

# Immune System Responses to Diseases/Disorders in the Dairy Animal and Potential Effects of Essential Fatty Acids

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## Introduction

Long chain fatty acids (LCFA), primarily in the form of phospholipids, make up a significant component of the cell membrane structure in animals. These fatty acids can affect membrane fluidity and can serve as signaling molecules and as precursors for the synthesis of eicosanoids, just to name a few roles. In fact, the fatty acids have been called “gatekeepers” of cell regulation (Yaqoob, 2003). In addition, cells dedicated to fight and prevent health problems in animals also contain LCFA in their cell membranes. These LCFA within cells are very dynamic, with the fatty acid profile of these cells open to modification by the fatty acid profile of the diet of the animal. Therefore there is strong interest in conducting human and animal research to determine how dietary fatty acids can influence the immune system. Most of this work has been done in nonruminant species, with little focus on ruminant animals. This paper will give a very brief overview of the effects of the essential fatty acids, the  $\omega$ -6 (linoleic) and  $\omega$ -3 (linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid) fatty acids, on the cells and compounds involved in immune preservation primarily of bovine.

## Immune System Overview

Edward Jenner is considered to be the founding scientist of immunology. In 1796 he discovered that if cowpox (called vaccinia) was injected into humans, they would be protected against human smallpox, an often deadly disease. He called this process “vaccination,” a word that is still used today to describe the inoculation of healthy individuals with weakened strains of disease agents to protect them against such diseases.

The body cells dedicated to fighting infection are classified as white blood cells (WBC; leucocytes). They are produced in the bone marrow and moved to the peripheral blood and lymph systems. Between 7,000 and 25,000 WBC are found in a drop of blood. The major types of WBC are monocytes, macrophages, neutrophils, and lymphocytes. **Monocytes** migrate from the circulation to healthy tissues (e.g. lung, GIT, liver) where they mature into **macrophages** continuously. Macrophages are the first cells to encounter pathogens which they can kill by engulfing them (a process called phagocytosis) using nitric oxide, superoxide anion, and hydrogen peroxide. These

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macrophages help secrete compounds called **cytokines** which include **tumor necrosis factor alpha (TNF $\alpha$ )**, interferon gamma (IFN $\gamma$ ), and a number of interleukins (e.g. IL-1 $\beta$ , IL-6, IL-12). Cytokines set up a state of inflammation in the tissue to deal with the local pathogens. Inflammation involves dilation of small blood vessels which slows down blood flow and allows neutrophils and fluid to come into the tissue causing the characteristic redness, swelling, heat, and pain associated with inflammation. Macrophages also produce lipid mediators of inflammation such as prostaglandins and leukotrienes, using certain fatty acids found in phospholipids of cell membranes as precursors. The **neutrophils** are the first WBC to arrive at the infection in large numbers. They recognize, ingest, and destroy pathogens with the same chemicals used by the macrophages. They usually die after destroying the pathogens and are a major component of pus. Macrophages also produce **chemokines** that induce changes in the number of adhesion molecules (**L-selectin** and  **$\beta$ 2-integrin**) on the outer membrane surface of neutrophils. Adhesion molecules are so named because they adhere and anchor neutrophils in the bloodstream to the blood vessel wall and help move them into the infected tissue, a process known as extravasation. These adhesion molecules are expressed by the endothelial cells of the blood vessels under the influence of proinflammatory cytokines like TNF $\alpha$ . TNF $\alpha$  induces the liver to produce proteins called **acute phase proteins** because they are produced in response to an acute inflammation or stress. These proteins are released into the blood by the liver and can mimic the action of antibodies but are nonspecific, that is, they can bind to a broad range of pathogens. Some of the acute phase proteins are **fibrinogen**, **ceruloplasmin**, **haptoglobin** and **acid soluble protein**. They help clear the blood of endotoxins produced by the gram-negative bacteria. **Fibrinogen** is a sticky, fibrous protein used to make fibrin to serve a key role in blood clotting and tissue repair. **Ceruloplasmin** is a protein that carries 90% of the copper in blood and helps oxidize ferrous iron to ferric iron for transportation by transferrin in blood and then uptake of iron by tissues. It may help prevent oxidative damage to endothelial cells during inflammation. It also helps **Haptoglobin** binds hemoglobin so that the iron in hemoglobin is not available to bacteria for replication. The liver may not be the only tissue able to synthesize haptoglobin in that mRNA expression of haptoglobin was detected in the alveolar epithelium of the bovine mammary gland (Thielen et al., 2007). However, **acid soluble protein** contains mostly  $\alpha_1$ -acid glycoprotein and is an anti-inflammatory agent that controls inappropriate or extended activation of the immune system. (See Jafari et al., 2006 for periparturient responses of  $\alpha_1$ -acid glycoprotein.)

Living organisms have both an innate immune system and an adaptive immune system. The innate immune system responds to pathogenic invasion immediately, fighting to destroy the infection without any prior “experience” with that pathogen. The adaptive immune system may take 4 to 7 days to take effect. In the adaptive immune system, specific antibodies (immunoglobulins) are produced against specific pathogens that provide life-long protective immunity to reinfection by that same pathogen. **Lymphocytes** belong to the adaptive immune system. Lymphocytes that are produced in the bone marrow but mature in the thymus are called T-lymphocytes. Like the macrophages, they are able to produce cytokines to create an inflammatory state and activate macrophages and B-lymphocytes. Lymphocytes that mature in the bone

marrow are called B-lymphocytes and they produce antibodies. (Janeway et al. (2005) was used as a source of information for this overview section.) Researchers will often isolate blood lymphocytes from experimental animals and stimulate them to proliferate and produce cytokines to identify differential response to experimental treatments.

### **Disease of the Dairy Animal, Performance, and Immune Markers**

The periparturient period of the dairy cow is a time of significant stress on her immune system (Goff and Horst, 1997) such that her immune response is suppressed, resulting in an increased risk of disease. As expected, the reproductive and production performance of cows that experience disease are reduced.

Mastitis. In a study involving 2087 cows, those that had clinical mastitis during the first 45 d postpartum were at 2.7 times greater risk of abortion within the next 90 d compared to those without mastitis (Risco et al., 1999). Coliform organisms that can cause mastitis liberate lipopolysaccharide endotoxin which in turn can cause an inflammatory response by the cow so that she releases  $\text{PGF}_{2\alpha}$ . High enough levels of  $\text{PGF}_{2\alpha}$  in the blood can result in luteolysis and therefore early embryo loss if the cow is pregnant. Although mastitis-causing gram positive bacteria do not produce endotoxins, the peptidoglycans comprising their cell wall can elicit an inflammatory response by the cow as well. Cows having mastitis after their first AI required an extra AI for pregnancy, thus having more days open than those without mastitis (Barker et al., 1998). Incidence of mastitis occurring close to first AI resulted in lower pregnancy rates for those cows compared to cows without such untimely mastitis in the Netherlands (Loeffler et al., 1999). Also the 'risk' of pregnancy (odds ratio) was reduced if cows experienced displaced abomasum (0.25;  $P = 0.036$ ), retained fetal membranes (RFM) (0.55;  $P = 0.004$ ), and loss of 1 BCS (0.80;  $P = 0.007$ ) but not milk fever (0.85;  $P = 0.12$ ) (Loeffler et al., 1999).

Milk was collected from 62 dairy cows and classified according to 4 concentrations of somatic cells, namely, <12,000, 12,000 to 100,000, 100,000 to 350,000, and > 350,000 per ml of milk (Sarikaya et al., 2006). As the concentration of somatic cells increased, the proportion of neutrophils increased. Neutrophils made up <10%, ~30%, ~50%, and ~65% of the somatic cells with increasing concentration of SCC. Expression of mRNA for  $\text{TNF}\alpha$  increased approximately 4-fold and COX-2 (an enzyme involved in the synthesis of prostaglandins from fatty acids) increased approximately 2 to 4-fold as SCC concentration increased from lowest to highest. Authors concluded that "natural stimuli like pathogens cause the effect of an upregulation of all inflammatory factors."

Cows with a new intramammary bacterial infection during the first 8 weeks of lactation had lower plasma concentrations of cholesterol (127 vs. 174 mg/100 ml) and albumin (36.0 vs. 38.4 mg/ml) and an unexpected lower concentration ( $P = 0.06$ ) of plasma haptoglobin at week 1 postpartum (Rezamand et al., 2007).

Endometritis. Most or all cows experience uterine inflammation after calving. Those cows that are unable to resolve the inflammation develop infections (metritis and endometritis) and suffer lowered reproductive performance. The incidence rate of non-specific uterine infections in dairy cattle herds ranges between 10 and 50% (Lewis and Wulster-Radcliffe, 2006). They are classified as non-specific infections because the bacteria involved in the infection were not identified. Endometritis occurs later than metritis. Endometritis is defined as the inflammation of the uterine endometrium without any signs of systemic infection but the diagnosis of endometritis varies widely and thus so does the incidence rate. Rectal palpation and a vaginal discharge are often used for diagnosis. A vaginal speculum examination enhanced the sensitivity of the diagnosis between 20-33 DIM (LeBlanc et al., 2002). Failure to use vaginoscopy would have missed 44% of the clinically relevant cases in a study of 1865 cows in 27 herds. Between 40 and 60 days postpartum, the uterus of 141 cows housed on 5 New York dairies was flushed and analyzed for neutrophils as evidence of endometritis. Incidence of endometritis ranged from 37 to 74% across farms based upon a cut-off point of 5% of total cells as neutrophils (Gilbert et al., 2005). Animals having endometritis had more days to first service (101 vs. 80; cows bred to observed estrus) and a lower pregnancy rate (69 vs. 90%). The relationship of parity and incidence of endometritis has been quite variable but most report a lack of relationship (Grohn et al., 1990; Gilbert et al., 2005). However LeBlanc et al. (2002) reported that 21% of cows in their 3<sup>rd</sup> or later parity had endometritis compared to 13% and 12% for cows in their 2<sup>nd</sup> or 1<sup>st</sup> parity.

The percentage of neutrophils in the uterus of 228 lactating, healthy Holstein cows on two Canadian dairy farms was determined at 2 time points: 20 to 33 days in milk and 34 to 47 days in milk (Kasimanickam et al., 2004). A cytobrush was used to brush the uterine wall and collect cells. Cows were diagnosed as having subclinical endometritis when the proportion of neutrophils exceeded 18% or 10% at the first and second examination, respectively. 37% (80/215 cows) of cows had endometritis. The first service pregnancy rate of cows diagnosed with subclinical endometritis at the second examination was 18% compared to 32% for healthy cows. Likewise the overall pregnancy rate (35 vs. 61%) and number of days open (100 vs. 162 days) was worse for cows having endometritis. Plasma concentrations of PGFM were greater at 0.5 and 1.5 days after calving in cows diagnosed with “heavy” versus “mild” endometritis.

Based upon the activity of plasma paraoxonase (a liver protein with hydrolase activity that is reduced when the liver is damaged), Friesian dairy cows (n=67) were retrospectively grouped into 4 categories (Bionaz et al., 2007). Cows with the highest activity of paraoxonase had the lowest concentration of haptoglobin and bilirubin but the highest concentration of albumin at 7 and 14 DIM. Plasma ceruloplasmin concentrations were not different among groups. 94% of cows in the low paraoxonase group had a health problem compared to 12% for the high paraoxonase group, with much of the difference due to greater incidence of metritis.

Fatty liver. Holstein dairy cows (n=10) with fatty liver (~11% lipid, wet weight) had lower concentrations of plasma TNF- $\alpha$  at 12, 14, and 22 DIM but greater concentrations of haptoglobin at 3, 8, and 12 DIM compared to cows with normal livers

(Ametaj et al., 2005). Plasma concentrations of PGE<sub>2</sub> were lower on day 8 of lactation of cows with fatty liver. Increased lipolysis and plasma NEFA were associated with the acute phase response (Hardardottir et al., 1994).

Calf health and stress. Haptoglobin was not detected in the serum of Friesian calves until they were orally given Salmonella, after which the mean serum concentration rose to 212 ug/ml at 3 days after infection (Deignan et al., 2000). Concentrations returned to normal by day 5.

Holstein bull calves (n=120) that had greater plasma concentrations of IgG on days 7 and 14 of life also had greater plasma concentrations of TNF $\alpha$ , suggesting that calves that had a successful passive transfer of immunoglobulins were better able to mount a good immune response to pathogens (Quigley et al., 2006).

Plasma concentrations of fibrinogen and haptoglobin decreased in bull and steer calves (205 kg of BW average) between the first day of arrival at a feedlot and 28 days later, indicating that these acute phase proteins were indicative of elevated stress by the animals (Berry et al., 2004). In addition, calves that developed bovine respiratory disease and were treated one or more times with antibiotics had greater plasma concentrations of haptoglobin than healthy calves.

### **Fatty Acids and Animal Immune Responses**

Changing the LCFA profile of cells. Changing the LCFA profile of the diet can directly change the LCFA profile of cells within the body and the way they function. The proportions of  $\omega$ -3 fatty acids were increased in the uterine endometrium of beef cows fed fish meal (Burns et al., 2003), in the caruncles of dairy cows fed fish oil (Mattos et al., 2004) and in the plasma and liver of dairy cows fed linseed oil (Amaral et al., 2006). Often the increase in proportion of  $\omega$ -3 fatty acids in tissues comes at the expense of  $\omega$ -6 fatty acids (linoleic and/or arachidonic acids) as documented with uterine endometrium (Burns et al., 2003), caruncles (Mattos et al., 2004), and plasma (Amaral et al. 2006). These changes in turn affected tissue function. Replacing uterine linoleic acid with  $\omega$ -3 fatty acids was likely responsible for reducing the production of PGF<sub>2 $\alpha$</sub>  by the uterus (Mattos et al., 2004).

In addition, the LCFA profile of immune cells can be changed by the fatty acid environment that they live in. Macrophages were collected from the peritoneal cavity of mice 4 days after interperitoneal injection of thioglycollate broth (Calder et al., 1990). The macrophages were incubated with various LCFA at physiological concentrations in order to determine if the composition of the cell membranes and the rate of phagocytosis could be modified. The proportion of LCFA in the phospholipid fraction of macrophage cell membrane was increased for each LCFA tested; namely, from 21 to 34% for C16:0, from 18 to 31% for C18:0, from 15 to 37% for C18:1, from 5 to 38% for C18:2, from 0 to 16% for C18:3, from 14 to 28% for C20:4, from 0 to 12% for C20:5, and from 1 to 13% for C20:6. The macrophages enriched in these LCFA were then exposed to zymosin, an insoluble carbohydrate of yeast cell wall, over a 2 hour period at 37°C in

order to test their ability to phagocytize zymosin. In comparison to the macrophages having the LCFA profile as coming directly from the mice, those macrophages enriched in the saturated fats or oleic acid phagocytized at a slower rate (72 to 89% of controls) whereas those enriched in the polyunsaturated fatty acids phagocytized at a faster rate (126 to 148% of controls). This improvement was likely due to an increased fluidity of the membrane which can influence receptor number and affinity. Changing the fatty acid profile of the membranes of liver cells (Luly and Shinnitsky, 1979), adipose cells (Gould et al., 1982) and red blood cells (Amatruda and Finch, 1979) strongly influenced the number of insulin receptors and their affinity for insulin (as cited by Calder et al., 1990).

Prostaglandin F<sub>2α</sub>. Prostaglandins are mediators of inflammation as they are produced by macrophages and are thought to be an attractant for neutrophils to the site of infection (Hoedemaker et al., 1992). PGF<sub>2α</sub> is produced by the uterus at the time of parturition and its concentration is measured as its metabolite (PGFM) in plasma. The secretion of PGF<sub>2α</sub> is associated with involution of the uterus and to help reduce the risk of uterine infection during this vulnerable time. Cows that have uterine infections usually have higher concentrations of circulating PGFM since prostaglandins are part of the inflammatory response (Del Vecchio et al., 1994). Dairy cows (n=14) diagnosed with heavy endometritis (fetid sanguine-purulent lochia) had greater concentrations of plasma PGFM and uterine fluid PGE<sub>2</sub> than cows with mild endometritis (mucopurulent to purulent lochia) (Mateus et al., 2003). However this does not mean that cows with greater concentrations of PGFM following parturition are more prone to uterine infections. On the contrary, those cows exhibiting greater plasma concentrations of PGFM around parturition may be better able to combat pathogenic microorganisms due to a greater production of PGF<sub>2α</sub>. Cows that peaked higher in plasma PGFM and had prolonged plasma concentrations of PGFM postpartum did not develop metritis after 15 days postpartum compared to those that did get metritis (Seals et al., 2002).

The precursor of PGF<sub>2α</sub> is arachidonic acid (C20:4) which is made from linoleic acid. With increased intake of linoleic acid, the uterus may produce more PGF<sub>2α</sub> around the time of calving. PGF<sub>2α</sub> likely stimulates phospholipase A<sub>2</sub> which releases arachidonic acid from phospholipids in the endometrium which in turn is synthesized into PGF<sub>2α</sub>. TNFα stimulated production of PGF<sub>2α</sub> by stromal cells of the uterus (Skarzynski et al., 2000). Supplying arachidonic acid with TNFα to the media dramatically increased synthesis of PGF<sub>2α</sub>.

Feeding supplemental linoleic acid prepartum to dairy cows elevated plasma PGFM postpartum and may have contributed to better performance. Calcium salts of vegetable oil enriched in linoleic acid (Megalac-R, Church and Dwight, Inc.) were fed to Holstein cows (n = 47) at 2% of dietary DM starting at 28 days prior to parturition, at the day of calving, at 28 days postpartum, or not at all (Cullens, 2005). Cows stayed on diets through 100 DIM. Blood samples were collected on Monday, Wednesday, and Friday from calving through the first AI (72 ± 3 DIM) and analyzed for numerous metabolites and hormones. Initiating fat supplementation during the prepartum period appeared to have several advantages. These cows tended to produce more milk than

cows started on fat after calving (93.0 vs. 81.8 lb/day). Although concentration of fat was greater in the liver (23 vs. 10.4%, DM basis) of cows fed fat prepartum, it did not seem to negatively affect health or performance. Cows fed the fat source prepartum had a slower decrease in plasma PGFM the first 14 days postpartum than cows not fed fat prepartum. However this difference did not affect uterine involution, in that the size of the previous pregnant horn and the size of the cervical os at 21 and at 28 DIM were not different. In addition, the amount and quality of the vaginal discharge at 21 and 28 DIM were not affected by treatments. On the contrary, a significantly lower proportion of the cows fed fat prepartum were diagnosed with a disease (mastitis, metritis, or retained fetal membranes) compared to cows not fed fat prepartum (1/12 vs. 15/35). Another indicator that cows fed fat prepartum were experiencing better health was a tendency toward elevated concentrations of plasma albumin during the first 4 wk postpartum (2.89 vs. 2.67 g/100 ml). Concentration of serum albumin falls in an acute disease state. Plasma concentrations of bilirubin were greater in cows fed fat prepartum (0.36 vs. 0.23 mg/100 ml) which may have been due to the greater concentration of fat in the livers of cows fed fat prepartum. Bilirubin increases due to severe hepatic damage but the values of cows on this study were within the normal range of 0 to 0.5 mg/100 ml for cattle (Merck Veterinary Manual, 1997). Plasma concentrations of the acute phase proteins, haptoglobin (31.9 vs. 17.0 mg HbB/100 ml) and ceruloplasmin (24.4 vs. 20.7 mg/100 ml), were greater for heifers compared to cows but were not affected by fat-feeding treatments. However, plasma concentrations of fibrinogen did not follow the same pattern. Fibrinogen was greater in heifers than cows if not fed fat (132 vs. 116 mg/100 ml); but for heifers fed fat prepartum or starting at parturition, fibrinogen was lower than or similar to that of cows, respectively. Are greater plasma concentrations of acute phase proteins in primiparous cows indicative of a more stimulated and protective immune system? Based upon neutrophil activity, older cows are more susceptible to disease than younger cows. The function of neutrophils collected from the blood of dairy cows in their fourth or greater lactation was more impaired when stimulated in vitro than those from cows in their third or less lactation (Gilbert et al., 1993). However neutrophil numbers were not affected by parity. Likewise, the viability of polymorphonuclear WBC in milk and the number of immature neutrophils in blood were lower from cows in their 4<sup>th</sup> to 5<sup>th</sup> lactation compared to their 1<sup>st</sup> lactation (Mehrzhad et al., 2002). Therefore, the higher plasma concentrations of the acute phase proteins in heifers may be indicative of a better responding immune system.

In a second Florida study, early postpartum concentrations of plasma PGFM peaked higher for lactating multiparous cows when fed a calcium salt of a mixture of trans C18:1 fatty acids (EnerG TR, Virtus Nutrition) compared to cows fed a saturated fat source (Rumen Bypass Fat, Cargill) (Rodriguez-Sallaberry et al., 2007); however, concentrations of plasma PGFM of lactating heifers were not affected by fat source (diet by parity by days interaction). In this study involving 30 animals, the incidence of metritis was not different between the two groups (1/15 vs. 4/15). These trans fatty acid isomers are not known precursors of the prostaglandins and so this response and mechanism on PGFM needs further research. In a third Florida study (Amaral et al., unpublished), the pattern of plasma concentrations of PGFM during the first 10 days postpartum were not different between cows not fed fat and those fed a calcium salt of

safflower oil, an excellent source of linoleic acid, at 1.5% of dietary DM starting 4 weeks prepartum. This lack of effect of feeding a calcium salt of safflower oil on plasma PGFM postpartum was repeated on a commercial dairy farm with the control cows being fed a calcium salt of palm oil distillate (Silvestre et al., unpublished). Two other studies have reported increased plasma PGFM of dairy cows fed oils enriched in linoleic acid. Dairy cows fed whole sunflower seeds (9.6% of diet) as a source of linoleic acid experienced a greater increase in plasma concentrations of PGFM after an oxytocin challenge than cows fed whole flaxseed, calcium salt of palm oil distillate, or no fat (Petit et al., 2004). Likewise, dairy cows fed supplemental rumen-protected linoleic acid (Soypreme, steam-treated soybeans) had a greater rise in plasma PGFM after an oxytocin challenge than cows fed rumen-protected linolenic acid (Linpreme, steam-treated linseeds) or no fat (Robinson et al., 2002). Therefore feeding additional linoleic acid to dairy cows may provide additional precursors for the synthesis of PGFM; however, whether this increase results in improved health is yet to be adequately documented.

White Blood Cells and Proinflammatory Molecules. The effect of lipid source on immune response was tested by feeding Holstein cows diets containing one of the following fat sources as a percentage of the diet prepartum and postpartum, respectively: 5.9 and 9.7% whole flaxseed, 2.7 and 4.7% calcium salt of palm oil distillate, or 9.4 and 20.3% micronized soybeans between 6 weeks before and 6 weeks after calving (Lessard et al., 2004). Thus the dietary concentration and intake of C18:3 (flaxseed), C16:0 & C18:1 (palm oil), and C18:2 (soybeans) were different among the 3 diets. An injection of ovalbumin was given s.c. at 6 and 2 weeks prepartum. Multiparous cows fed the fat source enriched in C18:2, soybeans, had a greater antibody response against ovalbumin in colostrum but not in blood compared to cows fed the other 2 fat sources, but heifers responded similarly across fat sources. Blood samples were collected at approximately 6, 3, and 1 week before and after calving. Peripheral blood mononuclear cells (PBMC) were isolated from the blood and stimulated to proliferate. Concentrations of TNF $\alpha$  and PGE $_2$  were measured in the media and were not affected by fat source. However the proliferation of PBMC were lower in animals fed soybeans. Likewise there was a significant inhibition of the mitogen-stimulated proliferative responses of lymphocytes when PBMC were incubated with high concentrations of linoleic acid (125 and 250  $\mu$ M) (Thanasak et al., 2005). However, increasing amounts of linoleic acid had no effect on lymphocyte proliferation. Similarly feeding safflower seeds high in linoleic acid (0 or 9.5% of diet) to nursing Angus cows resulted in fewer serum antibodies in their calves injected with ovalbumin (Lake et al., 2006). Rectal temperatures and average daily gain (0.90 vs. 0.66 kg/d for safflower seed-fed group and controls, respectively) did not differ. Elevated intake of C18:2 may not be the causative agent because in a second study using 3-year-old beef cows, production of serum antibodies against ovalbumin injections of calves tended to be lower by calves fed safflower seeds enriched in either C18:1 or C18:2 (~8% of dietary DM) compared to control calves fed beet pulp instead. Simply overfeeding oil can have a negative effect on the immune system. Elevated NEFA in plasma may be antagonistic to the immune system. Lymphocytes of heifers were collected and incubated in vitro with increasing concentrations of NEFA (Lacetera et al., 2004). Synthesis of DNA was diminished when concentrations of NEFA were at a clinically

ketotic level. Secretion of IgM and production of INF-gamma by lymphocytes were lower even at moderate concentrations of NEFA. Since blood NEFA concentrations often are elevated when fat is supplemented, care should be taken to not overfeed fat.

Multiparous Holstein cows at a commercial dairy farm in Florida were supplemented (1.5% of dietary DM) with calcium salts of palm oil distillate (47% C16:0) or of safflower oil (64% C18:2) (Virtus Nutrition) from approximately 30 days prior to calving through ~30 days post calving in 2007-08 (Silvestre et al., unpublished). At ~30 days post calving, cows were switched to calcium salts of palm oil distillate or calcium salts enriched in fish oil (11% C20:5 + C22:6) (Virtus Nutrition) through 2 AI sequences if needed. Therefore the 4 treatment sequences were the following: 1) palm oil-palm oil, 2) palm oil-fish oil, 3) safflower oil-palm oil, and 4) safflower oil-fish oil. Blood samples were collected daily during the first 10 DIM and Monday-Wednesday-Friday thereafter. Intake of C18:2 increased by approximately 74 grams prepartum and by 118 grams postpartum by cows fed safflower oil compared to those fed palm oil. Intake of C20:5 + C22:6 increased by approximately 19 grams per day by cows fed fish oil. Plasma concentrations of PGFM during the first 10 days (n=32 cows) were not different due to supplementing with safflower oil. Vaginoscopy using a disposable speculum was performed at 8 DIM to evaluate cervical discharge (n = 1116). Vaginal mucus was scored from 0 to 3 based upon appearance (0 = clear, 1 = flecks of white pus, 2 = <50% white mucopurulent, or 3 = >50% white/red purulent) using the scoring system of Sheldon et al. (2006). The frequency of diagnosis of uterine infection (score of 0+1 vs. 2 or 3) did not differ between cows fed palm oil (57% vs. 14.4% and 28%) or safflower oil (59% vs. 10% and 30%). Blood samples were collected at approximately -35, 0, 4, and 7 DIM to evaluate phagocytic and oxidative burst of neutrophils in whole blood using a dual color flow cytometry method. In addition, the expression of adhesion molecules, L-selectin and  $\beta$ 2-integrin, that bring neutrophils and monocytes from the blood into the tissues to kill pathogens was evaluated on the same days. The proportion of neutrophils that phagocytized with oxidative burst when exposed to *E. coli* did not differ between dietary treatments (n = 47) but phagocytosis did increase with increasing DIM, from 38% to 53% and 57% for 0, 4, and 7 DIM, respectively. Despite the lack of difference in neutrophil activity, cows fed additional linoleic acid from safflower oil did show evidence of improved immune response. The mean fluorescence intensity (MFI) of L-selectin associated with neutrophils coming from cows fed safflower oil was greater (P < 0.08) than that coming from cows fed palm oil on 4 and 7 DIM (n = 45). The MFI of  $\beta$ 2-integrin associated with neutrophils or monocytes was not affected by diet or DIM. However, the number of monocytes positive for L-selectin was greater at 4 (6467 vs. 5129) and 7 (6213 vs. 5304) DIM for cows fed safflower oil and the number increased with increasing DIM to a greater degree when safflower oil was fed (diet by day interaction). Monocytes positive for  $\beta$ 2-integrin was not different between treatments. Another measurement of neutrophil activity was to assess their ability to produce the cytokine TNF $\alpha$  after stimulation with bacterial lipopolysaccharide (LPS). At 30 DIM, neutrophils were isolated from blood samples (n = 16) and were cultured with or without LPS stimulation. Concentration of TNF $\alpha$  was greater in culture from neutrophils collected from cows fed safflower oil compared to those fed palm oil under both the nonstimulated (52.7 vs. 30 pg/ml) and LPS-stimulated (107 vs. 63.2 pg/ml)

conditions. At 80 DIM, concentration (42.5 vs. 82.7 pg/ml) and mass increase (4.6 vs. 47.5 pg/ml) of TNF $\alpha$  from LPS-stimulated neutrophils was less from cows fed fish-oil compared to cows fed palm oil. In agreement, growing pigs fed menhaden fish oil (5% of diet) had lower serum concentrations of TNF $\alpha$  than pigs fed corn oil when treated with LPS (Gaines et al., 2003). Another potential indication of a more active immune system in cows fed supplemental C18:2 was the increase in plasma concentrations of both acute phase proteins, haptoglobin (0.034 vs. 0.020 OD) and fibrinogen (248.8 vs. 205.3 mg/100 ml). Although physiological indicators of immune health were improved in cows fed supplemental safflower oil in a calcium salt form, health disorders were not different among the four groups of cows over the complete study or between the palm oil and safflower oil-fed groups during the first 30 days postpartum.

The same calcium salts of safflower oil and fish oil were used in a trial at the University of Florida dairy research farm in 2007 (Amaral et al., unpublished). In this study, 45 Holstein cows (16 primiparous and 29 multiparous cows) were fed one of 3 diets containing 0 or 1.5% calcium salt of safflower oil or fish oil starting at ~35 days prepartum through 7 weeks postpartum. Based upon milk and caruncle analysis, some of the target fatty acids were leaving the rumen intact and available for metabolism by the cows. The linoleic acid concentration in milk fat (3.94 vs. 3.49%) and caruncles (14.9 vs. 11.7%) sampled from cows fed safflower oil were greater than those of control cows. Likewise, the concentrations of C20:5 (0.09 vs. 0.02%) and C22:6 (0.094 vs. 0.001%) in milk fat and caruncles (1.22 vs. 0.86% for C20:5 and 1.06 vs. 0.41% for C22:6) were greater from cows fed fish oil compared to controls. Using a metricheck tool, a sample of vaginal mucous was collected on 5 and 10 DIM from each cow and the sample visually scored from 0 to 3 according to the level of infection (Sheldon et al., 2006) with 3 being the most infected. The proportion of animals that scored 3 was 10/14, 10/16, and 11/16 when sampled at 5 DIM and was 9/16, 7/16, and 12/16 when sampled at 10 DIM for controls, safflower oil, and fish oil, respectively.

Not all of the acute phase proteins responded in the same manner to fat supplementation. Concentrations of fibrinogen, haptoglobin, and acid soluble protein all increased postpartum indicating an inflammatory response associated with parturition. Only ceruloplasmin concentrations were not different across days. As in the Silvestre study, feeding the omega-6 fat increased plasma concentrations of fibrinogen (214, 259, and 206 mg/100 ml for control, safflower oil, and fish oil respectively) of multiparous cows but not primiparous cows (272, 226, and 254 mg/100 ml; treatment by parity interaction). Haptoglobin concentrations were numerically but not significantly increased as in the previous study of Silvestre (0.035, 0.040 vs. 0.039 OD) by feeding a supplemental omega-6 fat. In addition, plasma concentrations of acid soluble protein were greater during the first 3 weeks postpartum in multiparous cows fed omega-6 (63.2 ug/ml) or omega-3 (56.3 ug/ml) fats compared to controls (42.9 ug/ml) but this was not true for primiparous cows (parity by treatment by days interaction) ( $P = 0.06$ ). The exact role of acid soluble protein is not well understood, although it is thought to be anti-inflammatory. If that is the case, an increase in plasma acid soluble protein postpartum in cows fed linoleic acid may indicate the host's response to try to minimize the

proinflammatory effects of linoleic acid but be reflective of the anti-inflammatory effects of fish oil.

Blood was collected at ~18 days prepartum and at 0, 7, and 40 DIM and analyzed for concentration of WBC (using hemocytometer), percentage of WBC as neutrophils (Diff-quick staining) and neutrophil activity using flow cytometry. Neither the concentration of WBC (11,694 and 11,290 WBC/ul) nor that of neutrophils (3528 vs. 3461 per ul) differed between cows not fed fat and those fed omega-6 fatty acids. The proportion of WBC that were neutrophils averaged 30% and did not differ among dietary treatments but did differ by day of collection, being 29.7, 33.9, 26.3, and 29.6% for DIM -18, 0, 7, and 40, respectively. However the decrease in neutrophil number between days 0 and 7 was less for cows fed omega-6 compared to cows not fed fat and those fed fish oil, indicating that the feeding of omega-6 helped minimize the typical drop in blood neutrophils shortly after parturition. When presenting the isolated neutrophils with *E. coli in vitro*, the proportion of neutrophils that underwent phagocytosis and oxidative burst was not affected by dietary treatment. Evidence of anti-inflammatory effects of the fish oil was detected. Cows fed the omega-3 fats tended to have a lower concentration of WBC in blood (8796 vs. 11,290/ul), no difference in neutrophils as a % of WBC (28.5 vs. 30.3%), and a significantly lower concentration of neutrophils (2463 vs. 3461/ul) compared to the other treatments. In addition the intensity of phagocytosis tended to be lower in cows fed omega-3 compared to cows fed omega -6 (260 vs. 323) as measured using mean fluorescence intensity (MFI) indicating that each neutrophil consumed less bacteria. Some acute phase proteins (ceruloplasmin and fibrinogen) also indicated that the feeding of fish oil may be anti-inflammatory. Plasma concentrations of ceruloplasmin was lower in heifers fed fish oil compared to those fed safflower oil (11.1 vs. 10.5 ug/ml) but concentrations were not different in cows (11.1 vs. 11.4 ug/ml; parity by safflower vs. fish interaction). Plasma concentrations of fibrinogen were lower in heifers fed fish oil compared to those fed safflower oil (206 vs. 259 mg/100 ml) but concentrations were not different in cows (254 vs. 226 mg/100 ml; parity by safflower vs. fish interaction).

At ~33 DIM, the previously pregnant horn of the uterus was flushed with 20 ml of sterile saline. A sample of the collected fluid was placed in a hemacytometer in order to count total WBC and to determine cell viability. Cells not stained were considered alive and cells stained with trypan blue were considered dead. A further sample was stained (Protocol Hema3) to count cells as WBC, neutrophils, or epithelial cells. Severity of endometritis was graded according to the average number of neutrophils per ml of uterine flush. Of the animals detected with alive WBC (n=36), the concentration of live WBC in the uterine flush appeared greater in cows not fed fat compared to those fed safflower oil or fish oil (21,820, 4,201, and 1,419 x 1000/ml respectively), a large standard error prevented the detection of treatment differences. In a Florida study in which linseed oil (source of omega-3) was fed at 1.35% prepartum and at 1.5% postpartum, primiparous cows tended to have fewer neutrophils in a uterine flush compared to those fed high oleic acid sunflower oil, calcium salts of trans C18:1isomers, or Megalac-R (Amaral et al., 2005).

Further potential benefits of omega-3 fats or CLA. The anti-inflammatory action of fish oil may be associated with its partially inhibiting effects on the production of proinflammatory cytokines. Dietary fish oil was compared to corn oil (both at 7% of dietary DM) as a means to modulate the immune response of cross-bred pigs injected intraperitoneally with *E. coli* lipopolysaccharide (LPS) or saline (Liu et al., 2003). LPS can stimulate macrophages which in turn secrete proinflammatory cytokines. These cytokines can act systemically on organs and tissues to change metabolism, behavior, and neuro-endocrine secretions, possibly inhibiting growth. If dietary nutrients are partitioned away from growth toward assisting the immune response, efficiency of animal productivity will be reduced. Proliferation of lymphocytes and antibody production against bovine serum albumin were not affected by oil source. As expected, pigs given the LPS challenge responded with elevated plasma concentrations of interleukin-1 $\beta$  and cortisol but lower plasma concentrations of IGF-1. It has been suggested that reduced IGF-1 concentrations reflect the shifting of nutrients from normal growth to immunity needs and lowered IGF-1 is a causative factor for lowered weight gain during disease or stress. Therefore the elevation of proinflammatory cytokines may impair IGF-1 production. The feeding of fish oil attenuated these effects. That is, the LPS-stimulating effect on interleukin-1 $\beta$ , PGE<sub>2</sub>, and cortisol and its suppressing effect on IGF-1 were reduced when pigs were fed fish oil compared to corn oil. Feeding calcium salts of LCFA enriched in fish oil (Virtus Nutrition) to bST-injected lactating dairy cows resulted in elevated plasma concentrations of growth hormone and greater milk production but lower plasma IGF-1 (Bilby et al., 2006).

Conjugated linoleic acid (CLA) has affected some immune responses similarly as omega-3 fatty acids. Baby chicks were fed diets of 0, 2.5, 5, or 10 g of CLA (equal *cis*-9, *trans*-11 and *trans*-10, *cis* 12 isomers) for 6 weeks (Zhang et al., 2005). Although growth was not affected, chicks fed increasing amounts of CLA had increasing proliferation of peripheral mononuclear cells stimulated with LPS or ConA and greater production of antibodies.

In 2007, Caldari-Torres et al. analyzed the effects of two isomers of CLA on cytokine responses in cultured bovine PBMC. This study focused on the short term-effect of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers of CLA on interleukin-4 (IL-4), TNF $\alpha$ , and INF- $\gamma$  production by ConA-stimulated bovine PBMC. Interleukin-4 is considered an anti-inflammatory cytokine that shifts the immune system towards antibody production (a humoral response) whereas TNF $\alpha$  and INF- $\gamma$  are both pro-inflammatory and tend to drive cell-mediated responses. Bovine PBMC were isolated from nonpregnant lactating Holstein cows through density gradient centrifugation, and treated with ConA (10  $\mu$ g/mL) and the appropriate CLA isomer (100  $\mu$ M) at time of plating. Differences in IL-4 or TNF $\alpha$  due to fatty acid treatment were not detected. In contrast, co-incubation with the *trans*-10, *cis*-12 CLA isomer decreased ConA-induced INF- $\gamma$  production in cultured bovine PBMC (unpublished data). These *in vitro* findings provide no evidence for CLA-mediated improvement of immune functions in cattle. Whether these findings reflect the physiological effects of these fatty acids *in vivo* warrants further investigation.

## Summary

1. Dietary fats can change the fatty acid profile of tissues and WBC.
2. Parturition results in a proinflammatory condition based upon rapid increases in plasma concentrations of several acute phase proteins.
3. Acute phase proteins of primiparous cows respond differently to supplemental fats than those of multiparous cows.
4. Feeding supplemental linoleic acid can increase the release of prostaglandin F metabolite from the uterus of cows because it is a precursor for its synthesis. This may be advantageous around parturition when risk of disease is high because  $\text{PGF}_{2\alpha}$  is proinflammatory and can help attract neutrophils to fight infections.
5. Supplemental linoleic acid has often influenced the immune responses of bovids. On the immuno-stimulatory side, cows fed supplemental linoleic acid had 1) greater concentration of colostrum antibodies, 2) improved intensity of the adhesion molecule, L-selectin, for movement of monocytes and/or neutrophils out of the blood to the sites of infection, 3) increased production of TNF by neutrophils, and 4) increased plasma concentrations of haptoglobin, fibrinogen, and acid soluble protein. Providing supplemental linoleic acid to bovids in vivo or in vitro lowered proliferation of stimulated lymphocytes and lowered antibody concentration in plasma.
6. Feeding omega-3 fatty acids starting in the prepartum period has had anti-inflammatory effects. The number of WBC and neutrophils in blood were lower compared to cows not fed fat. The TNF $\alpha$  synthesis by isolated blood neutrophils was lowered at 70 DIM compared to cows fed calcium salts of palm oil distillate. The intensity of neutrophil phagocytosis was lower and the plasma concentration of some acute phase proteins were lower in heifers than cows when compared against animals fed an omega-6 supplement.

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# **SESSION NOTES**