Effects of altering the ratio of dietary n-6 to n-3 fatty acids on spontaneous luteolysis in lactating dairy cows

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ABSTRACT

Objectives were to evaluate the effects of altering the dietary ratio of omega-6 (n-6) to omega-3 (n-3) fatty acids on the profile of fatty acids and expression of genes related to the prostaglandin biosynthesis on endometrial tissue, uterine secretion of PGF_{2α}, and timing of spontaneous luteolysis in dairy cows. Multiparous lactating Holstein cows (n = 45) were blocked based on milk yield and, within each block, assigned randomly to 1 of 3 dietary treatments at 14 d postpartum for 90 d. Diets were supplemented with a mixture of Ca salts of fish, safflower, and palm oils to create 3 different ratios of n-6 to n-3 fatty acids, namely R4, R5, and R6, which resulted in 3.9, 4.9, and 5.9 parts of n-6 to 1 part of n-3 fatty acids, respectively. Blood was sampled every 2 h from d 16 to 23 of the estrous cycle and assayed for concentrations of progesterone and the PGF_{2α} metabolite 13,14-dihydro-15-keto-PGF_{2α} (PGFM). In a subsequent estrous cycle, endometrial tissue was collected for biopsy on d 8 and endometrial fatty acids profile and gene expression were quantified. The proportion of arachidonic acid of the endometrial fatty acids increased as the dietary ratio n-6 to n-3 fatty acids increased (R4 = 9.05, R5 = 11.64, and R6 = 13.41%). On the other hand, proportions of eicosapentaenoic (R4 = 2.85, R5 = 2.14, and R6 = 2.02%) and docosahexaenoic (R4 = 3.30, R5 = 1.57, and R6 = 1.08%) decreased as the ratio of n-6 to n-3 fatty acids in the diet increased. Increasing the ratio of dietary n-6 to n-3 fatty acids increased mRNA expression of estrogen receptor 1, oxytocin receptor, cyclooxygenase 2, prostaglandin E and F synthases, and steroidogenic acute regulatory protein in endometrium, but decreased expression of peroxisome proliferator-activated receptor gamma and insulin-like growth factor-1. The changes in endometrium gene expression caused by dietary treatments

were associated with changes in the ratio of n-6 to n-3 fatty acids in the endometrium. As the ratio increased from R4 to R6, the number of PGFM pulses (R4 = 5.6, R5 = 4.3, and R6 = 3.8 ± 0.6 pulses; least squares means ± standard error of the means) decreased, but the amplitude of the greatest PGFM pulse increased (R4 = 226, R5 = 267, and R6 = 369 ± 38 pg/mL). Luteolysis by d 23 of the estrous cycle was observed in 79.6% of the cows (R4 = 11/14; R5 = 13/15; and R6 = 11/15) and day of spontaneous luteolysis did not differ among treatments (R4 = 20.8; R5 = 21.1; and R6 = 21.0 ± 0.4). Three pulses of PGFM was the best predictor of luteolysis in dairy cows. Collectively, supplying the same quantity of fatty acids in the diet of lactating dairy cows, but altering the ratio of n-6 to n-3 fatty acids, influenced the endometrial fatty acids profile and gene expression and altered the pattern of prostaglandin synthesis; however, the changes were not sufficient to alter the length of the estrous cycle.

Key words: dairy cow, fatty acid, luteolysis, prostaglandin

INTRODUCTION

Supplemental fat is generally incorporated into dairy cattle diets to increase energy intake while attempting to reduce body fat mobilization and fed during transition period to minimize the incidence of early-lactation disorders (Damgaard et al., 2013). Altering dietary fatty acids intake has been shown to influence various reproductive measures in dairy cattle. Staples et al. (1998) reported that fat supplementation has advantageous effects on reproductive performance of dairy cows, and the benefits of fat feeding go beyond the increase in energy intake. In fact, some of the positive effects seem to be influenced by the type of fatty acids fed (Santos et al., 2008).

High intake of omega-6 (n-6) fatty acids, such as linoleic acid (C18:2 *cis*-9, *cis*-12), has the potential to alter the profile of fatty acids of the phospholipids present in cell membranes, resulting in increased proportion

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of arachidonic acid (C20:4 *cis*-5,*cis*-8,*cis*-11,*cis*-14), which, in turn, might favor the synthesis of series 2 prostaglandin (PG) and eicosanoids (Silvestre et al., 2011), thereby favoring a more proinflammatory state (Calder, 2012). On the other hand, high intake of omega-3 (n-3) fatty acids, especially eicosapentaenoic acid (EPA; C20:5 *cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17) and docosahexaenoic acid (DHA; C22:6 *cis*-5,*cis*-8,*cis*-11,*cis*-13,*cis*-16,*cis*-19), would increase the proportion of these fatty acids in membrane phospholipids, which are expected to decrease synthesis of series 2 PG and eicosanoids (Mattos et al., 2004), thereby exerting anti-inflammatory properties (Calder, 2012). It has been postulated that attenuation of PGF_{2α} synthesis by feeding n-3 fatty acids might be one of the mechanisms by which these fatty acids benefit reproduction (Mattos et al., 2000; Santos et al., 2008).

Silvestre et al. (2011) reported improved pregnancy and decreased pregnancy loss for cows fed Ca salts enriched with fish oil during the breeding period compared with cows fed Ca salts of palm oil composed of mostly SFA or MUFA. The fertility benefit from feeding n-3 fatty acids in the breeding period was enhanced when cows were fed Ca salts rich in n-6 fatty acids during late gestation and early lactation (Silvestre et al., 2011). More recently, Sinedino et al. (2017) showed that incorporating 10 g of DHA into the diet of dairy cows increased pregnancy per AI and rate of pregnancy. Those authors suggested that increasing dietary n-3 fatty acids may influence embryo development based on changes in interferon-stimulated gene expression when DHA was fed, as fatty acids are important regulators of conceptus development (Ribeiro et al., 2016); however, it also has been speculated that fatty acids might influence luteal lifespan (Mattos et al., 2000; Santos et al., 2008), which could allow less developed conceptuses to develop and establish the needed cross-talk to inhibit the pulsatile uterine secretion of PGF_{2α} and luteolysis. In support of this concept, in vitro production of PGF_{2α} by bovine endometrial cells was suppressed when the culture media was supplemented with n-3 fatty acids (Mattos et al., 2003). Indeed, when both n-6 and n-3 fatty acids were present in the culture media, increasing the ratio of n-6 to n-3 fatty acids increased the synthesis of PGF_{2α} (Caldari-Torres et al., 2006). Similarly, cows on d 15 of the estrous cycle challenged with oxytocin had increased PGF_{2α} release when fed diets with increased ratio of n-6 to n-3 fatty acids (Mattos et al., 2002; Petit et al., 2004; Dirandeh et al., 2013). In randomized experiments in humans, consumption of 2.7 g/d of n-3 fatty acids extended gestation length (Olsen et al., 1992), presumably by altering the balance of stimulatory and inhibitory PG that influence

uterine contractility and parturition. Nevertheless, it is unknown if altering the diet of cattle by supplementing either more n-6 or more n-3 fatty acids is capable of influencing luteal lifespan that might have implications to fertility.

The hypotheses of the current experiment were that diets containing different ratios of n-6 to n-3 fatty acids would alter the endometrial fatty acids profile and the expression of genes involved in the luteolytic signaling, thereby affecting the spontaneous pulsatile pattern of PGF_{2α} release that could influence luteal lifespan and the length of the estrous cycle. Our objectives were to determine the effects of altering the ratio of n-6 to n-3 fatty acids in the diet of Holstein cows on the fatty acids profile and expression of genes related to PG biosynthesis in endometrial tissue and timing of spontaneous luteolysis.

MATERIALS AND METHODS

The experiment was conducted at University of Florida Dairy Unit (Hague) from December 2011 to July 2012. All procedures for animal handling and care were approved by the University of Florida Animal Care and Use Committee (ARC # 014-11ANS).

Experimental Design, Cows, Housing, and Feeding

Details referring to cows, facilities, chemical composition of ingredients, and the respective profiles of fatty acids were described elsewhere (Greco et al., 2015). Briefly, weekly cohorts of cows in their second (n = 23) or greater lactation (n = 22) were blocked by parity (2 vs. >2) and milk yield from d 6 to 10 postpartum and, within each block, randomly assigned to 1 of 3 dietary treatments at 14 d postpartum (n = 15/treatment). The diets were offered as a TMR twice daily at 0730 and 1300 h, and the ratios of n-6 to n-3 fatty acids were manipulated by altering the supplemental fat added to the diet. The first ratio was of 3.9 parts of n-6 to 1 part of n-3 fatty acids in the diet (**R4**). The second ratio was of 4.9 parts of n-6 to 1 part of n-3 in the diet (**R5**). The final ratio was of 5.9 parts of n-6 to 1 part of n-3 in the diet (**R6**). All diets were isocaloric and isonitrogenous, contained the same total concentration of fatty acids, and were formulated to minimize the difference in concentrations of total PUFA (Table 1). Calcium salts enriched in palm oil fatty acids, safflower oil fatty acids, or fish oil fatty acids were used to manipulate the concentration of n-6 and n-3 fatty acids in the diets. These Ca salts were blended such that they represented 1.43% of the diet DM. The amount of supplemental fatty acids was a reasonable quantity

fed to early-lactation cows to alter tissue fatty acid composition, but also to minimize the risk of milk fat depression. This was particularly important because EPA and DHA, known to be potent disruptors of rumen biohydrogenation of UFA, favor the synthesis of CLA that suppress lipogenesis (Whitlock et al., 2002). The selected amount is similar to that fed to lactating cows by Silvestre et al. (2011) that showed changes in tissue composition of fatty acids and improvements in reproduction because of reduced pregnancy loss.

Estrous Cycle Synchronization to Characterize Spontaneous Luteolysis

Estrous cycles were synchronized (Figure 1) starting at 40 ± 3 DIM with an i.m. injection of 25 mg of PGF_{2α} (dinoprost tromethamine; Lutalyse sterile solution, Zoetis, Florham Park, NJ) followed by an i.m. injection of 100 µg of GnRH (gonadorelin hydrochloride; Factrel, Zoetis) administered 2 d later, at 42 DIM. Seven days later, at 49 DIM, cows received a GnRH injection, and

Table 1. Dietary ingredients and nutrient composition of diets

Item	Treatment ¹		
	R4	R5	R6
Ingredient, % of DM			
Corn silage	18.7	18.7	18.7
Bermuda silage	9.0	9.0	9.0
Alfalfa hay	6.1	6.1	6.1
Corn grain, finely ground	13.8	13.8	13.8
Citrus pulp	10.1	10.1	10.1
Soybean hulls	20.3	20.3	20.3
Soybean meal, solvent extract	10.1	10.1	10.1
Soybean meal, heat-treated ²	5.7	5.7	5.7
Molasses	1.6	1.6	1.6
Vitamin-mineral-protein premix ³	3.0	3.0	3.0
Ca salts of palm oil ⁴	0.73	0.65	0.53
Ca salts of safflower oil ⁵	0	0.37	0.70
Ca salts of fish oil ⁶	0.70	0.41	0.20
Nutrient, DM basis (±SD)			
NE _L , ⁷ Mcal/kg	1.62	1.62	1.62
CP, %	16.6 ± 0.8	16.6 ± 0.8	16.5 ± 0.8
Starch, %	17.3	17.3	17.3
NFC, ⁸ %	35.4 ± 1.9	35.4 ± 2.0	35.5 ± 1.9
ADF, %	16.0 ± 0.9	15.9 ± 0.9	15.6 ± 0.9
NDF, %	38.4 ± 2.3	38.4 ± 2.3	38.1 ± 2.3
NDF from forage, %	17.1 ± 0.7	17.1 ± 0.7	17.1 ± 0.7
Total fatty acids, %	3.66 ± 0.15	3.82 ± 0.17	3.88 ± 0.16
Total n-6 fatty acids, %	1.260 ± 0.10	1.447 ± 0.08	1.587 ± 0.08
Total n-3 fatty acids, %	0.327 ± 0.01	0.298 ± 0.01	0.269 ± 0.01
Ratio of n-6 to n-3	3.9	4.9	5.9
Ca, %	0.87 ± 0.07	0.89 ± 0.07	0.90 ± 0.08
P, %	0.33 ± 0.03	0.33 ± 0.03	0.33 ± 0.03
Mg, %	0.31 ± 0.02	0.31 ± 0.02	0.31 ± 0.02
K, %	1.48 ± 0.05	1.48 ± 0.05	1.48 ± 0.05
Cl, %	0.29 ± 0.03	0.28 ± 0.03	0.29 ± 0.03
Na, %	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.01

¹Treatments represent the ratio between n-6 and n-3 fatty acids in the diet. R4 is a ratio of 3.9 parts of n-6 to 1 part of n-3; R5 is a ratio of 4.9 parts of n-6 to 1 part of n-3; R6 is a ratio of 5.9 parts of n-6 to 1 part of n-3.

²AminoPlus (Ag Processing Inc., Omaha, NE).

³Contains (DM basis) 30.0% ProvAA1 LysAAMet (blend of blood meal and protected lysine and methionine, Venture Milling, Salisbury, MD), 28.5% sodium sesquicarbonate, 13.0% potassium carbonate, 7.0% dicalcium phosphate, 7.0% magnesium oxide, 3.5% sodium chloride, 1.2% Availa-4 (Zinpro Co., Eden Prairie, MN), 0.3% Sel-Plex 2000 (Alltech Biotechnology, Nicholasville, KY), 0.06% vitamin trace mineral premix, and 0.22% Rumensin 90 (Elanco Animal Health, Greenfield, IN). Each kg contains 27.8% CP, 5.2% Ca, 1.6% P, 4.1% Mg, 6.8% K, 10.7% Na, 2.3% Cl, 680 mg of Zn, 235 mg of Cu, 422 mg of Mn, 6.6 mg of Se, 23 mg of Co, 13.8 mg of I, 116,000 IU of vitamin A, 35,000 IU of vitamin D, 1,170 IU of vitamin E, and 450 mg of monensin.

⁴EnerGII, Ca salts of palm oil fatty acids (Virtus Nutrition, Corcoran, CA).

⁵Prequel21, Ca salts enriched in safflower oil fatty acids (Virtus Nutrition).

⁶StrataG113, Ca salts enriched in fish oil fatty acids (Virtus Nutrition).

⁷NE_L according to NRC (2001) using analyzed feed values and calculated at 24 kg of DMI/d.

⁸Calculated as 100 – (NDF + CP + fatty acids + ash).

Figure 1. Diagram of estrous cycle synchronization and blood sampling. Biopsy = endometrial tissue collected for biopsy. BS = blood sampling; CIDR = controlled internal drug-release containing 1.38 g of progesterone; GnRH = i.m. injection of 100 µg of gonadorelin; PGF_{2α} = i.m. injection of 25 mg of PGF_{2α}; US = ultrasonography of the ovaries.

then 12 h after the GnRH a controlled internal drug-release (**CIDR**; Eazi-Breed CIDR, Zoetis) insert containing 1.38 g of progesterone for intravaginal use. The CIDR insert was kept for 6.5 d, until 56 DIM. One hour after the CIDR removal, cows received an i.m. injection of PGF_{2α} followed by another injection of PGF_{2α} 24 h later. A GnRH injection was administered at 58 DIM to induce ovulation and this day was considered d 0 of the estrous cycle, which was confirmed by ultrasonography based on disappearance of a preovulatory follicle within 48 h of treatment. Cows that did not ovulate to the final GnRH were re-enrolled in the same protocol for synchronization of the estrous cycle.

Progesterone Clearance After an Injection of PGF_{2α}

At 56 DIM, 2 h after the CIDR removal (Figure 1), cows received an injection of 25 mg of PGF_{2α} and blood was sampled to evaluate progesterone clearance. A rectangle of approximately 15 × 5 cm, longitudinal to the mammary vein (i.e., caudal superficial epigastric vein) in both sides of the cow was shaved, cleaned, and disinfected with 70% alcohol solution. Blood was withdrawn in evacuated tubes containing K₂EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) at 0, 0.5, 1, 2, 4, 6, 12, and 24 h relative to PGF_{2α} injection, alternating between the sides of the cow between samples. Samples were immediately placed on ice, and within 30 min plasma was separated by centrifugation at 2,095 × *g* for 15 min at 7°C and then stored at -20°C for further

analyses. Progesterone concentrations were analyzed by RIA using a commercial kit (Coat-a-Count, Siemens Healthcare Diagnostics, Los Angeles, CA). The sensitivity of the assay was 0.05 ng/mL, calculated at 2 standard deviations below the mean counts per minute at maximum binding. Plasma samples containing moderate (1.6 ng/mL) or high (7.4 ng/mL) concentrations of progesterone were included throughout all assays and used to calculate the coefficients of variation. The intra-assay coefficients of variation were 5.4 and 4.4% and the interassay coefficients of variation were 6.6 and 5.3%, respectively, for the moderate and high concentration control samples.

Pharmacologically Induced Luteolysis

To characterize the pharmacologically induced luteolysis, 2 nonlactating, nonpregnant Holstein cows in mid diestrus bearing a functional corpus luteum (**CL**) were selected. An indwelling catheter was placed in the jugular vein 3 h before sampling started. Two blood samples were collected at 30 min and immediately before cows had been given 25 mg of PGF_{2α} i.m. Blood was then sampled at 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105, 120, 150, 180, 210, 240, 270, 300, 330, and 360 min relative to PGF_{2α} injection. Samples continued to be collected at 2-h intervals until 24 h, and then every 6 h until 48 h of the PGF_{2α} injection. All blood samples collected were immediately placed in ice and plasma was separated by centrifugation (2,095 × *g* for

15 min at 7°C), harvested, and stored at -20°C until analyses. Progesterone concentrations were measured as described above. Concentrations of the PGF_{2α} metabolite 13,14-dihydro-15-keto-PGF_{2α} (PGFM) were quantified by an enzyme immune assay as described by Ginther et al. (2010). The intra- and interassay coefficients of variation were 9.1 and 10.6%, respectively.

Spontaneous Luteolysis and Endogenous Release of PGF_{2α} Based on PGFM

On d 15 of the estrous cycle, when cows were approximately 73 DIM (Figure 1), an indwelling catheter was placed in the tail vessel following the technique described by Sears et al. (1978). Briefly, the ventral base of the tail was surgically cleaned with soap and disinfected with alcohol. The ventral vertebral groove, where the coccygeal vessels lay, was manually identified and, at approximately 5 cm from the base of the tail, the vessel was punctured with a thin wall 14 gauge × 5 cm needle (Monoject, Mansfield, MA). Better access to the vessel was achieved when the angle between the tail and the needle was nearly 60°. Once copious amount of blood started flowing through the needle, approximately 30 cm of tubing (polyethylene tubing, sterile, PE90, 0.86 mm ID, 1.70 mm OD; Becton Dickinson) was inserted in through the vessel, cranially. The needle was then removed from the tail and a blunt cannula (18 gauge × 2.5 cm; Monoject) was attached to a PRN 0.1-mL adapter (Becton Dickinson) connected to the extremity of the tubing to seal the tubing. The entire system was flushed with 10 mL of sterile heparinized saline solution (50 IU/mL, Sigma-Aldrich, Saint Louis, MO). A cotton patch was wrapped around the tail, avoiding contact of the external part of the tubing and the skin of the animal, and the tubing was placed between 2 layers of cotton patch. An extra protection wrap with self-adhesive plastic bandage (Sure Flexx, Stone Mfg. & Supply co. Inc., Kansa City, MO) was placed to protect from humidity and dirtiness. The bandage was stapled on to the skin to avoid the wrapping falling and misplacing the catheter. To protect against feces and urine, the tail was dressed with a pair of handleless sleeves glued to the base of the tail. Finally, a blood sample was taken to verify the catheter was working, and then tubing was rinsed with 15 mL of the heparinized saline solution.

Serial blood samplings, starting at 0600 h on d 16 of the estrous cycle, were collected every 2 h for 7 d until d 23 of the cycle (Figure 1). Blood was withdrawn using a 12-mL syringe (Monoject), the first 20 mL of blood and saline was discarded, and 8 mL of blood was then collected and immediately transferred to an evacuated

tube containing K₂EDTA (Vacutainer, Becton Dickinson), mixed by carefully swirling the tube, and placed in ice bath. Samples were centrifuged at 2,095 × *g* for 15 min at 7°C and plasma was harvested and stored at -20°C for further analyses. Progesterone and PGFM concentrations were measured following the procedures described above.

Estrous Cycle Synchronization for Endometrial Tissue Collection for Biopsy

On d 5 of a new estrous cycle, when cows were approximately 85 DIM, ovaries were scanned by ultrasonography for detection of a CL and measurements of the largest follicle. Cows with a visible CL received 2 i.m. injections of PGF_{2α} 24 h apart, followed by a GnRH injection 2 d later (Figure 1). The GnRH injection was considered d 0 of the estrous cycle based on ovulation of a follicle confirmed by ultrasonography. Twelve hours after the GnRH injection, cows received a CIDR insert that was maintained until d 6 of the estrous cycle. Progesterone was supplemented through the use of CIDR during metestrus and early diestrus to increase the incorporation of fatty acids in the endometrium, which would resemble late diestrus when luteolysis occurs (Brinsfield and Hawk, 1973; Boshier et al., 1987).

On d 8 of the estrous cycle, endometrial tissue was collected for biopsy from the ipsilateral uterine horn to the CL. Briefly, the cow was restrained in a chute and received an epidural block with 4 mL of lidocaine hydrochloride 2% solution (Lidocaine Hydrochloride Injection 2%, Agrilabs, St. Joseph, MO). The vulvar area was thoroughly cleaned with a 7.5% povidone-iodine soap scrub (Povidone Iodine Scrub, First Priority Inc., Elgin, IL) and rinsed with alcohol. The vaginal area was inspected and cleansed with gauze pads embedded in diluted chlorhexidine solution (2% chlorhexidine gluconate, First Priority) to remove any manure or deposited mucus. The reproductive tract was transrectally palpated to locate the cervix and the uterine horns. A sterilized endometrial forceps (Miltex Hi-Light forceps 30-1485 Eppendorfer, Miltrex, York, PA) was introduced in the closed position through the vagina and cervix and positioned midway through the horn ipsilateral to the CL. The forceps was placed carefully in position to avoid any damage to the uterine wall. The forceps jaw was opened and, with the hand positioned in the rectum of the cow, the endometrium was pressed against the lower jaw and the handle closed to sample the tissue. The forceps was removed through the cervix and vagina and the tissue removed and placed on filter paper and rinsed with sterile PBS

to remove any blood. The specimen was split in 2 and placed in RNase-free tubes with RNA later solution (Fischer Scientific, Hampton, NH). Tubes were plunged in liquid N, transported to the laboratory, and stored at -80°C for later analyses.

Endometrium Fatty Acid Profile and Gene Expression

Endometrial tissue was analyzed at the Clinical and Translational Science Institute – Biomedical Mass Spectrometry Core, University of Florida (Gainesville). Briefly, endometrial tissue was homogenized and lipids extracted as previously described (Bligh and Dyer, 1959). The fatty acid *cis*-10 C17:1 was added as the internal standard. Fatty acid methyl esters were prepared by a modification of the 2-step procedure of Nuernberg et al. (2007) using a combination of sodium methoxide followed by boron trifluoride in methanol. Fatty acid methyl esters were quantified using an Agilent 5973 GC-MS (Agilent, Framingham, TX), equipped with a Trace TR-5MS column ($30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$, Thermo Scientific, Rockford, IL). One microliter of sample was injected via the autosampler into the column. The oven temperature was set initially at 150°C for 2 min, increased by $5^{\circ}\text{C}/\text{min}$ up to 320°C , and held for 8 min. The MS was set to full scan, m/z 30 to 600, at 2.6 scan/sec.

A second specimen of endometrial tissue was used to quantify gene expression. The total RNA was extracted using Trizol reagent (Sigma-Aldrich) with the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA), according to instructions provided by the manufacturer (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/purelink_rna_mini_kit_man.pdf). Following extraction, concentration and purity of isolated RNA were assessed using a NanoDrop 200 spectrophotometer (Thermo Scientific). The DNase (Applied Biosystems, Foster City, CA) treatment was applied for 30 min at 37°C to remove genomic DNA and subsequently heat-denatured at 75°C for 15 min. Total RNA (250 ng/reaction) was reverse transcribed to complementary DNA using the high-capacity cDNA Reverse Transcriptase kit (Applied Biosystems) following manufacturer's instructions (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_042557.pdf). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was chosen as the reference gene. The list of genes and primers used are depicted in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-15065>). Genes of interest included those involved in PG metabolism, such as estrogen receptor 1 (*ESR1*), oxytocin receptor (*OXT*), cyclooxygenase 1 (*COX1*) and 2 (*COX2*), PGE (*PGES*) and PGF

(*PGFS*) synthase, and PGE₂ 9-ketoreductase (*CBR1*); steroid and fatty acid metabolism, steroidogenic acute regulatory protein (*STAR*), peroxisome proliferator-activated receptor α (*PPARA*), *PPARG*, and *PPARD*; and genes involved in the growth hormone or insulin-like growth factor axis, growth hormone receptor-1A (*GHR1A*), insulin-like growth factor-1 (*IGF1*), and insulin-like growth factor binding protein-3 (*IGFBP3*). Quantitative, reverse transcription-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). The cycling conditions applied were activation/denaturation at 60°C for 2 min and 95°C for 10 min; 40 cycles of 2 steps amplification protocol at 95°C for 15 s and 60°C for 1 min; and dissociation at 55 to 95°C . Primer efficiency ranged from 94 to 103%. Melting curve analysis and agarose gel of PCR products were performed and demonstrated the amplification of a single product of expected size for all genes investigated. Each PCR was performed in triplicate and the abundances of the selected genes were calculated using the cycle threshold (*Ct*) relative to the *Ct* of *GAPDH*, which was stable in all treatments. Data are presented using the comparative method developed by Livak and Schmittgen (2001) using treatment R4 as the reference for relative expression of mRNA abundance.

Calculations

Spontaneous luteolysis was considered when progesterone concentration dropped below 1.0 ng/mL in sequential blood samples (Mann and Lamming, 2006; Ginther et al., 2007). A pulse of PGFM was defined when concentration in plasma was greater than the mean basal concentration plus 2 standard deviations (Mann and Lamming, 2006). Basal PGFM concentration was calculated as the average of all PGFM values until a pulse was detected. Area under the curve of PGFM pulses was calculated following the formula described by Pruessner et al. (2003).

To characterize spontaneous luteolysis, the events were retrospectively subdivided based on changes in concentrations of progesterone as preluteolytic, luteolytic, and postluteolytic periods according to Ginther et al. (2007). Briefly, the luteolytic period was defined as the period of progressive decrease in progesterone concentration down to 1.0 ng/mL. The preluteolytic period was considered the 24 h preceding the luteolytic period. The postluteolytic period was considered the 36 h after progesterone concentrations declined below 1.0 ng/mL (Ginther et al., 2007).

The number of PGF_{2 α} pulses needed to elicit luteolysis was calculated using the estimates of agreement among the numbers of pulses of PGFM to predict luteal

regression. The gold standard used was concentration of progesterone below 1.0 ng/mL in sequential samples. Sensitivity (**Se**), specificity (**Sp**), positive predictive value (**PPV**), negative predictive value (**NPV**), and accuracy were calculated.

The Se was expressed as number of cows correctly detected with luteolysis based on the respective number of pulses of PGFM divided by the number of cows that underwent luteolysis based on progesterone concentration [(number of cows correctly detected with luteolysis using pulses of PGFM/number of all cows with luteolysis) $\times$ 100; i.e., truly positive test/(truly positive test + false negative test)]. The Sp was expressed as number of cows correctly identified without luteolysis based on the respective number of pulses of PGFM divided by the number of cows that did not undergo luteolysis based on progesterone concentration [(number of cows correctly detected without luteolysis using pulses of PGFM/number of all cows without luteolysis) $\times$ 100; i.e., truly negative test/(truly negative test + false positive test)]. The PPV was calculated as the number of cows correctly identified with luteolysis based on the respective number of pulses of PGFM divided by all cows detected with luteolysis [(number of cows correctly detected with luteolysis based on the respective number of PGFM pulses/number of all cows detected with luteolysis based on the respective number of PGFM pulses) $\times$ 100; i.e., truly positive test/(truly positive test + false positive)]. The NPV was calculated as the number of cows correctly identified without luteolysis based on the respective number of pulses of PGFM divided by all cows detected without luteolysis [(number of cows correctly detected without luteolysis based on the respective number of PGFM pulses/number of all cows detected without luteolysis based on the respective number of PGFM pulses) $\times$ 100; i.e., truly negative test/(truly negative + false negative)]. Accuracy was calculated as the proportion of correct outcomes [(number of correct tests based on the respective number of PGFM pulses/number of total tests performed) $\times$ 100].

Statistical Analysis

Data were analyzed using the GLIMMIX procedure of SAS (SAS ver. 9.4, SAS Inst. Inc., Cary, NC) fitting either a Gaussian or Poisson distribution according to the type of data. Tests for normality of residuals and homogeneity of variances were conducted for each dependent variable subjected to ANOVA after model fit. Transformation was applied when needed before statistical analyses to normalize the residuals or stabilize

the variance. Data with repeated measurements over time within the same experimental unit were analyzed with cow nested within treatment as a random effect for testing the effects of treatment. All models included the fixed effects of treatment, parity (2 or >2), time, and interactions between treatment and time, treatment and parity, and treatment and parity and time. The time reference for the models was hour or day relative to an event. Polynomial orthogonal contrasts were performed to determine linear or quadratic effects of altering the ratios of n-6 to n-3 fatty acids in the diet. The covariance structure (compound symmetry, heterogeneous compound symmetry, autoregressive 1, heterogeneous autoregressive 1, Toeplitz) that resulted in the lowest correct Akaike's information criterion was selected as best fit for each model. For unequally spaced measurements, the spatial power covariance structure was used. When a single measurement was determined for each cow, the model included the effects of treatment and parity.

Data from quantitative PCR were analyzed with a mixed model using the results of delta Ct. The statistical models included the fixed effect of treatment and the random effects of block and plate used in the PCR assays. The delta-delta Ct were obtained from delta Ct least squares means differences of pairwise comparisons among treatments and the reference treatment R4 (Yuan et al., 2006). The relative expression of each gene for each treatment was obtained by raising the PCR amplification efficiency ($E = 2$) to the power of delta-delta Ct (Yuan et al., 2006) to express the fold change relative to the reference treatment. Confidence limits were computed for graphical representation of relative expression from the lower and upper confidence interval after adjustment by the method of Tukey obtained for delta Ct least squares means differences described by Yuan et al. (2006). Additional analyses of gene expression were performed using endometrial fatty acids as the explanatory variable. The fatty acids in the endometrial were categorized into 3 percentiles based on the observed proportion of n-6 to n-3 fatty acids as low (mean = 4.45, range = 2.65 to 5.6), medium (mean = 8.81, range = 8.12 to 9.91), or high (mean = 11.10, range = 10.06 to 14.27) ratio.

The agreement between the number of PGF_{2 α} pulses needed to elicit CL regression with the proportion of cows undergoing or not luteolysis was calculated by kappa statistic in PROC FREQ of SAS. The kappa value and the respective 95% confidence intervals are depicted. Statistical significance was declared when $P \leq 0.05$ and tendency to differ when $0.05 < P \leq 0.10$. Results are presented as least squares means $\pm$ standard error of the means.

RESULTS

Detailed descriptions of DMI and lactation performance are presented elsewhere (Greco et al., 2015). The intake of fatty acids did not differ among treatments and averaged 953 ± 24 g/d. Increasing the ratio of n-6 to n-3 fatty acids in the diet increased ($P < 0.001$) the intake of linoleic acid ($R4 = 298.1$, $R5 = 329.5$, and $R6 = 369.4$ g/d), but decreased ($P < 0.001$) that of EPA and DHA combined ($R4 = 21.3$, $R5 = 14.9$, and $R6 = 10.0$ g/d).

Concentrations of PGFM after PGF_{2α} Injection

Administration of 25 mg of PGF_{2α} generated an enormous pulse of PGFM in plasma detected within 5 min after the injection (Figure 2). Concentrations of PGFM reached almost 4,000 pg/mL in cow 7432 and over 6,000 pg/mL in cow 15891. Both cows experienced CL regression and concentrations of progesterone dropped below 1.0 ng/mL within 24 h and remained below 0.5 ng/mL at 48 h of PGF_{2α} injection, when blood sampling stopped. No additional PGFM pulse was detected in

cow 7432, whereas a second pulse was observed at 105 min of PGF_{2α} injection in cow 15891.

Progesterone Clearance After PGF_{2α} Injection

After administration of PGF_{2α}, the concentrations of progesterone in plasma decayed for the first 1 h, from 5.6 to 3.2 ng/mL, after which it increased to 4.2 ng/mL at 2 h and then declined to basal concentrations below 1.0 ng/mL by 24 h of PGF_{2α} injection (Figure 3). The pattern of progesterone decline after administration of PGF_{2α} did not differ with treatment.

Luteolysis and Endogenous Release of PGFM

One cow from R4 treatment was excluded from the statistical analyses because the estrous cycle was not synchronized. The remaining 44 cows had the estrous cycle synchronized and were included in all analyses. During late diestrus, progesterone concentrations presented a pulsatile pattern that was observed in cows that underwent luteolysis as well as in cows that maintained the CL up to d 23 of the estrous cycle. A representative

Figure 2. Concentrations of progesterone and 13,14-dihydro-15-keto-PGF_{2α} metabolite (PGFM) in plasma of nonlactating, nonpregnant Holstein cows (cows 7432 and 15891) bearing a functional corpus luteum, before and after an i.m. injection of 25 mg of dinoprost as tromethamine salt.

Figure 3. Progesterone concentrations in plasma of lactating Holstein cows fed a ratio of 3.9 to 1 (R4; $n = 15$ cows), 4.9 to 1 (R5; $n = 15$ cows), and 5.9 to 1 (R6; $n = 15$ cows) n-6 to n-3 fatty acids in the diet, after an i.m. injection of 25 mg of $\text{PGF}_{2\alpha}$. Effect of treatment ($P = 0.83$), time ($P < 0.001$), and interaction between treatment and time ($P = 0.99$). Linear ($P = 0.72$) and quadratic ($P = 0.61$) effects of increasing the ratio of n-6 to n-3 fatty acids in the diet. Mean progesterone concentrations during the sampling period were 2.80 ± 0.41 , 3.15 ± 0.40 , and 3.01 ± 0.39 ng/mL, respectively, for cows receiving the R4, R5, and R6 diets. Error bars represent SEM.

cow that underwent luteolysis and one that maintained the CL is depicted in Figure 4. In general, cows that underwent luteolysis had 2 pulses of PGFM during the preluteolytic period, 2 pulses during the luteolytic period, and 1 pulse during the postluteolytic period.

The proportions of cows undergoing luteolysis by d 20 or 23 of the estrous cycle did not differ among treatments (Table 2). For cows undergoing luteolysis, the basal concentration of PGFM was similar among treatments and averaged 30.7 ± 3.8 pg/mL (Table 2). The total number of pulses of PGFM decreased ($P < 0.01$) with an increasing ratio of n-6 to n-3 fatty acids. Number of preluteolytic and luteolytic pulses tended ($P < 0.10$) to decrease with an increasing ratio from R4 to R6. The area under the curve of the PGFM pulses within the preluteolytic, luteolytic, or postluteolytic periods did not differ among treatments (Table 2). However, increasing the ratio from R4 to R6 increased ($P = 0.05$) the area under the curve of the greatest pulse of PGFM. During the preluteolytic period, the greatest PGFM pulse was greater for cows fed R4 than those fed R5 or R6 ($P < 0.05$; Figure 5A). The second greatest pulse of the preluteolytic period did not differ among treatments. The luteolytic period was characterized by at least 2 major PGFM pulses in most cows, and the greatest pulse was greater ($P < 0.05$) for R6 than R5 or R4 (Figure 5B). The second greatest pulse of the luteolytic period had smaller amplitude and only minor differences were observed. During the postluteo-

lytic period, cows had, on average, 1 pulse of PGFM and the amplitude of the pulse was greater ($P < 0.05$) for cows fed R6 than those fed R4 or R5 (Figure 5C).

One cow underwent luteolysis with 2 PGFM pulses, however, the measures of reliability indicated that a minimum of 3 pulses of $\text{PGF}_{2\alpha}$ were required to induce CL regression with the highest accuracy and with the best kappa value (Table 3). For the 8 cows that did not undergo luteolysis by d 23, the basal PGFM averaged 36.0 ± 6.5 pg/mL and the number of pulses and the area under the curve for those pulses did not differ among treatments (Table 2).

All cows, with no exception, had progesterone profiles with a pulsatile pattern as exemplified for cows in Figure 4, but the average concentrations of progesterone showed an elevation immediately before the beginning of the luteolytic period that was followed by a sharp decline to below 1.0 ng/mL in the next 36 h (Figure 6). Concentrations of progesterone did not differ among treatments during the preluteolytic period and averaged 6.16 ± 0.43 , 5.41 ± 0.40 , and 6.14 ± 0.42 ng/mL in the 24 h preceding the luteolytic period in cows fed R4, R5, and R6, respectively. During the luteolytic period, concentrations of progesterone were greater ($P < 0.05$) for cows fed R4 than R5 or R6, particularly between 8 and 24 h after onset of luteolysis, which averaged 3.74 ± 0.33 , 2.93 ± 0.31 , and 2.90 ± 0.32 , respectively. During the postluteolytic period, concentrations remained below 1.0 ng/mL in all 36 cows that underwent luteo-

olysis. Of the cows that underwent luteolysis, 1 R4, 1 R5, and 4 R6 had progesterone concentrations during the postluteolytic phase that did not drop below 0.3 ng/mL. The remaining 30 cows with regressed CL had progesterone concentrations that remained below 0.3 ng/mL during the postluteolytic period.

Endometrium Fatty Acid Profile and Gene Expression

Altering the ratio of n-6 to n-3 fatty acids in the diet resulted in changes in the profile of fatty acids of the endometrium of lactating dairy cows (Table 4). The concentrations of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), eicosadienoic acid (C20:2 *cis*-11, *cis*-14), and dihomo- γ -linolenic acid (C20:3 *cis*-8, *cis*-11, *cis*-14) did not differ among treatments. The proportion of heptadecanoic acid (C17:0) was greater ($P = 0.02$) in the endometrium of cows fed R4 than R5

or R6. Although cows fed R6 consumed an additional 71 g of linoleic acid daily compared with cows fed R4, the proportion of linoleic acid was not altered by treatments and averaged 13.5% of the total endometrial fatty acids. However, the concentration of arachidonic acid, a product of desaturation and elongation of linoleic acid, in the endometrial tissue increased ($P < 0.01$) as the ratio of dietary n-6 to n-3 fatty acids increased. On the other hand, the concentrations of EPA and DHA decreased linearly ($P < 0.01$) as the ratio of dietary n-6 to n-3 fatty acids increased. The proportions of total n-6 and n-3 fatty acids in the endometrium were affected ($P < 0.01$) by dietary treatments, and they followed opposite directions as the ratio of dietary n-6 to n-3 fatty acids increased. Because of such changes in fatty acids composition of endometrial lipids, the ratio of n-6 to n-3 fatty acids in the endometrium increased ($P < 0.01$) from 4.53 to 9.85 as the dietary ratio increased from R4 to R6.

Figure 4. Progesterone and 13,14-dihydro-15-keto-PGF_{20α} metabolite (PGFM) in plasma from d 16 to 23 of the estrous cycle of a representative cow that underwent luteolysis (no. 7389) and a cow that maintained the corpus luteum by d 23 (no. 7381).

Table 2. Effect of altering the ratio of dietary n-6 to n-3 fatty acids on timing of luteolysis and PGF_{2α} metabolite 13,14-dihydro-15-keto-PGF_{2α} (PGFM) concentrations and pulses of lactating Holstein cows

Item	Treatment ¹				P-value ²		
	R4	R5	R6	SEM	TRT	Linear	Quad
Luteolysis, % (n/n)							
By d 20	42.9 (6/14)	26.7 (4/15)	46.7 (7/15)	—	0.50	0.85	0.26
By d 23	78.6 (11/14)	86.7 (13/15)	80.0 (12/15)	—	0.83	0.93	0.56
Beginning of luteolysis, d	18.9	19.8	19.4	0.4	0.32	0.39	0.21
End of luteolysis, d	20.5	20.8	20.7	0.5	0.91	0.84	0.70
Duration of the luteolytic period, h	39.3	23.4	29.5	3.6	0.01	0.07	0.01
PGFM cows undergoing luteolysis							
Basal, pg/mL	28.7	34.5	29.0	3.8	0.53	0.96	0.26
Pulses, n							
Total	6.7	4.9	4.6	0.5	0.02	<0.01	0.24
Preluteolytic period	2.4	2.3	1.4	0.4	0.17	0.10	0.40
Luteolytic period	3.0	1.9	2.3	0.3	0.04	0.09	0.06
Postluteolytic period	1.5	0.7	0.9	0.3	0.11	0.16	0.11
Area under the curve, pg/h per milliliter							
Total	3,514	3,501	4,175	623	0.60	0.57	0.42
Preluteolytic period	1,118	1,678	752	390	0.45	0.22	0.78
Luteolytic period	1,794	1,597	2,386	366	0.25	0.30	0.21
Postluteolytic period	880	954	1,616	418	0.40	0.22	0.58
Greatest pulse	793	981	2,060	368	0.12	0.05	0.62
PGFM cows with no luteolysis							
Basal, pg/mL	34.6	34.2	39.2	6.5	0.83	0.62	0.76
Preluteolytic pulses, n	1.7	0.5	0.7	0.7	0.45	0.31	0.48
Total area under the curve, pg/h per milliliter	1,572	567	1,312	797	0.77	0.84	0.57

¹R4 = ratio of 4 to 1 of n-6 to n-3 fatty acids; R5 = ratio of 5 to 1 of n-6 to n-3 fatty acids; R6 = ratio of 6 to 1 of n-6 to n-3 fatty acids.

²TRT = effect of treatment; Linear = linear effect of altering the ratio of n-6 to n-3 fatty acids; Quad = quadratic effect of altering the ratio of n-6 to n-3 fatty acids.

Altering the ratio of n-6 to n-3 fatty acids on the diet of lactating dairy cows influenced the expression of genes associated with the luteolytic cascade, fatty acid metabolism, and growth hormone/IGF axis in the endometrium (Figure 7). Increasing the dietary ratio of n-6 to n-3 fatty acids resulted in a linear increase in the mRNA abundance of *ESR1* ($P < 0.001$), *OXTR* ($P = 0.01$), *COX2* ($P < 0.001$), *PGES* ($P = 0.04$), *PGFS* ($P = 0.02$), *STAR* ($P < 0.001$), and *IGFBP3* ($P = 0.03$); however, the same increase in the dietary ratio of n-6 to n-3 fatty acids resulted in a linear decrease in the mRNA abundance of *PPARG* ($P < 0.001$) and *IGF1* ($P = 0.10$). Treatment did not influence endometrial expression of *PLC*, *COX1*, *CBR1*, *PPARA*, *PPARD*, and *GHR1A* in dairy cows (Figure 7).

Linear positive relationships were observed between the expression of selected genes and the ratio of n-6 to n-3 fatty acids analyzed in the endometrium of dairy cows (Figure 8). Scatter plots depicting the delta Ct relative to endometrial fatty acids showed that the mRNA expressions of *ESR1*, *OXTR*, *COX2*, *PGES*, and *PGFS* increased ($P < 0.01$) with increased ratio of endometrial n-6 to n-3 fatty acids. On the other hand, the opposite response was observed for *PPARG* expression. As the ratio of n-6 to n-3 fatty acids decreased, expression of *PPARG* increased. Indeed, after grouping

cows according to the fatty acid composition of the endometrium as low (mean = 4.45, range = 2.65 to 5.6), medium (mean = 8.81, range = 8.12 to 9.91), or high (mean = 11.10, range = 10.06 to 14.27) ratio of n-6 to n-3 fatty acids, we observed that the relative expression of *ESR1* ($P < 0.001$), *OXTR* ($P = 0.08$), *COX2* ($P = 0.04$), *PGES* ($P = 0.02$), and *PGFS* ($P = 0.06$) in the endometrium increased as the ratio n-6 to n-3 fatty acids increased (Figure 9). Conversely, expression of *PPARG* increased ($P < 0.001$) as the ratio of n-6 to n-3 fatty acids in the endometrium decreased (Figure 9).

DISCUSSION

Prostaglandins are local mediators produced by many tissues, particularly the uterus, that play critical roles in female reproduction. One of the key roles of PG is the control of luteal lifespan; in particular, PGF_{2α} is known to be the molecule responsible for luteolysis in ruminants (McCracken et al., 1972). The precursor of uterine PGF_{2α} is arachidonic acid, which is present in the endometrial phospholipids and is formed from elongation and desaturation of linoleic acid (Leonard et al., 2004). After exposure to progesterone during diestrus, the endometrium accumulates arachidonic acid (Brinsfield and Hawk, 1973), which serves as precur-

Figure 5. Concentrations of 13,14-dihydro-15-keto-PGF_{2α} metabolite (PGFM) in plasma of lactating Holstein cows that underwent luteolysis and were fed a ratio of 3.9 to 1 (R4; n = 11 cows), 4.9 to 1 (R5; n = 13 cows), and 5.9 to 1 (R6; n = 12 cows) n-6 to n-3 fatty acids in the diet during the preluteolytic (A), luteolytic (B), and postluteolytic (C) periods. Within an hour, pairwise differences ($P < 0.05$) are represented as \$ = R4 vs. R5, € = R4 vs. R6, or ‡ = R5 vs. R6; tendencies ($P < 0.10$) are represented as * = R4 vs. R5, or † = R5 vs. R6. Error bars represent SEM.

sors of either PGF_{2α} or PGE₂. Experiments in vitro have shown that production of PGF_{2α} by bovine endometrial cells was suppressed when the culture media was supplemented with n-3 fatty acids (Mattos et al., 2003). In fact, when both n-6 and n-3 fatty acids were

present in the culture media, increasing the ratio of n-6 to n-3 fatty acids resulted in increased synthesis of PGF_{2α} (Caldari-Torres et al., 2006). Furthermore, in vivo experiments showed that diets with increased n-3 fatty acids suppressed the release of PGF_{2α} after

Table 3. Measures of reliability of number of 13,14-dihydro-15-keto-PGF_{2α} (PGFM) pulses to elicit luteal regression

Measure of reliability ¹	Number of PGFM pulses				
	>0	>1	>2	>3	>4
Sensitivity	100 (36/36)	100 (36/36)	97.2 (35/36)	77.8 (28/36)	61.1 (22/36)
Specificity	37.5 (3/8)	75.0 (6/8)	87.5 (7/8)	100 (8/8)	100 (8/8)
Positive predictive value	87.8 (36/41)	94.7 (36/38)	97.2 (35/36)	100 (28/28)	100 (22/22)
Negative predictive value	100 (3/3)	100 (6/6)	87.5 (7/8)	50.0 (8/16)	36.4 (8/22)
Accuracy	88.6 (39/44)	95.5 (42/44)	95.5 (42/44)	81.8 (36/44)	68.2 (30/44)
Kappa (95% CI)	49.5 (13.6–85.5)	83.1 (60.5–100)	84.7 (64.1–100)	56.0 (31.2–80.8)	36.4 (15.1–57.6)

¹Sensitivity = (number of cows correctly detected with luteolysis based on the respective number of PGFM pulses/number of all cows with luteolysis) × 100; specificity = (number of cows correctly detected without luteolysis based on the respective number of PGFM pulses/number of all cows without luteolysis) × 100; positive predictive value = (number of cows correctly detected with luteolysis based on the respective number of PGFM pulses/number of all cows detected with luteolysis) × 100; negative predictive value = (number of cows correctly detected without luteolysis based on the respective number of PGFM pulses/number of all cows detected without luteolysis) × 100; accuracy = (number of correct tests based on the respective number of PGFM pulses/number of total tests performed) × 100.

an oxytocin challenge (Mattos et al., 2002; Petit et al., 2004; Dirandeh et al., 2013). These and numerous other experiments led to the concept that altering the fatty acid profile of the diet might influence endometrial PG release, and feeding more n-3 fatty acids might attenuate the luteolytic signals and favor the maintenance of the CL and favor pregnancy in farm animals (Abayasekara and Wathes, 1999). Nevertheless, the PG response in cows fed diets supplemented with n-3 fatty acids following a challenge with oxytocin has not been consistent (Gulliver et al., 2012). Perhaps, lack of consistent attenuation in PG release after stimulation with oxytocin might be related to the amount of n-3 fatty acids reaching the duodenum for absorption and subsequent incorporation into reproductive tissues.

Although we noted an increase in n-3 fatty acids in the endometrium of cows fed R4, the length of the luteal phase and the day of CL regression was not altered in the current experiment. Similar to our findings, day of luteal regression was not altered in lactating dairy cows fed diets supplemented with fat sources differing in fatty acid profile (Hutchinson et al., 2012).

Silvestre et al. (2011) reported improved pregnancy per AI and reduced pregnancy loss for cows fed Ca salts enriched in EPA and DHA during the breeding period. Those authors and others (Abayasekara and Wathes, 1999; Santos et al., 2008) have speculated that one of the mechanisms by which n-3 fatty acids improve fertility in dairy cows is likely by attenuating the pulsatile release of PGF_{2α} during late diestrus, thereby having

Figure 6. Concentrations of progesterone in plasma of lactating Holstein cows that underwent luteolysis and were fed a ratio of 3.9 to 1 (R4; n = 11 cows), 4.9 to 1 (R5; n = 13), and 5.9 to 1 (R6; n = 12) n-6 to n-3 fatty acids in the diet during the preluteolytic, luteolytic, and postluteolytic periods. Within an hour, pairwise differences among treatments are represented as * $P < 0.05$, † $P < 0.10$.

protective effects on CL maintenance. Although cows fed R4, which consumed more n-3 fatty acids, primarily EPA and DHA, had some attenuation of the largest pulse of PGF_{2α}, they also had more total pulses because of an increase in number of pulses during the luteolytic period. Feeding n-3 fatty acids from fish (Mattos et al., 2002) or plant sources (Dirandeh et al., 2013) decreased the uterine synthesis of PGF_{2α} after a challenge with oxytocin, suggesting that these fatty acids have the ability to attenuate a large pulsatile release of PG. On the other hand, the increased number of pulses in cows fed R4 could be interpreted as favoring luteolysis (Schramm et al., 1983). In sheep, approximately 5 pulses of PGF_{2α} in a 25-h period were required to induce complete luteolysis (Schramm et al., 1983). The number of PGFM pulses has been characterized in heifers, as they typically have 2.4, 2.3, and 2.2 pulses during the preluteolytic, luteolytic, and postluteolytic periods, respectively (Ginther et al., 2010). These values are similar to those observed for lactating dairy cows in the current experiment. The average number of pulses varied with diet but, in general, cows undergoing luteolysis had 2.0 preluteolytic PGFM pulses, 2.4 luteolytic PGFM pulses, and 1.1 postluteolytic PGFM pulses. On the other hand, PG are important mediators of conceptus development (Dorniak et al., 2011), and smaller more frequent pulses might indicate increased supply of PG needed to mediate the effects of IFN- τ on the endometrium during conceptus elongation (Dorniak et al., 2011). Indeed, genes related to supply of precursors and synthesis of prostaglandins increase substantially in the trophectoderm during the onset of conceptus elongation, and were highly correlated with the expression of *PPARG* (Ribeiro et al., 2016). Inter-

estingly, endometrial expression of *PPARG* increased when cows were fed more n-3 fatty acids in the present experiment. Others have also shown that feeding fat supplements rich in n-3 fatty acids, flaxseed oil or fish oil, increased mRNA expression of *PPARG* in endometrial tissue in lactating dairy cows (Hutchinson et al., 2012). The negative linear association between mRNA expression of *PPARG* and the endometrial ratio of n-6 to n-3 fatty acids observed herein suggests that incorporating more n-3 fatty acids into the endometrium of dairy cows stimulates expression of *PPARG* that might have implications for conceptus development (Ribeiro et al., 2016) and pregnancy in cows supplemented with n-3 fatty acids (Silvestre et al., 2011; Sinedino et al., 2017).

For cows to undergo luteolysis, 3 pulses of PGF_{2α} were needed for progesterone to decline below 1.0 ng/mL. Only 1 lactating cow with only 2 pulses of PGF_{2α} had complete CL regression. Six of the 36 cows that underwent CL regression had progesterone concentrations between 0.4 and 0.7 ng/mL during the postluteolytic period compared with progesterone concentrations less than 0.3 ng/mL for the remaining 30 cows. The reasons for these relatively high concentrations of progesterone after luteolysis are either lack of complete CL regression, in which a portion of the luteal cells remain active, or simply late luteolysis, with not enough time for the death of the CL to result in concentrations of progesterone to drop below 0.3 ng/mL within the 23-d sampling period. Cows receiving different doses of PGF_{2α} on d 5 of the estrous cycle had, in some cases, incomplete luteal regression (Nascimento et al., 2014). In a group of cows receiving PGF_{2α} between d 8 to 13 of the estrous cycle, 1 cow had a decline followed by a

Table 4. Effect of altering the ratio of dietary n-6 to n-3 fatty acids on endometrium fatty acid profile of lactating Holstein cows on d 8 of the estrous cycle (LSM $\pm$ SEM)

Fatty acid, % of identified fatty acids	Treatment ¹			P-value ²		
	R4	R5	R6	TRT	Linear	Quad
C16:0	27.70 $\pm$ 0.80	27.88 $\pm$ 0.53	27.20 $\pm$ 0.46	0.62	0.59	0.55
C17:0	1.43 $\pm$ 0.07	1.16 $\pm$ 0.05	1.19 $\pm$ 0.04	0.02	0.01	0.03
C18:0	27.60 $\pm$ 1.61	25.19 $\pm$ 1.08	24.84 $\pm$ 0.93	0.34	0.15	0.48
C18:1 <i>cis</i> -9	12.18 $\pm$ 1.37	13.77 $\pm$ 0.91	12.55 $\pm$ 0.79	0.51	0.81	0.26
C18:2 <i>cis</i> -9, <i>cis</i> -12	12.40 $\pm$ 1.40	13.50 $\pm$ 0.93	14.44 $\pm$ 0.81	0.43	0.22	0.95
C20:2 <i>cis</i> -11, <i>cis</i> -14	0.80 $\pm$ 0.14	0.67 $\pm$ 0.09	0.71 $\pm$ 0.08	0.73	0.58	0.49
C20:3 <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14	1.33 $\pm$ 0.15	1.30 $\pm$ 0.10	1.33 $\pm$ 0.09	0.97	0.96	0.83
C20:4 <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16	9.05 $\pm$ 0.94	11.64 $\pm$ 0.63	13.41 $\pm$ 0.54	<0.01	<0.01	0.62
C20:5 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17	2.85 $\pm$ 0.21	2.14 $\pm$ 0.14	2.02 $\pm$ 0.12	<0.01	<0.01	0.12
C22:6 <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19	3.30 $\pm$ 0.53	1.57 $\pm$ 0.35	1.08 $\pm$ 0.31	<0.01	<0.01	0.20
Total n-6	23.55 $\pm$ 2.05	27.16 $\pm$ 1.36	29.89 $\pm$ 1.18	0.04	0.01	0.81
Total n-3	6.15 $\pm$ 0.62	3.72 $\pm$ 0.41	3.08 $\pm$ 0.36	<0.01	<0.01	0.12
n-6 to n-3 ratio	4.53 $\pm$ 1.12	7.99 $\pm$ 0.75	9.85 $\pm$ 0.65	<0.01	<0.01	0.43

¹R4 = ratio of 4 to 1 of n-6 to n-3 fatty acids; R5 = ratio of 5 to 1 of n-6 to n-3 fatty acids; R6 = ratio of 6 to 1 of n-6 to n-3 fatty acids.

²TRT = effect of treatment; Linear = linear effect of altering the ratio of n-6 to n-3 fatty acids; Quad = quadratic effect of altering the ratio of n-6 to n-3 fatty acids.

Figure 7. Expression of genes related to prostaglandin synthesis (*ESR1* = estrogen receptor 1; *OXTR* = oxytocin receptor; *PLC* = phospholipase C; *COX1* = cyclooxygenase 1; *COX2* = cyclooxygenase 2; *PGFS* = prostaglandin F synthase; *PGES* = prostaglandin E synthase; *CBR1* = prostaglandin E_2 9-ketoreductase); cholesterol and fatty acid metabolism (*STAR* = steroidogenic acute regulatory protein; *PPARA* = peroxisome proliferator-activated receptor α ; *PPARD* = peroxisome proliferator-activated receptor delta; and *PPARG* = peroxisome proliferator-activated receptor gamma); and somatotrophic axis (*GHR1A* = growth hormone receptor-1A; *IGFBP3* = insulin-like growth factor binding protein-3; and *IGF1* = insulin-like growth factor-1) in the endometrium of lactating Holstein cows fed a ratio of 3.9 to 1 (R4), 4.9 to 1 (R5), and 5.9 to 1 (R6) n-6 to n-3 fatty acids. The linear and quadratic effects of altering the dietary ratio of n-6 to n-3 fatty acids are depicted in each figure. Values represent the fold change relative to R6 and error bars represent the 95% CI.

Figure 8. Regression analyses between expression of selected genes (delta cycle threshold; Ct) and the ratio of n-6 to n-3 fatty acids in the endometrium of dairy cows. Expression of *ESR1* (estrogen receptor 1; $R^2 = 0.52$), *OXTR* (oxytocin receptor; $R^2 = 0.18$), *COX2* (cyclooxygenase 2; $R^2 = 0.32$), *PGES* (prostaglandin E synthase; $R^2 = 0.35$), and *PGFS* (prostaglandin F synthase; $R^2 = 0.16$) was positively associated ($P < 0.01$) with the ratio of n-6 to n-3 fatty acids observed in the endometrium, whereas the expression of *PPARG* (peroxisome proliferator-activated receptor gamma; $R^2 = 0.38$) was negative-associated ($P < 0.001$) with the ratio of n-6 to n-3 fatty acids in the endometrium of dairy cows.

rebound in progesterone concentrations (Chenault et al., 1976). To our knowledge, the same phenomenon has not been characterized in late diestrus with cows undergoing spontaneous luteolysis. Incomplete CL regression after a standard dose of $\text{PGF}_{2\alpha}$ treatment on d 5 was expected because many of the luteal cells remain unresponsive to the cell death signaling induced by $\text{PGF}_{2\alpha}$ typically observed in a more mature CL.

Substantial variability exists in the frequency and amplitude of $\text{PGF}_{2\alpha}$ pulses that induce spontaneous luteolysis in heifers, but typically 4 to 8 discrete pulses occur at intervals of 6 to 12 h (Kindahl et al., 1976; Silvia et al., 1991). Thatcher et al. (1986) studied 10 Friesian heifers in which 5 received saline and 5 received 3 mg of 17 β -estradiol on d 13 of the estrous cycle. Those authors identified 39 pulses of PGFM in the 10 heifers (3.9/heifer), of which 21 occurred preluteolysis. Kindahl et al. (1976) characterized 4 distinct $\text{PGF}_{2\alpha}$ pulses that occurred within a period of approximately 30 h for

luteolysis to occur in heifers. Although 1 lactating cow with only 2 pulses of $\text{PGF}_{2\alpha}$ had complete CL regression, results from the current experiment suggest that 3 $\text{PGF}_{2\alpha}$ pulses are required based on the measures of reliability of using $\text{PGF}_{2\alpha}$ pulses as a predictor of luteolysis. Infusion of low doses of $\text{PGF}_{2\alpha}$ to mimic the pulses observed during luteolysis depicts a similar pattern of PGFM pulses and subsequent CL regression in heifers as those observed during spontaneous luteolysis (Ginther et al., 2009).

Similar to findings observed by Ginther et al. (2007) in heifers, cows showed an initial increase in circulating concentrations of progesterone before a sharp decrease during the preluteolytic period. The individual profile of a heifer that underwent spontaneous luteolysis clearly showed the pulsatile pattern of progesterone concentrations during late diestrus with an increase in concentration preceding a sharp decline during luteolysis (Thatcher et al., 1986). Ginther et al. (2007)

observed that progesterone concentrations and blood flow to the CL increased and then decreased during the process of spontaneous luteolysis. A similar phenomenon was characterized in heifers induced to have luteolysis with infusion of low doses of $\text{PGF}_{2\alpha}$ to mimic the pulses observed during spontaneous luteolysis (Ginther et al., 2009).

When exogenous $\text{PGF}_{2\alpha}$ was administered to lactating cows in the present experiment, functional luteolysis occurred within 24 h and progesterone concentrations dropped below 1.0 ng/mL. Burke et al. (1997) found that feeding a diet enriched with n-3 fatty acids from fish meal increased the proportion of cows with progesterone concentrations in plasma above 1.0 ng/mL at 48 h after a $\text{PGF}_{2\alpha}$ injection, suggesting a protective effect of the n-3 fatty acids on the CL. Based on experi-

ments in vitro and work with exogenous administration of oxytocin and estradiol to induce pulses of $\text{PGF}_{2\alpha}$ (Mattos et al., 2000, 2003), one could expect that a diet enriched with n-6 fatty acids would favor luteolysis and a diet enriched with n-3 fatty acids would possess antiluteolytic properties. However, results from the present experiment, in which the dietary ratio of n-6 to n-3 fatty acids was altered, suggest that the changes implemented, within the ranges of n-3 fatty acids consumed by these cows, were not sufficient to exert any protective effects on the CL against an exogenous $\text{PGF}_{2\alpha}$ injection. Cows fed R4 consumed a diet containing 0.327% n-3 fatty acids, mostly EPA and DHA, which are potent regulators of cell metabolism (Calder, 2012). Nevertheless, this concentration of n-3 fatty acids is usually less than those of diets typically

Figure 9. Expression of selected genes according to the concentration of n-6 to n-3 fatty acids in the endometrium of dairy cows. Endometrium was categorized according to the ratio of n-6 to n-3 fatty acids observed by MS as low ratio (mean = 4.45, range = 2.65 to 5.6), moderate ratio (mean = 8.81, range = 8.12 to 9.91), or high ratio (mean = 11.10, range = 10.06 to 14.27). Genes differentially expressed included *ESR1* (estrogen receptor 1), *OXTR* (oxytocin receptor), *COX2* (cyclooxygenase 2), *PGES* (prostaglandin E synthase), *PGFS* (prostaglandin F synthase), and *PPARG* (peroxisome proliferator-activated receptor gamma). The linear and quadratic effects of ratio of n-6 to n-3 fatty acids analyzed in the endometrium by MS are depicted in each figure. Values represent the fold change relative to high ratio of n-6 to n-3 fatty acids and error bars represent the 95% CI.

consumed when cows graze pasture forages (Glasser et al., 2013). Cows grazing subtropical or temperate forages as the sole dietary ingredient consume diets with 1.1 to 2.2% total fatty acids, of which n-3 fatty acids, particularly α -linolenic acid, represent 30 to 60% of those fatty acids (Glasser et al., 2013). In those production systems, it would not be unusual for cows grazing pasture forages and supplemented with concentrates to consume diets with a 1 to 1 ratio of n-6 to n-3 fatty acids and ingest 100 to 200 g/d of α -linolenic acid, whereas cows in R4 ingested 20.3 g/d of EPA plus DHA and 76.3 g/d of total n-3 fatty acids (Greco et al., 2015). A 1 to 1 ratio of n-6 to n-3 fatty acids is difficult to achieve in TMR-based diets when the main forage is corn silage, which typically contains primarily n-6 fatty acids. It is possible that luteolytic responses to n-3 fatty acids might be dose-dependent in dairy cows, and larger quantities consumed might further increase the availability of those fatty acids in reproductive tissues (Zachut et al., 2010), thereby suppressing the pulsatile release of PGF_{2 α} and delaying luteolysis, although that remains to be demonstrated *in vivo*.

The pulse of PGFM observed with a luteolytic dose of 25 mg of PGF_{2 α} was massive in both nonlactating cows evaluated, reaching concentrations that were 10 to 15 fold those observed in the typical spontaneous PGFM pulse during the luteolytic period for lactating cows fed altered n-6 to n-3 ratios. Likewise, the duration of the concentration of PGFM above the thresholds observed for the peak of PGFM pulse in cows undergoing spontaneous luteolysis was long, lasting several hours (e.g., 8 h), either because the release and absorption of dinoprost from the site of injection was slow or because the half-life of PGFM is longer than the short half-life typically described for PGF_{2 α} . In pigs, after intravenous bolus injection of PGFM, the elimination half-life averaged ($\pm$ SD) 15.0 $\pm$ 1.3 min (Martin and Liptrap, 1981), whereas in heifers, the elimination half-life of PGFM averaged 18 min following intrauterine administration of 200 μ g of PGF_{2 α} (Williams et al., 1983). These values are 1.7 to 2 fold longer than the elimination half-life of 9.0 $\pm$ 0.9 min for PGF_{2 α} in heifers (Shrestha et al., 2012), which explains the long duration of high concentrations of PGFM after the injection of 25 mg of dinoprost.

Altering the dietary ratio of n-6 to n-3 fatty acids did not affect the concentrations of progesterone in plasma of dairy cows. All treatments were designed to have the same concentrations of fatty acids, and differences in PUFA concentrations among diets were small. Therefore, differences in progesterone concentrations typically observed with addition of dietary fat (Staples et al., 1998; Santos et al., 2008) or changes in progesterone clearance (Hawkins et al., 1995) were not

anticipated. In fact, the clearance of progesterone after induced luteolysis did not change among treatments. Interestingly, progesterone concentrations of lactating dairy cows during late diestrus displayed a pulsatile fashion. Ginther et al. (2011) showed a concurrent and coordinated pulse of LH that preceded the pulses of progesterone during the preluteolytic period in dairy heifers. Although LH was not quantified in plasma samples in our experiment, it is reasonable to suggest that a similar phenomenon also occurs in lactating dairy cows (Procknor et al., 1986). In mid diestrus, cows have LH pulses of high amplitude, varying by 1.2 to 7.0 ng/mL, and of low frequency, averaging 6 to 8 pulses per day, which was described as not having an inherent rhythmic pattern (Rahe et al., 1980).

The fatty acid profile of the endometrial tissue resembled that of the dietary fatty acids offered to cows. Others have also shown that the fatty acid profile of endometrial phospholipids is directly influenced by the fatty acids consumed in the diet (Bilby et al., 2006). In fact, altering the dietary profile of fatty acids influences numerous reproductive tissues, including the profiles of fatty acids in follicular fluid, granulosa cells, and the cumulus oocyte complex (Zachut et al., 2010), which can influence not only endometrial but also ovarian function that interplays with the uterus to induce luteolytic pulses of PGF_{2 α} . Increasing the ratio of n-6 to n-3 fatty acids, with concurrent increased intake of linoleic acid, resulted in a greater proportion of arachidonic acid in the endometrium. Arachidonic acid is the most biologically active n-6 fatty acid and can be synthesized by elongation and desaturation of linoleic acid (Abayasekara and Wathes, 1999; Leonard et al., 2004). Likewise, increasing the intake of EPA, by decreasing the ratio of n-6 to n-3 fatty acids in the diet granted greater proportion of EPA in the endometrium. Concentrations of EPA and DHA were greater in the caruncular tissue when cows were supplemented with 200 g/d of fish oil, starting at 21 d prepartum, compared with cows supplemented with olive oil (Mattos et al., 2004). Furthermore, cows supplemented with fish oil had increased concentrations of EPA and DHA in the endometrium on d 17 of the estrous cycle (Bilby et al., 2006; Childs et al., 2008). Therefore, results from the current experiment corroborate data in the literature suggesting that manipulation of the dietary fatty acids profile affects the fatty acids composition of the reproductive tissues. Furthermore, these changes in the fatty acid profile of the endometrium might lead to changes in gene expression. In late diestrus, progesterone downregulates its own receptors in the superficial epithelium, leading to a loss in ability to inhibit the expression of estrogen and oxytocin receptors in the endometrium (Silvia et al., 1991). Upregulation of en-

ometrial estrogen and oxytocin receptors and binding to respective ligands initiates the luteolytic cascade by activating endometrial phospholipases to release arachidonic acid from the membrane phospholipids (Mattos et al., 2000). As a result, arachidonic acid is available for the synthesis of PGF_{2α} and other PG (Mattos et al., 2000). In the current experiment, cows fed more n-6 relative to n-3 fatty acids had greater expression of estradiol and oxytocin receptors and enzymes involved in PG synthesis, which could be interpreted as favoring the luteolytic process. Feeding more R6 enriched the endometrium with increased concentration of linoleic and arachidonic acids, the substrate for PG synthesis. Waters et al. (2012) demonstrated that the estrogen receptor was downregulated on d 17 of the estrous cycle in the endometrium of cows with high n-3 fatty acid content compared with controls, which was also observed in cows fed R4 in the present experiment. Therefore, it is possible that changes in the pulsatile pattern of PGFM observed in the current experiment under spontaneous luteolysis, and that of cows induced to have PGF_{2α} pulses after treatment with estradiol and oxytocin, were influenced not only by the availability of fatty acid precursors for PG, but also by changes in the gene signaling that favor PG synthesis (i.e., R6 > R4). Likewise, steroidogenic acute regulatory protein is a rate-limiting enzyme responsible for the transport of cholesterol within the mitochondria for steroid synthesis (Manna et al., 2009). Several transcription factors are involved in the regulation of *STAR* (Manna et al., 2009), and arachidonic acid is involved in one of the signaling pathways that upregulates the expression and protein translation of the *STAR* enzyme (Stocco et al., 2005). Consistent with these findings, cows in the current experiment fed R6 had greater concentrations of arachidonic acid in the endometrium and increased abundance of *STAR* mRNA. Furthermore, positive relationships were observed between mRNA expressions of *ESR1*, *OXTR*, *COX2*, *PGES*, and *PGFS* with increased ratio of n-6 to n-3 fatty acids present in the endometrium.

CONCLUSIONS

Altering the ratio of n-6 to n-3 fatty acids in the diet of lactating dairy cows influenced the pattern of prostaglandin synthesis. Increasing the ratio of n-6 to n-3 fatty acids increased the amplitude but decreased the frequency of PGFM pulses. Furthermore, increasing the ratio of n-6 to n-3 fatty acids from R4 to R6 resulted in endometrial responses that included an increase in concentration of arachidonic acid and a decrease in concentration of EPA and DHA, increases in mRNA

expressions of estrogen and oxytocin receptors, cyclooxygenase 2, PGE and PGF synthase, steroidogenic acute regulatory protein, and insulin-like growth factor binding protein-3, but decreased the expression of peroxisome proliferator activated receptor gamma and insulin-like growth factor 1. These changes in endometrial gene expression were associated with changes in endometrial content of fatty acids. In spite of these dynamic changes caused by dietary fatty acids manipulations, length of the estrous cycle was not altered. Three pulses of PGFM was the best predictor that resulted in the highest combined sensitivity and specificity for luteolysis in lactating dairy cows.

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