

Diet supplementation with omega-3 affect reproductive traits and milk yield
of lactating dairy cows

by

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Abstract

Fatty acids can modulate important functions including metabolic pathways, health, and reproduction. Supplementation with fatty acids is a common and efficient practice to supply energy to the dairy cow. Several ingredients in dairy cattle diets, however, are rich in omega-6 fatty acids, with only a few ingredients supplying omega-3 fatty acids to the diet. This situation can create an imbalance that can impact milk production and reproduction because omega-6 fatty acids are related to pro-inflammatory responses, whereas omega-3 fatty acids are related to anti-inflammatory responses. In the first chapter of this thesis, a literature review of fatty acid classification, common sources of omega-6 and omega-3 fatty acid supplementation, important roles of fatty acids in cattle, and effects of omega-3 supplementation in milk production and reproduction elaborate on the importance of omega-3 fatty acid supplementation. In the second chapter, one experiment is described, which assessed effects of omega-3 supplementation on milk, oocyte quality, in vitro embryo quality, and in vivo conceptus development during the first 2 months of pregnancy in high milk-producing Holstein cows. In this experiment, 22 primiparous and 28 multiparous cows were enrolled weekly at 15 DIM and randomly assigned to either of two diets with different omega-6:omega3 (**n6:n3**) ratios: Control (6:1 n6:n3, n = 25) or Omega-3 (2:1 n6:n3, n = 25) and randomly allocated to 6 pens to consume the specific diet until approximately 140 DIM. We showed that omega-3 supplementation might help improve milk production in multiparous cows and increase quality of milk by reducing somatic cell count and n6:n3 ratio. Furthermore, we showed that omega-3 supplementation can impact pregnancy per AI by enhancing oocyte quality, cleavage rate, corpus luteum function, and placentation.

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Dedication

I want to dedicate this work to my wife, Julie, and my son, Cristian. They are my treasures. Thanks for being with me and helping me achieve my dreams. I hope to help you to achieve your dreams. Families can be together forever.

Chapter 1 - Review of Literature

Introduction

Fatty acids (FA) are a source of energy that can modulate various important systems including the reproductive system (Staples et al., 1998). Although FA supply a greater amount of energy than cereal grains, several ingredients in cattle diets are rich in omega-6 FA, but not many ingredients are a good source of omega-3 FA (Moallem, 2018). This imbalance favors omega-6 FA can have negative impacts on reproductive performance and milk production, in part, because of the pro-inflammatory products resulting from the omega-6 FA metabolic pathway (Calder, 2012). Thus, effects of omega-6 and omega-3 FA supplementation on reproductive performance and milk production in dairy cattle have been widely studied (Santos et al., 2008; Moallem, 2018). Omega-3 supplementation can have a positive effects on milk production and reproductive performance (Sinedino et al., 2017). No study, however, has assessed effects of omega-3 on in vivo embryo development in high milk-producing dairy cows.

Fatty Acid Classification

Fatty acids are a big family that can be classified according to the number of carbons and double bonds in their chemical structure. The FA have a carboxylic acid (COOH) group located in one end, termed α (alpha), and a methyl (CH₃) located at the other end, termed ω (omega; (Innis, 2008). According to the number of carbons in their structure, FA can be classified as short (less than 6 carbons), medium (between 6 to 12 carbons), long (between 13 to 21 carbons) and very long (more than 21 carbons; Saini and Keum, 2018). The FA are further classified as unsaturated FA (UFA) if they have a double bond between carbons in their chemical structure or saturated FA (SFA) if they have no double bonds (Wathes et al., 2007). These double bonds in the chemical structure of the FA bring physical flexibility, and for this reason, SFA are solid at

room temperature, whereas UFA are more liquid at the same temperature (Panickar and Bhathena, 2010). Further, UFA can be classified in monounsaturated FA (**MUFA**) if they only have one double bond in their chain or polyunsaturated FA (**PUFA**) if they have two or more double bonds between carbons in their chain.

Polyunsaturated FA can be separated into three groups: (1) omega-3 (**n3**); (2) omega-6 (**n6**); and (3) omega-9 (**n9**). Each name corresponds to the position of the first double bond starting on the methyl (omega) end (Wathes et al., 2007, Rincon-Cervera et al., 2021). In other words, the first double bond is in the third, sixth, and ninth carbon, starting the count in the omega end for omega-3, omega-6, and omega-9, respectively. All the FA belonging to the omega-3 and omega-6 families are PUFA, whereas most of the FA belonging to the omega-9 family are MUFA (Moallem, 2018). Alpha-linolenic acid (**ALA**, 18:3 n3) belonging to the omega-3 and linoleic FA (**LA**, 18:2 n6) belonging to the omega-6 family are called essential FA because they cannot be synthesized in the body, and for this reason, they need to be consumed in the diet (Burr and Burr, 1930).

It is important to review the pathways of the LA and ALA. The LA (18:2 n6) is the shorter FA in the omega-6 family. By action of the FA desaturase 2 (**FAD2**), LA can be transformed in gamma-linolenic acid (**GLA**, 18:3 n6), then by an elongase enzyme, GLA can be transformed in the dihomo-gamma-linolenic acid (**DGLA**, 20:3 n6) and by action of a FA desaturase 1 (**FAD1**), DGLA can be transformed into arachidonic acid (**AA**, 20:4 n6). The AA is the precursor of prostaglandins in the body. On the other hand, the shorter member of the omega-3 family is the ALA. By action of the same FAD2, ALA can be transformed into stearidonic acid (**SDA**, 18:4), then by action of similar elongases this SDA can be transformed into eicosatetraenoic acid (**ETA**, 20:4). By action of the same FAD1 ETA can be transformed into

eicosapentaenoic acid (EPA). By either elongases or desaturases EPA can be transformed in docosapentaenoic acid (**DPA**, 22:5 n-3), and finally into docosahexaenoic acid (**DHA**, 22:6 n-3) (Wathes et al., 2007; Saini and Keum, 2018). These two pathways compete in the use of these desaturases and elongases.

Common Sources of Omega-6 and Omega-3 Fatty Acids Supplementation

Several ingredients in the typical diet of dairy cattle in the U.S. such as corn, corn silage, soybean, cottonseed, and sunflower are sources of omega-6 FA. On the other hand, just a small number of feedstuffs normally utilized are rich in omega-3 FA, such as green forage, flaxseed, or fish oil (Moallem, 2018). Some of the most popular ingredients to supply omega-6 FA are soybeans and sunflower, and some of the most popular ingredients for supplementation of omega-3 FA are fish oil and flaxseed. Thus, the review will examine some of the principal characteristics of these feeds.

Soybean (Glycine max)

Soybean is one of the most important crops around the world for human and animal use. For example, soybean meal is the number one protein meal consumed (70.7%) in the world, followed by rapeseed and sunflower, with 12.1% and 6.1% of the total protein meal consumption, respectively. Soybean is also at the top of the world ranking for oilseed production, with 59% of the total world oilseed production. Brazil is the biggest producer of soybeans, with 36.4% of the world's production, followed by the U.S. with 34.4%, and Argentina with 12.1%. Soybeans represented approximately 33% of U.S. crop area planted in 2022 (SoyStats, 2023).

The importance of soybean is linked to being a good source of protein and fat, with approximately 40% of protein and 18% of total FA (Grummer and Luck, 1994). The FA composition of soybeans can vary according to the origin of the germplasm, but they are a rich

source of omega-6 FA with LA being the principal representative. Abdelghany et al. (2019) evaluated four different germplasm origins (China, Japan, Russia and USA) and reported that the FA profile range from palmitic acid (**PA**, 16:0, 8.2 to 17.2%), stearic acid (**SA**, 18:0, 2.7 to 5.2%), oleic acid (**OA**, 18:1, 13.5 to 31.9%), LA (45.6 to 63.9%), and ALA (3.4 to 12.8%).

Sunflower (*Helianthus annuus*)

Sunflower is another of the biggest crops around the world due to being an important source of omega-6 FA. Argentina, the European Union, and China are the biggest producers around the world, and the U.S. is sixth (National Sunflower Association, 2023). Ambrose et al. (2006) reported that sunflower seed contains 15.8% protein and 38.8% fat on a dry matter basis. The latter authors described that the FA profile of the sunflower seed was 74% for LA and just 0.12% for ALA. A similar composition of the FA profile was shown by Thangavelu et al. (2007). Zachut et al. (2010b) reported a FA composition of 53% SFA, 12% MUFA, 34% PUFA, with 33.4% of the total FA being LA.

Fish oil

Fish oil is a good source of omega-3 EPA and DHA. Approximately one-third of the total FA in fish oil is omega-3 long-chain PUFA, with EPA and DHA as the main FA (USDA, 2015). Supplementation of EPA and DHA is important because, while it is true that they can be synthesized from ALA, this synthesis is limited (Gulliver et al., 2012). The FA profile of fish oil is variable depending on the type of fish and its locational habitat. Data from the technical evaluation report compiled by ICF International for the USDA national organic program (2015) indicate that from 15 fish species, the percentage of FA from the total FA was less than 14% for ALA, less than 11% for LA, and from 2.5 to 42.5% for DHA, and from 4 to 26% for EPA. Moreover, Garg et al. (1988) reported that the percentage by weight in fish oil was 44.6% for

omega-3 FA and just 2.8% for omega-6 FA, being 6% for ALA, 8.9% for DHA, and 27.5% for EPA.

It is important to note that fish oil has a particular aroma that can reduce intake by cattle (Moallem 2018), and for this reason should be used in limited amounts. For example, a study (Donovan et al., 2000) indicated that feeding fish oil at 1% of the dry matter did not decrease the intake, but diets with 2 or 3% of fish oil diminished dry matter intake (**DMI**) of dairy cows.

Flaxseed (*Linum usitatissimum*)

Because of its high level of essential ALA, flaxseed is an important feed that can be used as a supplement in livestock. Flaxseed has approximately 40% fat, 25% protein, and 34% neutral detergent fiber on a dry matter basis. From the total fat, approximately 57% is ALA and just 16.1% is LA (Petit, 2002). Flaxseed is a source of plant lignin and is classified as a phytoestrogen, which can decrease fertility in cattle (Adams, 1995). In contrast, lignins in flaxseed do not decrease reproductive performance, rather they have been shown to improve fertility when 10.8% of flaxseed was included in diets (Ambrose et al., 2006). Feeding 10% of flaxseed based on dry matter will not reduce productivity in cows (Petit, 2010).

Important Roles of Fatty Acids in Cattle

The transition period (21 days before calving through 21 days after calving) is a crucial and challenging time for the metabolic system of a cow. Because of the space utilized by the fetus and the changing hormonal environment, dry matter intake decreases in dairy cows until parturition (Ingvarsten and Andersen, 2000). After parturition, DMI increases gradually but not sufficiently to sustain high milk production. This creates a negative energy balance during the first weeks after calving (Grummer, 1995). Severe negative energy balance can delay the onset of estrus and ovulation and can delay the establishment of pregnancy. Thus, FA supplementation

has been very beneficial in providing energy to cows, especially during this challenging time, by reducing the severity of negative energy balance. Although it is accepted that FA supplementation can help to reduce the negative energy balance, in recent years, it has also been shown that different profiles of FA can have varying results in the health and reproduction of dairy cows (Santos et al., 2008).

Fatty acids are a good source of energy, and make up part of every cellular membrane, and play an important role in many biological processes. In the cell membrane, different types of FA can change the fluidity of the lipid bilayer and, as a result, modulate the activity of some enzymes, expression of membrane receptors, and ion channels (Wahle et al., 2003). Fatty acids are also involved in the regulation of gene expression (Clarke, 2001). For example, supplementation with omega-3 FA is involved in the peroxisome proliferator-activated receptors (PPAR), which can activate genes to regulate the glucose and FA uptake during embryo elongation and other enzyme regulation (Giller et al., 2018; Bionaz et al., 2020). In addition, FA play an important role in the innate and adaptative immune system (Sordillo, 2016). Moreover, omega-6 FA are more related to pro-inflammatory responses, and omega-3 are related to anti-inflammatory responses (Saini and Keum, 2018).

Omega-3 Supplementation in Dairy Cattle

Dry Matter Intake

Although FA are a great source of energy, they can change dry matter intake (**DMI**). For instance, some studies have shown a reduction in DMI during FA supplementation (Choi and Palmquist, 1996; Schauff and Clark, 1992). Earlier studies feeding fish reported that omega-3 could limit the DMI because fish affects aroma and palatability of the diet (Donovan et al., 2000). In contrast, studies comparing different types of FA supplementation, such as omega-6 vs.

omega-3, have increased DMI. For example, Zachut et al. (2010a) using two isocaloric and isonitrogenous diets, showed that extruded flaxseed at 9.2% of diet dry matter during 100 d after calving increased DMI by 3.8% compared with controls. Moreover, in an elegant experiment, Greco et al. (2015) using three diets with different n6:n3 ratios reported that reducing the n6:n3 ratio increased the DMI. In another study (Ambrose et al., 2006), when comparing two isocaloric and isonitrogenous diets, one with a supplementation of rolled flaxseed (rich on ALA, omega-3 FA), and the other with a rolled sunflower seed (LA, omega-6 FA) supplementation at 9% and 8.7% of the dry matter (approximately 750 g of oil//cow daily), DMI tended to be greater for cows with the omega-3 supplementation during the 8 weeks of the study.

On the other hand, several other studies have shown no differences in DMI for diets enriched in omega-6 vs omega-3. Thangavelu et al. (2007), showed no differences in DMI between diets supplemented with flaxseed or sunflower seed. Similarly, Sinedino et al. (2017) reported no differences in DIM between diets enriched in omega-3 (100 g of an algae product, All-G-Rich, Alltech Inc., Nicholasville, KY, USA) vs control during between 27 to 120 d after calving. In agreement, Elis et al. (2016) found no differences for DMI of a diet rich in omega-3 with a rumen-protected encapsulated fish oil and a diet rich in omega-6 composed of toasted soybeans at 1% and 1.8%, fed from calving to 2 months after calving.

The different DMI results can be attributed to differences in the form of the supplement, aroma, acceptability, amount and duration of the supplementation, stage of lactation, composition of the diet, and total amount of fat (Zachut et al., 2010a; Moallem 2018). For example, when fish oil is used in small amounts DIM does not change, but greater amounts of fish oil reduce DMI.

Milk Production

Studies assessing milk production related to omega-3 supplementation also provided contrasting results. Omega-3 FA in their metabolic pathway is associated with anti-inflammatory responses, whereas omega-6 FA is associated with proinflammatory responses (Calder, 2012). All sequences of events produced by the immune system in an inflammatory response need energy. Consequently, in an inflammatory response, it is possible to detect a redirection of nutrients to the detriment of animal products such as meat or milk (Colditz, 2002). Therefore, cows consuming omega-3 FA supplements can have extra energy to increase milk production (Greco et al., 2015). For example, Petit et al. (2004) showed that the cows supplemented with whole flaxseed produced more milk than the cows supplemented with whole sunflower seeds. Moallem (2009) supplemented 96 lactating cows at 150 DIM (milked thrice daily) and reported a 2.7% increase in milk yield when cows were supplemented with flaxseed and wheat bran compared with controls. Zachut et al. (2010a) reported a 6.4% increase in milk production until 100 DIM in cows supplemented with flaxseed compared with controls. In agreement, Sinedino et al. (2017), using 739 lactating cows (milked twice daily) showed that cows supplemented with algae (omega-3 supplementation) increased milk production by 0.9 kg/d from 27 to 120 d after calving. Greco et al. (2015) used Ca salts of fish oil, safflower (Rich in LA), and palm oils (rich in palmitic and oleic FA) to create different diets with different n6:n3 ratios. They reported that milk production increased as the n6:n3 ratio in diets decreased (3.9 ratio produced 46.8 Kg/day, 4.9 ratio produced 44.8 Kg/day and 5.9 ratio produced 43.2 Kg/day).

Other studies in the literature have reported no differences in milk production. Ambrose et al. (2006) used 121 lactating cows for 8 weeks and reported no differences between the flaxseed (omega-3) and sunflower (omega-6) diets on milk production (36.7 and 36.0 Kg. per

cow per day, respectively). Elis et al. (2016) used 46 Holstein cows milked twice daily from calving to 22 weeks after calving and reported no differences in milk production between the group supplemented with protected fish oil (omega-3) vs cows supplemented with toasted soybean (omega-6) (33 ± 0.8 vs 34.4 ± 0.8 Kg/cow/day respectively). Freret et al. (2019) used 37 Holstein primiparous cows milked twice daily for 9 weeks starting 11 weeks after calving and reported no differences in milk production between cows whose diets were supplemented with microencapsulated fish oil (omega-3) compared with cows supplemented with microencapsulated soy oil (omega-6) (28.5 ± 1.5 vs 28.8 ± 1.5 Kg/cow/day respectively).

Milk Composition

Percentage and total production of fat and protein in milk is related to milk production and DMI in different studies that assessed FA supplementation. In some studies, it was possible to see that omega-3 supplementation increased DMI and more milk (Zachut et al., 2010a). Milk production has been highly associated with DMI (Harrison et al. 1990). In some studies, although decreasing the percentage of fat or protein in milk, the total production of fat and protein did not differ between treatments because total milk volume increased (Moallem et al., 2009; Moallem et al., 2013; Greco et al., 2015). In addition, it is important to consider that a large percentage of PUFA as unprotected oil and Ca salts of long chain FA can suffer biohydrogenation in the rumen. This biohydrogenation in rumen generated some intermediate isomers that can depress fat in milk (Piperova et al., 2004). Thus, the degree of protection of the omega-3 supplementation is relevant to determining its influence on milk components.

Fatty Acid Profile in Milk and Human Health

Just as with dairy cows, in the modern Western culture, it is normal for humans to eat many foods that contain omega-6 FA but just a few foods are rich in omega-3 FA. This leads to

an increase in the n6:n3 ratio in human diets. Wathes et al. (2007) mentioned that in Western societies, the n6:n3 ratio in the human diet has changed from 1:1 in a primitive diet to more than 10:1 in a modern human diet. Simopoulos (2002) described that Western societies' n6:n3 ratio is between 15:1 to 16.7:1 and that reducing this ratio in diets might help to decrease the risk of several important chronic diseases of high prevalence, such as cancer, cardiovascular, and inflammatory diseases. Because milk is one of the most consumed foods for humans around the world, decreasing the n6:n3 ratio in milk might reduce the total n6:n3 in the human diet and improve our health.

Omega-3 Effect on Cattle Reproduction

Cyclicality After Calving

After the cow calves, it is desirable that she resumes estrous cycles to increase the probability of pregnancy before 130 DIM (Middleton et al., 2019). Perhaps, a better balance between omega-3 and omega-6 FA could help the cow to recover sooner after parturition and, consequently, resume estrous cycles sooner. That postulate does not seem to be true because Silvestre et al. (2011) showed that the percentage of cycling cows at 63 DIM was not different between palm oil and fish oil supplemented cows. In addition, Elis et al. (2016) showed no differences in days at first postpartum ovulation between cows supplemented with omega-3 or omega-6 FA. A more recent study (Sinedino et al., 2017) also failed to support this idea of omega-3 FA influencing onset of postpartum estrous cycles. Recent studies have shown that inflammation is required for dairy cows to transition successfully after calving to good health and productivity (Horst et al., 2021).

Follicle Size

Size of the preovulatory follicle (**POF**) is related to the size of the future corpus luteum (**CL**). That is, a larger POF will result in a larger CL (Vasconcelos et al., 2001). Ambrose et al. (2006) reported that dairy cows supplemented with omega-3 FA using flaxseed had larger POF than cows supplemented with omega-6 FA using sunflower (16.9 ± 0.9 vs 14.1 ± 0.9 mm), respectively. Another study (Elis et al., 2016) indicated a tendency for dairy cows supplemented with omega-3 FA (fish oil) to have larger POF than cows supplemented with omega-6 FA using toasted soybeans. Moallem et al. (2013) did not detect differences in the diameters of POF between diets comparing omega-3 FA with SFA. Sinedino et al. (2017) reported that multiparous cows have larger POF than primiparous cows (16.2 vs 14.6 ± 0.7 mm), but the diameter of the POF did not differ between diets with an average of 15.4 ± 0.6 mm. These results indicate a lack of consistency addressing whether supplementation with omega-3 FA might increase the diameter of the POF and, therefore, obtain larger CL.

Corpus Luteum

Supplementation with omega-3 has been demonstrated to reduce $\text{PGF}_{2\alpha}$ secretion. This is a key observation because $\text{PGF}_{2\alpha}$ induces luteolysis and prevents secretion of progesterone by the CL essential for embryo development and maintenance of the pregnancy. The enzymes desaturases and elongase used to transform LA to AA acid are the same needed to transform ALA to EPA (Wathes, 2007). Thus, competition for these enzymes exists, and an increase in the omega-3 FA in the diet can alter the pathway from the AA to the EPA (Saini and Keum, 2018). Moreover, the enzyme prostaglandin–endoperoxide synthase (**PTGS**) transforms AA in the PGF_2 series (such as $\text{PGF}_{2\alpha}$) and EPA in the PGF_3 series, which are less active than PGF_2 and not involved in luteolysis. Mattos et al. (2003) showed that supplementation of omega-3 reduced the

release of $\text{PGF}_{2\alpha}$ in endometrial cell *in vitro*, whereas Mattos et al. (2004) showed that diets of cows supplemented with fish oil had less concentrations of PGF metabolite than cows supplemented with olive oil. *In vivo* studies have demonstrated that $\text{PGF}_{2\alpha}$ is decreased in postpartum cows supplemented with omega-3 (Petit et al., 2004), and that $\text{PGF}_{2\alpha}$ production is also decreased during the time of classic CL regression during the estrous cycle (Greco et al., 2018).

Embryo Development

Concentrations of progesterone are very important for embryo growth, development, and maintenance. Thangavelu et al. (2007) observed that morulas from cows fed with omega-3 FA tended to have more blastomeres than morulas from cows supplemented with omega-6 FA or SFA. Furthermore, at the stage of extended blastocyst, cows supplemented with omega-3 or omega-6 had more blastomeres than the SFA-supplemented cows. In agreement, Moallem et al. (2013) showed that cows supplemented with omega-3 FA had a greater incidences of embryo cleavage than cows supplemented with SFA. In addition, Freret et al. (2019) showed an enhancement in oocyte quality and a greater incidence on blastocysts in lactating dairy cows supplemented with omega-3 FA than those supplemented with omega-6 FA. In another study, Giller et al. (2018) reported that heifers supplemented with omega-3 FA had larger embryos at 15 d after AI than heifers supplemented with omega-6 FA. Nevertheless, the effect of omega-3 during early embryonic development stage and *in vivo* pregnancy development remains to be elucidated.

Summary

The current literature helps to understand the importance of FA supplementation in dairy cattle. Supplementation with omega-3 FA can reduce the omega-6:omega-3 ratio in the diet and in the

body of the dairy cow. This reduction can increase milk yield and milk quality. Furthermore, supplementation with omega-3 FA may increase fertility in dairy cows by improving oocyte quality, CL function, and embryo development.

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Chapter 2 - Omega-3 fatty acid supplementation affects oocyte quality and pregnancy development during the first two months of gestation in high-producing dairy cows

Abstract

Our aim was to assess benefits of omega-3 supplementation on milk yield and composition, oocyte quality, *in vitro* embryo quality, and *in vivo* conceptus development during the first two months of pregnancy. Holstein primiparous (**prim**, n = 22) and multiparous (**mult**, n = 28) were randomly assigned to diets (3 pens per diet) with 6:1 (**CON**, n=25) or Omega-3: 2:1 (**OMG3**, n=25) omega-6: omega-3 ratio from 15 until approximately 140 days in milk (**DIM**). Data were analyzed with procedure GLIMMIX (SAS 9.4). Omega-3-mult cows (60.8 ± 2.2 kg) tended ($P = 0.10$) to produce more milk than CON-mult cows (55.6 ± 2.2 kg) and somatic cell count was lower in OMG3 (54.8 ± 78.75) than CON (227.4 ± 76.26 ; $P = 0.10$). Percentage of protein and fat in milk did not differ between diets, but omega-6:omega-3 ratio was smaller ($P < 0.001$) in OMG3. Oocyte quality, assessed by a single oocyte harvest at 50 DIM, and number of recovered oocytes did not differ between diets, but OMG3-mult ($79.9 \pm 7.6\%$) had greater number of grade I and II oocytes/total oocytes recovered than CON-prim cows ($58.5 \pm 8.6\%$). Moreover, cleavage incidence tended to be greater in OMG3 than CON cows ($P = 0.07$). First and second timed artificial insemination (**TAI**) was conducted at 75 and 110 DIM, respectively, and weekly ultrasound was performed: (1) d 11-30 post-TAI to evaluate corpus luteum (**CL**) volume and blood flow; (2) d 30-60 post-TAI to evaluate size of CL, embryo, and amniotic vesicle. Only CON-prim (n=6), OMG3-prim (n=7), and CON-mult (n=9) cows that maintained

their pregnancy until 60 d of gestation from a single ovulation were compared post-TAI because only 1 OMG3-mult maintained the pregnancy until 60 d of gestation from a single ovulation. Progesterone did not differ between diets, but CL volume tended ($P = 0.10$) to be greater on d 11 in OMG3-prim cows than in CON-prim cows. Luteal blood flow was greater for CON-mult cows at 25 and 46 d post-TAI than CON-prim cows. Concentrations of PSPB was greater ($P = 0.01$) in the OMG3-prim than CON-mult and in CON-prim cows at 46 d post-TAI. Embryo-fetus and vesicle size did not differ ($P > 0.7$) between dietary treatments. In conclusion, omega-3 improved milk yield in multiparous cows and reduced somatic cell counts and omega-6:omega-3 ratio in milk. Moreover, it is likely that the omega-3 benefits in reproductive traits are related to oocyte and early embryo development, corpus luteum function, and placentation in high-producing Holstein cows.

Introduction

Fatty acids (**FA**) comprise a vast family of carboxylic acids with a carboxylic acid (COOH) group located in one end, termed α (alpha), and a methyl (CH₃) located in the other end, termed ω (omega; Innis, 2008). The FA can be classified by the number of double bonds in their chemical chain (Wathes et al., 2007). For example, FA without double bonds in their chemical structure are classified as saturated FA (**SFA**), whereas those with one or more double bonds in their chain are unsaturated FA (**UFA**). The double bonds in the chemical composition bring more flexibility to the structure, and for this reason, SFA are normally solid at the room temperature, whereas UFA are liquid at the same temperature (Panikar and Bhathena, 2010). The UFA can be subclassified in monosaturated FA (**MUFA**) if they have just one double bond or polyunsaturated FA (**PUFA**) if they have two or more double bonds in their chemical structure. The PUFA are more stable than MUFA (Rincon-Cervera et al., 2021). Moreover, PUFA can be

subclassified into omega-6 (also known as ω -6, n-6, or n6) if the first double bond is in the sixth-carbon counting from the omega end, or omega-3 (also known as ω -3, n-3, or n3) if the first double bond is in the third carbon counting from the omega end (Wathes et al., 2007; Rincon-Cervera et al., 2021).

Fatty acids are a source of energy that can play a role in several biological processes, such as cell function, metabolism, and responsiveness to hormones (Calder, 2015). For example, FA are actively involved in the innate and adaptive immune systems (Sordillo, 2016) and have been described to act in the regulation of the nuclear gene expression (Clarke, 2001). Moreover, FA are present in the lipid bilayer membrane of the cells, and different FA profiles can change the fluidity of the cell membranes and, thus, the activity of enzymes, expression of receptors, and ion channels (Wahle et al., 2003).

In modern U.S. dairies where cattle are fed total mixed ratios (**TMR**), it is common that several ingredients in the diet are rich in omega-6, with just a few ingredients normally rich in omega-3 FA (Moallem, 2018). This situation not only creates an imbalance between omega-6 and omega-3 FA in the diet but also in the body of the cattle (Greco et al., 2015; Moallem, 2018). This imbalance is important because omega-6 FA are associated with pro-inflammatory pathways, whereas omega-3 FA are associated with anti-inflammatory ones (Calder, 2012). An imbalance between omega-6 and omega-3 FA in dairy cattle diets has been associated with changes in health and reproductive performance (Moallem, 2018). Omega-3 diet supplementation increases pregnancy rate at first service of both primiparous and multiparous dairy cows (Sinedino et al., 2017), improve early embryo development (Thangavelu et al., 2007) and elongation (Giller et al., 2018), and reduce pregnancy loss (Ambrose et al., 2006; Silvestre et al., 2011).

Two important PUFA have been shown to be related to the benefits in reproduction: linoleic acid (**LA**, 18:2 n6) belonging to the omega-6 FA family and alpha-linolenic acid (**ALA**, 18:3 n3) belonging to the omega-3 FA family (Santos et al., 2008). These two FA are essential in the diet, and for this reason, must be consumed from the diet (Burr and Burr, 1930). The LA is converted into arachidonic acid (**AA**, 20:4 n6; Gulliver et al., 2012; Dyall et al., 2022), which is the precursor of prostaglandin $F_{2\alpha}$ in its luteolytic role during the estrous cycle, causing progesterone to decrease, which is otherwise required for embryo growth and maintenance of the pregnancy in mammals (Robinson et al., 2008). Because LA and ALA compete for their receptor in the body, increasing ALA in the diet could potentially decrease absorption of LA, and subsequently, prostaglandin $F_{2\alpha}$ secretion. Based on this mechanism, Mattos et al., 2003 and Mattos et al., 2004 demonstrated decreased omega-6 content in the diet of dairy cattle led to a reduction in prostaglandin $F_{2\alpha}$.

To the best of our knowledge, whether these effects carry over and are evidenced in conceptus development during the first two months of pregnancy remains to be elucidated. Thus, the objective of this study was to assess the benefits of omega-3 supplementation on milk yield and composition, oocyte quality, in vitro embryo quality, and *in vivo* conceptus development during the first two months of pregnancy in high-producing lactating dairy cows. Specifically, we hypothesized that omega-3 diet supplementation will improve milk yield, composition, and quality. Furthermore, we hypothesized that omega-3 supplementation will enhance oocyte quality, in vitro embryo development, and in vivo pregnancy development during the first two months of gestation in high-producing dairy cows.

Materials and Methods

Ethics Statement

Animal management was performed in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research and approved by the Institutional Animal Care and Use Committee of Kansas State University (protocol # 4590).

Cows, Location, and Experimental Design

Fifty lactating Holstein cows (primiparous, n = 22, multiparous, n = 28) with no apparent reproductive tract abnormalities or health disorders, as evaluated by ultrasound and ear tag automatized activity monitors (CowManager, Harmelen, Netherlands), respectively, were enrolled in the study. One multiparous cow was removed from the study at 133 DIM because of pneumonia and digestive issues.

The study was conducted from December 2021 through August 2022 at the Kansas State University Dairy Teaching and Research Center. Briefly, the housing facility comprised sand-bedded free-stall barns with roofs over the beds. Shades, fans, and sprinklers were used from May until the end of the experiment in August to alleviate heat stress during the warmer months. Cows in the herd are milked two or three times daily, depending on DIM and production, and the yearly average daily milk production is 44.1 kg per cow with percentages of protein and fat of 3.0% and 3.5%, respectively. All cows enrolled in the study were milked three thrice daily.

Cows were enrolled weekly at 15 ± 3 DIM and randomly allocated into each of six adjacent pens. Thus, 4 pens housed 8 cows and 2 pens housed 9 cows. Diets were randomly assigned to 3 pens per dietary treatment. The control diet (**CON**), balanced to deliver a 6:1 omega-6 to omega-3 ratio (**defined herein as n6:n3**), and Omega-3 diet (**OMG3**), balanced to deliver a 2:1 n6:n3 ratio by blending into the TMR GreatOPlus® (Naturally Better Omega-3

[NBO3], Manhattan, KS), an extruded feed product that combines flaxseed (a source of ALA) with *Nannochloropsis oculata* (a source of ALA and eicosapentaenoic acid (**EPA**)).

Diets were isocaloric and isonitrogenous (**Table 1**). Moreover, independent of the diet, all cows had ad libitum access to water and salt mineral blocks and were fed TMR once daily to meet or exceed the nutrient requirements for a lactating Holstein cow producing 50 kg of milk per day at 3.5% fat and 3.1% true protein when dry matter intake (**DMI**) was expected to be 24 kg/d (National Research Council, 2001). Cows received the CON or OMG3 diets until one of the three following conditions were achieved: (1) 60 d of gestation, (2) non-pregnant diagnosis after the second AI, or (3) until a pregnancy loss was diagnosed. The schematic experimental design of the study is shown in **Figure 1**.

Feed samples of all the ingredients in the diet were collected weekly during the experiment and stored frozen until assayed. Composited two months of weekly samples through the eight months of the study for all the ingredients were sent to a commercial laboratory (Dairy One, Ithaca, NY) to assay the nutrient composition by near-infrared reflectance spectroscopy (NIR). **Table 1** shows the diet contents.

Body Condition Score, Dry Matter Intake, Fatty Acid Profile, and Milk

Body condition score was evaluated using a scale of 1 (thin) to 5 (obese) with 0.25 cut points (Ferguson et al., 1994) at 21 d before the expected calving date, at calving, 21 d after calving, at ovum pick up (50 DIM), at timed artificial insemination (TAI; approximately 75 and 110 DIM for 1st and 2nd service), and at 30 and 60 d of gestation. Moreover, throughout the entire experiment, feed refusals were weighed weekly to estimate and compare DMI between dietary treatments.

Blood samples were collected at 15, 50, 75, 110, and 140 ± 3 DIM to determine fatty acid profile in serum. All blood samples in the experiment were collected from the coccygeal vessels using evacuated tubes containing sodium heparin (Vacutainer, Becton Dickinson, and Co., Franklin Lakes, NJ) and placed immediately on ice packs after collection. Samples were centrifuged at $1,800 \times g$ (J-6B centrifuge, Beckman Coulter Inc, Brea, CA), and the harvested plasma was stored at -20 °C until progesterone and pregnancy-specific protein B (**PSPB**) were assayed.

Milk samples from the first milking of the day were collected to assess milk fatty acid profile at 15 and 50 DIM. Milk components (protein, fat, and somatic cell count) were also assessed at 15, 50, 75, 110, and 140 DIM. Samples were collected in the milking parlor using a plastic sampling bottle directly attached to the milk line of each cow. The milk was gently mixed in the plastic bottle, and subsamples were placed in two vials, which were either frozen and stored at -20 °C without preservatives until fatty acid analysis were performed. Samples were shipped to the Dairy Herd Improvement laboratory (MQT Lab Services, Kansas City, MO) for milk composition and quality analysis. Finally, milk weights were recorded at each milking from 15 ± 3 DIM until a cow was removed from the experiment. Milk weights were converted to kg and weekly averages were used for analyses.

Postpartum Cyclicity, Oocyte Quality, and Embryo Development

From 21 to 45 ± 3 DIM, cows were evaluated twice weekly (Monday and Friday) by transrectal ultrasound to measure the diameter (mm) of the two largest follicles and any CL present to determine resumption of postpartum estrous cycles. Briefly, cows were restrained in a palpation rail, and follicle and CL diameters were determined with the caliper option of the ultrasound machine by averaging two perpendicular measurements of the structure at its apparent

maximal size. All ultrasound examinations in the study were performed with an Ibex-Evo II (E.I. Medical Imaging, Loveland, CO, US), equipped with a multilinear-array transducer set up at 7.5 MHz.

At 45 ± 3 DIM, a 100 μg injection of gonadorelin acetate (1 mL GONAbreed, Parnell Technologies PTY, LTD, Alexandria, New South Wales, Australia) was administered to all the cows with the purpose of ovulating a dominant follicle (if present) and start a new follicular wave as part of the OPU preparation. At 50 ± 3 DIM, all cows were submitted to a single OPU session (Pieterse et al., 1988). Briefly, cows were restrained in a chute and after clipping and disinfecting the area around the first and second caudal vertebra, an epidural anesthesia injection using 3.5 mL of lidocaine was administered in the space between the two vertebrae. Follicle content (≥ 3 mm) was aspirated subsequently using an ultrasound-guided technique and a vacuum system to generate continuous negative pressure (72 mmHg) attached to a 21-g needle. Follicle content was recovered into a 50-mL sterile Falcon vial with an OPU recovery media (BoviPlus®, Minitube, Germany) containing salts, bicarbonate, hepes, lactic acid, bovine serum albumin, heparin, and gentamicin. The media with the follicle content was maintained at 38 °C.

The recovered follicular fluid was transported to the Physiology and Endocrinology Laboratory at Kansas State University, washed, and filtered to facilitate oocyte search with a stereo microscope. The number and quality of recovered oocytes were evaluated and recorded. The cumulus-oocyte complexes were graded as 1 (cumulus present with more than three cell layers), 2 (cumulus not surrounding the oocyte or less than 3 cell layers thick), 3 (cumulus absent or partially present in scattered clumps in the matrix) or degenerated (oocyte damaged). All oocytes were allocated in cryotubes with maturation media and sent overnight in a portable incubator at 38 °C to an *in vitro* embryo research laboratory at University of Missouri. Lipid

content and mitochondrial activity were evaluated in the recovered oocytes from half of the cows (Ge et al., 2012). Red and green fluorescence were evaluated under a fluorescence microscope to detect high or low mitochondrial activity. Incidence of cleavage was evaluated in the remaining half of the cows.

Timed AI and Traits Associated with Pregnancy Development

All cows were enrolled at 50 ± 3 DIM into a PG-3-G TAI protocol (Peters and Pursley, 2002) in preparation for first services at 75 ± 3 DIM. The PG-3-G protocol consisted of 0.5 mg injection of cloprostenol sodium (2 mL estroPLAN, Parnell Technologies PTY, LTD, Alexandria, New South Wales, Australia), followed in 3 d by 100 μ g injection of gonadorelin acetate (1 mL GONAbreed) administered 7 d before the start of a modified Ovsynch-56 protocol (d 0: 1 mL GONAbreed; d 7 and d 8: 2 mL estroPLAN; d 9: 1 mL GONAbreed; and TAI 16 to 18 h later; Brusveen et al., 2008). At 32 d post-TAI, pregnancy diagnosis was performed via ultrasound to confirm the presence of an embryo with a heartbeat. Cows diagnosed as nonpregnant were immediately enrolled into one of two resynchronization protocols depending on their ovarian status as follows. If the cow had a CL, a Short-Synch protocol (Sauls-Hiesterman et al., 2019) was performed (d 0 [day of pregnancy diagnosis]: 2 mL estroPLAN; d 1: 2 mL estroPLAN; d 2: 1 mL GONAbreed; and TAI 16 to 18 h later). If the cow did not have a CL on the day of the pregnancy diagnosis but had a follicle ≥ 10 mm (Sartori et al., 2001), an Ovsynch-56 protocol was performed as previously described. Cows were resynchronized a single time for the purpose of this study so that only data from the first or second services were used.

Figure 1B illustrates the assessed pregnancy-related traits. Regardless of service number, weekly blood samples were collected to assess progesterone and PSPB concentrations from 11 d post-TAI until the next pregnancy diagnosis at 32 d post-AI. Ultrasound examinations were

performed at the same time to measure diameter of the CL and CL cavity, and luteal blood flow via Doppler (Ginther, 2014). The volume of CL was calculated using the mathematical formula to calculate the volume of a sphere ($[\frac{4}{3}] * \pi * r^3$) subtracting the CL cavity which was calculated using the same formula. If a cow was diagnosed pregnant (conceptus with a heartbeat) at 32 d post-TAI, weekly blood samples continued to be collected to measure progesterone and PSPB concentrations from 32 to 60 d post-TAI. Moreover, from 32 to 53 d post-TAI, weekly ultrasound examinations were performed to evaluate diameter of the CL and luteal blood flow via Doppler, conceptus crown-rump length, conceptus width, and amniotic vesicle length and width (Pierson and Ginther, 1984; Ginther, 2014). On d 60 post-TAI, the fetus head length, and width were measured instead of the entire fetus (Riding et al., 2008) because of the difficulty of fitting the larger entire fetus in a single ultrasound image. At each ultrasound evaluation, conceptus heartbeat was evaluated to corroborate pregnancy viability. If pregnancy loss was evidenced by the absence of a viable conceptus, the cow was removed from the experiment.

Concentrations of progesterone were determined by solid-phase RIA kit containing antibody-coated tubes and 125I-labeled progesterone (ImmuChem Coated Tube progesterone 125I RIA Kit, MP Biomedicals) following manufacturer's procedures. Sensitivity, intra-assay coefficient of variation (**CV**), and inter-assay CV were 0.07 ng/mL, 7.8%, and 8.3%, respectively. Concentrations of PAG were determined by ELISA (BioPRYN Flex; BioTracking LLC) following manufacturer's procedures. Intra- and inter-assay CV were 7.7 and 9.8 %, respectively.

Data Handling and Statistical Analyses

All statistical analyses were performed in SAS 9.4 (SAS Institute Inc., Cary, NC). The BCS, DMI, blood and milk fatty acid profile, and milk components (protein, fat, somatic cell

count) were analyzed using the procedure GLIMMIX with pen as the experimental unit. For each trait, a single model was developed using diet (CON and OMG3), parity (primiparous and multiparous), and DIM set as fixed effects. The two and three way interactions of diet, parity, and DIM were also assessed. Specifically for milk production, DIM was substituted by weeks in milk in the model. Moreover, pen within diet and week of enrollment were set as random effects. When one of the main effects or their interactions were significant, a Tukey test was used to assess the specific differences among main effects and interactions. For illustrative purposes, return to cyclicity (**Table 3**), oocyte quality (**Table 4**), milk composition (**Table 5**), milk yield (**Figure 2**), and pregnant cow reproductive parameters (**Figure 3**) are depicted separately for the interaction effects of diet and parity (control primiparous [**CON-prim**], control multiparous [**CON-mult**], omega-3 primiparous [**OMG3-prim**], omega-3 multiparous [**OMG3-mult**]) regardless of the interaction significance. Oocyte mitochondrial activity measured by fluorescence intensity, cleavage incidence, follicle size, day of first postpartum CL appearance, and oocyte-related traits (quantity and quality) were analyzed with procedure GLIMMIX using pen as the experimental unit. For each trait, a single model was developed using diet (CON and OMG3) as a fixed effect and pen within diet and week of enrollment as random effects.

For the pregnant cow comparisons, luteal blood flow, CL volume, progesterone and PSPB concentrations, embryo/fetus, and vesicle volume were analyzed. Only data from cows with single ovulations and a viable conceptus until d 60 of pregnancy were analyzed. From the 49 Holstein cows in the study, we obtained a total of 34 pregnancies at day 32 post-TAI (1st and 2nd service combined) in the CON-prim (n = 10), CON-mult (n = 11), OMG3-prim (n = 7), and OMG3-mult (n = 6). From those, the following cows were removed: (1) cows with twin pregnancies that remained pregnant until d 60 post-TAI (1 CON-mult, and 1 OMG3-mult); (2)

cows with multiple ovulations and single embryo (2 CON-prim, and 1 OMG3-mult); and (3) cows with pregnancy losses (2 CON-prim, 1 CON-mult, and 3 OMG3-mult). After these deletions, the OMG3-mult group was removed from the analysis because only 1 cow remained. Thus, only CON-prim (n = 6), OMG3-prim (n = 7), and CON-mult cows (n = 9) were considered in the subsequent analysis. The groups were compared for the aforementioned traits using the procedure GLIMMIX with pen as the experimental unit. For each trait, a model was developed using groups (CON-prim, OMG3-prim, and CON-mult) and days post-AI as fixed effects and the interaction of group by day. Week of enrollment was included as a random effect. When one of the main effects or their interactions were significant, a Tukey test was used to assess specific differences among groups.

All results are expressed as LS means \pm SEM. In all cases, statistically significant effects were reached when $P \leq 0.05$ and tendencies when $0.05 < P \leq 0.10$.

Results

Days in Milk, Dry Matter Intake, and Body Condition Score

A weekly comparison of DIM did not differ among pens assigned to each diet, which confirmed equal randomization of cows throughout the experiment. Moreover, for the DMI (**data not shown**), no differences ($P = 0.43$) in diet, parity ($P = 0.52$), or interaction diet by parity ($P = 0.59$) were observed.

Body condition score did not differ between diets ($P = 0.15$), and the interaction of diet by DIM also was not different ($P = 0.72$). Nevertheless, regardless of the diet, BCS at d -21 (3.0 ± 0.07) and 0 DIM (3.0 ± 0.07) were greater ($P < 0.001$) than at 21 d (2.8 ± 0.07), 50 d (2.7 ± 0.07), 75 d (2.7 ± 0.07) and 110 DIM (2.8 ± 0.07).

Omega-6 to Omega-3 Fatty Acid Ratio in Blood and Milk

Table 2 displays the n6:n3 ratio in plasma obtained from blood samples collected at 15, 50, 75, 110, and 140 DIM. As expected, the average n6:n3 ratio in plasma throughout the experiment was reduced ($P < 0.0001$) in the OMG3 (9.7 ± 0.2) compared with the CON (13.4 ± 0.2). Moreover, primiparous cows demonstrated a tendency ($P = 0.06$) of a smaller ratio (11.3 ± 0.2) compared with multiparous cows (11.7 ± 0.2). The interaction between diet and lactation also tended ($P = 0.06$) to differ, mainly driven by OMG3-prim (9.6 ± 0.3) having a smaller ratio than CON-prim (13 ± 0.3) and OMG3-mult (9.7 ± 0.2) having a smaller ratio than CON-mult (13.9 ± 0.2). Nevertheless, the OMG3-prim and OMG3-mult ratios did not differ. The interaction of DIM and diet was detected ($P < 0.0001$) for the plasma n6:n3 ratio. The interaction was mainly explained by a similar ratio of the CON (9.8 ± 0.3) and OMG3 (10.1 ± 0.3) at 15 DIM, but a smaller ratio in the OMG3 cows compared with to the CON cows at 50 DIM (8 ± 0.3 vs 11.5 ± 0.3), 75 DIM (9.6 ± 0.3 vs 14.7 ± 0.3), 110 DIM (10.1 ± 0.3 vs 15.2 ± 0.3), and 140 DIM (10.4 ± 0.3 vs 15.8 ± 0.3).

Table 2 displays the n6:n3 ratio in milk samples collected at 15 and 50 DIM. The average ratio over the days was smaller ($P = 0.03$) in OMG3 than CON cows (5.9 ± 0.18 vs 6.8 ± 0.19). Primiparous cows also had a lower ($P = 0.005$) ratio compared with multiparous (6.1 ± 0.16 vs 6.5 ± 0.14), and the main effect of DIM was also significant ($P < 0.0001$). The interaction diet by parity was not statistically different ($P = 0.62$). In contrast, the interaction of diet by DIM differed ($P < 0.0001$) and was explained by a smaller ratio in OMG3 cows compared with CON cows at 50 DIM (4.3 ± 0.2 vs 6.4 ± 0.2), but not at 15 DIM.

Milk Yield, Composition, and Quality

Figure 2 shows weekly milk yield from weeks 1 to 18 (i.e., 14 to 140 DIM). During this time, milk yield averaged 48.7 ± 1.9 and 50.4 ± 1.9 kg/d, respectively, and did not differ ($P = 0.50$) between CON and OMG3 cows. As expected, milk yield was less ($P = 0.001$) in primiparous (41.2 ± 1.67 kg/day) than multiparous cows (57.9 ± 1.6 kg/day). Moreover, the main effect of DIM was significant ($P < 0.0001$) with peak daily milk yield occurring at approximately 60 DIM for multiparous and 100 DIM for primiparous cows. The interaction of diet by parity was detected ($P < 0.0001$) because greater milk production was observed in the OMG3-mult cows (60.5 ± 2.0 kg/day) compared with the CON-mult cows (55.3 ± 2.0 kg/day), whereas greater ($P < 0.0001$) milk yield of those two groups occurred compared with the OMG3-prim cows (40.3 ± 1.98 kg/day) and CON-prim cows (42.1 ± 1.98 kg/day). The interaction of diet by DIM and the three-way interaction of diet by parity by DIM were not different, although the interaction between parity and DIM was detected ($P = 0.0001$).

Table 5 displays the results related to milk composition and quality. In summary, fat (3.3 ± 0.1 vs $3.2 \pm 0.1\%$), protein (2.8 ± 0.04 vs $2.9 \pm 0.04\%$), lactose (4.9 ± 0.03 vs $5.0 \pm 0.03\%$), solids-not-fat (8.6 ± 0.09 vs $8.7 \pm 0.09\%$), and milk-urea-nitrogen (12.0 ± 0.4 vs 11.6 ± 0.4 mg/dL) were not different between OMG3 and CON. Moreover, the interactions of diet by parity, diet by DIM, and diet by parity by DIM did not differ. In contrast, somatic cell counts of OMG3 vs CON tended ($P = 0.10$) to differ ($58,810 \pm 78,751$ vs $227,410 \pm 76,259$), whereas parity and DIM main effects and all interactions did not differ ($P > 0.60$).

Post-partum Cyclicity, Oocyte and Embryo Quality

Table 3 summarizes the diameters of the largest and second largest follicle, as well as when the first postpartum CL was detected from 20 to 45 DIM in the CON-mult, CON-prim,

OMG3-mult, and OMG3-prim groups. The main effect of diet, parity, and the interaction of diet by parity were not significant for the maximum follicle sizes, but the maximum follicle size tended ($P = 0.09$) to occur later in CON-prim cows (37.5 ± 3.7 DIM) than in CON-mult cows (27.8 ± 3.5 DIM). In contrast, OMG3-prim (28.8 ± 4.1 DIM) and OMG3-mult (29.0 ± 3.5 DIM) were not different at the DIM at maximum follicle size. First postpartum detected CL did not differ ($P = 0.46$) between diets and was not affected by parity ($P = 0.46$) or the interaction diet by parity ($P = 0.73$).

Table 4 displays the number of aspirated follicles as well as the quality of recovered oocytes for CON-mult, CON-prim, OMG3-mult, and OMG3-prim groups. For the number of aspirated follicles per cow, the number of grade 1, grade 2, and number of degenerated oocytes, the main effect of diet and the interaction diet by parity were not different. In contrast, the main effect of parity tended ($P = 0.06$) to differ for the number of aspirated follicles and differed ($P < 0.02$) for the number of grade 1, grade 2, and number of degenerated oocytes per cow. The interaction diet by parity tended ($P = 0.09$) to be detected for the recovery rate in which OMG3-prim tended to be greater than CON-prim cows but did not differ between multiparous cows. Moreover, when combining the grade I and II categories divided by the total of recovered oocytes, the OMG3-mult had a greater percentage of combined grade I and II oocytes/total number of recovered oocytes compared with CON-prim, but the OMG3-prim and CON-mult were not different.

A total of 166 oocytes were evaluated by fluorescence intensity. For the deep red mitochondrial intensity (CON: 42.5 ± 8.7 vs OMG3: 40.6 ± 8.2 units) and green mitochondrial intensity (CON: 7.7 ± 3.1 vs OMG3: 8.0 ± 3.1 units), the main effect of diet was not different. Moreover, incidence of embryo cleavage evaluated in the *in vitro*-produced embryos ($n = 22$)

showed that OMG3 (47%) cows tended ($P = 0.07$) to have a greater percentage incidence of cleavage than CON cows (33%).

Progesterone, Corpus Luteum, and Conceptus Development

In each of the following analyses in pregnant cows on day 60, CON-prim ($n = 6$), OMG3-prim ($n = 7$), and CON-mult cows ($n = 9$) were evaluated. Progesterone concentrations on d 0, 11, 18, 25, 32, 39, 46, 53, and 60 post-TAI (**Figure 3A**), the main effect of group and the interaction of group by days did not differ. As expected, the main effect of days was significant because concentrations of progesterone were smaller at TAI and increased from day 11 to 60 post-AI. In contrast, for CL volume from day 11 to 60 post-AI (**Figure 3B**), the interaction of group by days tended ($P = 0.08$) to differ, mainly driven by greater volume of the CL on day 11 in the OMG3-prim cows, intermediate volume in the CON-mult cows, and smaller volume in the CON-prim cows. Moreover, on day 32 post-AI, the CL volume of CON-mult cows was greater compared with CON-prim and OMG-3 prim cows. For the CL blood flow from day 11 to 60 post-AI (**Figure 3C**), an interaction of group by day was detected ($P = 0.05$) because of greater blood flow in the CON-mult cows, intermediate in the OMG3-prim cows, and decreased flow in the CON-prim cows on day 25 and 46 post-AI.

Figure 4A displays the vesicle volume and the embryo/fetus volume from day 32 to 53, as well as the fetal head volume on day 60 post-AI. For the embryo/fetus and the amniotic vesicle volumes, the main effect of group and interaction of group by day were not different, but as expected, the embryo/fetus and the amniotic vesicle increased steeply as day of pregnancy advanced regardless of the group. In addition, no difference was detected among the groups for the fetal head volume on day 60 post-AI. Figure 4B displays the PSPB concentrations from day 18 to 60 post-AI. An interaction of group by-day was detected ($P = 0.01$) because greater PSPB

concentrations were observed in the OMG3-prim cows compared with CONT-prim cows and CONT-mult cows on day 46 post-AI.

Discussion

Diet fat supplementation is commonly used in the dairy industry (Rodney et al., 2015). Such supplementation is done to achieve greater energy density, especially at times when DMI is limited, such as during the transition period (between 21 days before and 21 days after parturition). When supplementing commercial fat products, however, omega-6 is favored over omega-3 FA. In addition to this issue, the fatty acid profile of other components of the TMR are also richer in omega-6 than omega-3 (Moallem, 2018). This creates an imbalance, in which inflammation pathways can be favored over anti-inflammatory pathways (Calder, 2012). Therefore, previous studies have assessed the effect of omega-3 on different reproductive traits (e.g., early embryonic development, CL function, pregnancies per AI), but not on *in vivo* embryo development. Thus, the objective of our study was to assess the benefits of omega-3 supplementation on oocyte quality, *in vitro* embryo quality, and *in vivo* conceptus development during the first two months of pregnancy in high-producing lactating dairy cows.

The DMI in our study did not differ between CON and OMG3 pens, based on feed refusals weighed once weekly throughout the experiment. Although initial studies have reported lesser DMI when diets were enriched in omega-3 (Doreau and Chilliard, 1997; Donovan et al., 2000; Martin et al., 2008) our findings agree with other studies in which there were no differences in DMI between omega-3 fed and controls (Ambrose et al., 2006; Thangavelu et al., 2007; Silvestre et al., 2011; Elis et al., 2016; Sinedino et al., 2017; Freret et al., 2019). Such differences could be partially explained by the source of omega-3 and its palatability. Although initial studies using fish oil seemed to decrease DMI (Doreau and Chilliard, 1997), other sources,

such as flaxseed, algae, and even GreatOplus used in this study, seem to be as palatable as control diets (Ambrose et al., 2006; Thangavelu et al., 2007; Sinedino et al., 2017). Although with a small number of animals enrolled in our study, BCS was monitored at a cow level at -21, 0, 21, 50, 75, and 110 DIM, and it was possible to observe differences in BCS over time regardless of the diet consumed, but no differences were detected between cows fed the CON and OMG3 diets. It is worth mentioning that we only enrolled cows at 15 DIM if they did not present health issues, and so, the benefits or risks of feeding a diet rich in omega-3 (i.e., potentially more anti-inflammatory effects) during the first 2 weeks after calving were not assessed in this study.

As expected, the n6:n3 ratio in plasma was not different between CON and OMG3 at 15 DIM (enrollment day) but was reduced in the OMG3 group compared with CON on 50, 75, 110, and 140 DIM. Because our diets were isocaloric, a lower n6:n3 ratio indicates an increase in omega-3 availability. This is a key result in the study that demonstrates that our OMG3 diet with flaxseed and algae could provide more absorbable omega-3 content. Other studies using exclusively flaxseed and fish oil have also succeeded in increasing systemic concentrations of omega-3 in cattle (Elis et al., 2006; Moallem et al., 2013). The advantage of using a product that combines flaxseed with *Nannochloropsis oculata* (GreatOPlus) is that flaxseed is a source of ALA and *Nannochloropsis oculata* provides ALA and EPA. Having a source of EPA is particularly important because the efficiency of endogenous conversion of ALA to EPA is low (Gulliver et al., 2012). Moreover, GreatOPlus has been associated with benefits in poultry, beef, and swine.

In addition to the plasma FA profile, we also assessed the milk FA profile at 15 and 50 DIM. Like the plasma results, the n6:n3 ratio in milk was not different between CON and OMG3

diets. In contrast, the ratio decreased from 15 to 50 DIM in both CON and OMG3 diets but was lesser in the OMG3 than the CON diet at 50 DIM. The 67% reduction in n6:n3 ratio in the OMG3 diet is comparable with the 41% reduction demonstrated by Elis et al. (2016) using a rumen-protected encapsulated fish oil. Moreover, our results agree with previous studies that demonstrated that omega-3 can be metabolized and deposited in milk within 4 weeks of consuming the diet (Thangavelu et al. 2007). Comparable with our results, Ambrose et al. (2006) observed a 187% increase in ALA milk concentration in dairy cattle receiving a flaxseed-supplemented diet compared with only a 22% increase in a sunflower oil-supplemented diet. Omega-3 content reportedly increased in several different tissues during omega-3 dietary supplementation. For example, Bilby et al. (2006) demonstrated that supplementation with a calcium salt of fish oil in dairy cows reduced the n6:n3 ratio in endometrium (confirmed by Greco et al., 2018), liver, mammary gland, and muscle tissues compared with supplementing with whole cotton seed from 17 to 194 DIM. In addition, Mattos et al. (2004) showed that fish oil supplementation increased the omega-3 fatty acids in caruncles in comparison with cows supplemented with olive oil.

The first part of our working hypothesis was partially supported; the omega-3 diet supplementation improved yield and quality of milk. Support for the hypothesis came from the interaction of diet by parity, which was mainly driven by greater milk yield in multiparous but not in the primiparous cows fed OMG3 diet compared with CON. The effect of omega-3 on milk production is controversial in the literature because some studies have shown no differences compared with control diets (Ambrose et al., 2006; Thangavelu et al., 2007; Elis et al., 2016; Freret et al., 2019), whereas others have observed increased milk yield (Petit et al., 2004; Moallem, 2009; Sinedino et al., 2017). One should note that in some studies omega-3 and

controls diets were not isocaloric as in the present study. The rationale for greater milk production in omega-3-supplemented cows is that omega-3 fatty acids are related to anti-inflammatory pathways and responses, whereas omega-6 fatty acids are related to proinflammatory responses (Calder, 2012). Moreover, all the sequences of events produced by the immune system's inflammatory responses require energy. Consequently, in an inflammatory response, it is possible to observe a redirection of nutrients to the detriment of animal products such as meat or milk (Colditz, 2002). Therefore, cows consuming omega-3 fatty acids could have extra energy that might be used to produce milk (Greco et al., 2015).

Partial support for the hypothesis of an increase in milk quality in high-producing lactating cows supplemented with omega-3 came from the tendency for a reduction in the somatic cell count in milk from cows receiving the OMG3 vs CON diet. The rationale for this observation also comes from the anti-inflammatory pathways associated with omega-3 metabolism. Nonetheless, there were no differences in milk percentages of fat, protein, and lactose between OMG3 and CON cows. These results agree with previous reports in which total protein or fat in milk did not differ among diets. For example, Thangavelu et al. (2007) showed no differences in the percentage of protein or fat in milk between cows supplemented with flaxseed (rich in omega-3 FA), sunflower (rich in omega-6 FA) or saturated fatty acids. Ambrose et al. (2006) showed no difference in the percentage of protein or fat in milk between cows supplemented with flaxseed or sunflower. More importantly, the increase in omega-3 contents (decrease n6:n3 ratio) in milk, should be considered an enhancement of milk quality, because it could have the potential to benefit human health and would diminish the addition of omega-3 during milk processing.

Based on the omega-3 or omega-6 anti-inflammatory or pro-inflammatory pathways, one might suspect that onset of estrous cycles could be different between diets. However, there were no differences in the largest follicle sizes, DIM at detection of those follicles, or even first postpartum CL detection between CON and OMG3 cows when evaluated until 45 DIM or 6.5 weeks after calving. Our results agree with Silvestre et al. (2011) who found no differences at 63 DIM in the percentage of cyclic cows that received palm or fish oil supplementation from 30 to 160 DIM. In addition, Elis et al. (2016) showed no differences at first postpartum ovulation between cows supplemented with protected fish oil or toasted soybean. Sinedino et al. (2017) reported a greater incidence of estrus expression in primiparous cows fed a supplemented algae diet when compared with primiparous cows fed a control diet, but no differences were observed in responses of multiparous cows.

The second part of our hypothesis was also partially supported, that omega-3 supplementation will enhance oocyte quality, *in vitro* embryo development, and *in vivo* pregnancy development during the first two months of gestation in high-producing dairy cows. Support for the hypothesis came from a greater combined percentage of oocyte grades 1 and 2 per total number of recovered oocytes in OMG3-mult, an increase in the cleavage incidence in OMG3 vs CONT cows, larger CL diameter in OMG3-prim vs CONT-prim on day 11 post-TAI, less luteal blood flow fluctuation in OMG3-prim than CONT-prim, and greater PSPB concentrations in OMG3-prim vs CONT-prim and CONT-mult on day 46 post-AI.

The combined grade I and II oocytes per total number of recovered oocytes parity by diet interaction was mainly explained by a greater percentage of oocytes grades I and II in OMG3-mult cows. Omega-3 content can be altered in different compartments of the ovary, such as follicular fluid, granulosa cells, and cumulus-oocyte complex (Zachut et al., 2010), and might

suggest a special role or a different metabolism of the PUFA in various tissues (Moallem, 2018). Notably, this selective uptake could affect the quality of the oocyte and embryos (Santos et al., 2008). Nonetheless, our results failed to support an increase in mitochondrial activity between diets but supported a tendency to increase the cleavage rate in OMG3 cows vs. CON counterparts.

Although previous studies have shown no differences in progesterone concentrations resulting from feeding omega-3 vs. omega-6 supplemented on lactating dairy cows (Ambrose et al., 2006; Thangavelu et al., 2007; Sinedino et al., 2017), others have reported an increase in progesterone concentrations when supplementing omega-3 FA (Petit et al., 2006). Our results failed to support an increase in progesterone concentrations in pregnant cows receiving the OMG3 diet. The discrepancy between the studies showing greater progesterone concentrations and our study could be related to the fact that we assessed cows known to be pregnant through day 60 of gestation, whereas other studies either used cows regardless of their pregnancy status (Petit et al., 2006) or collected samples on days 0, 21 and 24 post-AI as a means of early pregnancy diagnosis (Ambrose et al., 2006). Using progesterone concentrations to determine pregnancy status before an ultrasound pregnancy diagnosis after day 30 post-AI was the approach used in several studies (Ealy and Seekford, 2019). It increases the rate of false positive pregnant cows (Starbuck et al., 2004). Greco et al. (2018) showed that the day of luteolysis was not different between three diets with three different n-6:n-3 ratios. Nonetheless, Mattos et al. (2004) showed reduced $\text{PGF}_{2\alpha}$ metabolite in cows supplemented with fish oil vs olive oil. Such reduction is likely associated with a reduction in arachidonic acid, the precursor of $\text{PGF}_{2\alpha}$, which lyses the CL. Thus, the benefits of supplementing diets with omega-3 have been associated with

the idea of decreased $\text{PGF}_{2\alpha}$, and theoretically this reduction on $\text{PGF}_{2\alpha}$ release can help to avoid luteolysis.

In our study, the OMG3-prim cows had greater CL size on d 11 post-AI than CON-prim. Moreover, the larger size is unlikely associated with $\text{PGF}_{2\alpha}$ secretion because luteolysis occurs after days 17-19 in cattle. Potentially, the larger CL could be explained by a larger preovulatory follicle (Vasconcelos et al., 2001), but we did not evaluate preovulatory follicle size at the time of AI. Furthermore, we did not observe any differences in maximal follicle size when assessing follicular diameters from 20 to 45 DIM. Previous studies have shown greater preovulatory follicle size in cows supplemented with omega-3 (Ambrose et al., 2006; Elis et al., 2016), while others have not found differences (Moallem et al., 2013; Sinedino et al., 2017). Thus, it is unclear if omega-3 fatty acid supplementation can help to increase the size of the preovulatory follicles and, as a result, increased CL size and theoretically greater progesterone concentrations, which may improve pregnancy rate. Nevertheless, the larger CL was not accompanied with greater progesterone concentrations in the current study. Progesterone concentration in blood plasma is related to its metabolic clearance rate (Sangsritavong et al., 2002; Sartori et al., 2002; Sartori et al., 2004), which was not assessed in this study. Moreover, luteal blood flow was greater in CONT-mult than in CONT-prim, and intermediate and like the two previous groups in OMG3-prim. These results could be interpreted as less fluctuation in the OMG3-prim than CON-prim cows, as evidenced in Figure 3C.

In our study, PSPB was higher in OMG3-prim than CONT-prim and CONT-mult on day 46 post-AI. This observation occurred concomitantly with lesser luteal blood flow in the CONT-prim cows. Although the function of PSPB remains to be elucidated, its concentration in maternal blood has been used to evaluate embryo presence. The binucleate giant cells from the

trophoblast migrate to the endometrial epithelium at approximately day 20 of gestation in cows (Wooding, 1992). The binucleate cells synthesize and store PSPB until it is released into maternal circulation (Zoli et al., 1992). Thus, PSPB concentrations from days 20 to 28 post-AI has been associated with pregnancy viability (Gábor et al., 2016). Peak PSPB approximately day 32, as observed in our study and in previous reports (Pursley et al., 2023) and will slowly decrease over the subsequent days. It is unknown what a greater PSPB concentrations on day 46 could mean, but tentatively, would reflect greater placental functionality. Finally, our study failed to support a direct effect of omega-3 on the embryo/fetus and amniotic vesicle volumes from days 32 to 53 post-AI, as well as on the fetus head size on day 60 post-AI.

Summary and Conclusions

In summary, multiparous Holstein cows supplemented with omega-3 fatty acids tended to produce more milk than herd mates who received a control diet rich in omega-6. This result was associated with a trend for fewer somatic cells in milk in the omega-3 cows compared with the control diet. Likely, using less energy for inflammation could explain the increase in milk production. Nonetheless, omega-3 supplementation did not change the percentage of protein or fat in milk but reduced the omega-6 to omega-3 ratio in milk and plasma showing that the omega-3 supplementation in the diet was absorbed and distributed through the body. The increase of omega-3 in milk is likely to play a role in human health and milk market, but this was not assessed in our study. Multiparous cows supplemented with omega-3 fatty acid had greater oocyte quality as demonstrated by a greater percentage of oocyte grade I and II combined over the total number of recovered oocytes. Moreover, cows fed the omega-3 diet also demonstrated greater incidence of oocyte cleavage. Although progesterone was not different between diets, the CL was greater on day 11 in the primiparous omega-3-supplemented cows compared with

primiparous controls. In fact, CL volume and luteal blood flow seem to have remained less variable in primiparous omega-3-supplemented cows than in primiparous controls. Although the embryo-fetus, and amniotic vesicle volumes were not different between control primiparous, control multiparous, and omega-3 primiparous cows, the omega-3 primiparous presented greater pregnancy specific protein B on day 46 post-AI, which could indicate greater placental function. In conclusion, the increase in the oocyte quality, and greater pregnancy specific protein B is evidence that omega-3 supplementation may improve conceptus development during the first two months of pregnancy in high producing lactating dairy cows.

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Table 1. Dietary ingredients and nutrient composition of diets

Item	Treatments	
	Control	Omega-3
Ingredients	% of dry matter	
Corn silage	34.7	32.8
Alfalfa hay	19	19
Corn grain ground	15.3	15.9
Wet corn gluten feed	17.7	17.7
Whole cottonseed	1	0
Soy Plus	8.5	7.7
GreatOPLUS	0	3.5
Megalac	0.6	0
Sodium bicarbonate	1.1	1.1
MKC dairy micro	1.3	1.3
Calcium carbonate	1	1
Nutrient		
NE _l ¹ , Mcal/kg	1.7	1.7
Crude protein, %	17.5	17.5
Starch, %	22.9	23.5
Nonfibrous carbohydrate, %	36.9	38.1
Acid detergent fiber, %	18.3	18.1
Neutral detergent fiber(NDF), %	31.4	30.3
NDF from forage, %	18.4	17.7
Ether extract, %	4.7	4.3
Ca, %	1.1	1.1
P, %	0.5	0.5
Mg, %	0.4	0.4
K, %	1.6	1.6
Na, %	0.6	0.7
n-6:n-3 (13) ratio ²	6:1	2:1

¹Net energy for lactation.

²Omega-6:omega-3 ratio.

Table 2. Omega-6 to omega-3 fatty acid ratios in plasma and milk samples¹

DIM	Plasma			Milk ²		
	CON	OMG3	<i>P</i> -value	CON	OMG3	<i>P</i> -value
15	9.8 ± 0.3	10.1 ± 0.3	0.99	7.2 ± 0.2	7.5 ± 0.2	0.82
50	11.5 ± 0.3	8.0 ± 0.3	< 0.0001	6.4 ± 0.2	4.3 ± 0.2	< 0.0001
75	14.7 ± 0.3	9.6 ± 0.3	< 0.0001	–	–	–
110	15.2 ± 0.3	10.1 ± 0.3	< 0.0001	–	–	–
140	15.8 ± 0.3	10.4 ± 0.3	< 0.0001	–	–	–

¹Control and OMG3 cows received a diet with 6:1 and 2:1 n6:n3 ratio, respectively. Blood samples were taken at 15, 50, 75, 110 and 140 DIM and the plasma fatty acid ratio was compared for diet ($P < 0.0001$); DIM ($P < 0.0001$); parity ($P = 0.06$); diet by parity ($P = 0.06$); and diet by DIM ($P < 0.0001$).

²Milk samples were collected at 15 and 50 DIM and the milk fatty acid ratio was compared for diet ($P = 0.03$); DIM ($P < 0.0001$); parity ($P = 0.005$); diet by parity ($P = 0.62$); and diet by DIM ($P < 0.0001$).

Table 3. Follicle diameters and days when largest follicle and first postpartum corpus luteum was detected¹

Item	CON- prim	CON- mult	Total	OMG3- prim	OMG3- mult	Total	P-value		
							Diet	Parity	Diet × parity
n	10	14	24	11	14	25	-	-	-
Largest follicle, mm	17.8 ± 1.0	18.2 ± 0.9	18.1 ± 0.6	18.5 ± 1.1	19.5 ± 0.9	18.8 ± 0.7	0.6	0.48	0.71
Second largest follicle, mm	15.6 ± 1.2	15.1 ± 1.1	15.5 ± 0.8	16.6 ± 1.4	16.0 ± 1.2	16.1 ± 0.9	0.5 3	0.61	0.99
DIM at maximum largest follicle size	37.5 ± 3.7	27.8 ± 3.5	33.1 ± 2.9	28.4 ± 4.1	29.0 ± 3.5	28.8 ± 2.6	0.4 2	0.11	0.09
DIM at first CL	24.1 ± 3.9	28.1 ± 3.6	25.4 ± 3.3	26.7 ± 4.7	28.2 ± 3.3	28.1 ± 2.7	0.4 6	0.46	0.73

¹Control and OMG3 cows received a diet with 6:1 and 2:1 n6:n3 ratio, respectively. Transrectal ultrasound was performed twice weekly from 21 to 45 DIM to evaluate diameters of follicles and any corpus luteum.

Table 4. Number of aspirated follicles and quality of recovered oocytes per cow for CON-mult, CON-prim, OMG3-mult, and OMG3-prim groups¹

Item	CON-prim	CON-mult	Total	OMG3-prim	OMG3-mult	Total	<i>P</i> -value		
							Diet	Parity	Diet × parity
N	10	14	24	11	13*	24	-	-	-
Aspirated follicles	16.5 ± 4.5	26.6 ± 4.1	21.5 ± 3.7	26.3 ± 4.2	29.1 ± 4.2	27.7 ± 3.5	0.28	0.06	0.26
Recovered oocytes									
Grade 1	1.3 ± 1.3	2.7 ± 1.2	1.7 ± 1.1	2.5 ± 1.2	4.4 ± 1.2	3.5 ± 1.1	0.37	0.02	0.7
Grade 2	0.9 ± 0.7	3.0 ± 0.6	1.9 ± 0.5	1.5 ± 0.7	2.3 ± 0.6	1.9 ± 0.5	0.96	0.02	0.26
Grade 3	1.1 ± 0.5	1.5 ± 0.2	1.3 ± 0.3	0.8 ± 0.5	1.1 ± 0.4	0.9 ± 0.3	0.48	0.48	0.88
Degenerated oocytes	0 ± 0.3	1 ± 0.2	0.5 ± 0.2	0.3 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0.82	0.01	0.19
Recovered oocytes (%)	30.8 ± 14.2	44.6 ± 12.8	37.6 ± 12.8	50.0 ± 13.2	44.5 ± 12.9	47.2 ± 12.5	0.39	0.46	0.09
Grade 1 and 2 per total oocytes recovered (%)	58.5 ± 8.6 ^a	61.0 ± 8.0 ^{ab}	59.7 ± 7.9	61.2 ± 7.8 ^{ab}	79.9 ± 7.6 ^b	70.5 ± 7.5	0.25	0.0007	0.007

¹Control and OMG3 cows received a diet with 6:1 and 2:1 omega-6:omega-3 ratio, respectively. Oocyte pick up was performed at 50 days in milk. One cow was not submitted to follicle aspirations.

Table 5. Milk composition (fat, protein, somatic cell count [SCC], lactose, solids-not-fat [SNF], and milk urea nitrogen [MUN]) from cows supplemented with omega-3 fatty acids and controls cows from 14 to 140 DIM¹

Item	Fat, %	Protein, %	SCC	Lactose, %	SNF, %	MUN, mg/dL
CON	3.3 ± 0.11	2.8 ± 0.04	227.4 ± 76.26	4.9 ± 0.03	8.6 ± 0.09	12.0 ± 0.37
OMG3	3.2 ± 0.12	2.9 ± 0.04	54.8 ± 78.75	5.0 ± 0.03	8.7 ± 0.09	11.6 ± 0.38
				<i>P</i> -value		
Diet	0.77	0.45	0.15	0.25	0.50	0.43
Parity	0.09	0.01	0.92	0.03	0.01	0.71
DIM	<0.0001	<0.0001	0.86	0.004	<0.0001	<0.0001
Diet × parity	0.42	0.52	0.62	0.47	0.98	0.87
Diet × DIM	0.49	0.80	0.84	0.89	0.65	0.80
Parity × DIM	0.68	0.09	0.47	0.51	0.00	0.26
Diet × parity × DIM	0.75	0.40	0.42	0.79	0.41	0.39

¹Control and OMG3 cows received a diet with 6:1 and 2:1 omega-6:omega-3 ratio, respectively.

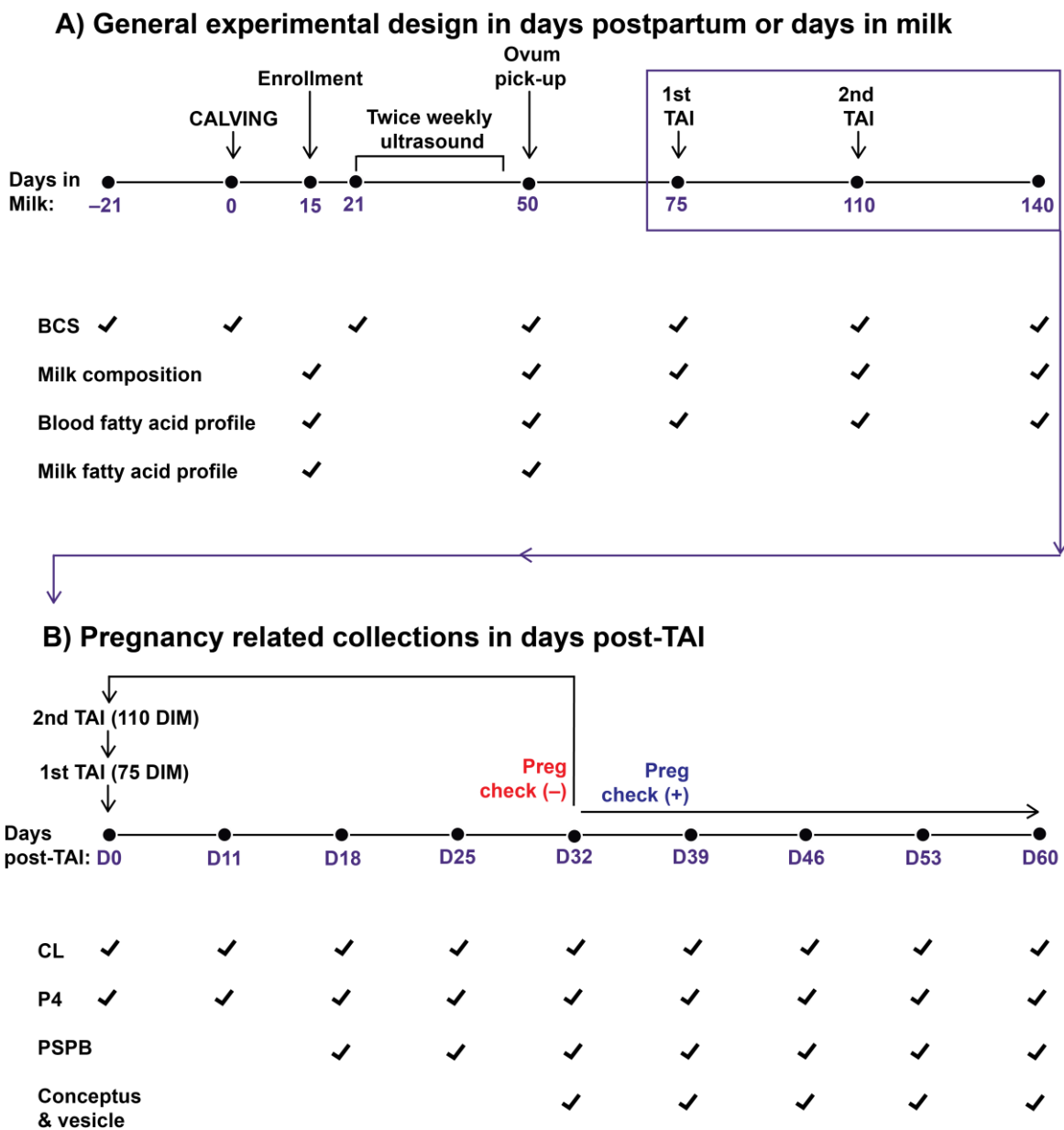


Figure 1.

Figure 1. Schematic of the experiment design. Milk and blood samples collection in days postpartum (A). Schedule for collection of pregnancy related traits in days post-AI (B). BCS = body condition score. CL: Ultrasound evaluation of any corpus luteum (B-mode for diameter and color doppler to measure the blood flow). DIM = Days in milk. TAI = Timed artificial insemination. Progesterone: Blood samples to measure progesterone concentration in plasma. PSPB: Blood samples to assay pregnancy-specific protein B concentration in plasma.

Weekly milk production per diet and parity

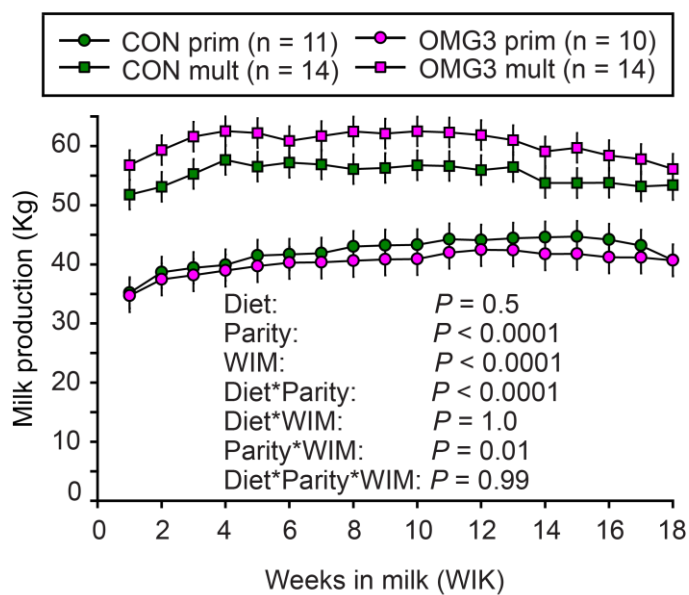


Figure 2. LS means \pm SEM for weekly milk production (kg) per diet and parity from 14 to 140 DIM. Control and omega-3 diets received an omega-6:omega-3 ratio of 6:1 and 2:1, respectively.

Reproductive parameters in pregnant cows

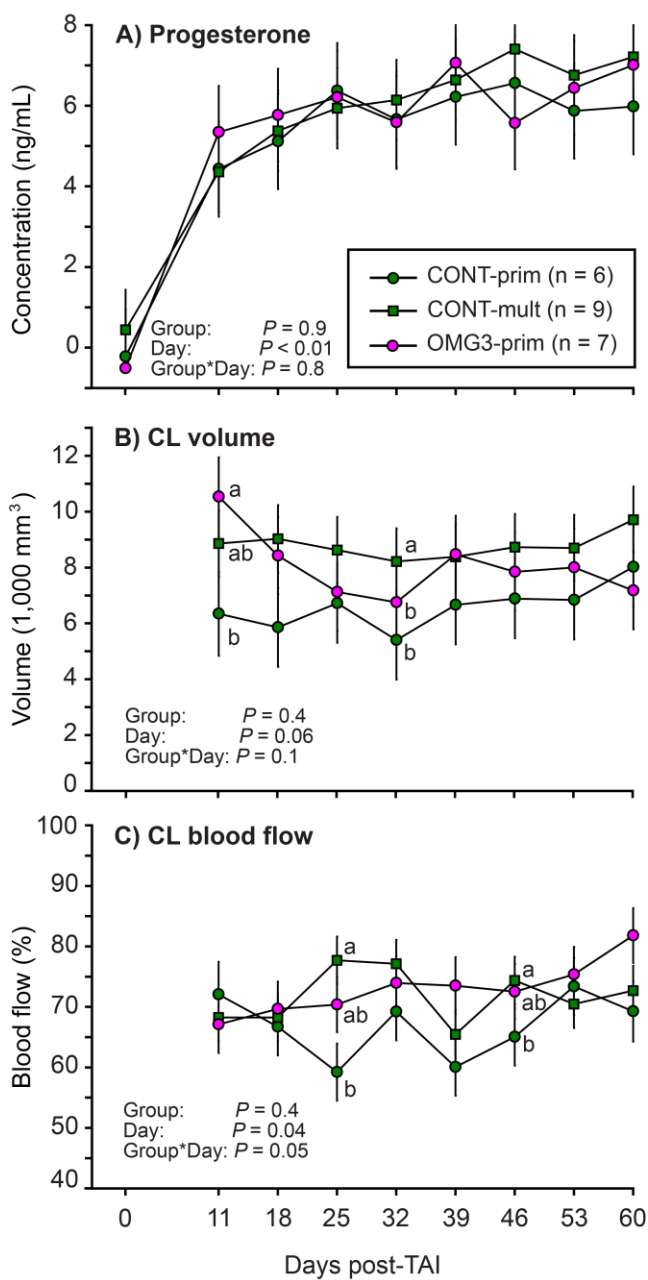


Figure 3. LS means \pm SEM for (A) plasma concentrations of progesterone (ng/mL) from 11 to 60 d of gestation; (B) Corpus luteum (CL) volume (1,000 mm³) from d 11 to 60 post-AI; and (C) luteal blood flow percentage (%) from d 11 to 60 post-AI. Results are depicted for the control primiparous, control multiparous, and omega-3 primiparous groups. Letters denote a statistical difference among the three groups within day when the interaction of group by diet $P \leq 0.10$.

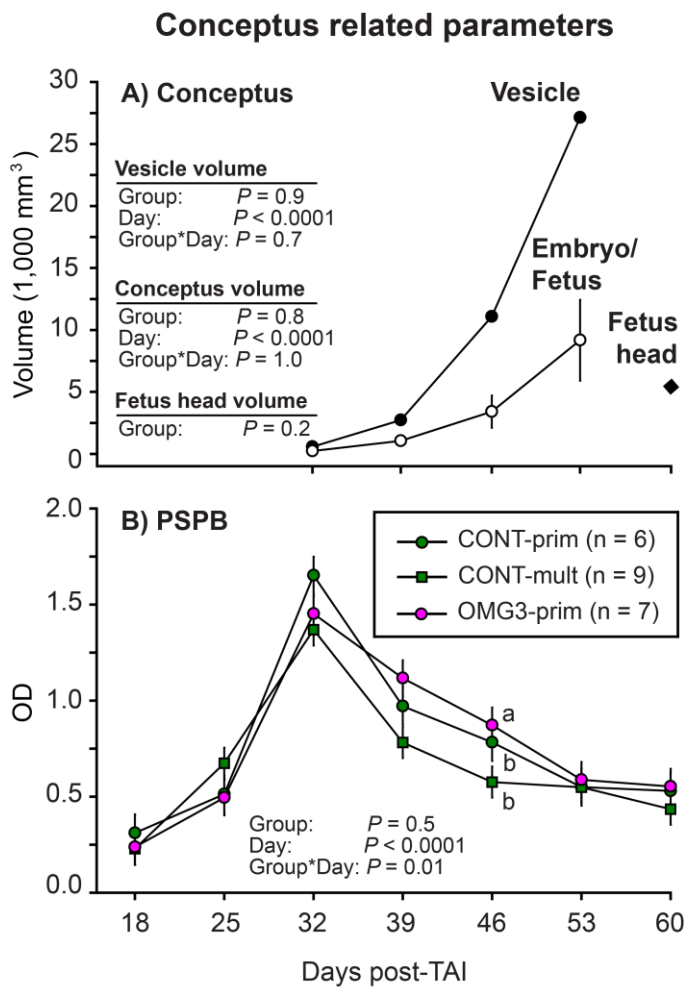


Figure 4. LS means \pm SEM for (A) mean embryo-fetal and amniotic vesicle volume (1,000 mm³) from d 32 to 60 of gestation for the control primiparous, control multiparous, and omega-3 primiparous groups; (B) Vesicle, embryo/fetus, and fetus head volume were compared by groups (Omega-3 primiparous, control primiparous and control multiparous cows). Letters denote a statistical difference when the interaction of group by diet $P \leq 0.05$.