07), *FABP4* (Q, P = 0.04), *FATP1* (Q, P = 0.06), *leptin* (Q, P = 0.02), and *resistin* (L, P = 0.05). Feeding pregnant ewes an increased amount of EPA and DHA in late gestation increased final BW (L, P = 0.01), ADG (L, P = 0.04; Q, P = 0.01), DMI (Q, $P \leq 0.01$), plasma glucose concentration (L, P = 0.04), and trended to decrease ghrelin concentrations (L, P = 0.07) in offspring during the finishing period. Dam supplementation did not affect G:F, nor plasma NEFA concentration ($P \geq 0.53$) of lambs. Therefore, increasing supplementation of EPA and DHA in pregnant ewes has an impact on offspring performance, increasing DMI, ADG, and BW.

Key words: adipose tissue, fatty acids, insulin resistance, obesity, sheep

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INTRODUCTION

Studies in livestock have shown that polyunsaturated fatty acids (PUFA) are effective in improving performance (Lopes et al., 2009). The beneficial effects observed when supplementing with PUFAs may be due to the ability of PUFAs to regulate gene expression of enzymes associated with lipid metabolism, such as upregulation of lipolytic genes and downregulation of lipogenic genes (Clark, 2001). Pregnant ewe growth and plasma metabolites do not change in ewes fed eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) when compared with ewes fed saturated (SFA) and monosaturated fatty acid (MUFA) (Coleman et al., 2018b). Also, the subcutaneous (SC) adipose tissue mRNA relative abundance of lipolytic enzymes were not affected the type of fatty acid in the diet, and only the enzyme *fatty acid synthase* (*FAS*) mRNA relative abundance increased in the EPA- and DHA-enriched diet compared with the SFA and MUFA diet (Coleman et al., 2018c). A possible explanation for the lack of differences in ewe growth might be the low dose of EPA and DHA used in the experiment (0.39% DM; Coleman et al., 2018c). Additionally, with this model, it is not clear whether the change in mRNA abundance of *FAS* in SC adipose tissue was an increase due to EPA and DHA, or a decrease due to SFA and MUFA in the diet.

Fatty acid (FA) supplementation during late gestation also changes the growth rate of offspring after weaning (Marques et al., 2017; Carranza Martin et al., 2018). In both studies, the treatments were a diet supplemented with a source of PUFA compared with a diet supplemented with a source of SFA and MUFA (Marques et al., 2017; Carranza Martin et al., 2018). Therefore, it is unknown if the difference of growth is because a positive effect of the EPA and DHA supplementation, or a negative effect of the SFA and MUFA supplementation. The mechanism that regulates offspring growth due to maternal FA supplementation is not known. The difference in growth observed previously (Carranza Martin et al., 2018) was not associated with changes in dry matter intake (DMI), despite a maternal by offspring diet interaction in the expression of *cocaine and amphetamine regulated protein* in the hypothalamus. However, the physiological mechanism that produced that change has not been evaluated.

Based on the cited literature, we hypothesized that increased supplementation of EPA and DHA would affect mRNA relative abundance of lipolytic and lipogenic genes on the SC adipose tissue of pregnant ewes without changes in body weight (BW). Also, we hypothesized that an increased dose of EPA and DHA during late gestation would increase growth and change plasma concentrations of metabolites and hormones in the offspring. The objectives of the present study are to evaluate the effect of increasing doses of EPA and DHA in the diet of ewes in the last third of gestation on their BW, mRNA relative abundance of genes associated with adipose tissue metabolism, and growth performance and plasma metabolites and hormones of their offspring during the finishing phase.

MATERIALS AND METHODS

Experimental Design and Sampling

This study was conducted at the Sheep Center of the Ohio Agricultural Research and Development Center, Wooster, OH (IACUC #2016A00000013). Seventy-two pregnant ewes (initial BW = 92.2 ± 2.94 kg, day 100 of gestation) were blocked, body condition scored, and randomly assigned to the different treatments. Ewes were divided into three groups (n = 24, 8 pens per group) which received a diet containing either 0, 1, or 2% of a Ca salt of fatty acids containing EPA and DHA (EPA+DHA, Strata G113, Virtus Nutrition LLC, Corcoran, CA). These doses were selected based on previous results (Coleman et al., 2018b) in which a lower dose (0.39% DMI) did not change offspring performance at weaning, but it increased offspring BW during the finishing period (Carranza Martin et al., 2018). Ewes received 2.02 kg/d of a mixed diet containing the different treatments (Table 1). The intake was fixed to meet the NRC (2007) requirements for ewes in late gestation. Ewes began receiving the treatment 50 d prior to the expected lambing day (day 50). At day 20 of the expecting lambing day (30 d of supplementation began), an SC adipose tissue sample was taken on 1 ewe per pen, as described by Coleman et al. (2018c). At lambing, the diet was discontinued and all ewes and lambs were moved to a common pen, where they received the same diet.

In previous experiments studying the effects of FA supplementation in late gestation (Garcia et al., 2014a; Marques et al., 2017; Carranza Martin et al., 2018; Coleman et al., 2018a, 2018b, 2018c) or late gestation and early lactation (Palmquist et al., 1977; Reynolds et al., 2006; Capper et al., 2007), the diets contained similar amounts of lipids, but with different profiles of FA. An explanation for the use of different lipids in the diets rather than a dose increase in lipid concentration might be due to the increase

Table 1. Diet ingredients and composition (% of DM basis) of diets containing 0, 1, or 2% of supplemented enriched sources of EPA and DHA, fed to pregnant ewes at 2 kg/d during the last 50 d of gestation

	% of DM basis		
	0%	1%	2%
Ingredients			
Corn silage	30.54	30.54	30.54
Alfalfa haylage	17.96	17.96	17.96
Ground corn	10.10	8.89	7.97
Soy hulls	30.65	30.88	30.81
Limestone	0.44	0.48	0.48
DDGS[1]	10.10	10.07	10.04
Mineral supplement[2]	0.20	0.18	0.18
Fatty acid supplement[3]	–	1.01	2.03
Composition			
Crude protein	15.48	14.41	14.47
NDF	39.21	41.27	41.63
EE	2.21	2.96	3.63
Ash	5.18	5.38	5.68

[1]DDGS = distiller's dried grains with solubles.

[2]Vitaferm Concept-Aid Sheep (BioZyme, St. Joseph, MO). Contains 15.5% Ca, 5% P, 16% NaCl, 4% Mg, 2% K, 10 ppm Co, 70 ppm I, 2,850 ppm Mn, 16.4 ppm Se, 2,500 ppm Zn, 130,000 IU/kg vitamin A, 7,500 IU/kg vitamin D_3, 550 IU/kg vitamin E.

[3]Strata G113, Virtus Nutrition LLC, Corcoran, CA.

of FA in the diet producing a confounding effect on energy intake. In the current study, ewes supplemented with 1 or 2% of EPA+DHA had more energy dense diets (NE_m, 0.54 or 1.07%, respectively) than the ewes supplemented with 0% EPA+DHA (FEDNA, 2010). Diets were not isoenergetic because to achieve that we would have to add a different feed ingredient such as corn, dried distillers grains, or fiber. Radunz et al. (2011) showed that offspring growth and metabolism can be changed by feeding their dams with isoenergetic diets, but with different feedstuff. Therefore, the addition of a new feedstuff to make the diet isoenergetic in the current experiment could confound the effects of the FA supplementation vs. effects elicited by another feedstuff. Despite the diets not being isoenergetic, an increase in NE_m intake of 0.54% per treatment is likely not enough to cause the changes observed in the present study.

Within 12 h following lambing, the lambs were weighed, and blood samples were collected (10 mL) from the jugular vein. At day 15 and 60 (weaning day) after lambing, lambs were weighed and blood samples were collected. The weights were used to calculate average daily gain (ADG). On day 15, milk production of the ewes was recorded by milking manually as described by Palmquist et al. (1977). A sample of milk was treated with bronopol and natamycin (Advanced Instruments, Norwood, MA) as preservatives and held at 4 °C until analysis. A sample of milk from each ewe was analyzed for milk fat, protein, lactose, somatic cell count (**SCC**), other solids (B200 Infared Analyzer, Bentley Instruments, Chaska, MN), and milk urea nitrogen (**MUN**; Skalar SAN Plus segmented flow analyzer, Skalar Inc., Norcross, GA) by DHI Cooperative Inc. (Columbus, OH). Milk net energy of lactation was calculated using the gross energy of the milk composition as described in the equation for dairy cattle (NRC, 2001).

Table 2. Diet formulation and composition (% of DM basis) fed to lambs born from pregnant ewes supplemented during the last 50 d of gestation with 0, 1, or 2% of Ca salts containing EPA and DHA

	% of DM basis
Ingredients	
Ground corn	61.09
Soy hulls	24.08
Soybean meal	11.09
Ca salts of fatty acids[1]	1.48
Mineral and vitamin mixed[2]	2.26
Composition	
Crude protein	15.6
NDF	16.24
EE	3.45
Ash	4.78

[1]EnerGII, Virtus Nutrition LLC, Corcoran, CA.

[2]Mineral and vitamin mixed contain (DM basis): 19.35% urea, 38.76% of limestone, 19.36% of sodium chloride, 0.4% of vitamin A, 0.4% of vitamin D_3, 1.96% of vitamin E, 3.76% selenium, 0.49% Bovatec 91, and 15.52% of ammonium chloride.

After weaning, lambs (n = 72) were blocked by sex and BW and randomly allocated to a pen. All lambs were fed the same diet, which contained 61.09% ground corn, 24.08% soy hulls, 11.09% soybean meal, 1.48% Ca salt of palm oil (Ener GII, Virtus Nutrition), and 2.26% mixed mineral vitamin (Table 2). Dry matter intake and refusal were measured daily, and BW and blood samples were conducted on day 1, 14, 28, 42, and 56. All blood samples were collected in polypropylene tubes containing solutions of disodium EDTA and benzamidine HCL (1.6 mg and 4.7 mg/mL of blood, respectively); and placed on ice. After centrifugation for 25 min at 1,800 × g and 4 °C, plasma was collected, aliquoted, and stored in individual polypropylene tubes at −80 °C until further analysis.

Sample Analysis

Subcutaneous adipose tissue samples were used to measure mRNA relative abundance of genes

involved in lipolysis and lipogenesis (Table 3). The method used for this analysis was the NanoString technology as described previously (Coleman et al., 2018c).

Plasma obtained from the lambs preweaning at day 0, 15, and 60; and postweaning at day 61, 74, 88, 102, and 116 were used to measure plasma glucose (Relling et al., 2010) and nonesterified fatty acid (NEFA) concentration (Johnson and Peters, 1993). Plasma samples from the lambs at day 56 postweaning were used to measure concentrations of ghrelin and glucose-dependent insulinotropic polypeptide (**GIP**) as described previously (Relling et al., 2010). Postweaning plasma samples were collected 30 min before the normal feeding time. Postweaning plasma samples were collected 30 min before the normal feeding time to account for differences in plasma ghrelin concentrations that are observed preprandially, but not postprandially (Relling et al., 2010). Plasma insulin concentration was measured using a bovine assay (Mercodia Ab, Uppsala, Sweden). We validated the insulin assay for use in sheep based on parallel displacement using serial additions of ovine plasma samples compared to the bovine insulin standard curves. The percent recovery was 98% ± 3%. All samples were run as duplicates, and ghrelin and GIP concentration were measured on the same day (assay). The interassay variation (CV%) for ghrelin, GIP, and insulin were 8.32, 7.16, and 6.85%, respectively. The minimum sensitivity for ghrelin and GIP were 0.0015 and 0.00022 pmol per tube, respectively.

Table 3. Gene names and GenBank accession numbers

Gene name[1]	Accession number
LPL	NM_001009394.1
ATGL	NM_001308576.1
HSL	NM_001128154.1
DGAT1	NM_001110164.1
DGAT2	XM_012096078.2
SCD	NM_001009254.1
Δ^5-desaturase	XM_012101996.2
Δ^6-desaturase	XM_015103138.1
ELOVL2	XM_012101293.2
ELOVL4	XM_015097304.1
ELOVL5	XM_012100862.2
FABP4	NM_001114667.1
FAS	XM_015098375.1
FATP1	XM_015095580.1
GIP Receptor	XM_015100601.1
Ghrelin Receptor	NM_001009760.1
Insulin Receptor	XM_004008549.3
PPAR alpha	XM_012175774.2
PPAR betaldelta	XM_004018768.3
PPAR gamma	NM_001100921.1
RXR alpha	XM_012117960.2
Adiponectin	NM_001308565.1
Leptin	XM_004008038.3
Resistin	NM_001306111.1
Cox-2	NM_001009432.1
5-lox	XM_015104505.1
Beta-actin	NM_001009784.1
Beta-2 microglobulin	NM_001009284.2
Ciclophilin A	NM_001308578.1
GAPDH	NM_001190390.1
PGK1	NM_001142516.1

[1]LPL = lipoprotein lipase; ATGL = adipose triglyceride lipase; HSL = hormone sensitive lipase; DGAT1 = diacylglycerol acyltransferase 1; DGAT2 = diacylglycerol acyltransferase 2; SCD = stearoyl-CoA desaturase; ELOVL2 = elongation of very long chain fatty acid 2; ELOVL4 = elongation of very long chain fatty acid 4; ELOVL5 = elongation of very long chain fatty acid 5; FABP4 = fatty acid binding protein 4; FAS = fatty acid synthase; FATP1 = fatty acid transport protein 1; GIP = glucose-dependent insulinotropic polypeptide; RXR = retinoid X receptor; Cox-2 = cyclooxygenase 2; 5-lox = 5-lipoxygenase; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; PGK1 = phosphoglycerate kinase.

Statistical Analyses

Ewe and lamb performance, and plasma glucose and NEFA concentration data were analyzed as a randomized complete block design with a repeated measurement procedure (SAS 9.4). The model considered the fixed effects of time, treatment and their interaction, and the random effect of ewes or lambs within the pens. For mRNA relative abundance and milk production and composition, plasma insulin, ghrelin, and GIP concentration, a similar model was used without time and its interactions. Number of lambs born from each ewe and the BW of the ewe at the time of the biopsy were used as a covariables for adipose tissue mRNA relative abundance. The effect of treatment on number of lambs born, lambs weaned, and sex distribution was evaluated using a similar model, but without repeated measurements. In the present study, no treatment effect was observed for those variables ($P > 0.10$). For the performance data of lambs preweaning, the model also included sex of the lambs and number of lambs at lambing (single, twin, or triplets), the actual time of sampling related to birth day, and the ewe BW as covariables, which were removed from the model if they were not significant ($P > 0.10$). In regard to post-weaning lamb data, the effect of the interaction of sex with diet and sex diet and time was analyzed to evaluate possible sexually dimorphic results for some variables. However, there was no sex by diet, sex by day, or sex by day by diet interactions. Therefore, the final model included the random effect of sex. If there

was no time by treatment interaction and for the samplings without multiple samplings, linear and quadratic polynomial contrasts were used for mean separation. Pearson correlations between day 48 lamb plasma glucose, NEFA, insulin, ghrelin, and GIP concentrations were estimated with Proc Corr of SAS (SAS/STAT ver. 9.4, SAS Institute Inc., Cary, NC).

RESULTS

Ewe Performance, and SC Adipose Tissue mRNA Relative Abundance

There was no time by treatment effect ($P > 0.20$) on ewe BW or body condition score from parturition to weaning due to different doses of EPA+DHA supplementation. Ewes supplemented with 1% of EPA+DHA trended to be heavier than the ewes with 0 or 2% EPA+DHA (quadratic effect: $P = 0.06$; Table 4). The difference in BW was due to a random allocation to the ewes to the treatment; BW was different on the first day of sampling and remained with that difference throughout the experiment. Increasing the dose of EPA+DHA during the last 50 d of gestation did not elicit differences in body condition score ($P > 0.21$; Table 4).

After 30 d of supplementation with the different treatments, there were no differences ($P > 0.12$) in SC adipose tissue mRNA relative abundance of *lipoprotein lipase* (*LPL*), *adipose triglyceride lipase* (*ATGL*), *hormone sensitive lipase* (*HSL*), *diacylglycerol acyltransferase 2* (*DGAT2*), *elongation of very long chain fatty acid* (*ELOVL*) 4, *fatty acid synthase* (*FAS*), *peroxisome proliferator-activated receptors* (*PPAR*) *β/δ*, *PPARγ*, *retinoid X receptor α* (*RXRα*), *adiponectin*, *5-lipoxygenase* (*5-LOX*), or *cyclooxygenase* (*COX*) *2* (Table 5) due to increasing amount of EPA+DHA supplementation. The relative abundance of mRNA for *diacylglycerol acyltransferase 1* (*DGAT1*) was greatest for the ewes supplemented with 0% EPA+DHA compared with the 1 or 2% EPA+DHA, while ewes supplemented with 1 or 2% EPA+DHA had similar concentrations (linear: $P = 0.04$; quadratic: $P = 0.09$; Table 5). There was a quadratic effect ($P \leq 0.05$) for *fatty acid binding protein 4* (*FABP4*), *PPARα*, *ELOVL2*, and *leptin*; and a quadratic tendency ($P \leq 0.09$) for *Δ^5-desaturase*. In all of these genes, the relative abundance of mRNA was greater for the ewes supplemented with 1% EPA+DHA, compared with ewes supplemented with 0 or 2% EPA+DHA (Table 5). There was a quadratic tendency ($P \leq 0.07$) for *Δ^5-desaturase* and *Δ^6-desaturase*, *fatty acid transport protein 1* (*FATP1*), and *ELOVL5* mRNA relative abundance in the adipose tissue to be least in the 1% EPA+DHA treatment when compared to ewes supplemented with 0 or 2% EPA+DHA (Table 5). There was a linear decrease ($P = 0.05$) in resistin mRNA relative abundance due to the increased supplementation of EPA+DHA.

When evaluating milk yield and composition on day 15 post-lambing, there were no differences ($P \geq 0.20$) on milk yield, fat (%), protein (%), MUN (%), or milk energy (Mcal/kg). There was, however, a linear increase in the lactose and total solid concentration due to increased EPA+DHA supplementation during the last 50 d of gestation (Table 6).

Lamb Performance, Plasma Hormones, and Metabolites Concentration

In the lambs from birth to weaning, there were no differences ($P \geq 0.17$) for initial or final BW, plasma glucose, or NEFA concentration (Table 7). There was, however, a time by treatment interaction ($P < 0.01$) for ADG. Lambs born from ewes supplemented with 1% EPA+DHA had a greater ADG during the first 15 d of life ($P < 0.05$) compared with the 0 and 2% EPA+DHA. From day 15 to 60 (weaning), the ADG was similar between the 3 treatments.

Once the lambs were weaned and placed on finishing diets, there was no treatment by time effect ($P \geq 0.44$) on BW, ADG, dry matter intake (DMI), or kg of gain/kg of DMI (G:F) due to maternal supplementation with increasing doses of EPA+DHA during the last 50 d of gestation (Table 8). Body weight and ADG increased (linear: $P < 0.05$; quadratic: $P < 0.08$; Table 8) due to increased EPA+DHA

Table 4. Effect of 0, 1, or 2% EPA- and DHA-enriched diet supplementation during the last 50 d of gestation on average body weight (BW) and body condition score (BCS) from 50 d before lambing until weaning in ewes

	Treatment[1]				*P*-value[2]	
Item	0%	1%	2%	SEM	Linear	Quadratic
Pens	8	8	8			
Ewes	24	24	24			
BW, kg	94.8	89.8	91.0	3.99	0.25	0.06
BCS	3.2	3.3	3.3	0.14	0.77	0.70

Diets containing 1 or 2% of EPA+DHA are more energy dense diets (NE_m, 0.54 or 1.07%, respectively) than the diet 0% EPA+DHA.

[1]Treatments on ewes supplemented with 0, 1, or 2% EPA- and DHA-enriched diet (Strata G113, Virtus Nutrition) during the last 50 d of gestation; body condition score measured on a scale of 1–5.

[2]L = linear effect of treatment; Q = quadratic effect of treatment.

Table 5. Effect of 30 d supplementation with 0, 1, or 2% EPA- and DHA-enriched diet on concentration of mRNA from the subcutaneous adipose tissue of ewes in late gestation

	Treatments[2]				*P*-value[3]	
Item[1]	0%	1%	2%	SEM	Linear	Quadratic
Pens (ewes)	8	8	8			
LPL	4,386.4	5,228.0	2,117.9	1,248.89	0.23	0.20
ATGL	1,501	1,446	1,211	235.1	0.24	0.67
HSL	5,041	4,643	3,830	704	0.13	0.76
DGAT1	1,162.27	609.85	695.46	194.35	0.04	0.09
DGAT2	4,335.3	6,850.8	4,868.0	1,556.58	0.81	0.25
SCD	9,795	14,409	13,150	3,583.1	0.50	0.46
Δ⁵-desaturase	158.25	119.71	187.68	37.45	0.35	0.06
Δ⁶-desaturase	85.29	51.45	99.52	20.49	0.56	0.07
ELOVL2	19.39	23.88	20.19	1.76	0.73	0.05
ELOVL4	42.07	46.72	98.86	34.95	0.26	0.58
ELOVL5	823.9	691.1	887.8	81.5	0.57	0.06
FABP4	137,835	171,332	126,268	15,610	0.59	0.05
FAS	11,822	11,648	11,361	3,410	0.91	0.98
FATP1	80.25	44.04	55.61	13.85	0.09	0.06
PPARα	177.4	148.5	187.9	16.5	0.55	0.02
PPARβ/δ	115.8	92.9	94.0	17.71	0.22	0.42
PPARγ	2,106.74	2,283.71	2,126.17	248.12	0.95	0.52
RXRα	270.94	199.83	282.07	44.88	0.84	0.12
Adiponectin	31,656	43,742	37,086	7,014	0.61	0.32
Leptin	138.0	219.2	75.42	36.4	0.25	0.02
Resistin	17.328	7.274	6.849	3.450	0.05	0.26
5-LOX	74.99	40.26	59.04	13.91	0.44	0.13
COX-2	8.84	9.66	10.42	1.41	0.44	0.98

Diets containing 1 or 2% of EPA+DHA are more energy dense diets (NE_m, 0.54 or 1.07%, respectively) than the diet 0% EPA+DHA.

[1]Gene expression is a relative value estimated using the mean of beta-actin, beta-2 microglobulin, ciclophilin A, GAPDH, and PGK1. LPL = lipoprotein lipase; ATGL = adipose triglyceride lipase; HSL = hormone sensitive lipase; DGAT1 = diacylglycerol acyltransferase 1; DGAT2 = diacylglycerol acyltransferase 2; SCD = stearoyl-CoA desaturase; ELOVL2 = elongation of very long chain fatty acid 2; ELVL4 = elongation of very long chain fatty acid 4; ELOVL5 = elongation of very long chain fatty acid 5; FABP4 = fatty acid binding protein 4; FAS = fatty acid synthase; FATP1 = fatty acid transport protein 1; RXR = retinoid X receptor; COX-2 = cyclooxygenase 2; 5-LOX = 5-lipoxygenase.

[2]Treatments on ewes supplemented with 0, 1, or 2% EPA- and DHA-enriched diet (Strata G133, Virtus Nutrition LLC, Corcoran, CA).

[3]L = linear effect of treatment; Q = quadratic effect of treatment.

Table 6. Effect of 0, 1, or 2% EPA- and DHA-enriched diet supplementation during the last 50 d of gestation on milk production and milk composition from a 3 hour period on day 15 post-lambing in ewes

	Treatment[1]				*P*-value[2]	
Item	0%	1%	2%	SEM	Linear	Quadratic
Pens	8	8	8			
Ewes	24	24	24			
Milk yield, mL in 3 h	294.9	299.1	307.8	24.93	0.75	0.94
Fat, %	8.1	7.8	7.8	0.38	0.66	0.74
Protein, %	4.1	3.9	4.1	0.10	0.88	0.20
Lactose, %	4.87	5.02	5.05	0.047	0.01	0.27
Total solids, %	5.776	5.96	6.00	0.059	0.02	0.29
MUN, %	14.75	13.75	13.32	1.341	0.51	0.87
Milk energy, Mcal/kg[3]	4.862	4.756	4.803	0.151	0.79	0.66
Milk energy, Mcal in 3 h[3]	0.343	0.340	0.353	0.035	0.85	0.86

Diets containing 1 or 2% of EPA+DHA are more energy dense diets (NE_m, 0.54 or 1.07%, respectively) than the diet 0% EPA+DHA.

[1]Treatments on ewes supplemented with 0, 1, or 2% EPA- and DHA-enriched diet during the last 50 d of gestation.

[2]L = linear effect of treatment; Q = quadratic effect of treatment.

[3]Milk net energy of lactation was calculated using the gross energy of the milk composition as described on the equation for dairy cattle (NRC, 2001).

Table 7. Effect of 0, 1, or 2% EPA- and DHA-enriched diet supplementation during the last 50 d of gestation in pregnant ewes on offspring lambs body weight (BW), average daily gain (ADG), and plasma glucose and NEFA concentrations

	Treatment[1]				*P*-value			
Items	0%	1%	2%	SEM	L	Q	Time	Time × treatment
Pens	8	8	8					
Lambs	38	37	35					
Lambing BW, kg	6.13	5.85	6.16	0.52	0.99	0.75	0.95	0.88
Weaning BW, kg	27.41	27.92	27.67					
ADG, day 15, kg/d	0.31	0.36	0.33	0.109	0.52	0.05	0.10	<0.01
ADG, day 60, kg/d	0.25	0.25	0.25					
Glucose	98.55	98.41	102.44	3.00	0.33	0.59	0.56	0.17
NEFA, µEq/L	581.0	514.4	566.0	52.78	0.89	0.37	0.63	0.86

Diets containing 1 or 2% of EPA+DHA are more energy dense diets (NE_m, 0.54 or 1.07%, respectively) than the diet 0% EPA+DHA.

[1]Treatments on ewes supplemented with 0, 1, or 2% EPA- and DHA-enriched diet during the last 50 d of gestation; Average daily gain on the lambs from day 0–15 and day 15–60.

Table 8. Effect of 0, 1, or 2% EPA- and DHA-enriched diet supplementation during the last 50 d of gestation in pregnant ewes on final body weight (BW), average daily gain (ADG), dry matter intake (DMI), and gain to feed ratio (G:F) during the finishing period (after weaning) in their offspring lambs

	Treatment[1]				*P*-value[2]	
Items	0%	1%	2%	SEM	L	Q
Pens	8	8	8			
Lambs	24	24	24			
Final BW, kg	49.2	49.6	52.6	1.03	<0.01	0.08
ADG, kg	0.42	0.41	0.46	0.02	0.04	0.01
DMI, kg	1.27	1.13	1.27	0.14	0.94	<0.01
G:F	0.328	0.343	0.344	0.07	0.56	0.53

Dam diets containing 1 or 2% of EPA+DHA are more energy dense diets (NE_m, 0.54 or 1.07%, respectively) than the diet 0% EPA+DHA.

[1]Treatments on ewes supplemented with 0, 1, or 2% EPA- and DHA-enriched diet during the last 50 d of gestation;

[2]L = linear effect of treatment; Q = quadratic effect of treatment.

supplementation in the dams' diet. There was a quadratic effect ($P < 0.01$) for DMI; lambs born from ewes supplemented with 0 or 2% EPA+DHA had a greater DMI than the lambs born from ewes supplemented with 1% EPA+DHA (Table 8).

There was a linear increase ($P = 0.04$) in plasma glucose concentration and a tendency for a linear decrease ($P = 0.07$) in plasma ghrelin concentration during the finishing period due to increased maternal EPA+DHA supplementation during the last 50 d of gestation (Table 9). Fatty acid supplementation to pregnant ewes did not change ($P \geq 0.17$) plasma concentrations of NEFA, insulin, or GIP in the offspring during the finishing period (Table 9).

Using Pearson correlation, plasma ghrelin concentration tended to be negatively associated with plasma insulin concentration ($r = -0.22$; $P = 0.07$; Table 10) and positively associated with plasma NEFA concentration ($r = 0.46$; $P < 0.01$; Table 10). Plasma insulin concentration was not correlated with plasma GIP concentration, but was positively correlated with plasma glucose concentration ($r = 0.32$; $P < 0.01$; Table 10), and negatively correlated with plasma NEFA concentration ($r = -0.45$; $P < 0.01$; Table 10). Plasma GIP concentration was not correlated ($P > 0.17$; Table 10) with plasma glucose or NEFA concentrations; and plasma glucose and NEFA concentrations were not correlated ($P = 0.12$; Table 10).

DISCUSSION

Ewe Performance, and SC Adipose Tissue mRNA Relative Abundance

Despite the diets not being isoenergetic, the increase in lipid concentration in the diet did not have an effect over time on BW or body condition score. As described previously, the tendency for a difference in BW was observed since day 1 of the experiment, and this difference was maintained throughout the entire 50-d supplementation period.

Table 9. Effect of 0, 1, or 2% EPA- and DHA-enriched diet supplementation during the last 50 d of gestation in pregnant ewes on plasma glucose, nonesterified fatty acids (NEFA), insulin, ghrelin, and glucose-independent insulinotropic polypeptide (GIP) concentrations during the finishing period (after weaning) in their offspring lambs

	Treatments[1]				*P*-value[2]	
Item	0%	1%	2%	SEM	L	Q
Pens	8	8	8			
Lambs	24	24	24			
Glucose, mg/dL	102.78	104.74	107.21	5.94	0.04	0.83
NEFA, µEq/L	210.63	212.22	218.87	59.51	0.70	0.80
Insulin, ng/mL	40.8	42.4	52.3	6.04	0.17	0.58
Ghrelin, pM	36.7	29.8	27.2	3.68	0.07	0.64
GIP, pM	71.2	73.5	74.2	3.7	0.58	0.86

Dam diets containing 1 or 2% of EPA+DHA are more energy dense diets (NE_m, 0.54 or 1.07%, respectively) than the diet 0% EPA+DHA.

[1]Treatments on ewes supplemented with 0, 1, or 2% EPA- and DHA-enriched diet during the last 50 d of gestation.

[2]L = linear effect of treatment; Q = quadratic effect of treatment.

Table 10. Pearson correlation of plasma hormone and metabolite concentrations of lambs feeding a high concentrate diet born from ewes supplemented with 0, 1, or 2% EPA- and DHA-enriched diets during the last 50 d of gestation

	Insulin	GIP[1]	Glucose	NEFA[1]
Lambs	69	62	71	71
Ghrelin	−0.22[a]	−0.09	−0.01	0.46[b]
Insulin		0.10	0.32[b]	−0.45[b]
GIP			−0.14	0.18
Glucose				−0.19

[1]GIP = glucose-dependent insulinotropic polypeptide; NEFA = nonesterified fatty acids.

[a]*P*-value for the correlation ≤ 0.10 and > 0.05.

[b]*P*-value for the correlation ≤ 0.01.

Garcia et al. (2014a) and Coleman et al. (2018b), feeding different amounts (up to 2%) of lipids during late gestation, did not report differences in dam BW. Nevertheless, when lipids were supplemented in late gestation and early lactation the results are inconsistent. Reynolds et al. (2006) did not report differences on dam BW when FA were supplemented in late gestation and early lactation; however, Capper et al. (2007) observed a decrease in BW after lambing when ewes were supplemented with Ca salts of palm oil instead of fish oil. The difference in results observed by Reynolds et al. (2006) and Capper et al. (2007) compared to the present study could be due to the doses used in the experiments. Reynolds et al. (2006) used 3 and 4.5% of the diet as FA, while Capper et al. (2007) used a diet with 6% of FA, which are greater than the doses used in the current experiment.

Based on previous literature (Clarke, 2001), we expected a dose response on the relative mRNA relative abundance for genes associated with lipid metabolism in the dam's SC adipose tissue. Particularly, an increase in the relative mRNA abundance of genes associated with FA oxidation and a decrease in the abundance of genes associated with FA synthesis. Coleman et al. (2018c) found differences in the mRNA abundance of *FAS* and *leptin* in SC adipose tissue after 30 d of supplementation with different sources of fatty acids. Also, it is stated in that study (Coleman et al., 2018c) that the lack of difference observed in other genes might be due to a low FA supplementation (0.39% of the diet in DM basis). In the present study, we supplemented with increasing doses of an EPA- and DHA-rich supplement at rates greater than Coleman et al. (2018c) to determine if the dose rate of FA supplementation is a factor behind how EPA and DHA may alter genes in SC adipose tissue.

In the current study, the relative mRNA abundance of the lipases, *ATGL* and *HSL*, and the lipogenic enzymes *LPL* and *FAS* did not change due to increased amounts of EPA and DHA in the diet. The relative mRNA abundance of the enzyme *DGAT1*, which is involved in triglyceride synthesis, decreased with the increased amount of EPA and DHA in the diet, which supports our hypothesis that increased concentrations of EPA and DHA would decrease mRNA relative abundance of genes associated with lipid synthesis. It is important to consider that despite the decrease in *DGAT1* mRNA abundance observed in the current experiment, no changes were observed in pregnant ewes after 30 d of supplementation with a lower dose (Coleman et al., 2018c) or in lambs supplemented for 42 d with 1.48% of Ca salts containing EPA and DHA (Coleman et al., 2018a). A difference between the

current experiment and the ones where no changes were observed due to EPA and DHA supplementation (Coleman et al., 2018a, 2018c) could be the control used in each experiment. In the current experiment, a dose increase was used in the design, but in the previous studies (Coleman et al., 2018a, 2018c) a Ca salt of palm fatty acid distillate was used as the control. Therefore, it is possible that the differences in relative mRNA abundance for *DGAT1* in the current experiment are not simply an effect of PUFA, but also an effect of lipid intake.

We expected that an increase of PUFA in the diet would decrease the mRNA abundance of desaturase enzymes (Nakamura and Nara, 2004). However, in the present study, we observed a quadratic increase for *Δ⁵-desaturase* and *Δ⁶-desaturase*. The supplementation with 2% of a Ca salt enriched with EPA and DHA had the greatest relative abundance of mRNA for the desaturases. However, the supplementation with 1% of Ca salts containing EPA and DHA decreased the abundance of the *Δ⁵-* and *Δ⁶-desaturases*. The expression of desaturases does not depend only on the FA profile of the diet, but on the plasma concentration of insulin and the induction of PPAR as well (Nakamura and Nara, 2002). In the current study, we did not measure the plasma concentration of insulin in ewe plasma; however, Coleman et al. (2018b) with a similar animal model, but lower dose of EPA and DHA, did not observe differences in plasma insulin concentration. However, in the present study, there was a quadratic response in the mRNA abundance of *PPARα* in response to PUFA supplementation. Therefore, and based on the association of the mRNA relative abundance in the current study, PPARα might have played a role in regulating abundance of *Δ⁵-desaturase*, and *Δ⁶-desaturase* (Nakamura and Nara, 2002; Kamal et al., 2018); however, evaluating the function of PPARα as a key regulator of gene expression was outside the scope of this paper. Previous studies have shown that lipid supplementation in beef (Waters et al., 2009) or sheep (Coleman et al., 2018a, 2018c) did not change mRNA relative abundance of *PPARα*. In the current experiment, the relative abundance of *PPARα* mRNA was greater for the ewes supplemented with 0 and 2% of Ca salts containing EPA and DHA compared with the ewes supplemented with 1%. The results reported by Waters et al. (2009) are based on a dose response to fish oil in muscle tissue of finished cattle; thus, the differences in tissue function and metabolism and animal model might explain the different results compared to the present study. The results from Coleman et al. (2018a, 2018c) are with a lesser concentration of EPA and DHA in the diet and comparing different sources of FA, which might also explain the different results. We do not have a physiological explanation for this finding, but it is worth mentioning that the mRNA relative abundance of *PPARα* has a similar pattern to the concentration of *Δ⁵-desaturase* and *Δ⁶-desaturase*.

Based on our hypothesis, we were expecting a decrease in *leptin* mRNA abundance, associated with an increase in fatty acid oxidation and decrease in lipid synthesis. *Leptin* mRNA abundance had a quadratic response, with a greater relative abundance in ewes supplemented with 1% of Ca salts with EPA and DHA, compared with ewes supplemented with 0 and 2% of Ca salts with EPA and DHA. Leptin is associated with adiposity (Delavaud et al., 2000), and though we did not evaluate adiposity, body condition score was similar between treatments. Rats fed a diet with 8.9% DHA had no difference in plasma leptin concentration, but they had a lower *leptin* mRNA concentration than the control group fed lard (Reseland et al., 2001). When different sources of Ca salts of FA (0.39% of the diet) were fed to pregnant ewes, the supplementation with EPA and DHA increased *leptin* mRNA relative abundance in the SC adipose tissue (Coleman et al., 2018c). The differences observed in results comparing previous studies (Reseland et al., 2001; Coleman et al., 2018c) and the quadratic response observed in this experiment may be due to the interaction of the amounts and types of FA in each experiment (Yang et al., 2016), and the interaction with stage of gestation (Szczesna et al., 2018). The mechanism of how different FA at different concentrations regulate *leptin* mRNA relative abundance remains unclear.

Subcutaneous adipose tissue *resistin* mRNA relative abundance linearly decreased with increased PUFA in the diet. Resistin has previously been associated with plasma NEFA concentration in dairy cows (Reverchon et al., 2014). Coleman et al. (2018c) did not find differences in SC adipose tissue *resistin* mRNA relative abundance or in plasma NEFA concentration when pregnant ewes were supplemented with different sources of FA. In the current experiment, we did not measure plasma NEFA concentration in ewes; however, Haugen et al. (2005) showed that an increase in arachidonic acid and EPA in culture media increased the relative abundance of *resistin* in adipose tissue in vitro. Therefore, it is possible that the decrease in *resistin* mRNA relative abundance in the present study may

be associated with an increase in plasma PUFA concentration.

The enzymes ELOVL2 and 5 elongate PUFA to very long PUFA (Zhang et al., 2016). In the present study, the mRNA relative abundance for both enzymes have opposite responses due to EPA+DHA supplementation with *ELOVL2* abundance being greatest with 1% EPA+DHA supplementation, while ELOVL5 had the lowest relative mRNA abundance with 1% EPA+DHA. Lambs supplemented with PUFA have decreased ELOVL2 mRNA relative abundance (Coleman et al., 2018a). However, growing goats supplemented with heated linseed oil have a greater concentration of ELOVL5 in the adipose tissue, which was associated with an increased concentration of n-3 FA in the adipose tissue (Wang et al., 2019). While dietary supplementation plays a role in regulating ELOVLs function (Zhang et al., 2016), we do not have a physiological explanation for the reason of opposite response of ELOVL2 and 5 to increased DHA and EPA in the diet of pregnant ewes.

Previous studies in sheep have observed no changes in milk yield and composition with supplementation of various sources of PUFA during gestation (Coleman et al., 2018b) and lactation (Kitessa et al., 2003; Capper et al., 2007; Gallardo et al., 2014). In the present study, milk yield, fat percent, protein percent, and MUN did not change. There was a linear increase in lactose concentration, which increased the concentration of total solids. One of the limiting steps for lactose synthesis is the activity of the enzyme lactose synthase. Lactose synthase is formed by 2 proteins: α-lactalbumin and galactosyltransferase, with galactosyltransferase being the limiting step in lactose synthesis (Kuhn et al., 1980). An increased supply of EPA and DHA during gestation into the mammary gland might have changed mammary gland gene expression of lactose synthase. However, this experiment was not designed to evaluate changes in mRNA relative abundance in the mammary gland.

Lamb Performance, Plasma Hormones, and Metabolites Concentration

Lamb BW did not change at lambing or weaning due to treatment. Nevertheless, ADG had a time by treatment interaction in which lambs born from ewes supplemented with 1% of Ca salts of EPA and DHA had a greater ADG during the first 15 d of life compared with the lambs born from ewes supplemented with 0 and 2% of Ca salts of EPA and DHA. Studies in ruminants comparing different sources of FA in late gestation and its effect on offspring BW show different results. Lambs born from ewes supplemented with SFA or PUFA (Palmquist et al., 1977; Coleman et al., 2018b) or fish oil (Capper et al., 2007) did not show differences in BW during the preweaning period. However, Garcia et al. (2014a) reported that dairy calves born from cows supplemented with SFA or Ca salts containing PUFA in late gestation had greater birth weights than calves born from cows fed no supplemental fat. Calves born from dams supplemented with SFA had a greater DMI, BW gain, and ADG than calves born from PUFA-supplemented cows from birth to 60 d of age (Garcia et al., 2014b). The difference in results between the studies could be attributed to the differences in the fat sources, FA profile, and FA amount. Also, the difference in ADG at day 15 in the lambs was not associated with changes in milk yield, or milk fat, protein, or lactose concentrations. In the present study, despite the difference over time in ADG, there were no differences in plasma glucose and NEFA concentration.

Despite lamb weaning weight being similar between treatments, their finishing weight was linearly increased due to maternal supplementation with Ca salts of EPA and DHA during late gestation. Carranza Martin et al. (2018) and Marques et al. (2017) showed an increase in offspring BW when they were born from dams supplemented with a diet containing Ca salts of EPA and DHA, compared with the offspring born from dams supplemented with a source of Ca salts containing SFA and MUFA. Therefore, based on the results of the current experiment, the differences in growth observed by previous studies (Marques et al., 2017; Carranza Marin et al., 2018) might be due to a beneficial effect of the PUFA supplementation, rather than being due to an adverse effect of the SFA and MUFA. The increase in growth during the finishing period was not associated with changes in DMI or feed conversion (G:F). Dry matter intake showed a quadratic effect due to dam supplementation with Ca salts containing EPA and DHA; lambs born from ewes supplemented with 1% of Ca salts containing EPA and DHA ate less than the lambs born from ewes supplemented with 0 and 2% of Ca salts containing EPA and DHA. The mechanism for the improvement in growth and the quadratic effect on DMI due to increased EPA and DHA in the maternal diet is not known. Changes in offspring hypothalamus mRNA abundance of genes associated with DMI and energy partitioning due to PUFA supplementation to pregnant ewes (Carranza Martin et al., 2018) might be involved

in the regulation of growth and DMI (Mastorakos and Zamanti, 2004); however, more studies need to be conducted to understand the mechanism that regulates changes in weight and DMI due to maternal FA supplementation.

Plasma glucose concentration during the finishing phase increased as dam EPA and DHA supplementation increased during late gestation. Carranza Martin et al. (2018) did not find differences in plasma glucose concentration due to supplementation of pregnant ewes with different types of FA, but the dose of FA used was 0.39% of the DM. Plasma glucose concentration in the current experiment seems to be positively associated with BW, but not with DMI. Plasma glucose concentration depends on glucose synthesis by the liver (Reynolds, 2002) and the use of glucose by tissues, which is mainly dependent on plasma insulin concentration. Despite the lack of treatment effects on plasma insulin concentration, plasma glucose and insulin concentrations were positively correlated. Normally, an increase in plasma glucose concentration triggers the secretion of insulin. In a healthy animal, the increase of plasma insulin concentration decreases plasma glucose concentration to maintain glucose homeostasis. As mentioned previously, plasma glucose concentration and DMI were not associated; therefore, the increase in plasma glucose might not be driven by the increased uptake of glucose or its precursors in the current experiment. Therefore, it is possible that the supplementation with PUFA during gestation changed glucose metabolism. Previous studies in sheep evaluating the effect of maternal nutrition showed changes in glucose metabolism of the offspring (Gardner et al., 2005; Ford et al., 2007; Radunz et al., 2011). It might be possible that the differences in BW observed in the present study could be due to an unpaired glucose-insulin system (Tschöp et al., 2001); however, the design of the current experiment does not allow us to confirm such an assumption.

Plasma ghrelin concentration showed a tendency for a linear decrease as EPA and DHA concentration increased in the maternal diet. Plasma ghrelin concentration was also negatively associated with plasma glucose and insulin concentrations. Ghrelin is a hormone that has been associated with DMI in ruminants (Wertz-Lutz et al., 2006; Relling et al., 2010); however, the use of ghrelin or its agonists do not increase daily DMI (Wertz-Lutz et al., 2006; Roche et al., 2008). In the current study, we saw a negative association between DMI and plasma ghrelin concentration. The mechanism of the regulation of ghrelin secretion remains unknown, but it is possible that the increase in DMI decreased the secretion of ghrelin and led to a decrease in plasma ghrelin concentration. It is also possible that the maternal supplementation with PUFA changed ghrelin metabolism. In nonruminants, another known function of ghrelin is its role in energy balance homeostasis (Chabot et al., 2014), and it has been associated with obesity (Tschöp et al., 2001). In ruminants, plasma ghrelin concentration has also been associated with energy balance (Roche et al., 2006; Bradford and Allen, 2008). Therefore, it is possible that the changes in glucose concentration could be due to the changes in ghrelin concentration, leading the lambs to an increase in BW. This study shows only the association between the variables but cannot explain cause-effect or the physiological mechanism that lead to observable changes in the offspring growth and metabolism when PUFA was supplemented to pregnant ewes. Thus, more work is needed to uncover the mechanisms by which PUFA may regulate offspring metabolism and growth.

In conclusion, dam supplementation during late gestation with increasing amounts of a Ca salt with EPA and DHA did not change ewe growth, but it did change SC adipose tissue relative mRNA abundance of genes associated with lipid metabolism. The changes in relative mRNA abundance did not always have a linear response to the increase of EPA and DHA in the ewe diet. Dam supplementation increased offspring BW, which was not associated with DMI, but positively associated with plasma glucose concentration and negatively associated with plasma ghrelin concentration. The current experiment was not designed to evaluate insulin resistance, but it is possible that changes in glucose, insulin, and ghrelin homeostasis might the reason for the increase in lamb BW.

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