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Original Research

Dietary Supplementation of Algae-Derived Omega-3 Fatty Acids Influences Endometrial and Conceptus Transcript Profiles in Mares

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ABSTRACT

Maternal diet has been shown to impact fetal development across a variety of species. Given the reported effects of omega-3 long-chain polyunsaturated fatty acid (n-3 LCPUFA) supplementation on health across species, it was hypothesized that supplementation of algal-based n-3 LCPUFA to mares around conception would alter the maternal uterine environment and modify gene expression in early conceptuses. Nonlactating, light horse mares were supplemented with concentrate (n = 6) or an isocaloric diet containing 0.06 g/kg body weight n-3 LCPUFA (primarily docosahexaenoic acid [DHA], n = 7) at least 60 days before first sample collection. Four consecutive ovulatory cycles were monitored. Uterine endometrial samples were obtained 12.5 days postovulation from cycles 1 (uninseminated), 3, and 4 (bred to a common stallion); embryos were flushed from cycles 3 and 4. Biopsies from supplemented mares were greater in DHA and total omega-3 fatty acid concentrations ($P < .05$), indicating successful tissue incorporation. Uninseminated n-3 LCPUFA supplemented mares displayed increased *PTGFS* and reduced *IL6* gene expression ($P < .01$) than controls. Supplemented pregnant mares had increased mRNA abundance of *PTGES* ($P = .05$), *PTGFS* ($P = .03$), and *SLCO2A1* ($P = .01$), while supplemented barren mares exhibited lower *PTGFS* ($P = .05$) and *IL6* ($P < .01$) expression than control mares. In the conceptus, relative transcript abundance of trophoblast and endoderm markers (*GATA3*, *GATA4*, *GATA6*, *TFAP2A*, *ELF3*) was greater ($P < .05$) in embryos from supplemented mares. These results indicate algae-derived n-3 LCPUFA supplementation prior to breeding alters the maternal uterine environment and modifies expression of genes in preimplantation equine conceptuses.

Animal welfare/ethical statement: All procedures were approved by the Virginia Tech IACUC under the approval 13-031 APSC.

Conflict of interest: The authors declare no conflicts of interest.

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1. Introduction

Emerging evidence across a wide range of species suggests that environmental factors near the time of conception, such as maternal diet, can induce permanent changes in the structure, physiology, and metabolism of the conceptus. In turn, these changes may alter the fetal health trajectory, potentially predisposing offspring to chronic, degenerative diseases later in life [1–4]. Despite suggestions that similar phenomena exist in the horse [5–7], studies have largely focused on glucose and insulin response to dietary modification [8,9]. No studies have specifically tested hypotheses regarding effects of periconceptual diet on offspring health or uterine function.

Long-chain polyunsaturated omega-3 fatty acids (n-3 LCPUFAs), primarily docosahexaenoic (DHA) and eicosapentaenoic acid (EPA), have beneficial effects on reproduction [10–14] by modifying prostaglandin synthesis and metabolism [15] and by regulation of genes important to uterine function [10]. In cattle, dietary inclusion of DHA/EPA altered prostaglandin synthesis pathways and improved embryo quality and number [14]. Through mediating inflammatory, metabolic, and vascular mechanisms, n-3 LCPUFA may play a role in oocyte potential [16] and embryonic development [17].

Incorporation of n-3 LCPUFA into equine diets has a number of physiological effects including the incorporation into skeletal muscle and red blood cell membranes as well as beneficial effects on insulin sensitivity and joint health; however, the effects of n-3 LCPUFA on reproduction in the horse are largely unknown [18–20]. The objective of this study was to evaluate the effects of n-3 LCPUFA supplementation on fatty acid composition and expression of genes in equine uterine tissue, and expression of genes related to developmental stage in equine conceptuses. It was hypothesized that n-3 LCPUFA fed around conception would incorporate into uterine tissue, alter gene expression related to prostaglandin and inflammatory pathways, and modify expression of genes involved in early embryogenesis.

2. Materials and Methods

2.1. Animals and Diets

All animal procedures were approved by the Institutional Animal Care and Use Committee (Protocol 13-031-APSC) at Virginia Tech. Horses were housed at the Virginia Tech Middleburg Agricultural Research and Extension Center in Middleburg, Virginia. Prior to assigning horses to study groups, fasting blood samples were obtained from all horses via jugular venipuncture and used to determine plasma glucose and insulin concentrations to assess insulin sensitivity as described previously [21,22]. Glucose levels were evaluated via a glucose analyzer (YSI Life Sciences, Yellow Springs, OH), while insulin levels were determined via a colorimetric ELISA (Mercodia, Uppsala, Sweden).

Thirteen nonlactating, light horse mares were matched by age (mean ± standard error of the mean; age = 13.56 ± 0.11 years), BCS (BCS; 7.07 ± 0.21) [23], parity and insulin sensitivity, and randomly assigned to one of two groups. It is important to note that the average BCS of 7.07 is considered overweight. The relevance of this fact will be described later. Mares in the n-3 LCPUFA supplemented group (n = 7) were fed a diet containing 0.06 g/kg body weight (BW) algae-derived n-3 LCPUFA (DHA Gold DSM Nutritional Products Inc. Ames, IA) mixed with a commercially available concentrate diet (Southern States, Richmond, VA; 1.28 ± .03 kg/day), while mares in the control group (n = 6) received an isocaloric diet consisting solely of the concentrate (Southern States; 1.56 ± .05 kg/d). The DHA supplement was provided by DHA Gold.

All diets were stored and prepared in a temperature and humidity controlled room to ensure nutrient preservation. Body weights and body conditions were recorded weekly, and diets were adjusted on a weekly basis to account for any body weight differences. After an initial 20-day acclimation period, during which horses were slowly introduced to the concentrate and DHA supplement, diets were fed 60 days prior to the first sample collection. Horses also remained on the same pastures during the acclimation as they did during the experimental protocol. All mares were housed on a single pasture containing mixed fescue and orchardgrass with free choice access to water and a vitamin/mineral mix. Dietary supplementation began in June and proceeded through November. Pasture samples were collected every 2 weeks and analyzed for nutrient composition. Pasture samples obtained were representative of available forage types and selected from areas with evidence of grazing. Pasture samples were analyzed individually by collection time point, and results were grouped into three categories: mid-summer (mean of samples in June and July), late summer (mean of samples in August and September), and fall (mean of samples in October and November). Concentrate aliquots were collected every 2 weeks and stored at −20°C until further analysis for nutrient composition. Concentrate and supplement samples were analyzed individually (Dairy One, Ithaca, NY) by collection time point, and results were pooled by batch. All nutrient requirements for mares were met by the diets. Nutrient composition of feedstuffs is outlined in Table 1.

2.2. Experimental Procedure and Schedule

Prior to enrollment in the study, all mares had experienced at least one spontaneous ovulation during the current breeding season. Estrous synchronization took place 45 days following the start of dietary supplementation, so that mares would have at least 60 days of dietary treatment prior to the first sample collection. Initial synchronization of estrous cycles in mares was accomplished using a single i.m. dose of cloprostenol (250 mcg, Estrumate, Schering-Plough Animal Health, Kenilworth, NJ) followed by daily oral administration of 0.044 mg/kg BW altrenogest (Regumate, Intervet Inc, Summit, NJ) for 2 weeks and finally a second i.m. dose of cloprostenol injected on day 15. Thereafter, mares were monitored daily via transrectal ultrasound, and ovarian follicular activity as well as uterine edema were recorded for four consecutive ovulatory cycles. Ovulation was confirmed via transrectal ultrasonography as the presence of a corpus luteum on the ovary in place of the dominant follicle. At 12.5 days following ovulations 1, 3,

Table 1
Nutrient composition of feedstuffs.

Nutrients and Feedstuffs			Pasture		
Nutrient[a]	Concentrate	DHA Supplement	Mid-Summer[b]	Late Summer[c]	Fall[d]
DM %	90.20	97.20	32.20	36.50	34.80
CP %	14.40	10.20	16.15	12.10	14.70
ADF %	19.40	1.50	35.00	36.20	32.40
NDF %	35.70	2.50	52.00	54.10	53.80
Ca %	1.12	0.03	0.87	0.82	1.82
P %	0.71	0.15	0.28	0.22	0.24
Zn mg/kg	211.00	4.00	ND[e]	ND	ND
Cu mg/kg	55.00	5.00	ND	ND	ND
Total fatty acids	4.72	52.28	1.48	0.95	1.15
C18:2 n-6 (LA)	2.47	ND	0.21	0.15	0.15
C18:3 n-3 (ALA)	0.21	ND	0.77	0.50	0.65
C20:5 n-3 (EPA)	ND	0.81	ND	ND	ND
C22:6 n-6 (DHA)	ND	20.76	ND	ND	ND

Abbreviations: DM, dry matter; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber; LA, linoleic acid; ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.
[a] Except for DM, all values presented on a 100% DM basis.
[b] Mid-Summer = mean of samples obtained in June and July.
[c] Late Summer = mean of samples obtained in August and September.
[d] Fall = mean of samples obtained in October and November.
[e] ND = not detected during analysis.

and 4, endometrial biopsies were obtained transcervically using an equine uterine biopsy instrument (Kruuse, Denmark) and divided into three aliquots. Two aliquots were immediately snap frozen in liquid nitrogen and then stored at −80°C until needed for RNA extraction and fatty acid analysis. A final aliquot was fixed in formalin and paraffin embedded. From these samples, 5-μm tissue sections were cut and affixed to glass slides. Tissues were stained with hematoxylin and eosin under standard procedures and then graded by an experienced theriogenologist blinded to treatment at Auburn University School of Veterinary Medicine (author A.K.J.). Biopsy scores were assigned as outlined by Kenney and Doig, 1986. Briefly, this scoring system takes into consideration inflammation and fibrosis of the endometrium, assigning a score indicating the mare's ability to conceive and maintain a pregnancy. Ultimately, this evaluation establishes a scoring system to determine the quality of the endometrial tissue. Following the biopsy, a single IM dose of cloprostenol was administered to induce luteolysis, which was confirmed via ultrasonography. No endometrial biopsies were obtained following ovulation 2 to minimize stress on the uterine environment. A fourth ovulatory cycle was added to the study to allow for the collection of additional conceptus samples.

2.3. Embryo Recovery

In the third and fourth estrous cycles, mares with follicles ≥35 mm in diameter and appropriate edema were artificially inseminated every other day until ovulation using fresh, extended (INRA 96, IMV Technologies, Normandy, France) semen from a single stallion of known fertility of at least 500 million motile spermatozoa per insemination. At 12.5 days after ovulation, transrectal ultrasound was used to observe the presence or absence of an equine conceptus. Regardless of ultrasonography outcome, all mares underwent embryo flush protocol. Briefly, a modified nasogastric tube was passed transcervically to the opening of the uterus. Approximately 2 L of Bio Life "Advantage" Complete embryo flush media (AgTech Inc, Manhattan, KS) was infused into the uterus. Transrectal uterine massage was used to ensure complete uterine filling, and media was flushed out of the uterus into a collection vessel. The process was repeated twice or until a conceptus was observed in the collection vessel. Following embryo recovery, all mares received a single i.m. dose of 20 IU oxytocin (AgriLabs, St. Joseph, MO) to ensure uterine fluid clearance and a single i.m. dose of cloprostenol (250 mg, Estrumate) to ensure luteolysis.

2.4. Total RNA Isolation and cDNA Synthesis

Total RNA was isolated from endometrial tissues using TRIzol reagent and PureLink RNA isolation columns (Life Technologies, Carlsbad, CA) according to the manufacturer instructions, while total RNA was isolated from conceptus tissues using Qiagen AllPrep DNA/RNA Kit (Qiagen, Hilden Germany) according to the manufacturer instructions. For both tissues, RNA parameters were determined using a NanoDrop 2000 Spectrophotometer. Further, conceptus RNA quality was determined using Experion RNA Standard Sensitivity Chips (Bio-Rad, Hercules, CA) per manufacturer recommendations. All RNA samples were stored at −80°C until analysis.

2.5. Gene Expression Analysis by qRT-PCR

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed on candidate genes of interest listed in Table 2 for endometrial samples. Total RNA (50 ng) was treated with DNase I amplification grade, and first-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit

Table 2
Forwards and reverse primer sequences for genes utilized for qRT-PCR analysis of endometrial tissues.

Gene	Forward (F) and Reverse (R) Primer (5′-3′)	Function
PTGS1	F: GCCTGACTCCTTCAGAGTGG	Prostaglandin signaling
	R: TCTCGGGATTCCTTGATGAC	
PTGS2	F: TATCCGCCCACAGTCAAAGACA	Prostaglandin signaling
	R: TGTTGTGTTCCCGCAGCCAAAT	
PTGES	F: GGAACGACATGGAGACCATCTAC	Prostaglandin signaling
	R: GAAGGGATGCCCAATCCCCTAG	
PTGFS	F: AAGCCAGGGCTCAAGTACAA	Prostaglandin signaling
	R: AGCACCGTAGGCAACTAGGA	
HPGD	F: GTTGCACAGCAGCCTGTTTA	Prostaglandin signaling
	R: CATCGATGGGTCCAAAATTC	
SLCO2A1	F: CGTTTTCTCTCTGCAAACCA	Prostaglandin signaling
	R: GAGCGGTACTCCACTCCATT	
PPARA	F: AGTGGTCCAGGATCAGATGG	Prostaglandin signaling
	R: AGGCATGAACTCCGTAATGG	
PPARD	F: ACGACATCGAGACATTGTGG	Prostaglandin signaling
	R: TGATCTCCTTGTAGGGTGGC	
PTGER2	F: CCTCCAAGCCCTTAGGTTTC	Prostaglandin signaling
	R: TATCCACAAGGGCCAGCTAC	
PTGFR	F: CGTGTGCTTGTTTGCTGTTT	Prostaglandin signaling
	R: ATGGCATTGCACAAGAATGA	
PLA2G4A	F: AGGGACAGCAACATTTACCCT	Prostaglandin signaling
	R: GAGGTCTGGGCACGAACAAA	
OXTR	F: TCTTCTTCGTGCAGATGTGG	Prostaglandin signaling
	R: ACAGCATGTAGATCCAGGGG	
PLA2G3	F: CACAGACTGTCTCGCCCTTT	Prostaglandin signaling
	R: CTGGAACCTGGCATCACAGT	
ESR1	F: GATAATCGACGCCAGGGAGG	Steroid signaling
	R: CTTCGTAGCATTTGCGGAGC	
ESR2	F: TCCTTTCTCACGTCAGGCAC	Steroid signaling
	R: GCCGTCTTTGCTCTCACTCT	
PGR	F: CCCAGCATGTCGCCTTAGAA	Steroid signaling
	R: TGATCAGTGGGGGCATCAAC	
FABP3	F: GGTCAAGTCCCTTGTGACACT	Fatty acid metabolism
	R: GAGGCAATCTGGTGCTGAGT	
APOA1	F: GGGAAAACAGCTGAACCTGA	Fatty acid metabolism
	R: GGAAATCGTCCAGGTAGGGC	
SAA	F: GTCATCAGCGATGCCAGAGA	Fatty acid metabolism/ inflammatory signaling
	R: GTACTTGTCAGGCAGGCCAT	
CRP	F: GCAGCCGGTGCAAGATAGAA	Inflammatory signaling
	R: TTCCAAATCCCCAGGCCATC	
IL1B	F: CGGCCGGGACATAACTGACT	Inflammatory signaling
	R: GCCTGCAGCATGTTCAAACC	
IL6	F: GGCACCCAGTCTGAGAACAG	Inflammatory signaling
	R:TCTCAGGCTGAACTGCAGGAA	
IL10	F: GGCACCCAGTCTGAGAACAG	Inflammatory signaling
	F: TGGCAACCCAGGTAACCCTTA	
TNFA	F: GGCCCAGACACTCAGATCAT	Inflammatory signaling
	R: TTGGGGGTTTGCTACAACAT	
NFKB	F: GCCAACCCAAGTCTCTCTCC	Inflammatory signaling
	R: ATTACTGACAGCCCTTGCCC	
UTEROCALIN	F: CCCGGATGTCATGTGGATGT	Nutrient transport
	R: GTGGAGGCACCGATCAGTTT	
ACTB	F: GGGACCTGACGGACTACCT	Reference gene
	R: CCGTGGTGGTGAAGCTCTA	
GAPDH	F: CATCATCCCTGCTTCTACTGG	Reference gene
	R: TCCACGACTGACACGTTAGG	

Abbreviation: qRT-PCR, Quantitative reverse transcriptase polymerase chain reaction.

(Life Technologies) according to the manufacturer instructions. Each primer pair was validated by examining primer efficiencies. Also, amplification of single products was ensured by melting curve analysis. Reactions lacking template were included in each assay to ensure the absence of contaminated reagents. All qRT-PCR on endometrial samples was conducted using the 7500 Fast Real-Time PCR System (Life Technologies). Each sample was carried out in duplicate using SYBR Green Master Mix (Life Technologies). The PCR cycle and conditions were as follows: 1 cycle at 95°C for 10 minutes, followed by 40 cycles of 30 seconds of denaturation at 95°C, and 30 seconds of annealing at 60°C. Endometrial samples were normalized using *ACTB*. Both *GAPDH* and *ACTB* were evaluated as reference transcripts based on previous validation efforts [24], and *ACTB* was chosen due to its lower coefficient of variation. Relative expression level of target genes was calculated using ΔΔCt method [25].

For conceptus samples, qRT-PCR was performed on genes of interest depicted in Table 3. All qRT-PCR on conceptus samples was conducted using the Eppendorf RealPlex Mastercycler ep Gradient S (Eppendorf, Germany). Each 20 μL reaction was carried out in duplicate using 10 μL of SYBR Green Master Mix (Life Technologies). The PCR cycle and conditions were as follows: 1 cycle at 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C, 15 seconds at 55°C, and 20 seconds at 68°C. Conceptus samples were normalized using *GAPDH,* which had been verified previously as a suitable reference for equine embryos [24]. Both *GAPDH* and *18S* were analyzed as reference genes, and *GAPDH* was utilized due to its lower coefficient of variation. Abundance of this reference transcript was not altered based on diet, pregnancy status, or other measured parameters. Relative expression level of target genes was calculated using ΔΔCt method [25].

Genes evaluated were selected based on specific molecular pathways of relevance to the study including those related to prostaglandin signaling, inflammation, fatty acid metabolism, and tissue lineage specifications [10,26,27].

Table 3
Forwards and reverse primer sequences for genes utilized for qRT-PCR analysis of conceptus tissues.

Gene	Forward (F) and Reverse (R) Primer (5′-3′)	Function
SLC17A5	F: GCAGATTTTGGAGTTGGAGC R: AAGCTTGCTTCTCTCAAGCG	Capsule formation
TGM3	F: GGTGTCTGTGAACATGACGG R: ATGAGATCTTCACGGGATGC	Capsule formation
REX1	F: GACGGGAAAGGCCTGGATA GAAG R: GGCGGTAAGAAGCTGTTGA GAAAGG	ICM specification
SALL4	F: AGAACTTCTCGTCTGCCAGC R: TTTCCTTGGGAAACATCTCG	ICM specification
CDX2	F: GGAGCTGGAGAAGGAGTTTC R: GGAGGGAAGACACAGGACTC	TE specification
HAND1	F: TGCCCACGAGGTTCATGTTG R: GCAGAACTCAAGAAGGCGGA	TE specification
GATA3	F: GCATCCAGACCAGAAACCGA R: ATGGTGAGGTCCGAAGGAGA	TE specification
ELF3	F: ACAGCAAGCTCTTCTCCAGC R: GACACTTCTCCAGGCAGACC	TE specification
EOMES	F: CTAAAAGAAGGTGCCAAAGC R: CTTAAGACCCAGCCCTTCTC	TE specification
TFAP2A	F: AATGCTTTGGAAACTGACGG R: ATTGACCTACAGTGCCCAGC	TE specification
GATA6	F: CGAGCGCTGTTTGTTTAGGG R: ACTTCTAGCTCCTCGGGTGG	PE specification
GATA4	F: CTGACAAAGCCCAGAAGACC R: CGTGGATTTCCTGACAGACC	PE specification
PTGS1	F: GCCTGACTCCTTCAGAGTGG R: TCTCGGGATTCCTTGATGAC	Prostaglandin signaling
PTGS2	F: TATCCGCCCACAGTCAAAGACA R: TGTTGTGTTCCCGCAGCCAAAT	Prostaglandin signaling
PTGES	F: GGAACGACATGGAGACCAT CTAC R: GAAGGGATGCCCAATCCCCTAG	Prostaglandin signaling
PTGFS	F: AAGCCAGGGCTCAAGTACAA R: AGCACCGTAGGCAACTAGGA	Prostaglandin signaling
HPGD	F: GTTGCACAGCAGCCTGTTTA R: CATCGATGGGTCCAAAATTC	Prostaglandin signaling
SLCO2A1	F: CGTTTTCTCTCTGCAAACCA R: GAGCGGTACTCCACTCCATT	Prostaglandin signaling
PPARA	F: AGTGGTCCAGGATCAGATGG R: AGGCATGAACTCCGTAATGG	Prostaglandin signaling
PPARD	F: ACGACATCGAGACATTGTGG R: TGATCTCCTTGTAGGGTGGC	Prostaglandin signaling
PLA2G3	F: CACAGACTGTCTCGCCCTTT R: CTGGAACCTGGCATCACAGT	Prostaglandin signaling
FABP3	F: GGTCAAGTCCCTTGTGACACT R: GAGGCAATCTGGTGCTGAGT	Fatty acid metabolism
SAA	F: GTCATCAGCGATGCCAGAGA R: GTACTTGTCAGGCAGGCCAT	Fatty acid metabolism
APOA1	F: GGGAAAACAGCTGAACCTGA R: GGAAATCGTCCAGGTAGGGC	Fatty acid metabolism
IL10	F: GGCACCCAGTCTGAGAACAG R: TGGCAACCCAGGTAACCCTTA	Inflammatory signaling
CRP	F: GCAGCCGGTGCAAGATAGAA R: TTCCAAATCCCCAGGCCATC	Inflammatory signaling
IL1B	F: CGGCCGGGACATAACTGACT R: GCCTGCAGCATGTTCAAACC	Inflammatory signaling
GAPDH	F: CATCATCCCTGCTTCTACTGG R: TCCACGACTGACACGTTAGG	Reference gene

Abbreviations: PE, primitive endoderm; qRT-PCR, Quantitative reverse transcriptase polymerase chain reaction; ICM, inner cell mass; TE, trophectoderm.

2.6. Fatty Acid Analysis

Fatty acid extraction and analysis were completed by the University of Missouri-Columbia Agricultural Experiment Station and Chemical Laboratories (Columbia, MO). Briefly, fatty acids were extracted using the Folch extraction method for total lipids from at least 25 mg of endometrial tissue per sample [28]. Fatty acid analysis was completed using Association of Official Agricultural Chemists and American Oil Chemists' Society official methods by gas-liquid chromatography [29].

2.7. Statistical Analysis

All statistical analysis was performed and analyzed using SAS (v9.3, SAS Inst. Inc, Cary, NC). Body condition score, body weight measurements, and endometrial fatty acid composition were first tested for normality, transformed if necessary via Box Cox transformation, and then analyzed using generalized linear models. Endometrial biopsy scores were analyzed using the Wilcoxon–Mann–Whitney test. Transcript abundances were analyzed using nonparametric Kruskal–Wallis test. Differences were considered significant at an alpha level of 0.05.

3. Results

3.1. Diet Consumption and Overall Animal Health

All horses remained clinically healthy over the course of the experiment. Body weight and body condition remained constant through the course of supplementation (Table 4). Following the acclimation period, horses readily consumed both diets. Only one example of refusal in a single mare (n-3 LCPUFA group) during the estrus period of one estrous cycle was observed. The refusal was approximately 12% of the total diet offered during that period.

3.2. Endometrial Fatty Acid Composition

Endometrial biopsies obtained approximately 12.5 days postovulation 1 were analyzed for fatty acid composition. Mares from the n-3 LCPUFA supplemented group had increased incorporation of DHA and total n-3 fatty acids into endometrial tissue as compared to control mares (Table 5), both as a percentage of total fat ($P < .001$) and wet tissue weight ($P = .015$). The ratio of total n-3 to total n-6 fatty acids in biopsies from supplemented mares was higher than those from control mares ($P = .017$).

3.3. Endometrial Biopsy Scores

Endometrial biopsy scores from n-3 LCPUFA supplemented mares (n = 7) were less following the first ovulation ($P = .010$) than those obtained from control mares (n = 6) (Fig. 1). Mares were undergoing dietary treatment for approximately 48 days at the time of tissue collection. Thereafter, biopsy scores between groups remained similar.

3.4. Endometrial Gene Expression in Cycling Mares

Endometrial biopsies taken postovulatory cycle 1 were obtained from nonbred, cycling animals, to evaluate tissue differences independent of influences due to insemination. Results of gene expression analysis are depicted in Fig. 2.

Table 4
Body weights and body condition scores.

Measureable	Start of Supplementation[a]		End of Supplementation[b]	
	DHA	Control	DHA	Control
BCS (1–9)	6.93 ± 0.20	7.00 ± 0.28	7.07 ± 0.30	7.08 ± 0.31
BW (kg)	645.00 ± 54.00	652.00 ± 45.00	642.00 ± 52.00	661.00 ± 30.00

Abbreviations: BCS, body condition score; BW, body weight; DHA, docosahexaenoic acid.
[a] June 20, 2013.
[b] October 24, 2013.

Endometrial samples obtained from n-3 LCPUFA supplemented mares displayed reduced abundance of *IL6* ($P = .004$) and increased transcript abundance of *PTGFS* ($P = .004$) relative to those obtained from control animals. No differences among other transcripts were observed.

3.5. Endometrial Gene Expression in Inseminated Mares

Endometrial samples obtained from mares approximately 12.5 days following ovulation in cycles 3 and 4 could be categorized into those from both pregnant animals (n-3 LCPUFA group n = 5; control group n = 4) and those that were bred but did not become pregnant (n-3 LCPUFA group n = 8; control group n = 5). Results of gene expression analysis are depicted in Figure 3 for pregnant mares and Figure 4 for mares that were bred but did not become pregnant. Among pregnant animals, samples from mares supplemented with n-3 LCPUFA contained increased mRNA abundance of *PTGES* ($P = .05$), *PTGFS* ($P = .028$), *SLCO2A1* ($P = .014$), *OXTR* ($P = .050$), *UTEROCALIN* ($P = .028$), *SAA* ($P = .014$), and *IL10* ($P = .050$) relative to control animals. Samples obtained from mares that were bred but did not become pregnant displayed increased transcript abundance of *PTGFS* ($P = .047$) and reduced *IL6* ($P = .003$) in nonsupplemented control animals as compared to those obtained from n-3 LCPUFA supplemented mares. No differences among other transcripts were observed.

3.6. Conceptus Recovery and Gene Expression

Thirteen conceptuses were recovered from mares at 12.5 days postovulation in cycles 3 and 4. All conceptuses were recovered intact with the acellular membrane intact.

Table 5
Endometrial fatty acid composition.

Measureable	Control	DHA	*P*-Value
DHA (μg)	6.41 ± 2.37	24.99 ± 2.45	.0002
DHA (% total fat)	1.80 ± 0.35	3.29 ± 0.38	.0155
DHA (% wet weight)	8.15 ± 2.02	19.62 ± 2.18	.0027
[1]Omega 3 (μg)	14.79 ± 3.16	42.20 ± 3.42	.0001
[1]Omega 3 (% total fat)	4.02 ± 0.35	5.48 ± 0.37	.0154
[1]Omega 3 (% wet weight)	18.77 ± 2.35	32.73 ± 2.54	.0020
[2]Omega 6 (μg)	100.5 ± 17.2	202.4 ± 18.5	.0020
[2]Omega 6 (% total fat)	26.63 ± 0.67	26.01 ± 0.73	.5506
[2]Omega 6 (% wet weight)	15.55 ± 2.01	12.69 ± 1.89	.1011
[1]Omega 3: [2]Omega 6	0.15 ± 0.02	0.21 ± 0.02	.0174

Abbreviation: DHA, docosahexaenoic acid.
[1]Omega 3 = linolenic (18:3n3) + clupanodonic (22:5n3) + DHA (22:6n3).
[2]Omega 6 = linoleic (18:2n6) + arachidonic (20:4n6).

Conceptuses were processed immediately to preserve the integrity of the RNA/DNA so measurements of conceptus size were omitted and are recognized as a limitation of the current study. However, all conceptuses were of similar size and visible morphology, and due to similar breeding protocol, it can be assumed that all conceptuses were of similar age. Following RNA extraction and quality evaluation, 10 conceptuses were found suitable for gene expression analysis (DHA n = 5; control n = 5) (Fig. 5). Those conceptuses not utilized for transcriptional evaluation were done so due to insufficient RNA quantity and/or quality. Conceptus gene expression was evaluated for candidate genes listed in Table 3. Differential expression of genes related to trophectoderm (TE) and primitive endoderm (PE) was observed (Fig. 5). Embryos from supplemented mares contained increased transcript abundance of *GATA3, TFAP2A,* and *ELF3*, markers of TE differentiation ($P < .01$), while embryos from n-3 LCPUFA supplemented mares contained increased transcript abundance of the PE differentiation related genes *GATA4* and *GATA6* ($P < .01$). Additionally, increased expression of *CRP* ($P = .009$) was observed among embryos from supplemented mares, relative to those from control mares.

4. Discussion

The current study demonstrates that dietary supplementation of an algal source of DHA successfully

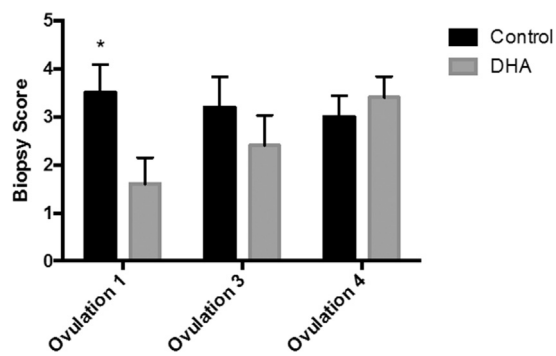

Fig. 1. Endometrial biopsy scores of tissue obtained approximately 12.5 days post-ov-1. On average, mares were on dietary treatment for 48 days at time of tissue collection. Endometrial biopsy scores as analyzed by an independent investigator at Auburn University College of Veterinary Medicine utilizing a modified Kenney Scale. Scores represent a scale of 1–5 with a score closer to 1 being indicative of healthier tissue. Tissues scoring 2a and 2b were reassigned a score of 3 or 4, respectively, to allow for statistical analysis. An asterisk indicates $P \leq .05$. DHA, docosahexaenoic acid.

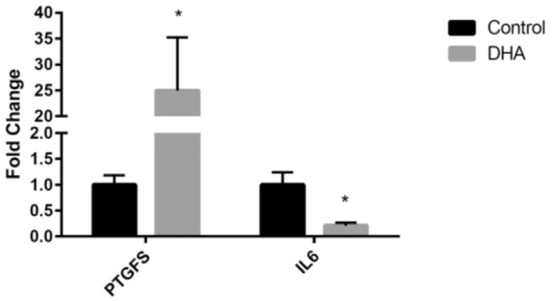

Fig. 2. Endometrial gene expression in cyclic mares. Gene expression analysis of equine endometrial tissues obtained from supplemented (n = 7) and control (n = 6) animals postovulatory cycle 1. Values are expressed as fold changes. An asterisk indicates $P \leq .05$. DHA, docosahexaenoic acid.

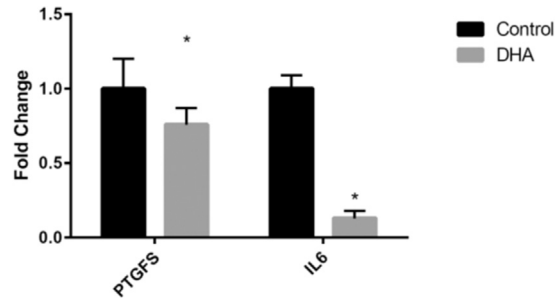

Fig. 4. Postovulatory cycle 3 and 4 endometrial gene expression. Gene expression analysis of equine endometrial tissue obtained from supplemented (n = 8) and control (n = 5) animals bred but not pregnant approximately 12.5 days postovulation following ovulatory cycles 3 and 4. Values are expressed as fold changes. An asterisk indicates $P \leq .05$. DHA, docosahexaenoic acid.

incorporates into equine endometrial tissue altering the fatty acid composition and affecting changes in gene expression. Due to the inefficient conversion of the parent fatty acid, alpha-linolenic acid, into the long-chain fatty acids EPA and DHA, direct supplementation with DHA is necessary to facilitate the desired alterations in fatty acid balance [30]. Several studies have demonstrated incorporation of dietary n-3 LCPUFA (primarily DHA and EPA) into various equine tissues, including red blood cells, skeletal muscle, synovial fluid [30,31], and in this study, uterine endometrium. The amount of n-3 LCPUFA fed to mares (0.06 mg/kg BW) in this study, and duration of supplementation, was consistent with that described previously [18,30]. Alterations in the fatty acid composition, namely the increased incorporation of n-3 fatty acids, likely provided substrates of an anti-inflammatory or lesser inflammatory nature to the uterine environment [10,32]. Additional research is necessary to fully understand the implications of altering membrane composition on endometrial health.

It is important to note that the current study utilized a group of overconditioned mares (BCS > 6.5) with a previous history of insulin resistance. Obesity may modify reproductive function through direct alteration in endometrial health or function through perturbations in implantation and embryonic receptivity of the endometrium [33]. Studies have demonstrated that maternal obesity or metabolic dysfunction in the mare leads to an altered follicular environment, aberrant cyclicity, decreased fertility, and early embryonic loss [34,35]. While a lean control group was not included in the current study, the results described herein may provide insight into mitigating the negative effects of equine maternal obesity and associated metabolic perturbations on endometrial health and subsequent offspring development.

Endometrial health, regardless of body condition, is of utmost importance to the development and maintenance of a successful equine pregnancy, and endometrial biopsy scoring is a commonly utilized tool to evaluate uterine health. Among uninseminated cycling mares, a lower biopsy score was reported among n-3 LCPUFA treated mares compared to control mares following the first ovulatory cycle. This difference lost its significance as biopsy scores for n-3 LCPUFA mares worsened linearly over time, although no change was noted across cycles for control mares. To ensure a clean and representative uterine environment, no biopsies were taken prior to dietary treatment, so it is impossible to know if the difference that was observed existed before treatment began. While no

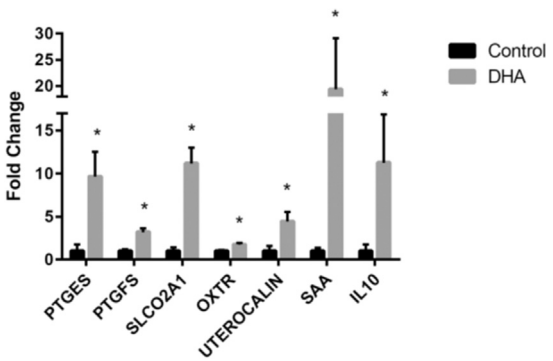

Fig. 3. Postovulatory cycle 3 and 4 endometrial gene expression. Gene expression analysis of equine endometrial tissue obtained from pregnant supplemented (n = 5) and control (n = 4) animals approximately 12.5 days postovulation following ovulatory cycles 3 and 4. Values are expressed as fold changes. An asterisk indicates $P \leq .05$. DHA, docosahexaenoic acid.

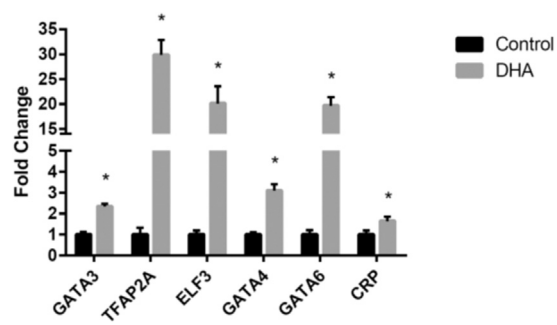

Fig. 5. Conceptus gene expression. Gene expression analysis of day 12.5 equine conceptuses obtained from supplemented (n = 5) and control (n = 5) animals. Values are expressed as fold changes. An asterisk indicates $P \leq .05$. DHA, docosahexaenoic acid.

preliminary biopsies were obtained, all mares had demonstrated a successful estrous cycle with no uterine fluid or abnormalities observed via transrectal ultrasound, indicating that the endometrium was likely healthy prior to sample collection. During the course of sampling, increasing stress was placed on the reproductive tract of the mares through breeding and tissue collection, which may be responsible for the loss of significance over time. It is important to note that the current study observed no differences between supplemented and control groups in relation to pregnancy rates.

Alterations in the expression of prostaglandin biosynthesis genes were observed in both cycling and inseminated mares regardless of pregnancy success. Cycling mares supplemented with n-3 LCPUFA exhibited higher expression of *PTGFS* relative to unsupplemented mares. While an increase in transcriptional abundance of *PTGFS* in n-3 LCPUFA supplemented mares may seem contradictory to published results in cattle [10], the samples taken from these mares were from 12 days postovulation, a different relative time point. Further, studies in cattle and sheep have indicated a reduction in the pathways associated with $PGF_{2\alpha}$ secretion or overall reductions $PGF_{2\alpha}$ production or activity in n-3 LCPUFA supplemented animals. The differences in estrous cyclicity related to timing and overall actions of prostaglandins between cattle and horses may be responsible for these contradictory results [36,37]. Indeed, mRNA expression of *PTGFS* in cycling mares has been shown to peak in late diestrus [38,39]. PTGFS is necessary for the conversion of PGH_2 into $PGF_{2\alpha}$, and this peak coincides with the increase of $PGF_{2\alpha}$ released from the uterus into the uterine vein on day 14 of diestrus [40]. Thus, increased gene expression of *PTGFS* among n-3 LCPUFA supplemented, cycling mares could indicate more rapid priming of the uterus for subsequent estrous cycles. However, no differences were observed in interovulatory period between control and supplemented mares. It is possible that the administration of cloprostenol may have altered cyclicity such that cycle length was not altered between groups.

The enzymes PTGES and PTGFS catalyze synthesis of PGE_2 and $PGF_{2\alpha}$, and upregulation of mRNA expression of both PTGES and PTGFS in early pregnancy versus cyclic mares has been observed previously [38,41]. In horses, the mobile conceptus suppresses the ability of the endometrium to secrete $PGF_{2\alpha}$ between days 9 and 16 and secretes its own $PGF_{2\alpha}$ and PGE_2 to stimulate uterine contractions and facilitate essential intrauterine movement [42]. Maternal PGE_2, however, seems to play a necessary role in early equine pregnancy, as a luteotprotective factor involved in processes necessary for establishment of pregnancy [43]. Therefore, increases in gene expression of *PTGES*, and the nonselective prostaglandin transporter *SLCO2A1*, among supplemented mares compared to control diet mares may reflect a beneficial effect by which n-3 LCPUFA facilitates a more hospitable uterine environment for embryonic development.

Uterine contractions as a result of oxytocin secretion are known to play a role in facilitating necessary early equine embryonic motility throughout the uterus [44]. Among pregnant, supplemented mares, the increased expression of *OXTR*, compared to that of pregnant control mares, may indicate an increased ability of the developing conceptus to interact with the maternal endometrium. Pregnant, supplemented mares also displayed increased mRNA abundance of *UTEROCALIN,* a progesterone-dependent lipocalin produced by the endometrium, responsible for fatty acid transportation during the preattachment phase, relative to pregnant control mares [45,46].

Differential expression between treatment groups was observed in both pregnant mares and those that were bred, but did not become pregnant, for genes related to inflammation and immune response. Mares that were bred but did not become pregnant can be further classified as those that failed to fertilize and those that fertilized successfully but did not effectively maintain pregnancy. While impossible to distinguish between the two classifications in this study, early embryonic loss is of increasing concern and results of the current study may provide insights into alterations in uterine dynamics [47]. Similar to patterns observed in cycling mares, pregnant mares supplemented with n-3 LCPUFA displayed increased mRNA abundance of the anti-inflammatory cytokine *IL10*. Further, supplemented mares that became pregnant displayed increased mRNA abundance of *SAA*. These results coupled with decreased expression of the proinflammatory cytokine *IL6* in nonpregnant supplemented mares potentially indicate an altered inflammatory response of the endometrial tissue due to DHA incorporation. Proper integration of acute phase proteins and local cytokine expression facilitates embryo receptivity, maternal-embryo communication, and maintenance of pregnancy [48]. Disturbances in this delicate balance can cause pregnancy loss or alter developmental trajectory of the offspring.

Due to the delayed implantation of equine embryos, the milieu of hormones and other factors secreted from the endometrium may play an important role in modulating embryonic development and results from this study support that hypothesis. Conceptuses obtained from supplemented mares displayed increased mRNA abundance of *GATA3, ELF3,* and *TFAP2A*, genes that are associated with TE lineage specification, tissues that will develop into extraembryonic membranes [26,27,49]. Further, conceptuses obtained from supplemented mares displayed differential expression of *GATA4* and *GATA6,* genes related to the differentiation of PE, cells that are responsible for the formation of internal fetal structures [50–52]. Finally, expression of *CRP* in conceptuses obtained from n-3 LCPUFA supplemented mares was greater compared to control mares. C-reactive protein has been implicated for its role in mediating successful fertilization and pregnancy maintenance possibly because of improved maternal-fetal communication [53]. While alteration in gene expression is a critical step in modulation of physiological function, it must be understood that differences in mRNA concentration may arise as a result of a number of processes including but not limited to the proliferation of immune cells in the endometrium and changes in cellular composition. Alterations in the expression patterns of these genes could, however, indicate a potential benefit to embryonic development and maternal-fetal interaction of

n-3 LCPUFA supplementation, as a precursor to protein translation; however, more work is necessary to fully understand the extent of maternal-fetal communication in the horse during early pregnancy and its relevance to embryonic development.

5. Conclusion

While the effect of n-3 LCPUFA supplementation, specifically DHA and EPA continues to be investigated in a variety of species with favorable results [16,54,55], the current study, to the authors' knowledge, is the first to evaluate the effects of supplementing an algae-derived omega-3 fatty acid on endometrial and subsequent embryonic gene expression in horses. Additionally, as far as the authors are aware, this is the first study to report the incorporation of supplemental dietary fatty acids, specifically DHA, into endometrial tissue in the horse. Previous studies have found that supplementation of an EPA and DHA source altered red blood cell membrane, skeletal muscle, and synovial fluid composition in horses [18,30]. The current study demonstrated alteration of inflammatory signaling and prostaglandin signaling in the endometrial tissue obtained from DHA supplemented mares. The most significant alterations in expression were found in genes responsible for inflammatory signaling and prostaglandin synthesis in endometrial tissues. Finally, genes related to lineage specification were differentially expressed between conceptuses obtained from supplemented mares and those obtained from control mares. Taken together, these results indicate that an algae-derived omega-3 fatty acid supplement administered to mares during the preconceptual and periconceptual period effects changes in uterine composition, subsequently altering the uterine environment and impacting conceptus development. The results from this study indicate that opportunities may exist to potentially improve the reproductive health of mares through supplementation of marine-derived omega-3 fatty acids around the time of conception and that maternal diet during this period can have a significant effect on conceptus maturation.

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