Title: Effect of fish meal supplementation on spatial distribution of lipid microdomains and on the lateral mobility of membrane-bound prostaglandin F_{2α} receptors in bovine corpora lutea¹

Running Title: Lipid microdomains and FP receptor mobility

5 **Summary Sentence:** Fish meal supplementation disrupts lipid microdomains and increases FP receptor mobility on bovine corpus luteum plasma membranes

Key Words: bovine, corpus luteum, omega-3 fatty acids, lipid microdomains, FP receptor

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Abstract

This study examined the effects of fish meal supplementation on spatial distribution of lipid microdomains and lateral mobility of prostaglandin $F_{2\alpha}$ (FP) receptors on cell plasma
25 membranes of the bovine corpus luteum (CL). Beef cows were stratified by BW and randomly assigned to receive a corn gluten meal supplement ($n = 4$) or fish meal supplement ($n = 4$) for 60 d to allow incorporation of fish meal derived omega-3 fatty acids into luteal tissue. Ovaries bearing the CL were surgically removed **between days 10 to 12** post-estrus corresponding to approximately **day 60** of supplementation. A 200 mg sample of luteal tissue was analyzed for
30 fatty acid content using GLC. The remaining tissue was enzymatically digested with collagenase to dissociate individual cells from the tissue. Cells were cultured to determine effects of dietary supplementation on lipid microdomains and lateral mobility of FP receptors. Luteal tissue collected from fish meal supplemented cows had increased omega-3 fatty acids content ($P < 0.05$). Lipid microdomain total fluorescent intensity was decreased in dissociated luteal cells
35 from fish meal supplemented cows ($P < 0.05$). Micro and macro diffusion coefficients of FP receptors were greater for cells obtained from fish meal supplemented cows ($P < 0.05$). In addition, compartment diameter of domains was larger while resident time was shorter for receptors from cells obtained from fish meal supplemented cows ($P < 0.05$). Data indicate that dietary supplementation with fish meal increases omega-3 fatty acid content in luteal tissue
40 causing disruption of lipid microdomains. This disruption leads to increased lateral mobility of the FP receptor, increased compartment sizes, and decreased resident time which may influence prostaglandin signaling in the bovine CL.

1. Introduction

Luteinizing hormone from the anterior pituitary gland causes the release of the ovum from the follicle and differentiation of the theca and granulosa cells into small and large luteal steroidogenic cells, respectively [1-3]. Luteal steroidogenic cells secrete progesterone which is essential for early pregnancy in mammals. In the non-pregnant cow, uterine prostaglandin (PG) $F_{2\alpha}$ is released late in the estrous cycle in a series of 5 to 8 pulses, causing regression of the corpus luteum (CL) allowing for return to estrus [4-6]. Prostaglandin $F_{2\alpha}$ (FP) receptors are heterotrimeric G-protein coupled receptors located on steroidogenic luteal cells of the CL [7]. Binding of $PGF_{2\alpha}$ to its receptor triggers a complex intracellular signaling cascade to initiate regression of the CL. In the pregnant female, the conceptus inhibits uterine synthesis and release of $PGF_{2\alpha}$, preventing regression of the CL, allowing for continued secretion of progesterone, and establishment of pregnancy [8]. Loss of pregnancy can occur when a viable conceptus fails to adequately regulate $PGF_{2\alpha}$ secretion, leading to regression of the CL [9, 10]. Therefore, diminishing or altering sensitivity of luteal cells to $PGF_{2\alpha}$ may prevent regression of the CL during early pregnancy.

The plasma membrane of mammalian cells is composed of a lipid bilayer which is highly dynamic. It also contains unique regions called lipid microdomains, ranging in size from 10 to 200 nm in diameter that are high in cholesterol and sphingolipids [11, 12]. These domains are well-organized, which cluster to form larger, ordered platforms. The order, or spatial distribution of these domains, favor specific protein-protein interactions, resulting in the activation of signaling cascades [13]. There are two distinct lipid microdomain structures associated with

plasma membranes – linear domains referred to as lipid rafts and invaginated domains referred to as caveolae. These microdomains have been postulated to regulate vesicle trafficking/sorting, endocytosis of membrane-bound proteins, cholesterol homeostasis, and serve as platforms to facilitate co-localization of intracellular signaling proteins during agonist-induced signal transduction [11]. Numerous studies have shown that ligand-bound membrane receptors often coalesce into lipid microdomains following addition of an agonist resulting in the activation of intracellular signaling pathways [14-16]. Additionally, the disruption of lipid microdomains, either by beta-methyl cyclodextrin (β -MCD) or inhibiting transcription of specific microdomain structural proteins, has been shown to exert major effects on G-protein coupled receptor signaling. This disruption has been reported to effect the localization, trafficking, and signaling of the G-alpha subunit [17]. Therefore, altering lipid microdomain structure on the plasma membrane of luteal cells may reduce $\text{PGF}_{2\alpha}$ signaling in bovine CL.

Long-chain polyunsaturated fatty acids such as omega-3 fatty acids can be incorporated into glycerophospholipids and increase membrane fluidity [18, 19]. Changes in membrane order or fluidity have been reported to affect ligand affinity and subsequent ion flux for the acetylcholine receptor [20]. In addition to altering membrane fluidity, these fatty acids have been reported to disrupt lipid microdomain composition, affect mobility of membrane-bound receptors, and decrease cell signaling [21-23]. We recently reported that inclusion of fish meal in the diet of non-lactating beef cows increased blood plasma [24-26] and luteal [26] content of omega-3 fatty acids. Therefore, it is hypothesized that omega-3 fatty acids in fish meal will incorporate into the plasma membrane of luteal cells altering lipid microdomains and lateral mobility of FP receptors in cells of the bovine CL. The objectives of the current study were to examine the effects of fish meal supplementation on 1) plasma and luteal omega-3 fatty acid

composition, 2) organization and spatial distribution of lipid microdomains on cells of the bovine CL and 3) lateral mobility of the membrane-bound FP receptors.

2. Materials and methods

2.1 Animals and tissue collection

All animal procedures described herein were approved by the Colorado State University Institutional Animal Care and Use Committee (Approval # 13 - 4440A). Beef cows of mixed breeds were purchased at a local sale barn in Fort Collins, Colorado and housed at the Colorado State University Animal Reproduction Biotechnology Laboratory Foothills campus. Reproductive organs were palpated per rectum for presence of gross anatomical abnormalities (cystic follicles) and adhesions. Transrectal ultrasonography was performed on ovaries for presence of CL and uteri for absence of a fetus. Cows with adhesions, cystic follicles, absence of a CL, or pregnant were removed from the study.

Cows were stratified by BW and randomly assigned to receive corn gluten meal (n = 4; controls) or fish meal (n = 4; SeaLac, Omega Protein). Diets were delivered daily to cows at 2% BW on a dry matter intake basis that met or exceeded NRC requirements [27]. The ration consisted of 95% mixed hay and 5% pelleted supplement of either fish meal or corn gluten meal. Diets were formulated to be isocaloric and isonitrogenous (Tables 1 and 2) and cows were fed for approximately 60 d to allow for adequate time to incorporate omega-3 fatty acids into blood and reproductive tissues in the fish meal supplemented animals. Cows were housed in a dry lot and were individually penned (3.7 × 3.0 m) between 0600 and 1000 each day to receive supplements and hay. After consumption of rations, cows were then turned out to have *ad*

libitum access to water. Body weights were collected weekly to monitor changes in weight and diets were adjusted as needed.

Jugular blood samples were collected immediately before supplementation commenced and weekly thereafter to measure changes in plasma fatty acid composition. Samples were
115 collected in 3-mL blood tubes containing 5.4 mg EDTA (BD Vacutainer, Becton and Dickson Co, Franklin Lakes, New Jersey, USA) and immediately placed on ice. Samples were centrifuged at $1500 \times g$ for 15 min, after which plasma was then collected and stored at -80°C until GLC analysis.

Cows were administered 25 mg injections of $\text{PGF}_{2\alpha}$ (Lutalyse, Pharmacia & Upjohn Co,
120 MI, USA) on **day 36 and 50** of the supplementation period to synchronize estrous cycles. Ovaries bearing the CL were surgically removed by standing flank procedure, as previously described [28] **between days 10 to 12** post-estrus following the second $\text{PGF}_{2\alpha}$ injection (approximately **day 60** of the supplementation period). After collection, the ovary was placed in $1\times$ sterile PBS and transported on ice to the laboratory at the University of Northern Colorado.
125 Superficial sterilization of the ovary was performed by immersing into a 70% ethanol solution.

2.2 Cell preparation

Using sterile techniques under a laminar flow hood, the CL was removed from the ovary and a 200 mg sample of tissue was placed in a 1.7-mL micro centrifuge collection tube and stored at -80°C until GLC analysis. The remaining tissue was cut into 500 μm slices using a
130 Stadie-Riggs microtome. Slices of tissue were placed into a 60-mm² Petri dish containing $\text{Ca}^{+2}/\text{Mg}^{+2}$ -free Hank's balanced salt solution (HBSS) that was supplemented with 20 mM HEPES, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 mg/mL amphotericin B (pH

= 7.34) until enzymatic digestion. Tissue was then placed in T-25 culture flasks containing 5 mL dissociation medium (HBSS supplemented with 2000 units type 1 collagenase per g of tissue and 0.1 % BSA). Flasks were incubated in a water bath at 37°C with agitation for 45 min. Following incubation, the suspension was removed and transferred to a sterile 15-mL conical tube. Cells were washed 3 × in 5 mL of sterile 1× PBS. In brief, cells were centrifuged at 500 × g for 5 min at 4°C, the supernatant was removed, and cell pellets were re-suspended in fresh 5 mL 1× PBS. Following the final wash, the cell pellet was re-suspended in 10 mL of Ham's F12 culture medium, supplemented with 5% fetal bovine serum, insulin-transferrin-selenium-supplement (51300044; Invitrogen, Carlsbad, CA, USA), 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 mg/mL amphotericin B (pH = 7.34), and placed on ice. Fresh dissociation medium was added to the remaining undigested tissue and incubated with agitation for additional 45 min. Cells were collected, washed 3 × with PBS as above, and then combined with the previous sample. Viability of the cells was determined using trypan blue exclusion, and cell number was estimated using a hemocytometer. Viability of the cell populations was $96 \pm 1.8\%$.

2.3 Cell culture

Cells were cultured in 35-mm round-bottom culture dishes with poly-D-lysine coated coverslips (MatTex Corporation, Calhoun GA, USA). To facilitate cellular adhesion to coverslips, biomatrix (BD matrigel matrix basement membrane, BD Biosciences MI, USA) was added to dishes immediately prior to addition of 5×10^4 viable cells/mL per manufacturer's protocol. Cells were maintained under an atmosphere of humidified air and 5% CO₂ at 37°C for approximately 12 h prior to lipid microdomain staining or FP receptor labeling.

2.4 Lipid microdomain staining and analysis

Five dishes were prepared from each CL for lipid microdomain analysis. Two dishes were chosen at random and treated for 1 h with 10 mM β -MCD to remove membrane cholesterol which leads to **spatial disruption** of microdomains, thus serving as a positive control. Lipid microdomains were labeled as previously described [29].

Cells were viewed using a Zeiss confocal microscope equipped with a 40 $\times$ water immersion objective (1.2 N.A). A 555 nm laser was used to excite Alexa-555 fluorophore, and emission of light was collected between 560 to 1000 nm. Approximately 30 cells were randomly selected from each dish, and 1 μ m slice images were generated from bottom to top of each cell. Three-dimensional images were generated, and whole cell fluorescent intensity was determined using ImageJ software. A total of 366, 80, 578 and 117 dissociated cells were analyzed from tissue collected from controls, controls treated with β -MCD, fish meal supplemented cows, and fish meal supplemented cows treated with β -MCD, receptively.

2.5 Prostaglandin FP receptor labeling, single particle tracking, and analysis

Receptors were prepared for single particle tracking experiments using biotinylated antibody as previously described [29]. Three to five dishes were prepared from each CL for single particle tracking. Biotin was conjugated to FP receptor rabbit polyclonal antibody (product no. 101802; Cayman Chemical, Ann Arbor, MI, USA) using a DSB-X biotin protein labeling kit (Life Technologies, Carlsbad, CA, USA) per manufacturer's instructions.

Experiments were conducted using a Zeiss confocal microscope equipped with a high-speed camera (Hamamatsu Photonics, Japan). Ultraviolet light was used to excite quantum dots and emission of light was collected at 605 nm. Images were collected using 100× oil objective (N.A. = 1.4) and acquisition image size of 512×512 pixel ($33.3 \mu\text{m} \times 33.3 \mu\text{m}$). Individual receptors
180 were recorded for 29 s (30 fps) for a total of 870 frames per video. Receptors containing a minimum of 10 s of recordings were used for the final analysis.

Video Spot Tracker v08.01 software (Computer Integrated Systems for Microcopy and Manipulation, University of North Carolina, Chapel Hill, NC, USA) was used to generate individual receptor trajectories. The resolution was $0.07 \mu\text{m}/\text{pixel}$, and the XY coordinates
185 obtained in pixels from Spot Tracker were converted to μm^2 . Receptor trajectories were visualized by plotting XY coordinates using Excel software. Mean square displacement (MSD) for each trajectory was calculated using the equations reported by Daumas [30] and then plotted against time. Micro diffusion coefficient was determined from the slope of the fitted line equation at $0\Delta\text{time}$ and $1\Delta\text{time}$. Macro diffusion coefficient was estimated from the slope of the
190 fitted line through the entire MSD vs. time plot. A statistical variance model was used to calculate domain size and resident time as previously described [29]. In brief, a sliding window analysis was performed to determine the normalized variance in the position of the receptor within the time windows. Windows were then translated alongside of particle trajectories. The time of the inter-domain jumps were indicated by peaks and collected as $n + 1$. The average
195 diameter of an individual domain and resident time was calculated as previously described [29, 31, 32]. A total of 122 and 104 receptor trajectories were analyzed from cells collected from control and fish meal supplemented cows, respectively.

2.6 Plasma, tissue, and supplement fatty acid analysis

Plasma (500 μ L), luteal tissue (200 mg), or dietary supplement (100 mg) was added to a
200 16 $\times$ 100 mm reaction tube and freeze-dried for 24 h prior to methylation. Samples were covered
and maintained in the dark to minimize light-induced oxidation of fatty acids. Fatty acids were
methylated using direct methylation as previous described [26]. Long-chain fatty acid
composition was determined using an Agilent 7890A Series GLC (Wilmington, DE) with a MS
detector. The instrument was equipped with a 30-m $\times$ 0.20-mm (i.d.) fused silica capillary
205 column (Supelcowax10; Supelco Inc., Bellefonte, PA, USA). The fatty acid methyl ester
preparations were injected (1 μ L) using the splitless mode. The carrier gas was helium and the
oven temperature was programmed from an initial temperature of 140°C that was held for 10
min and then increased to a final temperature of 250°C at the rate of 2.5°C/min. The final
temperature was held for 10 min for a total run time of 65 min. Chromatograms were generated
210 with a computing integrator (ChemStation Plus Chromatograph Manager; Agilent Technologies,
Boulder, CO, USA). Standard fatty acid methyl ester mixtures were used to calibrate the GLC
system using reference standard GLC 68-D (Nu-Chek Prep, Inc. Waterville, MN, USA). Palmitic
acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) α -
linolenic acid (18:3), arachidonic acid (20:4), eicosapentaenoic acid (20:5), and docosahexaenoic
215 acid (22:6) were identified by comparing the mass spectrometry analysis and relative retention
times of fatty acid methyl ester peaks of samples with those of standards. These peaks were then
calculated as normalized area percentages of fatty acids.

2.7 Statistical analysis

All data are reported as least square means $\pm$ standard error of the mean and significance
220 was declared at $P < 0.05$. Effects of dietary supplementation on initial and ending BW, luteal
fatty acid composition, lipid microdomain staining intensity, lateral mobility of FP receptors

(micro and macro diffusion coefficients), domain sizes and resident time of receptors were analyzed using 1-way analysis of variance. The model included dietary supplementation, cow, and residual error as sources of variation. Cow was considered a random variable in the model.

225 Calculations were made using the mixed model procedure of SAS and means were compared by *t*-tests using the PDIFF option of SAS. The effects of dietary supplementation on plasma fatty acid content was analyzed using 1-way analysis of variance with repeated measures. The statistical model included dietary supplementation, day, cow, supplementation $\times$ day, and residual error as sources of variation. Cow was considered a random variable in the model. Calculations
230 were made in SAS using mixed modeling procedure and the repeated statement. A heterogeneous autoregressive covariance structure was used in the repeated model to account for heterogeneous variation among samples. Pre-planned pairwise *t*-test comparisons were used to determine differences using the PDIFF option of SAS.

235 **3. Results**

3.1 Changes in body weight

Initial BW was 463 ± 11.3 kg for control cows and 478 ± 11.3 kg for fish meal animals and did not differ ($P > 0.05$). All cows gained BW during the supplemental period. Likewise, ending body weight did not differ ($P > 0.05$) between supplementation groups and was $515 \pm$
240 18.1 kg for control cows and 521 ± 18.1 kg for fish meal supplemented animals.

3.2 Plasma and luteal fatty acid composition

There was no effect of dietary supplementation, day, or dietary supplementation $\times$ day on plasma fatty acid content of palmitic, palmitoleic, stearic, oleic or arachidonic acids ($P > 0.10$; data not shown). Plasma linoleic acid was greater in cows receiving control supplement as compared to cows receiving fish meal supplement ($P < 0.05$; data not shown). The effects of dietary supplementation on plasma omega-3 fatty acid composition during the supplemental period are shown in Figure 1. There was no effect of dietary supplementation, day, or dietary supplementation $\times$ day interaction on plasma α -linolenic ($P > 0.05$). However, there was an effect of dietary supplementation, day, and dietary supplementation $\times$ day interaction on plasma eicosapentaenoic and docosahexaenoic acids ($P < 0.05$). Plasma eicosapentaenoic or docosahexaenoic acid did not differ ($P > 0.10$) between controls or fish meal supplemented cows at the beginning of the experiment (day 0). However, cows supplemented with fish meal had higher plasma eicosapentaenoic acid starting at day 28 and docosahexaenoic acid starting at day 21 and both remained higher for the remainder of the supplemental period ($P < 0.05$).

The effects of dietary supplementation on luteal fatty acid composition are shown in Figure 2. There was no effect of dietary supplementation on luteal content of palmitic, palmitoleic, stearic, oleic, linoleic, and arachidonic acids ($P > 0.05$). However, luteal content of α -linolenic, eicosapentaenoic, and docosahexaenoic acids were greater in tissue obtained from fish meal supplemented cows as compared to control cows ($P < 0.05$).

3.3 Spatial distribution of lipid microdomains in dissociated luteal cells

Antibody cross-linking of monosialotetrahexosylganglioside (GM₁) resulted in formation of distinct patches on the plasma membrane of live, non-fixed dissociated cells. Representative cells from tissue acquired from a control and a fish meal supplemented cow are shown in Figure

265 3. The spatial distribution of microdomains of cells obtained from fish meal supplemented cows were disrupted resulting in a 52% decrease in total cell fluorescent intensity as compared to cells obtained from controls (Fig. 3; $P < 0.05$). Cells were also treated with β -MCD to examine the effects of cholesterol on lipid microdomain structure in these cells. Regardless of source of tissue (control or fish meal supplemented animals), depletion of cholesterol by β -MCD resulted
270 in reduced total cell fluorescent intensity when compared to untreated cells ($P < 0.05$). Cells from control animals that were depleted of cholesterol with β -MCD resulted in a 62% decrease in total cell fluorescent intensity; cells from fish meal supplemented cows that were depleted of cholesterol had a 56% reduction in total cell intensity ($P < 0.05$).

275 *3.4 Lateral mobility of FP receptors on dissociated luteal cells*

Representative FP receptor trajectories of cells from CL obtained from control and fish meal supplemented cows are shown in Figure 4. There was an effect of dietary supplementation on both micro and macro diffusion coefficients. Micro diffusion of FP receptors on cells obtained from fish meal supplemented animals was increased by 152% as compared to receptors
280 on cells from controls (Fig. 5; $P < 0.05$). Furthermore, macro diffusion was increased by 178% in cells obtained from fish meal supplemented animals (Fig. 5; $P < 0.05$).

Dietary supplementation affected diameter, also referred to as corals, of lipid microdomains. Domains associated with FP receptors were 2-fold larger for cells obtained from fish meal supplemented animals as compared to cells obtained from controls (Fig. 5; $P < 0.05$).

285 Dietary supplementation also affected the time a receptor resided within a domain. Receptors of cells from CL obtained from cows supplemented with fish meal were retained for 22% less time

in a domain as compared to receptors of cells from tissue obtained from controls (Fig. 5; $P < 0.05$).

4. Discussion

290   The present study was undertaken to examine the influence of dietary supplementation of fish meal on lipid microdomain structure and lateral mobility of FP receptors obtained from dissociated cells of the bovine CL. Inclusion of rumen-grade menhaden fish meal in the diet of beef and lactating dairy cows has been reported to improve pregnancy rates [33-35]. However, the mechanism by which fish meal improves pregnancy is still largely unknown. Many studies
295   have been conducted that examine influences of fish meal on uterine $\text{PGF}_{2\alpha}$ metabolism [25, 36, 37], but little work regarding CL function has been conducted. Menhaden fish meal contains approximately 11% crude fat with a high percentage of long-chain polyunsaturated omega-3 fatty acids, eicosapentaenoic and docosahexaenoic acid (Table 2). This class of fatty acids has been shown to affect lipid microdomain structure [22, 38] and lateral mobility of membrane-
300   bound receptors [29]. However, to our knowledge, the influence of these fatty acids on lipid microdomains or receptor mobility have not been investigated with cells of the bovine CL *in vivo*.

      Cows supplemented with fish meal had increased plasma and luteal eicosapentaenoic and docosahexaenoic fatty acids which is in agreement with previous studies from this [24-26] and
305   another laboratory [39]. Nutrition, especially energy balance, can have a significant impact on follicular growth and subsequent CL function in beef and dairy cows [40-42]. However, cows in this study had similar gain in BW during the supplementation period and differences in plasma

and luteal omega-3 fatty acid composition were due to changes in dietary fatty acids received from the supplementation and not attributed to plane of nutrition.

310   One of the objectives of this study was to examine the effects of dietary fish meal supplementation on lipid microdomain structure of dissociated cells of the CL. Lipid microdomains are microscopic regions ranging in size from 10 to 200 nm in diameter that are rich with cholesterol and sphingolipids, primarily sphingomyelin and GM₁ [11]. These are well-organized domains, which cluster to form larger, ordered platforms to facilitate membrane
315   trafficking (both endocytosis and exocytosis) and cell signaling. Binding of cholera toxin to GM₁ followed by cross-linking with antibodies is a common approach that can be used to visualize microdomains on plasma membranes of cells [43]. In this study using live, non-fixed dissociated cells of the CL, lipid microdomains were detected as punctated patches on membranes from cells collected from both supplemented groups. Spatial distribution of
320   microdomains on membranes of cells from tissue collected from fish meal supplemented animals were more dispersed (i.e., less clustering of GM₁ on the plasma membrane) resulting in decreased total cell fluorescent intensity. Similar effects of omega-3 fatty acids on lipid microdomain structure have been reported for cells of the CL [29] and immune cells [22, 44, 45] treated with fish oil. Cholesterol also plays an important role in lipid microdomain integrity [46,
325   47]. Removal of cholesterol by culturing cells in the presence of β -MCD resulted in dispersion of these domains in cells from tissue collected from controls, which is in agreement with other studies [29, 46]. Likewise, cells obtained from fish meal supplemented cows, cultured in the presence of β -MCD, resulted in further disruption of microdomains. A limitation of this study was use of dissociated cells. The CL is glandular tissue composed of many cell types which
330   include steroidogenic (large and small), endothelial, fibroblasts, pericytes, and immune [1-3].

Therefore, it was not possible to determine the specific cell type that was influenced by dietary supplementation in the current study. However, it was apparent that most all cells collected from fish meal supplemented animals had considerable disruption of lipid microdomains. Enrichment of specific populations of cell types of the CL and effects of omega-3 fatty acids on lipid microdomain structure is warranted in future studies.

A second objective of this study was to investigate the influence of fish meal supplementation on lateral mobility of FP receptors using single particle tracking. Micro and macro diffusion coefficients, domain size, and resident time of receptors on FP positive cells obtained from controls were within values reported in the literature for membrane-bound receptors using single particle tracking methodology [16, 48-54]. Here in, we show that altered mobility of FP receptors on the plasma membrane was associated with an increase in omega-3 fatty acids in bovine luteal tissue. Lateral mobility of the FP receptor, as determined by micro and macro diffusion, was increased in FP positive cells of tissue collected from cows supplemented with fish meal. Furthermore, the average time each receptor resided in a domain was decreased compared to receptors on cells obtained from controls. Likewise, increased domain sizes were observed in cells collected from fish meal supplemented animals which is consistent with a recent *in vitro* study from this laboratory showing fish oil increased domain size [29]. In the cow, FP receptors are expressed on the plasma membrane of steroidogenic (both large and small) [55, 56] and endothelial cells [7, 57]. Therefore, it was not possible to identify the specific cell type that expressed FP receptors in the current study and warrants additional investigation.

Results from this study clearly demonstrate that fish meal supplementation increases luteal omega-3 fatty acid content in bovine CL and has a dramatic impact on lipid microdomain

structure and FP receptor mobility. The mechanisms by which these fatty acids alter membrane
355 dynamics and lipid microdomain structure are largely unknown. As opposed to bulk lipids of the
plasma membrane, a characteristic of sphingolipids in lipid microdomains is the increased
unsaturated fatty acyl chains giving rise to a more quasi-liquid ordered state. However,
glycerophospholipids within the plasma membrane have unsaturated or polyunsaturated fatty
acid acyl chains and are loosely packed together in a quasi-liquid disordered fashion giving the
360 membrane its fluid property [58-60]. Therefore, one mechanism is that eicosapentaenoic and
docosahexaenoic fatty acids from dietary marine oils become esterified to glycerophospholipids
and increase the quasi-liquid disorder of the plasma membrane, which in turn increases fluidity
of the membrane and lateral mobility of the FP receptor.

Another possible mechanism is that incorporation of long-chain polyunsaturated fatty
365 acids into lipid microdomains of biological membranes and potentially alters protein distribution,
protein-lipid association, and/or protein-protein interactions. Reports in the literature show
polyunsaturated long-chain fatty acids including eicosapentaenoic and docosahexaenoic acids
can be incorporated into sphingolipids and thereby influence the quasi-liquid state of the
membrane lipid microdomain from ordered to more disorder [22, 38]. This disruption of quasi-
370 liquid ordered state may displace membrane-bound receptors from lipid microdomains. Single
particle tracking data from the current study show a reduction in resident times, supporting this
hypothesis.

In conclusion, results from the present study show that inclusion of fish meal in the diet
of cows affected CL membrane lipid microdomains and mobility of FP receptors, likely
375 impacting membrane fluidity and microdomain structure. Alteration of lipid microdomain

structure and mobility of FP receptors may reduce PGF_{2α} signaling in the bovine CL. Reduction in PGF_{2α} signaling within the CL in early pregnant females may improve fertility.

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Figure legends

Figure 1. Effects of dietary supplementation on relative composition of plasma omega-3 fatty acids. Panel A: α -Linolenic acid; dietary supplementation ($P > 0.05$), day ($P > 0.05$), and dietary supplementation $\times$ day interaction ($P > 0.05$). Panel B: Eicosapentaenoic acid; dietary supplementation ($P < 0.05$), day ($P < 0.05$) and dietary supplementation $\times$ day interaction ($P < 0.05$). Panel C: Docosahexaenoic acid; dietary supplementation ($P < 0.05$), Day ($P < 0.05$) and dietary supplementation $\times$ day interaction ($P < 0.05$). [#]Significance differences within day of supplementation, $P < 0.05$. *Significance differences within day of supplementation, $P < 0.001$.

Figure 2. Effects of dietary supplementation on relative composition of luteal long-chain fatty acids. Panel A: Relative composition (weight percent) of palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and arachidonic acid (20:4). Panel B: Relative composition (weight percent) of luteal omega-3 fatty acids α -linolenic acid (18:3), eicosapentaenoic acid (20:5), and docosahexaenoic acid (22:6). *Significant difference within class of fatty acid; $P < 0.05$. Corn gluten meal supplemented animals (n = 4; open bars); fish meal supplemented animals (n = 4; solid bars).

Figure 3. Effects of dietary supplementation on lipid microdomain structure on the plasma membrane of dissociated bovine luteal cells. Panel A: Representative cell obtained from (A) corn gluten meal supplemented animal, (B) individual cell obtained from same corpus luteum of corn gluten meal supplemented animal and subsequently treated with 10 mM beta-methylcyclodextrin (β -MCD), (C) individual cell obtained from fish meal supplemented cow

and, (D) individual cell obtained from same corpus luteum of fish meal supplemented animal and subsequently treated with β -MCD. Panel B: Mean total cell intensity for cells obtained from corn gluten meal cows ($n = 4$; $n = 366$ cells), cells from corn gluten meal cows and treated with β -MCD ($n = 80$ cells), cells from fish meal supplemented animals ($n = 4$ cows; $n = 578$ cells), and cells from fish meal supplemented animals and treated with β -MCD ($n = 117$ cells). $*P < 0.05$; corn gluten meal vs. fish meal; $\#P < 0.05$; corn gluten meal vs. corn gluten meal treated with β -MCD, and fish meal vs. fish meal treated with β -MCD.

Figure 4. Prostaglandin $F_{2\alpha}$ (FP) receptor trajectories.

Three individual FP receptor trajectories (A,B,C) on cell membranes obtained from a corn gluten meal supplemented cow and fish meal supplemented cow. The table shows calculated micro diffusion coefficient (D_{micro}), macro diffusion coefficient (D_{macro}), domain size, and residence time for each individual receptor.

Figure 5. Effects of dietary supplementation on lateral mobility of the prostaglandin $F_{2\alpha}$ (FP) receptor. Distribution of micro diffusion coefficient (A), macro diffusion coefficient (B), residence time (C), and microdomain diameter (D) of FP receptors in luteal tissue collected from corn gluten meal supplemented cows ($n = 4$; open bar; $n = 122$ receptors) or fish meal supplemented cows ($n = 4$; solid bar; $n = 104$ receptors). Diffusion coefficients were calculated using mean square displacement and domain size using sliding window variance (see Material

and Methods). The inset in each panel shows the mean $\pm$ standard error of the mean (* $P < 0.05$).

Figure 1

Figure 2

A

Figure 3

Effects of fish meal on lateral mobility of prostaglandin $F_{2\alpha}$ receptors in bovine luteal cells.				
Treatment	Diffusion Coefficient		Domain Characteristics	
	D_{micro} ($\mu m^2/s$)	D_{macro} ($\mu m^2/s$)	Residence Time(s)	Domain Size (μm)
Corn Gluten Meal A	0.106	0.00002	4.79	0.019
Corn Gluten Meal B	0.111	0.00002	7.26	0.024
Corn Gluten Meal C	0.040	0.00002	4.78	0.019
Fish Meal A	0.638	0.002	4.25	0.154
Fish Meal B	0.743	0.033	2.78	0.607
Fish Meal C	0.608	0.010	4.42	0.433

Figure 5

Table 1. Chemical composition of mixed grass hay

Dry matter intake, %	95
Chemical Analysis	
Crude Protein, %	15.2
Acid Detergent Fiber, %	37.0
Neutral Detergent Fiber, %	56.7
Water Soluble Carbohydrates, %	8.7
Simple Sugars, %	5.3
Starch, %	1.0
Non Fiber Carbohydrates, %	11.1
Crude Fat, %	3.5

Table 2. Ingredient, chemical composition, and long-chain fatty acid profile of supplements

Item	Experimental Diet	
	Corn Gluten Meal	Fish Meal
Dry Matter Intake, %	5	5
Ingredient of Pelleted Supplement, %		
Corn gluten meal	59.3	0
Fish meal	0	60.0
Wheat midds	19.2	17.4
Wheat – ground	7.3	7.8
Limestone	7.4	7.4
Molasses cane	4.0	4.0
Salt	1.3	1.3
Soybean oil-mixer	0.25	0.8
Magox	0.3	0.3
Monocal 16 21	0.25	0.25
Zinc sulfate	0.2	0.2
Selenium	0.1	0.1
Manganese sulfate	0.1	0.1
Copper sulfate	0.1	0.1
Vitamin A 30/3	0.1	0.1
Ranch-o-dine	0.02	0.02
Vitamin E 125	0.02	0.02
Vitamin D 0/30	0.02	0.01
Chemical Analysis		
Crude Protein, %	39.8	40.1
Undegradable Intake Protein, %	33.7	34.2
Degradable Intake Protein, %	29.5	29.9
Total Digestible Nutrients, %	68.8	69.6
Crude Fat, %	3.0	3.5
Fatty Acid Composition of Supplement, wt %		
Palmitic Acid	14.3	26.4
Palmitoleic Acid	1.5	8.7
Stearic Acid	17.8	4.6
Oleic Acid	38.8	12.5
Linoleic Acid	2.2	11.6
Alpha-Linolenic Acid	2.2	2.5
Arachidonic Acid	<0.5	1.1
Eicosapentaenoic Acid	<0.5	9.0
Docosahexaenoic Acid	<0.5	8.9