Dietary n-3 polyunsaturated fatty acid supplementation alters the expression of genes involved in the control of fertility in the bovine uterine endometrium

Sinéad M. Waters,1 Gerard S. Coyne,1,2 David A. Kenny,1,2 David E. MacHugh,2,3 and Dermot G. Morris4

1Teagasc, Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Grange, Dunsany, Co. Meath, Ireland; 2Animal Genomics Laboratory, UCD College of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin, Ireland; 3UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin, Ireland; and 4Teagasc, Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Mellows Campus, Athenry, Co. Galway, Ireland

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Reproductive performance is a critical factor influencing the productive and economic efficiency of beef and dairy herds (17). In lactating dairy cows in particular, a well-documented worldwide deterioration in reproductive performance has been observed (18). The modern high-producing dairy cow is therefore considered subfertile (56), and there is a growing impetus by animal scientists to characterize the underlying causes of this poor fertility and to develop strategies to improve reproductive performance in both beef and dairy cattle (21, 49, 56, 77). Nutrition is known to have a major impact on many aspects of the reproductive process in cattle (29), and recent evidence has suggested that supplemental fat may help to ameliorate the current decline in bovine fertility (87).

A number of studies demonstrate that supplementation with dietary n-3 polyunsaturated fatty acids (n-3 PUFA) may improve reproductive performance in cattle (1, 25, 47, 71). These fatty acids are believed to act as specific regulators of some key reproductive processes including ovarian function (48), steroidogenesis (8, 87), and suppression of uterine prostaglandin F2α (PGF2α) synthesis (62). The long chained (LC) n-3 PUFAs, eicosapentaenoic (EPA), and docosahexaenoic acids (DHA) are known to be regulators of gene transcription in many tissues (5, 86). Data from our own group has shown that n-3 PUFA supplementation resulted in an increase in systemic insulin-like growth factor (IGF)-1 and cholesterol concentrations and a decrease in the number of degenerate embroyos in beef heifers (8). Furthermore, our group has demonstrated that dietary inclusion with LC n-3 PUFA can regulate the expression of a number of genes in the uterine endometrium that may influence uterine function (13, 14) and consequently embryo survival.

Improving our knowledge of uterine endometrial function is key to understanding the factors, including diet, that influence both the establishment of pregnancy and the causes of early embryo loss. The endometrium, a glandular mucous membrane, secretes numerous growth factors and proteins into the lumen of the uterus, which in turn play a pivotal role in uterine-embryo interactions. Failure of an embryo to prevent luteolysis is a major cause of reproductive wastage in cattle (63). Embryonic interferon tau suppression of endometrial pulses of the luteolytic PGF2α is essential to the establishment of pregnancy (51). It has been suggested that diet-derived n-3 PUFA have the potential to positively influence pregnancy as they have an effect on many key enzymes involved in prostaglandin (PG) metabolism (13). Dietary PUFA can also influence the transcriptional regulation of a wide variety of genes (16) that may play an important role in the regulation of many biological and physiological processes that are key to normal uterine function. Therefore, the pattern of global gene expression in the endometrium in response to PUFA is important in understanding critical molecular pathways that influence biological functions controlling reproduction.

In a review of microarray technology, Evans et al. (20) identified numerous studies that implicated several cellular processes in the regulation of reproductive tissues in cattle. In bovine endometrium, microarray studies have analyzed changes in gene expression in pregnant versus cyclic animals (84), in fertile versus infertile cattle during pregnancy (83), and also in response to estradiol and/or progesterone treatment (24, 69).

The response of reproductive tissue to alterations in diet is likely to effect the expression of a large number of genes. However, with high-throughput transcriptomic approaches, there is little information available on the effect of dietary n-3 PUFA on the expression of genes involved in the control of fertility in the bovine uterine endometrium.
PUFA supplementation on expression profiles particularly those that affect fertility in the uterine endometrium of any species. In this study, a nonlactating beef heifer model was used to ascertain the effects of diet, independent of the metabolic stress associated with negative energy balance. Both diets were formulated to be isocaloric to establish the effects of n-3 PUFA independent of their role as energy substrates. Hence, the objective of this study was to determine, with microarray technology, the effects of dietary supplementation with n-3 PUFA on gene expression profiles involved in the regulation of fertility in uterine endometrial tissue of cattle.

**MATERIALS AND METHODS**

*Animals and experimental design.* Endometrial tissue was collected as part of the study of Childs et al. (8) and prepared as described by Coyne et al. (13). All procedures were carried out under license in accordance with European Community Directive 86-609-EC. In the current study, tissue from only the high n-3 PUFA and the control diets were used. In brief, in the former study estrus cycles were synchronized in reproductively normalnulliparous crossbred beef heifers by intramuscular administration of two injections of 500 µg of PGF2α analog (Cloprostanol, Estrumate; Schering-Plough, Hertfordshire, UK) 11 days apart. Twenty animals were offered a barley straw [1.4 kg dry matter (DM)], moulases (0.28 kg DM), and concentrate (5.50 kg DM) based ration. This was supplemented with either a ruminally protected source of palmitic acid (Palmit80; Trouw Nutrition, Belfast, Northern Ireland), which acted as the control diet (CON; n = 10), or a high n-3 PUFA diet (n-3 PUFA; n = 10) consisting of 275 g of a partially rumen protected n-3 PUFA (EPA/DHA, 1.5:1) enriched supplement based on fish oil (Trouw Nutrition, Belfast, Northern Ireland). Concentrations of fish oil in the total DM offered were 0 for the CON diet and 4.15% for the n-3 PUFA diet. Both diets were formulated to be isonitrogenous and isolipid (8% added lipid DM). Ingredient composition and the chemical analysis of the treatment diets, including their fatty acid concentration, have been outlined by Coyne et al. (13). All animals were individually fed their respective treatment diets for 45 days prior to slaughter and tissue collection.

*Endometrial tissue collection and fatty acid analysis.* Animals were slaughtered in a licensed abattoir (Jennings, Ballinrobe, Co. Mayo, Ireland) on day 17 (day 45 of the trial) of the synchronized estrus cycle. Intercanicular endometrial tissue was collected by peeling from the underlying uterine myometrium, and samples of ~4 cm² and weighing 2.5 g were dissected. Samples were rinsed with RNase-free phosphate-buffered saline (PBS) (Sigma-Aldrich Ireland, Dublin, Ireland), snap-frozen in liquid nitrogen and stored at ~80°C. The fatty acid concentrations of the endometrial tissue used in this study were previously reported (13). This latter study demonstrated that concentrations of total n-3 PUFA were twofold higher in tissue from animals in the n-3 PUFA group than the CON. Concentrations of EPA and DHA were seven (P < 0.001) and twofold (P < 0.05) higher, respectively, in the n-3 PUFA versus the CON group. The 10 heifers in each of the CON and n-3 PUFA dietary groups were assessed on the basis of their endometrial concentration of LC n-3 PUFA, initiation of estrus, plasma estradiol concentrations, and morphology of preovulatory follicle and corpus luteum (CL). In brief, in the study of Childs et al. (8), animals offered the high n-3 PUFA supplement consumed an additional 264 g of EPA and DHA combined compared with no added n-3 PUFA in the control cattle. This evoked a twofold increase in the total n-3 PUFA content of the uterine endometrial tissue of the high n-3 PUFA compared with the control animals (0.33 vs. 0.16 mg n-3 PUFA per g tissue, SE = 0.019). There was no effect of diet on onset and the mean timing of estrus for the two groups of animals selected for the current study. Equally, there was no effect of treatment on plasma concentrations of estradiol on days 10, 14, or 16 of the estrous cycle the endometrial tissue was collected. CL diameter (25.2 ± 3.48 and 23.2 ± 1.42 mm for CON and high n-3 PUFA groups, respectively) on the day of slaughter did not differ between diets (P > 0.05). In addition there was no notable difference in morphology of the CL and follicle between the two groups. Endometrial tissue from seven animals within each of the CON and n-3 PUFA treatment groups were selected, balanced on these criteria, and used for the current study.

*RNA extraction and quality analysis.* Frozen endometrial tissue was fragmented under sterile conditions with a hammer. The frozen tissue sample was rapidly weighed, and ~100 mg of tissue was placed in 3 mL of TRI Reagent (Sigma-Aldrich Ireland) and homogenized. Total RNA was isolated from the homogenate with chloroform and subsequently precipitated with isopropanol. RNA samples were stored at ~80°C. We carried out determination of RNA quantity by measuring absorbance at 260 nm on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). We verified RNA quality by ensuring all RNA samples had a ratio 260/280 between 1.8 and 2. We treated 20 µg of total RNA from each sample with RNase-free DNase to remove any contaminating genomic DNA and then purified it with the RNeasy mini kit (Qiagen, West Sussex, UK). We further assessed RNA quality and quantity by measuring the 18S/28S ratio and RNA integrity number (RIN) on an Agilent Bioanalyzer 2100 with the RNA 6000 Nano LabChip kit (Agilent Technologies Ireland, Dublin, Ireland). Samples of RNA with 28S/18S ratios ranging from 1.8 and 2.0 and RINs of between 8 and 10 were deemed high quality and suitable for microarray analysis.

*Microarray hybridization.* Expression profiling was carried out with a 24,027 probe set bovine oligonucleotide array (Affymetrix), representing ~23,000 bovine transcripts based on the original mapping using Unigene build 57 (March 24, 2004). RNA from each animal was hybridized to a separate array, and all 14 RNA samples were hybridized and scanned by the German Resource Centre for Genomics Research, Germany (RZPD), according to the manufacturer’s instructions.

*Microarray analysis.* All microarray analyses including pre-processing, normalization, and statistical analysis was carried out with R (R Core Team 2007) version 2.6 and Bioconductor (31) version 2.1, as described by Morris et al. (57) and McCarthy et al. (54). Data quality assessed before and after normalization using a number of in-built quality assessed control methods implemented in the Bioconductor affycoretools and associated packages to identify problems if they existed with microarray hybridization, RNA degradation, and data normalization. Microarray data from all 14 arrays were preprocessed by the mmg-MOS normalization method (40, 61) employing the default settings and differential expression (DE) was analyzed by the puma-DE method both implemented in the Bioconductor package “puma” (44, 60, 61, 67). The puma method uses a Bayesian hierarchical model to calculate the probability of positive likelihood ratio (PPLR) of differential expression. The PPLR associates probability values of genes being differentially expressed, which is a measure of the false positive detection of differential expression, to each ratio and generates lists of genes ranked by the probability of DE. The PPLR was converted into “P-like values” by the recommended formula prior to subsequent analysis.

As many of the original annotations for the Affymetrix bovine chip are erroneous (15, 27), the remapped annotations were determined using the “bovineidaipusv006d” chip definition file (CDF), which returned “Entrez Gene” gene name identifiers. This annotation is based on the CDF-Merger procedure as described by de Leeuw et al. (16), which generates a hybrid CDF based on the standard Affymetrix CDF (version 26) and the custom Brainarray (version 11.0.1) CDF. This remapped annotation includes mapping to all RefSeq (mature RNA protein coding transcripts and validated complete coding sequences in GenBank). Annotations were also supplemented by interrogating the Ensembl Bos taurus database version 46 using the biomart package in Bioconductor and manual annotation where possible with recent entries in Entrez Gene.
Network analysis of differentially expressed genes with IPA. To examine the molecular functions and genetic networks, the microarray data were further analyzed with Ingenuity Pathway Analysis (IPA ver. 7.5; Ingenuity Systems, Mountain View, CA; http://www.ingenuity.com), a web-based software application that enables identification of pathways and functions most relevant to experimental datasets or genes of interest.

A dataset containing gene identifiers and corresponding expression and P-like values was uploaded into the application as described by McCarthy et al. (54). The hybrid annotated gene set was used as background in IPA with both up- and downregulated genes analyzed together. Briefly, each identifier was mapped to its corresponding gene object in the Ingenuity knowledge base. A set of genes was selected as “focus” genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity. Network analysis returns a score that ranks networks according to their degree of relevance to the network eligible molecules in the dataset. The score takes into account the number of eligible molecules in the network and its size, as well as the total number of network eligible molecules analyzed and the total number of molecules in the knowledge base that could potentially be included in networks.

cDNA synthesis. Using the same RNA samples that were analyzed by microarray technology, we synthesized first-strand complementary DNA (cDNA) by reverse transcription of 1 μg of DNase-treated RNA from each sample using random hexamers and the High-Capacity cDNA Reverse Transcription Kit according to manufacturer’s instructions (Applied Biosystems, Warrington, UK). Samples were stored at −20°C for subsequent analyses.

Primer design. Gene specific primer sequences (Table 1) were designed with the Primer3 online program (http://frodo.wi.mit.edu/) based on bovine mRNA sequences available from the National Center for Biotechnology Information (NCBI) GenBank sequence repository (http://www.ncbi.nlm.nih.gov/Genbank/). Primer alignment specificity and compatibility were determined with the Basic Local Alignment Search Tool from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). All oligonucleotides were commercially synthesized as highly purified, salt-free products (Sigma-Aldrich Ireland). PCR products obtained by amplification with gene-specific primers were sequenced to verify their identity. In the case of all genes examined in this study, DNA sequences were 100% identical to published sequences.

Real-time quantitative reverse transcription PCR. For validation experiments, real-time quantitative reverse transcription PCR (qRT-PCR) assays were designed to measure the relative expression of a number of genes and to confirm the expression in the samples used for microarray analysis. Using RNA extracted from the same endometrial

Table 1. Bovine oligonucleotide forward and reverse primer sequences (5'-3'), PCR product length, and amplification efficiency

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Product Size, bp</th>
<th>Accession No.</th>
<th>Efficiency*</th>
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<td>CDC42SE1</td>
<td>F: 5'-CAGAGCTCAAGAAGCTTCAAG-3'</td>
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<td>NM_001035112.1</td>
<td>1.08</td>
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<tr>
<td></td>
<td>R: 5'-CTGTTGCTGGCTTGCTGCTG-3'</td>
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<td>COP57B</td>
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<td>NM_001046612.1</td>
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<td>CPN1</td>
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*10^8 (-1/slope).
samples used in the current study, we tested the stability of the expression of several putative reference genes, including 18S rRNA (RN18S1), actin, beta (ACTB), peptidylprolyl isomerase A [cyclophilin A] (PPIA), ubiquitin (UBQQA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ribosomal protein S9 (RPS9) as reference genes for real-time qRT-PCR analysis. All the primers were validated with a pooled cDNA sample. A standard curve was generated with serial dilutions of pooled cDNA. In the current study, three reference genes, namely RPS9, ACTB, and UBQQA, were used to normalize gene expression data. The principle behind the selection of the reference gene is that the expression ratio of two perfect reference genes should be constant across all samples. We examined the expression stability of the reference genes with the software program geNorm version 3.5 by calculating the gene expression stability measure (M value). With three reference genes, the M values was 0.16, which was below the default minimum coefficient of 1.5, and the pairwise variation V value was 0.08, which was below the threshold level of 0.15 as specified by the geNorm program (82).

The real-time qRT-PCR reactions were carried out in a 96-well plate format in a total volume of 20 μl, containing 1 μl cDNA (10–50 ng of RNA equivalents), 10 μl Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 8 μl nuclelease-free H2O2, and 1 μl forward and reverse primers (250–1,000 nM per primer). Assays were performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with the following cycling parameters: 95°C for 10 min and 40 cycles of 95°C for 1 s, 60°C for 1 min followed by amplicon dissociation (95°C for 1 s, 60°C for 1 min, 95°C for 15 s and 60°C for 15 s). Primer concentrations were optimized for each gene by titrating 250, 500, and 1,000 nM per primer. Dissociation curves were examined for the presence of a single PCR product. The amplification efficiency (E) of the real-time qRT-PCR reaction was calculated for each gene by creating a standard curve from twofold serial dilutions of cDNA. The slope for each set of standards was used to determine E = 10^(-1/Slope). Only primers with PCR efficiencies between 90 and 110% were used. Expression of each target gene was normalized to the reference genes to calculate relative gene expression values for each gene. Fold change in gene expression between the CON and the n-3 PUFA groups were calculated using the 2^-ΔΔCT method (45).

Statistical analysis. Gene expression levels were recorded as Ct values, i.e., the number of PCR cycles at which the fluorescence signal is detected above the threshold value. The software package GenEx 4.3.5 (Multid ID Analysis, Gothenburg, Sweden) was used to calculate the Ct values, normalization to reference genes, calculation of quantities relative to the average, and log transformation of the expression values for all genes. All data were analyzed using the Statistical Analysis Systems (SAS) statistical software package version 9.1 (SAS Institute, Cary, NC). Gene expression data were examined to determine whether they were normally distributed (PROC UNIVARIATE, SAS). Differences in mean relative gene expression values between the two groups (CON and n-3 PUFA) were analyzed by ANOVA (PROC MIXED, SAS) with a randomized block design. Animal within treatment was used as the error term. The Tukey critical difference test was used to determine statistical difference between CON and n-3 PUFA mean values. Mean differences of P < 0.05 were considered to be significant. Spearman correlations between microarray and real-time qRT-PCR data were determined using the CORR procedure of SAS.

RESULTS

Gene expression. A microarray platform consisting of 20,489 Hybrid CDF annotated genes (representing ~94% of the probe content on the array) was used to compare the effects of the high n-3 PUFA diet versus a CON diet on gene expression in the uterine endometrium. The microarray data have been deposited in NCBI’s Gene Expression Omnibus ( GEO) and are accessible through GEO Series accession number GSE35212, http://www.ncbi.nlm.nih.gov/projects/geos/query/acc.cgi?acc=GSE35212.

The gene with the highest positive fold change in n-3 PUFA-fed animals was protease serine 2 (PRSS2), which exhibited a fold change of +13.1 (P < 0.05). Major histocompatibility complex, class II, DQ, alpha 1 and 2 (BOLA-DQA1 and BOLA-DQB2) genes both appeared in the top five with a +4.4- and +4.3-fold higher abundance in n-3 PUFA-fed animals, respectively (P < 0.05). The gene that displayed the largest downregulation in n-3 PUFA-fed animals was phospholipase A2, group III (PLA2G3) with a fold change of −26.9 (P < 0.05).

Pathway analysis of differentially expressed genes with IPA. Gene expression data were further analyzed through the use of IPA. From the 20,489 Hybrid CDF annotated genes, a total of 11,299 genes were mapped to the IPA database. Among the 1,807 differentially expressed genes (DEG), 1,058 genes were mapped to the IPA database. 494 genes were upregulated (Supplementary Table S1) and 564 genes were downregulated (Supplementary Table S2) in n-3 PUFA-fed animals compared with control animals. A total of 890 DEG were suitable for generating networks in IPA. Within IPA, functions and pathways were not significant using a Benjamini Hochberg-corrected P value of 0.05. However, a total of 50 networks were identified by IPA, 47 of which had a score (>log (P value)) of 10 or greater, with a range of 13 to 32 focus genes among the DEG (Supplementary Table S3). Table 2 lists some of the important networks affected by LC PUFA supplementation selected on the basis of relevance to fertility.

Real-time qRT-PCR validation of microarray results. For verification of the microarray results a total of 17 genes were analyzed by real-time qRT-PCR (Table 3). Candidates were chosen to be representative of those genes that were top ranked in the microarray DEG list, genes that were not differentially expressed, and genes with known functional importance to the biological question. Among genes analyzed, the direction and magnitude of expression between methods were reasonably consistent, with only one DEG exhibiting an opposing direction of fold change between the two methods. There was a significant (P < 0.05) correlation in the measurement of gene expression between the two technologies for all genes analyzed except TCF7. In addition, there was a >50% correlation between microarray and real-time qRT-PCR analysis for 12 of the DEG (Table 3).

DISCUSSION

To our knowledge, this is the first study to explore the effect of dietary n-3 PUFA supplementation on global gene expression in the bovine endometrium. Previous research conducted on endometrial tissues harvested from this animal model (13, 14) focused on the expression of candidate genes with putative roles in reproductive processes. However, following n-3 PUFA supplementation biological changes are likely to be mediated by a coordinated alteration in the expression of a large number of genes affecting a number of processes in the endometrium. In the current study, IPA identified 47 networks containing >13 focus genes that were differentially expressed due to supplemental PUFA. The top-scoring network is “RNA post-transcriptional modification, carbohydrate metabolism, lipid metabolism.” The presence of “lipid metabolism” in the top
scoring network and in eight others would be expected considering that in the current study while both diets contained fat, DEG was based on animal’s diet being enriched with LC PUFA. Of particular interest is that three of the networks refer to “embryo development” in their function, indicating that dietary PUFA alters the expression of genes that potentially facilitate embryo development thus improving fertility. Indeed, many cellular processes potentially important in the control of reproduction in cattle were found to be altered in the endometrium due to supplemental PUFA. These included PG biosynthesis, steroidogenesis, transcription factor regulation, maternal immune response, and tissue remodeling.

**PG biosynthesis.** A review by Wathes et al. (87) highlights the potential effects of PUFA intake on PG biosynthesis, steroidogenesis, and transcriptional regulation as the mechanisms likely to influence reproduction. Research from our own group, using real-time qRT-PCR technology, previously demonstrated the effect of n-3 PUFA supplementation on key genes controlling series-2 PG biosynthesis in the bovine uterus (13). In the current study, a global analysis of endometrial gene

### Table 2. Gene networks identified following network analysis with IPA

<table>
<thead>
<tr>
<th>Functions</th>
<th>Molecules in Network</th>
<th>Score*</th>
<th>Focus Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA posttranscriptional modification, carbohydrate metabolism, lipid metabolism</td>
<td>ARHGAP21, ARRB1, C12orf51, C16orf80, CHMP1B, DCP1A, DUB, EDC3, ENSA, FRY, GNM1, ING2, Insulin, KIF2A, LAPT5M, MAGOB, NIF1L1, PLA1A, RAB19, RNF168 (includes EG:16591) B, SAFB, SFRS1, SFRS6, SKIL, SMURF2, SNRNP70, SRRM2, STAT5A, STAT5a/b, THRAP3, UCP2, USP8, USP16, YWHAQ, ZC3H1</td>
<td>44</td>
<td>32</td>
</tr>
<tr>
<td>Cellular assembly and organization, cellular function morphology and maintenance</td>
<td>14-3-3, Akt, AKT2, AMPK, Ccdd88A, Ccld2, CGN, Cknsr3, Dock7 (includes EG:85440), Fgcr1a2a3a, Foxo4, Ggig1, Grb10, Hdl, Hpsi, Hspb8, Icam2, Jm, Jm2, Mltst8, Mp, Ppp2rb, Prkrs, Rgl2, Rps6ka1, Rptor, Sas, Sncb, Socs4, Tbc1d4, Tgfr3, Tsc1, Tsc2</td>
<td>33</td>
<td>27</td>
</tr>
<tr>
<td>Developmental disorder, embryonic development, genetic disorder</td>
<td>Ankrd10, Ap, Arhi, Arl4D, Azini, C20orf30, Ccnb2, Cdc2, Cdc7, Crcl, Cyclin B, DNA-directed RNA polymerase, Eif4e2, Eif4ef1, F2, Fibrin, Fkbp5, Krt20, Mboat2, Peptidylprolyl isomerase rase, Polr2g, Polr3a, Ppg, Ppl1, Proteasome, Prss23, Rap, Rassf5, Rpl4, Tcof1, Ube2c, Ube2e2 (includes EG:7328), Ube2es, Ypel1</td>
<td>29</td>
<td>26</td>
</tr>
<tr>
<td>Cell-to-cell signaling and interaction, cell-mediated immune response, cellular growth and proliferation</td>
<td>Actr6, Anxa11, Atg5 (includes EG:9474), Atg6v0a2, Atg6v0a1e1, Atg6v1e1, Atg6v1h, Cblc, Cd3, Cd3e, Csde1, Fcer1, Fcer1c, Fby, Gag, H+_transporting two-sector ATPase, Hla-dr, Hla-drbl1, Hpr1, Hu51, Lact, Lcp2, Mapk, Nfat (complex), Plc gamma, Rap3, Smpd3, Sos, Spingomyelinase, Straps, Syk, Zap</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>Gene expression, embryonic cell death</td>
<td>Acvr1, Acvr2b, Adgfp, Cbp/p300, Cbp, Cyp26a1, Cyp7a1, Dhx9, Hoxb9, Iak, Jnk1/2, Mtc72, N-cor, Ncoa4, Peg10, Pias, Pias2, Rar, Rxr, Sirt2, Smad, Sma2/3-smad4, Sop, Stat, Swi-nf, T3-tr-rxr, Tbr2, Jtgfr, Tgif1, Thyroid hormone receptor, Ubxn1, Zbtb16</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Cell cycle, cellular assembly and organization, DNA replication, and repair</td>
<td>Casp4, Cd2b2, Ddx3x, Dmnt3b, Eno1, Ep300, Flrt2, Hnrrnp1, Igbkp2b1, Igbp3, Ilk, Kcnmb1, Mir182 (includes EG:406958), Mir20a (includes EG:406982), Mthfd1l, Ncapd2, Ncapd3, Ncapg2, Ncapg (includes EG:64151), Ncaph, Ncaph2, Ne6, Pcbp1 (includes EG:5093), Pcd2, Phlda3, Polr2a, Puf60, Ras1l1b, Rap1a, Sma2c, Smc2, Smc4, Smyd2, Vps26a, Ywhaq, Znf532</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Embryonic development, tissue development</td>
<td>Actb, Ap, Cbx5, Cds2, Ifi44, Ifi44i1, Ifi44i1, Elavl1, Fam174b (includes EG:400451), Fmr1, Ntr6d2, Gbx2, Gfi1b, Heca, Iffo1, Mir181a1, Mir214 (includes EG:406966), Mir29a (includes EG:407021), Mir301a, Mir361 (in cludes EG:404323), Nat12, Npy, Phc2, Rnf183, Scl25a28, Smg1, Snn, Tars2, Tmem70, Tnrc6c, Tp63, Usp1, Usp2, Usp10, Usp13 (includes EG:8975), Zmym3 (includes EG:9203)</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Cell-mediated immune response, embryonic development, antigen presentation</td>
<td>Atg3, Atg7, Atg4a, Capn8, Ccdc18, Fam134a, Gabaraapl2 (includes EG:11345), Herc5, Ifitm3, Ifna2, Ifna6, Ifna7, Ifna5 (includes EG:3442), Ifnz, Il15, Il18r1, Ira4, Irf7, Klrg1, Mir210 (includes EG:406992), Nme3, Nme4, Parp9, Rtn1, Tt3b, Sapa1, Synpo, Tbx21, Tmem53, Tor1b, Tntr1d1, Uba7, Ube2l6, Ulk3, Xaf1</td>
<td>11</td>
<td>14</td>
</tr>
</tbody>
</table>

*All networks had a score [log(P value)] of 10 or greater. The score is based on ranking of networks according to their degree of relevance to the network eligible molecules in the IPA dataset. For a full list of networks see Supplementary Table S3.*
expression by microarray data has comprehensively identified the effects of supplemental n-3 PUFA on the expression of genes involved in the regulation of PG biosynthesis, catabolism, transport, and receptor interaction.

The initial step in series-2 PG biosynthesis involves liberation of arachidonic acid (AA) from the sn-2 position of membrane phospholipids by the action of phospholipase A2 (PLA2) enzymes. In our previous study, real-time qRT-PCR analysis with identical RNA samples revealed a strong tendency for a decrease in mRNA expression of PLA2G2A (13). Numerous isoforms of PLA2 have been identified, and of the other PLA2 genes expressed on the microarray, the expression of PLA2G3 was found to be 26.9-fold downregulated by n-3 PUFA supplementation, displaying the single largest depression in the expression of any gene on the microarray. Studies have demonstrated that this enzyme is capable of enhancing the PG biosynthetic response in various cell types (58). The PLA2G3 enzyme, like PLA2G2A, belongs to the family of secreted PLA2 (sPLA2). Interestingly, in cells not expressing sPLA2, the majority of AA is mobilized by cytosolic PLA2 enzymes; however, in cells that express sPLA2, the majority of AA appears to be released by sPLA2 (6). An associated study from our laboratory found that endometrial AA concentrations were decreased in animals offered a high n-3 PUFA compared with a control diet (52). AA release has also been attributed to induction of the STAR protein is known to promote steroidogenesis (78). In the current study, we demonstrated the potential luteotrophic effects of dietary n-3 PUFA through increased mRNA expression of PTGES1, an enzyme critical in PGE2 production (13). The decreased expression of PTGR2 may provide further evidence of a preference for PGE2 production and activity following n-3 PUFA supplementation. However, the implication of a reduction in PTGR2 on PGE2 activity, if any, has to be considered and warrants further investigation.

The mRNA expression of the endothelin 1 gene (EDN1) and its gene for its primary receptor, endothelin receptor type A (EDNRA), was increased in the endometrium of n-3 PUFA-fed animals. Research has implicated endothelin-1 and EDNRA in increased PG production (70), and they were originally thought to be important in the mediation of PGF2α-induced luteolysis (55). In vitro studies have since demonstrated that endothelin-1 increases bovine luteal secretion of PGE2 (91), and it is now proposed that the effects of endothelin-1 are antiluteolytic rather than luteolytic by increasing the uterine PGE2/PGF2α ratio (89, 90). If this is the case, the n-3 PUFA-induced transcription of END1 and EDNRA observed here could further illustrate a shift to a more luteotropic environment in n-3 PUFA-fed animals.

**Steroidogenesis.** Steroid hormones are crucial for normal reproductive function (53), and the effect of PUFAs on steroidogenesis is another mechanism by which PUFAs are hypothesized to influence reproduction (87). An important rate-limiting step in steroid hormone production is the transport of cholesterol within the mitochondria by the steroidalogenic acute regulatory (STAR) protein (52). Studies have demonstrated that the luteolytic PGF2α can inhibit STAR gene expression in the CL and ovaries of various species (23). Conversely, the expression of STAR is augmented by PGE2 (2), and this has been shown to induce progesterone production in cultured endometrial stromal cells (81). Therefore, the increase in endometrial expression of STAR observed in the current study may indicate a reduction in local PGF2α and/or an increase in the luteotropic PGE2.

The transcriptional regulation of STAR is complex and multiple transcription factors have been implicated in its control (52). AA release has also been attributed to induction of STAR mRNA and protein expression (73). However, there is evidence of negative regulation of STAR gene expression through AA-mediated signaling pathways (85). Our findings suggest the latter, as we have previously demonstrated that AA concentrations were decreased in animals offered a high n-3 PUFA diet compared with a control diet (8). Coiled-coil alpha-helical rod protein 1 (CCHR1) through interaction with STAR protein is known to promote steroidogenesis (78). In the current study, microarray DEG analysis revealed that the expression of the gene encoding CCHR1 was also increased in n-3 PUFA-fed animals. This may suggest a role for CCHR1 in the upregulation of STAR expression and a potential increase in steroidogenesis in the endometrium of heifers supplemented with n-3 PUFA.

**Transcriptional regulation.** Dietary PUFA is known to mediate its effects on numerous molecular and cellular pathways through modulation of gene expression (39). Regulation of transcription factor activity is another mechanism through which PUFA can influence reproduction (87). PUFA can regulate gene expression through the activity of transcription

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**Table 3. Microarray validation with qPCR on selected genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change</th>
<th>P Value</th>
<th>Fold Change</th>
<th>P Value</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC43SE1</td>
<td>-2.18</td>
<td>0.036</td>
<td>-1.12</td>
<td>0.047</td>
<td>0.51</td>
</tr>
<tr>
<td>COPS7B</td>
<td>0.99</td>
<td>0.933</td>
<td>-1.00</td>
<td>0.960</td>
<td>0.25</td>
</tr>
<tr>
<td>CPN1</td>
<td>-2.47</td>
<td>0.031</td>
<td>-2.74</td>
<td>0.010</td>
<td>0.57</td>
</tr>
<tr>
<td>EDN1</td>
<td>1.39</td>
<td>0.016</td>
<td>1.31</td>
<td>0.003</td>
<td>0.57</td>
</tr>
<tr>
<td>ESR1</td>
<td>-2.41</td>
<td>0.023</td>
<td>-3.29</td>
<td>0.002</td>
<td>0.27</td>
</tr>
<tr>
<td>G0T1</td>
<td>-1.36</td>
<td>0.051</td>
<td>-1.77</td>
<td>&lt;0.001</td>
<td>0.52</td>
</tr>
<tr>
<td>INPP1</td>
<td>1.02</td>
<td>0.919</td>
<td>1.00</td>
<td>0.999</td>
<td>0.21</td>
</tr>
<tr>
<td>NPEPS</td>
<td>-1.08</td>
<td>0.240</td>
<td>1</td>
<td>0.999</td>
<td>0.25</td>
</tr>
<tr>
<td>PLIN2</td>
<td>-1.96</td>
<td>0.065</td>
<td>-2.06</td>
<td>&lt;0.001</td>
<td>0.64</td>
</tr>
<tr>
<td>PTGR2</td>
<td>-1.79</td>
<td>0.043</td>
<td>-1.20</td>
<td>0.025</td>
<td>0.62</td>
</tr>
<tr>
<td>SFRP2</td>
<td>2.71</td>
<td>0.023</td>
<td>2.23</td>
<td>&lt;0.001</td>
<td>0.71</td>
</tr>
<tr>
<td>SPAG9</td>
<td>-1.29</td>
<td>0.08</td>
<td>-1.36</td>
<td>&lt;0.001</td>
<td>0.69</td>
</tr>
<tr>
<td>SSFA2</td>
<td>-1.48</td>
<td>0.052</td>
<td>-1.25</td>
<td>0.001</td>
<td>0.68</td>
</tr>
<tr>
<td>STAR</td>
<td>2.25</td>
<td>0.021</td>
<td>1.34</td>
<td>&lt;0.001</td>
<td>0.22</td>
</tr>
<tr>
<td>TCF7</td>
<td>1.71</td>
<td>0.155</td>
<td>1.15</td>
<td>0.049</td>
<td>-0.03</td>
</tr>
<tr>
<td>WNT11</td>
<td>-2.72</td>
<td>0.052</td>
<td>-2.70</td>
<td>0.001</td>
<td>0.78</td>
</tr>
<tr>
<td>YWHAG</td>
<td>-1.42</td>
<td>0.045</td>
<td>-2.60</td>
<td>0.004</td>
<td>0.52</td>
</tr>
</tbody>
</table>
expression of uterus (14). In the current study we found a reduction in the mentation altered the expression of the IGF system in the changed due to n-3 PUFA supplementation. Using real-time qRT-PCR analysis (14), according to microarray ESR1 (65), and as in the current study similar to our previous suppression of production is unclear, as studies in cattle have suggested that real-time qRT-PCR has indicated that ESR1 was significantly downregulated in the n-3 PUFA-fed animals, while the expression of BOLA-DQA1 and BOLA-DQA2 was among the most upregulated genes in n-3 PUFA-fed animals, while the expression of BOLA-DQB1 was also significantly upregulated. The involvement of these molecules in transplant rejection has led to interest in their role in pregnancy (19), and there is evidence to suggest these genes could influence reproductive events (10, 11). The BOLA-DQA1 and BOLA-DQA2 genes encode for α- and β-chains of the heterodimer BOLADQ, a cell surface receptor important in immune recognition and antigen presentation. Furthermore, a microarray study revealed that C4 and SERPING1, which were differentially expressed in early pregnancy in the bovine endometrium, were involved in embryo-maternal immune modulation (3). Similarly our study found that C4 and a related SERPIN gene, SERPINF1, were differentially expressed, indicating a potential role for n-3 PUFA supplementation in providing an enhanced embryo-maternal response in cattle.

The complement system is a highly regulated network that consists of >30 proteins, and its activation results in a cascade of enzymatic reactions, known as complement activation pathways, which occur through three known pathways: alternate, classical, and lectin (42, 68). Activation of the complement system plays a crucial role in host defense and inflammation, resulting in opsonization of pathogens and their removal by phagocytes, as well as cell lysis (68). However, inappropriate complement activation can contribute to numerous diseases and pathological conditions (68). Previous studies have also found expression of complement system components in the endometrium during the window of implantation (84). Studies have implicated higher levels of numerous members of the complement system in recurrent abortions (75), and there is a hypothesis that either dysregulation of the complement system or higher synthesis of its components in the endometrium of women may be one of the reasons for unexplained, consecutive miscarriages (92).
In the current study, the expression of the genes encoding for complement component 1, q subcomponent binding protein (C1QB) and complement component 5 (C5), were both decreased in the endometrium of n-3 PUFA-fed animals. The C5 protein has been examined in rabbit uterine flushings in relation to reproductive state (37), and a functionally active complement system has been detected in mouse uterine secretion at the periovulatory stage (35). A study using a human endometrial cell line found C5 receptor protein expression significantly higher in subjects who experienced unexplained recurrent spontaneous abortions (43). Therefore, the downregulation of the complement system components as observed in the current study could have the beneficial effect of suppressing a complement-mediated immune response in the presence of fetal antigens. Consequently, the dietary n-3 PUFA modulation of the expression of both antigen presentation and complement-related genes may have a functional relevance in reproduction.

Tissue remodeling. Endometrial remodeling is crucial for successful pregnancy (3), and in our study, numerous genes associated with this process were differentially expressed following dietary n-3 PUFA intake. The expression of the matrix metalloproteinase (MMP) genes MMP2 and MMP3 was lower in n-3 PUFA-fed animals, with MMP3 displaying one of the greater fold changes between groups. MMP family members are involved in the breakdown of extracellular matrix (ECM) associated with endometrial remodeling around the implantation window (30). A recent microarray study found that severe negative energy balance resulted in higher levels of expression of several MMPs in the uterus of postpartum dairy cattle (88), while an in vitro study in BEND cells demonstrated that interferon-tau and progesterone may inhibit MMP2 production (31). Secreted phoshoprotein 1 (SPP1) is an ECM protein (37) that can regulate MMP2 expression and MMP3 enzyme activity (7, 22), and the expression of the SPP1 gene was lower in n-3 PUFA-fed animals. SPP1 is a multifunctional protein involved in a diverse array of biological processes in the uterus throughout the estrous cycle, and its regulation is thought to be important in the establishment of pregnancy (36). The SPP1 protein can also act as a ligand for the signaling receptor integrin alpha V beta 3 (αvβ3), and studies in cattle have suggested a role for integrin αvβ3 in the onset of luteolysis (41). Activation of integrin αvβ3 stimulates COX-derived PG production in coronary arterioles (32), and its activity can be increased by the presence of IGF-1 (50). Our group has previously demonstrated reduced IGFI expression in the endometrium of cattle following n-3 PUFA supplementation (14), and this, together with a reduction in SPP1 expression, could impact integrin αvβ3 signaling.

Transforming growth factor-β (TGF-β) members are abundantly expressed in the endometrium throughout the estrous cycle and are involved in a broad range of processes (38). This signaling pathway is associated with endometrial remodeling and immune function, which, as discussed earlier, could play a role in the establishment of pregnancy (69, 74, 84). Sugawara et al. (74) examined expression of TGF-β superfamily members including activin in bovine endometrial tissue. Specific expression of the activin gene was detected in the gravid horn of the endometrium during peri-implantation, and they suggest that the activity of TGF-β superfamily members including activin-like molecules plays a pivotal role in endometrial remodeling, which is an essential process in implantation and placentogenesis. TGF-β signaling is highly regulated, and in the present study numerous components of this pathway were differentially expressed, indicating a role for n-3 PUFA supplementation in their modulation. Our data also indicate that genes for numerous receptor types were differentially expressed, including activin A receptor, type I (ACVR1); activin A receptor type II B (ACVR2B); and transforming growth factor, beta receptor III (TGFB3). Furthermore, our study found that the CLDN5 gene was differentially expressed due to supplemental PUFA, while a related gene, CLDN4, is involved in tissue remodeling of the endometrium in early pregnancy (3). Moreover, 11 of the significant networks were involved in “cellular assembly and organisation,” “cellular growth and proliferation,” “cell development,” and “cell morphology,” functions that are intrinsically linked with tissue remodeling in the endometrium.

Comparison with related studies. In cattle, microarray studies have previously shown differences in endometrial gene expression between pregnant and cyclic animals (84) and between responses to estradiol and/ or progesterone treatment (24, 69). In a study by Salilew-Wondim et al. (66), microarrays were used to examine the utility of embryo and endometrial transcriptome signatures (on days 7 and 14 of the estrous cycle) as predictors of pregnancy success after embryo transfer. Gene expression differences between day 14 receptive and nonreceptive endometrium revealed differential expression of only 14 genes. In that study the solute carrier family 25 (aspartate/glutamate carrier), member 12 gene, SLC25A12 was found to be altered between receptive and nonreceptive endometrium (66), while in the current study, the related gene, SLC25A28, was differentially expressed in the endometrium of n-3 PUFA supplemented compared with control animals. Salilew-Wondim and colleagues (66) have also demonstrated that the GJAI gene, which codes for a gap junction protein, was differentially expressed between receptive and nonreceptive endometrium on day 14 and was also found to be altered in the current study. In a recent study by Walker et al. (83), gene expression in endometrial tissue of fertile versus infertile cows was examined on day 17 of pregnancy following embryo transfer on day 7 of the reproductive cycle. Similar to the present study they found an alteration in the expression of genes involved in the maternal immune response due to fertility state such as those of the complement system. Specifically, similar to the fertile pregnant animals in that study, in our study cattle supplemented with PUFA were also found to have increased expression of TLR3 in endometrial tissue.

Conclusion

In conclusion, the results presented here demonstrate that n-3 PUFA supplementation of cattle diets has a global impact on uterine endometrial gene expression. We have demonstrated that previously unreported genes and pathways are differentially affected by n-3 PUFA supplementation. We have provided further evidence to support findings of previous studies that associate dietary PUFA intake with aspects of reproductive physiology including PG biosynthesis, steriodogenesis, and transcription factor regulation. We have also observed an effect of n-3 PUFA supplementation on genes associated with maternal immune response and tissue remodeling, which has the
potential to influence a vast array of reproductive events, including implantation. The transcriptional regulation of numerous biological processes through n-3 PUFA supplementation may positively influence the uterine environment, and fortifying cattle diets with n-3 PUFA could provide new alternatives for the development of sustainable nutritionally focused management strategies to improve reproductive efficiency.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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