Understanding the role of diets enriched in long chain fatty acids on postpartum reproductive function and embryo development in dairy cows

by

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Abstract

The objectives of this work were to investigate the influence of diets enriched in long chain fatty acids on feed intake, calf birth weight, maternal and neonatal fatty acid profiles, milk production, postpartum reproductive function, early embryonic development and its transcriptome profile in dairy cows. In previous research, the interval from calving to first ovulation was longer in cows given prepartum diets supplemented with canola (high oleic acid) seed than in those fed diets supplemented with linola (high linoleic acid) or flax (high α-linolenic acid) seed. A longer interval from calving to first ovulation is associated with reduced postpartum fertility. Since canola is a common ingredient in dairy cow rations in western Canada, it was important to further investigate the reported detrimental effect of a canola-based diet on the interval from calving to first ovulation in dairy cows. My first study was designed to understand the mechanisms by which first ovulation was delayed in canola fed cows. I hypothesized that luteinizing hormone (LH) pulsatility and pituitary responsiveness to gonadotropin releasing hormone (GnRH), early postpartum, would be reduced in cows fed canola prepartum. Results of the first study did not support my hypothesis. In the second study, the effects of prepartum supplemental fat (no-oilseed vs. oilseed) and the source of fat (canola vs. sunflower seed) on gestation length, calf weight, milk production and postpartum reproductive function were evaluated. Prepartum oilseed supplementation reduced dry matter intake during pre and postpartum periods in multiparous cows with an associated decrease in milk yield. It also increased gestation length, female calf birth weight and postpartum reproductive disorders without affecting ovarian function including the interval from calving to first ovulation postpartum, and fertility. In the third study, I evaluated the effects of dietary fat (oilseeds vs. control) and the type of supplemental fat (canola vs. sunflower) during late gestation on maternal

and neonatal plasma fatty acids, and expression of fatty acid transporter genes in placental cotyledonary tissue. Maternal LCPUFA, neonatal total n-3 fatty acids, eicosapentaenoic (EPA) and docosahexaenoic (DHA) were reduced following fat supplementation prepartum. Additionally, fatty acid protein transporter 4 (*FATP4*) and fatty acid translocase (*FAT*/*CD36*) mRNA expression was also lower in cotyledonary tissues of cows fed supplemental fat than control. I concluded that reduced total n-3 fatty acids, EPA and DHA in plasma of neonates born of dams fed fat prepartum was likely due to reduced expression of placental *FATP4* and *FAT/CD36*. In the final study, I investigated the effect of dietary long-chain fatty acids on the development and gene expression of *in vivo*- and *in vitro*-derived embryos. Feeding cows a diet supplemented with flax seed reduced the proportion of degenerated embryos than those fed canola or sunflower possibly through altered expression of up to 175 genes, of which, many were involved in cell survival and viability. Supplementation of serum harvested from cows fed flax or sunflower seed to embryo culture medium *in vitro,* did not affect embryo development. Hence, I concluded that the reduced proportion of degenerated embryos seen in cows fed flaxseed likely resulted from enhanced oocyte competence than post-fertilization embryo development. In summary, a canola-based prepartum diet did not have any detrimental effects on postpartum ovarian function. Prepartum dietary oilseed supplementation not only reduced dry matter intake and milk production in multiparous cows but also increased birth weight, reduced essential fatty acids in newborn calf blood and increased postpartum reproductive disorders. Feeding cows a diet enriched in n-3 polyunsaturated fatty acids (flax seed) during the breeding period reduced the proportion of degenerated embryos likely by altering the expression of embryonic genes improving cell survival and viability.

Preface

Chapter 3 of this thesis has been published as **Salehi, R.,** Colazo, M.G., Oba, M., and Ambrose, D.J. (2015) "A prepartum diet supplemented with oilseeds high in oleic or linoleic acid reduced GnRH-induced LH release in dairy cows during second week postpartum" in **Reproductive Biology and Endocrinology** 13: 69-78; doi: 10.1186/s12958-015-0060-x. I participated in experimental design, sample collection and laboratory analysis. I managed and analyzed data, and drafted the manuscript. DJA conceived the study, designed the experiment, assisted with sample collection and contributed to the writing and editing of the manuscript. MGC participated in experimental design, performed jugular catheterization, assisted with sample collection, data analysis and manuscript revisions. MO designed the rations and offered editorial suggestions. All authors read and approved the manuscript.

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Chapter 5 of this thesis was submitted as **Salehi, R.,** and Ambrose, D.J. "Prepartum maternal diets supplemented with oilseeds altered fatty acid profile in bovine neonatal plasma possibly through reduced placental expression of fatty acid protein transporter 4 (*FATP4*), fatty acid translocase (*FAT/CD36*)" to the journal, Reproduction, Fertility and Development. I designed the experiment, performed sample analysis, managed and analyzed data, and drafted the manuscript. DJA participated in experimental design and contributed to the writing and editing of the manuscript.

Chapter 6 of this thesis submitted as **Salehi, R.,** Colazo, M.G., Tsoi, S., Behrouzi, A., Tsang, B., Oba, M., Dyck, M.K., and Ambrose, D.J. "Morphologic and transcriptomic assessment of bovine embryos exposed to long chain fatty acids" to the journal, **Reproduction**. I participated in experimental design, *in vivo* embryo collection, RNA extraction and microarray analysis. I performed *in vitro* embryo production, laboratory sample analysis, managed and analyzed data, and drafted the manuscript. DJA conceived the study, designed the experiment, assisted with *in vivo* embryo collection and evaluation, and contributed to the writing and editing of the manuscript. MGC participated in experimental design, assisted with *in vivo* embryo collection and manuscript revisions. AB assisted with *in vivo* embryo collection and animal feeding. ST assisted with microarray analysis and manuscript revisions. MO designed the rations and offered editorial suggestions. MKD designed and assisted with microarray analysis and offered editorial suggestions. BT offered editorial suggestions. All authors read and approved the manuscript.

Dedications

To my parents and my wife for their love, support and encouragement in all my endeavours

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List of abbreviations

1,25-Vit D	Calcitonin and 1,25-dihydrovitamin D
ACTB	Actin-β
ANTE (1)	First-order Ante Dependence
ARA	Arachidonic acid
ARH (1)	Heterogeneous Autoregressive
aRNA	Amplified RNA
AUC	Area under the curve
B2M	Beta-2-microglobulin
BCS	Body condition score
BH	Benjamini-Hochberg
BHBA	β-Hydroxybutyric Acid
BW	Body weight
CAN	Canola
CL	Corpus luteum
CLA	Conjugated linoleic acids
CLDN4	Claudin 4
COC	Cumulus–oocyte complexes
CREB1	cAMP responsive element binding protein 1
DHA	Docosahexaenoic acid
DIM	Days in milk
DMI	Dry matter intake
D-PBS	Dulbecco's phosphate-buffered saline

EE	Ether Extract
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid
FABPs	Intracellular fatty acid binding proteins
FASN	Fatty acid synthase
FAT/CD36	Fatty acid translocase
FATP4	Fatty acid protein transporter 4
FATPs	Fatty acid transport proteins
FC	Fold change
FDR	False Discovery Rate
FLX	Flax
FOS	FBJ murine osteosarcoma viral oncogene homolog
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin releasing hormone
H2AFZ	H2A histone family, member Z
IFNT	Interferon-tau
IGF-I	Insulin-like growth factor I
IL-1	Interleukin-1
IL1RN	Interleukin 1 receptor antagonist
IPA®	Ingenuity® Pathway Analysis
IUPAC	International Union of Pure and Applied Chemistry
IVC	*In vitro* culture
KRT18	Keratin 18
KRT19	Keratin 19

LCPUFA	Long-chain polyunsaturated fatty acids
LGALS3	Lectin galactoside-binding soluble 3
LH	Luteinizing hormone
MUFA	Monounsaturated fatty acids
MUN	Milk urea nitrogen
NANOG	Nanog homeobox
NEFA	Non-esterified fatty acids
PGF2α	Prostaglandin F2α
PLAC8	Placenta-specific 8
PMNL	polymorphonuclear leukocytes
PPARA	Proliferator-activated receptor-α
PPARD	Proliferator-activated receptor-δ
PPARG	Proliferator-activated receptor-γ
PPARs	Peroxisome proliferator-activated receptors
PRID	Progesterone releasing intravaginal device
PTGS2	Prostaglandin-endoperoxide synthase 2
PUFA	Polyunsaturated fatty acids
RPL19	Ribosomal protein L19
RT	Reverse transcription
SLC9A3	Solute carrier family 9, subfamily A, member 3
SRXN1	Sulfiredoxin 1
SUN	Sunflower
TAI	Timed-AI
TBX3	T-box 3

TGF-β1	Transforming growth factor beta 1
TMUFA	Total monounsaturated fatty acids
UN	Unstructured
VLDL	Very-low density lipoproteins

Chapter 1. General introduction

1.1. Introduction

Poor reproductive efficiency of lactating dairy cows is considered a major challenge to the dairy industry. Forty percent of pregnancy loss happened between Days 8-17 indicating early embryonic mortality is a key contributor to the reduced reproductive efficiency in dairy cows (Thatcher *et al*., 2001). During early embryonic development interferon tau (IFNT) inhibits uterine prostaglandin F2α (PGF2α) release (Robinson *et al*., 2006) otherwise released PGF2α resulted in regression of corpus luteum (CL) (Thatcher *et al*., 1995) and termination of pregnancy (Robinson *et al*., 2006). Hence the ability of embryo to produce enough IFNT and to communicate with the cells of uterus is critical for the normal establishment of pregnancy in lactating dairy cattle.

Dietary fat supplementation during transition period improved reproductive performance in dairy cows (Thatcher *et al.* 2006, de Veth *et al.* 2009) and improved energy balance (von Soosten *et al.* 2012). Feeding supplemental fat increased total number of follicles (Lucy *et al.* 1991, Wehrman *et al.* 1991, Thomas & Williams 1996, Beam & Butler 1997, Lammoglia *et al.* 1997), medium size follicles (Moallem *et al.* 2013) and increased the size of preovulatory follicles (Lucy *et al.* 1991, Lucy *et al.* 1993, Beam & Butler 1997, Oldick *et al.* 1997). The summary of previous research indicated that feeding cows a diet supplemented with fat increased dominant follicle diameter on average by 2.8 mm, representing a 20.1% size increase in fat supplemented cows. Increased size of preovulatory follicles might partially result from increased concentrations of plasma LH, stimulating the latter stage of follicular growth.

Our research group (Colazo *et al.* 2009) reported that cows fed a prepartum diet supplemented with canola seed (high in oleic acid) had a longer interval from calving to first ovulation compared with those fed diets supplemented with either linola (high in linoleic) or flaxseed (high in linolenic). The interval from calving to first ovulation is mainly influenced by energy balance (Canfield & Butler 1990), postpartum health disorder (Beam & Butler 1997, Opsomer *et al.* 2000), IGF-1 concentration and LH pulsatility (Roche 2006), whereas the longer interval to first ovulation observed in cows fed a prepartum diet supplemented with canola seed was not associated with energy balance, the incidence of health disorders or IGF-1 concentrations postpartum (Colazo *et al.* 2009). However, the effect of dietary fatty acids on LH pulsatility during early postpartum period and pituitary responsiveness to GnRH are not known. Moreover, the effects of prepartum dietary fatty acids on the interval to first ovulation need to be compared with a control (no fat) diet.

Long-chain polyunsaturated fatty acids (LCPUFA), such as eicosapentaenoic acid (EPA; C20:5 n-3) and docosahexaenoic acid (DHA; C22:6 n-3) are important for fetal growth and development in rodents and humans (Duttaroy 2000) and fetus mainly depends on maternal LCPUFA to meet its requirements. Although some substrates can be transferred to fetus through passive diffusion, it may not fully meet the fatty acid demand of developing fetus. Previous research in the human indicated that placenta contained several fatty acid transporters, responsible for a preferential transportation of LCPUFA from maternal to neonatal blood. Although, the bovine and ovine placentae are less permeable than the human placenta (Battaglia & Meschia 1988), it has been found that ovine fetuses mainly relied on maternal circulation to meet their fatty acid requirements (Campbell *et al.* 1994). However, limited information is

available regarding transfer of specific LCPUFA from dam to fetus and the expression of placental fatty acid transporters in the bovine species.

Several studies evaluated the effect feeding dietary fatty acids on oocyte competence and embryo development. It was reported that adding saturated fatty acids (stearic or palmitic acid) to *in vitro* maturation media reduced cumulus expansion, increased cumulus apoptosis and delayed progression to metaphase II in bovine oocytes (Leroy *et al.* 2005). Furthermore, blastocysts derived from matured oocytes in the presence of saturated fatty acids had lower cell number with greater apoptosis (Van Hoeck *et al.* 2011). On the other hand, it has been reported that polyunsaturated fatty acids (PUFA) had positive effects on embryo development in the human and bovine species (Wathes *et al.* 2007a). Our research group (Thangavelu *et al.* 2007) found that feeding a diet enriched in α-linolenic (flax seed) or linoleic acid (sunflower seed) to lactating dairy cows enhanced embryonic development through a significant increase in blastomere number compared to feeding a diet enriched in saturated fatty acid (high in stearic and palmitic acids). Feeding cows a diet enriched in α-linolenic acid (flaxseed oil) also increased the numbers of follicles, oocytes collected by transvaginal ultrasonography, enhanced the cleavage rate of *in vitro* fertilized oocytes and tended to improve blastocyst rate compared with a diet enriched in saturated fatty acid (Moallem *et al.* 2013). In another study, adding α-linolenic acid to *in vitro* maturation medium enhanced oocyte maturation and subsequent embryo development (Marei *et al.* 2009). Although these studies have provided significant information regarding the effect of fatty acids on *in vivo* embryo development or on oocyte competence, very limited information is available about the transcriptome profile of embryos exposed to different fatty acids during their development as well as how fatty acids specifically influenced post-fertilization embryo development.

Chapter 2. Review of literature

2.1. Metabolic changes during the transition to lactation

Transition period is defined as 3 week before to 3 week after parturition. During transition period, the major challenge is to meet the increase of energy requirements for fetal growth and milk production in dairy cows (Bell 1995). The dramatic increase of fetal growth during the last 35 days of gestation reduced ruminal capacity and dry matter intake (DMI) (Stanley *et al.* 1993). Moreover, increased estradiol concentration during last week of gestation is also involved in reducing DMI. Intravenous administration of 17β-estradiol reduced DMI and milk production in dairy cows (Grummer *et al.* 1990). Another hormone increased periparturition is Growth hormone (Grum et al., 1996). The greater concentration of growth hormone caused an increase in responsiveness of adipose tissue to lipolytic signals such as norepinephrine. Collectively, increased growth hormone and estradiol as well as reduced ruminal capacity and dry matter intake lead to increased mobilization of fatty acids from adipose tissue, providing energy to animals.

After calving, the initiation of milk synthesis increases nutrient demands to support milk production. However, the rate of DMI increase during postpartum period is slower than the rate of milk production increase. Hence, feed intake cannot meet the nutrient requirements for maintenance and milk production during early postpartum period (Ingvartsen and Andersen 2000, Hayirli *et al.* 2002). **Figure 2.1** clearly indicates that calculated utilization of net energy of lactation (A) and metabolizable protein (B) by mammary gland for milk production accounted for 97 and 83%, respectively, of intakes. During transition period, reduced DMI increases the lipolysis of adipose tissue and subsequently non-esterified fatty acids (NEFA) which could be

used as an energy source (Grummer 1995) to compensate negative energy balance. Therefore, the concentration of NEFA in blood could reflect the degree of adipose tissue mobilization (Pullen *et al.* 1989); the greater negative energy balance, the higher NEFA concentration. Thereafter, NEFA are transferred to the liver and through complete or partial oxidization of NEFA produce carbon dioxide or ketone bodies, respectively. These end products can be used as energy source for the liver or other organs. Moreover, NEFA also could be used to produce triglycerides and subsequently they can be stored or exported out of liver as very-low density lipoproteins (VLDL). However, ruminant liver has low ability to export synthesized VLDL (Kleppe *et al.* 1988), therefore, during early postpartum, severe negative energy balance results in excessive liver NEFA uptake and lipid accumulation (Grum *et al.* 1996).

The demand for glucose in dairy cows increases with the initiation of lactation. As DMI decreases during early postpartum, feed intake cannot meet the glucose requirements. Therefore, the two main adaptations to meet the animals' glucose demands are, first an increase in hepatic gluconeogenesis (Reynolds *et al.* 2003), and second, using the "glucose sparing mechanism". In "glucose sparing mechanism", glucose oxidation reduced in peripheral tissues and then spared glucose can be directed to the mammary gland for lactose synthesis. In ruminants, the main substrates for hepatic gluconeogenesis are: (1) propionate from ruminal fermentation; (2) lactate from lactic acid cycling; (3) amino acids from skeletal muscle breakdown; and (3) glycerol from adipose tissue lipolysis. However, reduced DMI during early lactation has cows depend on mainly gluconeogenesis by amino acids and glycerol. Using NEFA as fuel for organs such as skeletal tissue decreases dependence on glucose as energy source. Thus glucose can be used for milk synthesis in mammary glands.

During the transition period, the health disorder incidences affect milk yield during the time of illness and most likely for the entire lactation. For example, milk yield was reduced by 535 kg during a 305-d lactation in multiparous cows (parity 4 or greater) that had ketosis during transition period (Rajala-Schultz *et al.* 1999a, Rajala-Schultz *et al.* 1999b). Incidence of any health disorder during periparturition period reduced milk production by 7.2 kg per day during the first 20 d postpartum (Wallace *et al.* 1996). Clearly, the incidence of health disorders during periparturient period negatively influenced milk yield during that lactation, in addition to the cost of lost saleable milk during treatment for the disorder and costs of veterinary treatment. Negative energy or protein balance are the main contributing factors for immune dysfunction (Goff and Horst 1997). It was found that cows with excessive negative energy balance and loss of body condition are the most likely to face health disorders during the postpartum period (Goff and Horst 1997) and also affect their reproductive performance. Additionally, inadequate supply of metabolizable protein has been related to impaired immune function (Houdijk *et al.* 2001) and increased the incidence of retained placenta. It was reported that the supplementation of vitamins A, E and trace minerals such as selenium, copper and zinc can improve immune function (Goff & Horst 1997). Moreover, a tremendous increase in the demand for calcium soon after parturition and the beginning of milk synthesis causes most cows to experience some level of hypocalcemia (Horst *et al.* 2003). Parathyroid hormone, calcitonin and 1,25-dihydrovitamin D (1,25-Vit D) are some of the major endocrine factors regulating plasma Ca concentrations. A dramatic reduction of blood calcium at calving leads to parturient hypocalcemia (milk fever). Subclinical hypocalcemia results from smaller decreases in blood calcium and causes disorders such as displaced abomasum by decreased smooth-muscle function (Goff and Horst 1997). Hypocalcemia also increases the incidence of retained placenta (Goff 1999).

2.2. Long-chain fatty acids

Most naturally derived fatty acids have a chain with an even number of carbon atoms, from 12 to 28, and contain a methyl group and a carboxylic group at each of the end chain. Based on the presence of double bonds, fatty acids can be categorized to saturated or unsaturated fatty acids. Fatty acids also can be classified into short-chain, medium-chain and long-chain fatty acids containing less than six, six to twelve and longer than twelve carbons, respectively (Frackenfield 2000).

2.2.1. Fatty acid nomenclature

Saturated fatty acids are categorized as those without any double bonds in their carbon chain, whereas unsaturated fatty acids have at least one double bond (**Table 2.1**). Unsaturated fatty acids can be further divided into mono or polyunsaturated fatty acids (PUFA) containing only one double bond or more than one double bond in their carbon chain, respectively (Gurr *et al.* 2002). The carbon atom of the fatty acid chain is numbered based on distance from carboxyl group; for instance the first carbon after carboxyl group named C-1 (IUPAC-IUB, 1977). Moreover, carbon atoms in PUFA are identified by Greek letters. Unsaturated fatty acids also can be either *cis* or *trans* based on their configuration. In the *cis* or *trans* configuration adjacent hydrogen atoms are found on the same or opposite side of the double bond, respectively (Gurr *et al.* 2002). There are two systems for fatty acid nomenclature, i.e., systematic name and shorthand nomenclature. Both systems provide information about the chain length, degree of unsaturation, and location and configuration of the double bonds. For instance *cis*-9, *cis*-12 octadecadienoic acid is the systematic name for linoleic acid, an 18-carbon fatty acid with two *cis* double bonds at positions 9 and 12. The shorthand nomenclature consists of two numbers which is separated by a

colon for instance the shorthand nomenclature of stearic acid would be 18:0. The first number indicates the length of fatty acid carbon chain and the next one indicates the number of double bonds (Gurr *et al.* 2002). The position and type of double bond would be indicated after the number indicating double bond(s). For example, vaccenic acid containing a *trans* double bond at the 11^{th} carbon can be represented as 18:1, *trans*-11.

The position of double bond in PUFA should be represented in relation to the methyl group. For example linoleic acid which has two *cis* double bonds at positions 9 and 12 relative to carboxyl group would be identified as 18:2, n-6. Additionally, PUFA are mainly divided into n-3 or n-6 fatty acids depending on the position of double bond relative to methyl group. Moreover, the n-3 and n-6 fatty acids are only able to be converted to fatty acids from the same group; meaning n-6 PUFA can only convert to other members of n-6 PUFA (Bezard *et al.* 1994). Essential fatty acids are considered as those PUFA that cannot be synthesized endogenously including linoleic (18:2, n-6), α-linolenic (18:3, n-3), eicosapentaenoic (20:5, n-3) and docosahexaenoic (22:6, n-3) acid (Cheng *et al.* 2001, Sampath and Ntambi 2005).

Conjugated linoleic acids (CLA) are PUFA with two double bonds. These two double bonds are located on adjacent carbons and can be trans/trans, trans/cis, cis/trans or cis/cis (Bauman *et al.* 2004). For instance, in cis-9, trans-11 CLA the double bonds are located between carbons 9-10 and 11-12. However, this differs with a typical PUFA such as linoleic acid in which a methylene group (CH2) is located between the double bonds. Conjugated linoleic acids are produced through ruminal biohydrogenation (Harfoot and Hazlewood 1997); therefore, they can only be obtained from ruminant-derived products.

2.2.2. Long-chain fatty acid metabolism in ruminants

In the rumen, the lipid metabolism involves lipid hydrolysis and biohydrogenation of unsaturated fatty acids. Dietary lipids are hydrolyzed by rumen bacteria, salivary and plant lipases. Although the extent of hydrolysis is generally high (>85%), some factors such as decrease in rumen pH reduce rumen hydrolysis ability (for reviews please see (Doreau *et al.* 1997, Harfoot & Hazlewood 1997)).

"Rumen microbial biohydrogenation" is the mechanism by which unsaturated fatty acids become saturated fatty acids through the addition of hydrogen molecules. Based on metabolic pathways, bacteria involved in rumen biohydrogenation are classified into two groups, A and B (Kemp and Lander 1984). Having both groups of bacteria is required to complete biohydrogenation of PUFA. Group A bacteria can hydrogenate PUFA to trans 18:1 fatty acids and a large variety of bacteria is categorized as group A bacteria. Group B can hydrogenate trans 18:1 fatty acid to stearic acid and only a few bacterial species are characterized as group B (Harfoot & Hazlewood 1997).

In ruminants, some fatty acids derive from microbial phospholipids which originate from exogenous (uptake of dietary fatty acids) and endogenous (de novo synthesis) sources. The contribution of each source mainly depends on lipid content of the diet and rumen bacterial species. Increasing lipid concentration in the diet enhanced exogenous uptake by some microbes with formation of cytoplasmic lipid droplets (Bauchart *et al.* 1990).

Although most unsaturated fatty acids become saturated though rumen biohydrogenation, some biohydrogenation intermediates pass the rumen, go to the small intestine and then accumulate in tissue and milk. The most common dietary unsaturated fatty acids are linoleic and α-linolenic acids. Stearic acid is the product of complete biohydrogenation and vaccenic acid is

an intermediate biohydrogenation product of both linoleic and α-linolenic acids (Emken 1995). *Cis* 9, *trans* 11 and *trans* 7, *cis* 9 are the most abundant isomers of CLA in ruminant fat. They can be synthesized endogenously through the action of the 9-desaturase enzyme but their substrate is vaccenic acid (Griinari *et al.* 2000, Kay *et al.* 2004) and *trans*-7 18:1 (Corl *et al.* 2002), respectively.

Fatty acids are mainly absorbed in the jejunum. However, before absorption water-insoluble fatty acids are required to be solubilized into the aqueous environment. Micelle formation is the key of this process (Davis 1990). Both bile and pancreatic juice are required for micelle formation. Bile provides bile salts and lecithin, and pancreatic juice supplies the enzyme to convert lecithin to lysolecithin and the bicarbonate to raise the pH. Inhibition of bile secretion reduces fatty acid absorption (Moore and Christie 1984). Once fatty acids absorbed via epithelial cells of the jejunum, fatty acids are esterified into triglycerides and then packaged into chylomicrons for transport to the blood. Intestinally-absorbed triglycerides are the main sources of blood triglycerides in ruminants (Emery *et al.* 1992).

During the periparturient period, negative energy balance and hormonal changes related to parturition and the initiation of lactation lead to increased lipolysis in adipose tissues, greater NEFA concentration and subsequently increased NEFA uptake by the liver (Drackley and Andersen 2006). Non-esterified fatty acid in the liver can be oxidized to acetyl-CoA or CO_2 through β-oxidation or tricarboxylic acid (TCA) cycle, respectively. Acetyl-CoA can be used as the substrate either with oxaloacetate to form citrate for TCA cycle or with acetoacetyl-CoA for ketogenesis (Gurr *et al.* 2002). However, the low availability of oxaloacetate often results in acetyl-CoA being used to produce ketone bodies (acetoacetate, acetone and β-hydroxybutyrate) (Gurr *et al.* 2002). Thereafter, the ketone bodies produced can be used as an alternative fuel for

glucose in a "glucose sparing mechanism"; by this way, glucose can be used preferentially by the mammary gland to support milk production. Additionally, those remaining NEFA are re-esterified to cellular triglycerides, which may be subsequently exported from the liver as VLDL or deposited in the liver as lipid droplets. Secretion of very low-density lipoprotein-triglyceride from the liver is inherently low in ruminants, even under intense lipid mobilization periods leading to hepatic lipid accumulation, more commonly referred to as fatty liver.

2.2.3. Long-chain fatty acid incorporation in tissues

It is evident from several studies that feeding protected and unprotected fatty acids sources result in the incorporation of those fatty acids into tissues of both beef and dairy cattle. Feeding dairy cows a diet supplemented with fish oil (200 g/d from 21 days before and until 21 days after parturition) during periparturient period increased the concentration of total and individual n-3 fatty acids in milk fat and placental caruncular tissue compared to those fed an olive oil supplemented diet (Mattos *et al.* 2004). Similarly, feeding a diet supplemented with a partially rumen protected (calcium salts) fish oil (1.9% of dietary DM) for at least 8 weeks also increased concentration of n-3 fatty acids in endometrial, liver, and mammary tissue, as well as milk fat (Bilby *et al.* 2006b). More recently, Moallem et al. (Moallem *et al.* 2013) indicated that feeding cows during pre and postpartum periods, diets supplemented with saturated fat (240 and 560 g/day per cow, pre and postpartum respectively), flaxseed oil (300 and 700 g/day per cow pre and postpartum respectively) or fish oil (300 and 700 g/day per cow pre and postpartum respectively) increased the proportion of α-linolenic acid in follicular fluid, granulosa cells, and cumulus oocyte complexes of flax fed cows than in other groups. Our group (Dutra *et al.* 2015) also found that cows fed a diet supplemented with sunflower for at least 60 days had higher concentrations of linoleic acid (0.60 vs. 0.30, 0.32) and total n-6 FAs (0.62 vs. 0.31, 0.33) and

higher n-6/n-3 ratio (9.25 vs. 2.09, 3.84) in follicular fluid than those fed either flax or canola. Similarly, cows fed a diet supplemented with flax had higher concentrations of α-linolenic acid (C18:3; 0.14 vs. 0.08, 0.06), total n-3 FAs (0.16 vs. 0.09, 0.07) and higher n-3/n-6 ratio (0.49 vs. 0.10, 0.11) in follicular fluid than those fed either canola or sunflower (Dutra *et al.* 2015). Alterations in tissues fatty acid profile could affect productive and reproductive performance of dairy cows which will be discussed in section 2.6 "Effects of supplemental fat on reproductive performance".

2.2.4. Benefits of feeding fats to dairy cows

Since inadequate energy intake during early postpartum period results in negative energy balance in dairy cows, feeding fat supplemented diets has become a strategy, in the dairy industry, to mitigate negative energy balance during the transition to lactation (Curtis *et al.* 1985). Greater milk production and negative energy balance also contribute to a decrease in reproductive performance, specifically a reduction in occurrence and intensity of estrus as well as embryo survival (Thatcher *et al.* 2003).

Feeding dairy cows diets supplemented with fats reduced the incidence of metabolic disorders in dairy cows possibly through improving energy balance status during periparturition period (Grummer and Carroll 1991). Hepatic fatty acid oxidation and accumulation can be influenced by dietary fatty acid composition. Using *in vitro* studies, it has been found that eicosapentaenoic and docosahexaenoic acids had greater hepatic oxidation than oleic or palmitic acid (Mashek *et al.* 2002). Oleic or palmitic acids had greater incorporation for triglycerides synthesis, whereas the PUFA were poor substrates for incorporation into cellular lipids (Mashek *et al.* 2002). Treatments containing PUFA decreased palmitic acid metabolism to triglycerides

and total cellular lipids compared with treatments with oleic or palmitic acids (Mashek *et al.* 2002).

Previous studies have indicated that feeding dietary fatty acids could improve reproductive efficiency through mechanisms not related to energy levels. Studies indicate that feeding diets enriched in PUFA may improve oocyte development (Moallem *et al.* 2013) and fertilization (Cerri *et al.* 2009).

2.3. Effect of supplemental fat on feed intake and production

2.3.1. Dry matter intake

Garcia *et al.* (2014) fed cows diet supplemented with no fat (Control), enriched in saturated fatty acids (mainly stearic acid, 1.7% of DM; Energy Booster 100, Milk Specialties, Dundee, IL), or enriched in essential fatty acids (27.4% linoleic acid; 1.7% of DM; Megalac R, Church and Dwight, Princeton, NJ) during the last 8 wk of pregnancy. They reported that neither prepartum fat supplementation nor the type of fatty acid affected mean DMI during prepartum period, although cows fed essential fatty acids consumed 0.9 kg/d less DM compared with those fed diet enriched in saturated fatty acid (Garcia *et al.* 2014). The same laboratory reported no significant differences in pre- or postpartum DMI among cows fed a control (no fat), calcium salt of safflower oil (63% C18:2 n-6; 1.5% of DM), or calcium salt of palm and fish oil (5.4% C20:5 n-3 and 5.3% C22:6 n-3; 1.5% of DM) supplemented diet, although there was a trend ($P = 0.09$) for lower postpartum DMI in cows fed the n-3 supplement compared to cows fed the n-6 supplement (Amaral 2008). However, in both experiments above, they did not report the interaction of treatment by parity for DMI, whereas Petit *et al.* (2007) found a parity by treatment interaction for DMI. They assigned cows (n = 33 total; n = 15 primiparous, n = 18 multiparous)

to 1 of 3 diets supplemented with whole flaxseed (high in α-linolenic acid, 3.3 and 11.0% of DM in pre and postpartum diets, respectively), Energy Booster (high in saturated fatty acids; 1.7 and 3.5% of DM in pre and postpartum diets, respectively) or no added fat (control) from 6 weeks before until 4 weeks after calving. Although, dietary treatments did not affect DMI in primiparous cows during pre and postpartum periods, multiparous cows fed Energy Booster had lower DMI during postpartum period than those fed flaxseed or control.

Elliott *et al.* (1996) reported that DMI tended to be depressed by addition of 6.1% calcium salt of polyunsaturated fatty acid compared with addition of similar concentrations of hydrogenated palm fat that were either prilled or flaked. Firkins and Eastridge (1994) also evaluated treatment means from 11 experiments and reported that DMI decreased as degree of unsaturation of long chain fatty acid increased. Similarly, in a study by Mattos *et al.* (2004), feeding a diet supplemented with fish oil (6% C20:5 n-3 and 28% C22:6 n-3) reduced DMI in the prepartum and postpartum periods by 30.3 and 18.1%, respectively, compared to those fed diet supplemented olive oil (61% C18:1 n-9) diet. In that study (Mattos *et al.* 2004) pre and postpartum diets contained 2 and 1.8% oil (DM basis), respectively. In a study with 1,500 dairy cows fed calcium salt of fatty acids at 1.5 % of DM, mean group DMI was significantly greater for the cows fed a diet supplemented with safflower oil than those fed palm oil (46% C16:0) during pre- and postpartum periods (Silvestre 2008). Moreover, cows in this study had greater DMI during the breeding period when fed a diet supplemented with palm oil compared with those fed fish oil (5.4% C20:5 n-3, 5.3% C22:6 n-3) supplemented diet (Silvestre 2008). These results indicate that the degree of saturation affect the amount of DMI and Drackley *et al.* (1992) proposed that DMI is affected by the amount of unsaturated fat reaching the duodenum. Greater hypophagic effects, as amount of unsaturated FA reaching the duodenum increased, might be

from greater release of cholecystokinin stimulated by unsaturated fatty acids compared to saturated fatty acid. In addition, unsaturated fatty acid might be absorbed and oxidized in the liver more quickly, generating reducing equivalents and satiety faster than saturated fatty acid (Allen 2000).

2.3.2. Body weight and body condition score

It has been suggested that feeding dietary fatty acids improved conception or pregnancy rates through improved energy balance status. However, feeding dietary fatty acids enhanced conception rates either without affecting BW or BCS (Carroll *et al.* 1994, Garcia-Bojalil *et al.* 1998b, Garcia-Bojalil *et al.* 1998a) or even in some cases with increasing BW or BCS losses (Sklan *et al.* 1991, Burke *et al.* 1997). For instance, feeding cows a diet supplemented with tallow (3% of DM) increased pregnancy rate despite having a more negative energy balance from week 2 to 12 postpartum compared to controls (Son *et al.* 1996). More recently, Rodney *et al.* (2015) used meta-analysis approach to compare several published studies about the effect of diets supplemented with fat on pregnancy rate. They found that feeding cows diets supplemented with fats during the transition period increased the probability of pregnancy to insemination by approximately 27% and significantly reduced the interval from calving to pregnancy without affecting BW. Therefore, dietary fatty acids most probably influence reproductive performance by other ways than affecting BW and BCS.

2.3.3. Milk yield, milk fat, and milk protein

Previous studies have shown inconsistent results regarding milk production and composition in response to fat supplementation. Douglas *et al.* (2004), Ballou *et al.* (2009) and Mattos *et al.* (2004) found that prepartum fat supplementation did not affect milk yield and

composition. Similarly, feeding cows a basal diet (Control) or basal diet + 150 g/d of CLA mix, or basal diet + 150 g/d of trans-C18:1 mix from 4 weeks before to 7 weeks after calving did not affect milk yield or milk protein percentage during first 7 weeks postpartum (Selberg *et al.* 2004). Dietary treatments also did not affect milk fat percentage during first 4 weeks but feeding diet supplemented with calcium salt of CLA significantly reduced milk fat percentage from week 5 to 7 (Selberg *et al.* 2004). Additionally, Duske *et al.* (2009) and Petit *et al.* (2007) reported that feeding cows diets supplemented with fat prepartum decreased milk yield and lactose (%), but increased fat (%) during first 4 weeks postpartum.

Whitlock *et al.* (2002) fed cows diets supplemented with either 0% supplemental fat (control diet) or 2% added menhaden fish oil, 2% extruded soybeans, 1% fish oil and 1% extruded soybeans. They found that feeding either diet supplemented with fish oil reduced milk production and milk fat percentage. Similarly, when Donovan *et al.* (2000) fed cows diet supplemented with fish oil at 0, 1, 2, and 3% of DM, they found that milk yield increased as dietary fish oil increased from 0 to 1% but decreased linearly from 1 to 3% dietary fish oil. Additionally, fish oil did not affect milk protein percentages, whereas there were linear decreases for milk fat as fish oil concentration increased from 1 to 3%. A recent meta-analysis and meta-regression by Rabiee *et al.* (2012) also indicated that milk yield positively correlated with DMI and the significant reduction in milk yield reported by Donovan *et al.* (2000) and Whitlock *et al.* (2002) might be attributed to the reduction in intake associated with dietary fish oil.

2.4. Effect of supplemental fat on plasma metabolites and hormones of dairy cows

2.4.1. Non-esterified fatty acids and β-hydroxybutyric acid

In dairy cows, plasma concentration of NEFA has association with fat mobilization and energy balance during early postpartum (Grummer and Carroll 1991). Although plasma NEFA concentrations increase when fat is supplemented to the diet of lactating dairy cows (Grummer and Carroll 1991), this response is inconsistent. Some studies reported that fat supplementation did not affect plasma NEFA concentration (Mattos *et al.* 2004, Amaral 2008, Castaneda-Gutierrez *et al.* 2009). Additionally, the type of supplemented fat and physiological status of animals could influence lipid mobilization and NEFA concentration. It was found that saturated fatty acids have a more marked effect on lipid mobilization than unsaturated fatty acids (Selberg *et al.* 2004, Rodriguez-Sallaberry *et al.* 2007).

Grum *et al.* (1996) showed that prepartum fat supplementation increased NEFA without affecting β-Hydroxybutyric Acid (BHBA) and glucose. The data of (Grummer and Carroll 1991) indicated that the effects of fat supplementation on plasma BHBA were inconsistent. The elevation in plasma BHBA concentrations in response to fat supplementation seems to depend on the metabolic status of the cow; during the transition period when plasma NEFA concentrations increase, there is a greater probability of a coincidental increase in plasma BHBA concentrations than later in lactation (Grummer and Carroll 1991).

Feeding multiparous cows a diet supplemented with calcium salts of CLAs increased plasma NEFA and BHBA concentration during the first wk postpartum than cows fed a control (no fat) diet (Selberg *et al.* 2004). Similarly, multiparous cows fed a lipogenic diet or mix of glucogenic and lipogenic diet from 3 weeks before to 9 weeks after calving had higher plasma NEFA and BHBA concentrations than those fed the glucogenic diet (van Knegsel *et al.* 2007).

As explained above, the effect of fat supplementation on BHBA is inconsistent. Rodriguez-Sallaberry *et al.* (2007) reported that, although, feeding *trans* fatty acids increased

plasma NEFA concentration of multiparous cows, it did not affect plasma BHBA concentration in primi and multiparous cows. Similarly, Petit *et al.* (2007) found that feeding cows a diet supplemented with saturated fatty acid (high in palmitic and linoleic acids) from 6 weeks before until 4 weeks after calving increased NEFA concentrations during postpartum period in multiparous cows than those fed control or whole flaxseed, without affecting BHBA. In contrary with previous reports, Silvestre *et al.* (2011b) and Amaral (2008) reported no treatment effect on plasma NEFA or BHBA concentrations. In the study by Silvestre *et al.* (2011b) cows were fed either calcium salt of palm oil or safflower oil at 1.5% of DM, whereas Amaral (2008), supplemented diets with either calcium salt of n-3 or n-6 fatty acids.

The greater fat mobilisation during early postpartum period increased plasma NEFA concentration (Grummer and Carroll 1991). However, this NEFA could not be completely removed from the circulation by tissues or mammary glands (Grummer and Carroll 1991). Increased concentration of both NEFA and BHBA could happen when the liver is not able to completely oxidize fatty acids (Goff and Horst 1997). However, increased NEFA concentration without affecting BHBA concentration can be explained by enhancing fatty acids oxidation in the liver.

2.4.2. Glucose

Feeding multiparous cows a diet supplemented with either prilled fats (high in palmitic and stearic acid; 1.9% of DM), calcium salts of long chain n-6 fatty acids (2.24% of DM), propylene glycol (769 g/d) or no fat supplementation during 3 weeks before and after calving did not affect circulating glucose concentrations (Castaneda-Gutierrez *et al.* 2009). Feeding a diet supplemented with *trans* fatty acids also did not influence plasma glucose concentration in

multiparous Holstein compared with those fed a diet enriched in saturated fats (Rodriguez-Sallaberry *et al.* 2007). Silvestre *et al.* (2011b) reported that feeding transition diets rich in palm oil or safflower oil had no effect on plasma glucose concentration. Similarly, Amaral (2008) found that feeding cows a diet enriched in either n-6, n-3 or control (no fat) did not affect plasma concentration of glucose during pre and postpartum period.

2.5. Effects of supplemental fat on maternal and neonatal fatty acid profile

Linoleic and α-linolenic acid, n-6 and n-3 PUFA, are the main essential fatty acids as they cannot be synthesized endogenously. Oilseeds such as flax (high in α-linolenic acid), sunflower (high in linoleic acid) seed mainly contain these fatty acids. Fish oil also is the main source of LCPUFA such as DHA, EPA and arachidonic acid (ARA). The LCPUFA are involved in several biological functions such as immune inflammatory responses and nervous system development. For instance DHA is required for maintaining membrane fluidity, impulse propagation and synaptic transmission. Previous research has shown the last 30 days of gestation is the peak of brain development in human. Given that DHA is an integral component for neurogenesis, deficiency of DHA during late gestation may have detrimental effect on fetal brain development (Innis 2007).

During late gestation period, increased lipolysis resulted in a maternal hyperlipidemia, having a critical role to provide adequate supply for developing fetus (Helland et al. 2003, Jensen et al. 2005). Hence, it is very important that the dam receives and reserves adequate LCPUFA during early pregnancy to support the high demands of developing fetus later on. Dam and neonate fatty acid profiles at birth indicated that maternal plasma contained 30, 5 and 1.5% of linoleic acid, ARA and DHA, whereas neonatal cord blood had 10, 10 and 3% of linoleic acid,

ARA and DHA, respectively. These differences indicate that a preferential transportation of LCPUFA form maternal to neonatal blood occurs to satisfy the fetal demands, especially when high quantities of ARA and DHA are required for brain and retina development (Haggarty 2002, Haggarty 2004). Accordingly, studies using human placental choriocarcinoma (BeWo) cells (Campbell et al. 1996) and placental perfusion system (Haggarty et al. 1999) have confirmed a selective preferential transport of LCPUFA, especially for DHA and ARA in human placenta. Previous studies have shown that low fish intake during pregnancy increased the risk of poor cognitive and behavioural outcome (Oken et al. 2005, Hibbeln et al. 2007). A lower visual ability and neural maturation also was found in infants with low DHA blood level (Helland *et al.* 2001, Bakker *et al.* 2003, Malcolm *et al.* 2003).

Passive diffusion of free fatty acids through placental membranes is one mechanism by which fatty acids are transferred to the fetus (Hamilton 1998, Hamilton & Kamp 1999) although passive diffusion may not fully meet the fatty acid demand of developing fetus. Previous research in the human placenta and in BeWo cells, a human choriocarcinoma cell line (Campbell et al. 1997, Campbell et al. 1998b, Duttaroy 2000), revealed a selective transportation of LCPUFA over nonessential fatty acids to satisfy fetal demands. It has been reported that several fatty acid transporters such as fatty acid transport proteins (FATPs), fatty acid translocase (FAT/CD36) and intracellular fatty acid binding proteins (FABPs) facilitate fatty acid transport in human placenta (Campbell et al. 1998a, Duttaroy 2000). Fatty acid translocase or CD36 which has been found in placenta and basal membrane (Campbell et al. 1998a) is able to bind to several substrates as ligand such as free fatty acids and collagen (Duttaroy 2009). Previous research has shown the role of FAT/CD36 in fatty acid uptake in heart and skeletal muscle (Koonen et al. 2005) in human and sheep (Zhu et al. 2010) placenta; however, there is no information available

about the role FAT/CD36 in bovine placenta. Fatty acid transport proteins are a family with six members including FATP1 to FATP6 (Schaffer & Lodish 1994, Stahl et al. 1999). As the overexpression of FATPs increased the fatty acid uptake, FATPs are categorized as fatty acid transporters. Previous research has found the expression of FATPs in different human tissues (Stahl et al. 1999), human placenta (Campbell et al. 1998a, Larque et al. 2006a, Larque et al. 2006b) and more recently in sheep (Zhu et al. 2010) and mouse (Mishima et al. 2011) placenta, suggesting their role in fatty acid transportation between the maternal and fetal circulations. Larque et al. (2006b) indicated that among FATPs only FATP1 and FATP4 expression was significantly correlated with DHA with the uptake of maternal DHA into placental and cord blood phospholipids.

2.6. Effects of supplemental fat on reproductive performance

Several studies report improved reproductive performance in lactating dairy cows fed supplemental fat. Staples *et al.* (1998) suggested that the positive effects of dietary fat on fertility of dairy cows are probably not only a result of improved energy status but also because dietary fats could affect the pituitary, ovarian and uterine function to enhance fertility.

2.6.1. Effect of dietary fats on luteinizing hormone secretion, ovarian follicular dynamics and function of corpus luteum

The pituitary function, luteinizing hormone (LH) secretion and subsequently ovarian follicle development are regulated by energy balance in dairy cows. It was found that negative energy balance delayed postpartum estrus (Randel 1990) by reducing the frequency of LH pulses essential for follicle growth (Schillo 1992). Enhanced LH secretion in cows fed diets supplemented with fat could be a result of improved energy balance (Palmquist & Weiss 1994,

Harrison *et al.* 1995). Moreover, the mean concentrations of serum LH was greater in beef cows fed calcium salts of fatty acids (0.5% of body weight) compared to those fed an isocaloric control diet (Hightshoe *et al.* 1991). However, the mechanisms independent of energy by which dietary fatty acids could affect LH secretion has not yet been established in ruminants.

It was found that feeding dietary fatty acids could improve follicular growth (Lucy *et al.* 1993). Feeding cows a diet supplemented with calcium salt of long-chain fatty acids increased the number of medium (6–9 mm) sized follicles, follicles >15 mm and the largest follicle diameter (18.2 vs. 12.4 mm) in a synchronized oestrous cycle compared to those fed control diet (Lucy *et al.* 1991). The previous studies, as summarized in **Table 2.2**, indicate how diet supplemented with fats affect the size of the dominant follicle. Feeding cows a diet supplemented with fat on average increased dominant follicle diameter by 2.8 mm, representing a 20.1% increase in fat supplemented cows. Additionally, it was found that feeding supplemental fat increased total number of follicles (Lucy *et al.* 1991, Wehrman *et al.* 1991, Thomas and Williams 1996, Beam and Butler 1997, Lammoglia *et al.* 1997), that of medium size follicles (Moallem *et al.* 2013) and increased the size of preovulatory follicles (Lucy *et al.* 1991, Lucy *et al.* 1993, Beam and Butler 1997, Oldick *et al.* 1997). Increased size of preovulatory follicles may partially result from increased concentrations of plasma LH, stimulating the latter stage of follicular growth. However, the effect of dietary fatty acids on LH concentration during early postpartum period remains to be determined.

Increased size of ovulatory follicle size subsequently increases the size of corpus luteum (CL) which may enhance progesterone secretion. Additionally, feeding diets supplemented with fatty acids consistently increases plasma concentrations of cholesterol (Grummer and Carroll 1991), which is the precursor for progesterone synthesis. Feeding cows diet supplemented

sunflower or flax seed (high in α-linolenic acid) increased plasma progesterone concentration in cows during Days 7 and 8 post-ovulation compared to those fed a diet enriched in saturated fatty acids (Thangavelu *et al*. 2007). Moreover, Piccinato *et al.* (2010) evaluated the effects of different fatty acids on the hepatic metabolism of estradiol and progesterone in cattle and found that incubating bovine liver slices with α-linolenic acid increased (~60%) progesterone and estradiol half-life compared to palmitic, palmitoleic (C16:1) and oleic acid. Additionally, infusing an emulsion of soybean oil (high in linoleic acid) increased hepatic progesterone-half life, and decreased clearance of progesterone in non-lactating dairy cows (Sangsritavong *et al.* 2002). Hence, feeding diets supplemented with fatty acids reduced metabolic clearance of progesterone and/or increased progesterone synthesis.

Previous studies indicate that the type of fat has more potent effect on follicular growth in cows than fat supplementation *per se*. Feeding cows diets enriched in PUFA increased dominant follicle diameter compared with diets enriched in monounsaturated fatty acids (Staples *et al.* 1998, Bilby *et al.* 2006a). The mean diameter of the ovulatory follicle (Ambrose *et al.* 2006) and CL (Petit & Twagiramungu 2006) increased when dairy cows were fed diets high in n-3 PUFA. More recently, Colazo *et al.* (2009) reported that dry cows fed diets supplemented with linola (high in linoleic acid) or flax seed (high in linolenic acid) ovulated sooner after calving compared to those cows fed diets supplemented with canola (high in oleic acid) and also had a numerically lower incidence of ovarian cysts. Castaneda-Gutierrez *et al.* (2009) fed cows (n = 12/treatment) diets supplemented with prilled fatty acids (high in palmitic and stearic acid; 1.9% of DM), calcium salts of long chain n-6 fatty acids (2.24% of DM), propylene glycol (769 g/d) or no fat supplementation during 3 wk before and after calving. The ovaries were scanned after d 10 postpartum 3 times per week to detect ovulation. Juchem *et al.* (2010) fed one of two diets

consisting of calcium salt (2% of DM) of either palm oil (high in palmitic acid, n = 246) or transoctadecenoic acid (n = 255) from 25 d prepartum to 80 d of lactation. The ovaries were examined weekly by ultrasonography until the sixth week of lactation to determine the first postpartum ovulation based on the presence of a CL. In another study (Caldari-Torres *et al.,* 2011), cows were fed diets containing fat supplements enriched in saturated fatty acids (1.5% of DM, n = 10), calcium salts of transoctadecenoic fatty acids (1.8% of DM, n = 10) or calcium salts of safflower oil fatty acids (high in linoleic acid, 1.8% of DM, n = 9). Dietary treatments were initiated at approximately 28 d before expected calving date and continued through 49 d postpartum. The interval from calving to first ovulation after parturition was determined by elevated (> 1 ng/mL) plasma progesterone concentrations for 2 sampling days. For progesterone measurements, blood samples were collected 3 times per week from d 12 through 49 postpartum (Caldari-Torres *et al.* 2011). In all three studies, dietary treatments did not affect the interval from calving to first ovulation. Recently, Silvestre *et al.* (2011b) fed cows (n = 1055) either calcium salt of long-chain fatty acids of palm oil or of safflower oil from 2 weeks pre-partum to 4 weeks postpartum, and then half of the cows in each transition treatment group were switched to either calcium salt of long-chain fatty acids of palm oil or fish oil (264 ⁄ treatment). The proportions of cyclic cows at 63 days postpartum were not different among treatments. Taken together, these data demonstrate that type of supplemental fatty acids, whether more saturated or unsaturated does not consistently influence resumption of postpartum cyclicity in lactating dairy cows.

2.6.2. Effect of dietary fats on follicular fluid, oocyte competence and embryo development

Several studies have compared the fatty acids profile of serum with follicular fluid. It has been found that follicular fluid triacylglycerol and free fatty acid content correlated with those in

serum (Leroy *et al.* 2004a, Leroy *et al.* 2004b). Although linoleic, palmitic and oleic acid are the main fatty acids in bovine follicular fluid (Homa & Brown 1992, Tsujii 2001), oleic, palmitic and stearic acid are the predominant fatty acids in follicular fluid of lactating dairy cows (Leroy *et al.* 2005). Moreover, it was found that the fatty acid profile of follicular fluid could be associated with follicle development. Follicles with higher ratio of oestrogen:progesterone had a greater concentration of palmitic and oleic acids and lower concentrations of stearic and linoleic acids (Renaville *et al.* 2010).

Oocyte is a large cell containing high amount fat (Jeong *et al.* 2009) , and the darkness of the oocyte cytoplasm is associated with oocyte fatty acid profile (Jeong *et al.* 2009). It was found that under *in vitro* conditions, the cleavage and blastocyst formation rate of oocytes with darker cytoplasm was greater than of oocytes with lighter cytoplasm (Jeong *et al.* 2009). The evaluation of fatty acid profile of oocytes with different cytoplasmic darkness indicated that oocytes with lighter cytoplasm contained a higher percentage of oleic and linoleic (18:2) fatty acids, while the darker oocytes had more saturated stearic acid (Kim *et al.* 2001). However, both types had same amount of palmitic acid (Kim *et al.* 2001).

A study comparing the total lipid content of bovine, porcine and ovine oocytes indicated that the porcine species had approximately 160 ng total fat per oocyte, which is about 2.5 and 1.8 times more than that in bovine and ovine oocytes, respectively (McEvoy *et al.* 2000). Moreover, the fatty acid profile analysis revealed that palmitic, stearic and oleic acids were the most abundant fatty acids in the oocytes of cow, pig and sheep (Genicot *et al.* 2005). Comparing bovine granulosa cells and plasma, with oocytes from the same animals, indicated that cumulus-oocyte complex (Adamiak *et al.* 2006) and oocytes (Wonnacott *et al.* 2010) are enriched in saturated fatty acids, suggesting selective uptake and/or de novo fatty acid synthesis in oocytes.

Several studies evaluated the effect of feeding dietary fatty acids on follicular fluid and oocyte fatty acid profile. Zeron *et al.* (2002) reported that feeding ewes a diet supplemented with fish oil for 13 weeks increased linoleic and docosahexaenoic acid in follicular fluid and linoleic, linolenic, arachidonic and docosahexaenoic acid in cumulus cells without affecting oocyte fatty acid profile. However, feeding ewes a diet supplemented with fish oil improved oocyte membrane integrity in response to chilling (Zeron *et al.* 2002). Wonnacott *et al.* (2010) found that feeding ewes a mix of fish and vegetable oils (high in n-3 and n-6 PUFA) increased n-3 and n-6 fatty acids in granulosa cells and oocytes, respectively.

Individual fatty acids have distinct effects on oocyte maturation and subsequent developmental competence. Leroy *et al.* (2005) reported that adding stearic acid or palmitic acid to *in vitro* maturation media of bovine oocyte reduced cumulus expansion, increased cumulus apoptosis and delayed progression to metaphase II. Additionally, treatment of bovine oocytes with a mixture of palmitic, stearic and oleic acid during *in vitro* maturation resulted in up-regulation genes involved in energy metabolism and oxidative stress in oocytes. Furthermore, blastocysts derived from these oocytes had lower cell number and greater apoptosis (Van Hoeck *et al.* 2011).

Although saturated fatty acids had detrimental effects on oocyte competence and subsequent embryo development, adding oleic acid to *in vitro* maturation medium reversed the detrimental effects of palmitic acid and stearic acid on subsequent embryo development (Aardema *et al.* 2011). Thangavelu *et al.* (2007) demonstrated that feeding a diet enriched in α-linolenic (flax seed) or linoleic acid (sunflower seed) to lactating dairy cows enhanced embryonic development through a significant increase in blastomere number compared to feeding a diet enriched in saturated fatty acid (high in stearic and palmitic acids). Moreover,

cows that were fed a diet supplemented with a partially rumen protected n-3 PUFA (high in eicosapentaenoic and docosahexaenoic acids) had a lower proportion of degenerated embryos than those fed a diet supplemented with palmitic acid (Childs *et al.* 2008). It was found that PUFA have positive effects on oocyte developmental competence. In this regard, cows fed a diet enriched in α-linolenic acid (flaxseed oil) had a greater numbers of follicles, oocytes collected by transvaginal ultrasonography, cleavage and blastocyst rate of *in vitro* fertilized oocytes compared with those fed a diet enriched in saturated fatty acid (Moallem *et al.* 2013). In another study, adding α-linolenic acid to *in vitro* maturation medium enhanced oocyte maturation and subsequent embryo development, whereas linoleic acid significantly inhibited cumulus cell expansion, delayed the development of oocytes to the metaphase II stage and reduced cleavage and blastocyst rate (Marei *et al.* 2009, Marei *et al.* 2010). *In vitro* maturation of oocytes in the presence of conjugated linoleic acid (t10,c12CLA) also improved the morphology of bovine blastocysts (Lapa *et al.* 2011).

Thus, specific fatty acids have distinct effects on oocyte maturation and in general saturated fatty acids, particularly palmitic acid and stearic acid, which are elevated in follicular fluid in some metabolic contexts are detrimental, while the presence of unsaturated fatty acids such as oleic acid and α-linolenic acid can counteract these detrimental effects and promote developmental competence.

2.7. Summary

Previous studies have shown that dietary fatty acids did not consistently influence resumption of postpartum cyclicity in lactating dairy cows (Wathes *et al.* 2007a, Santos *et al.* 2008, Wathes *et al.* 2013). In one study, the interval from calving to first ovulation was longer in

cows given prepartum diets supplemented with canola (high oleic acid) seed than in those fed diets supplemented with linola (high linoleic acid) or flax (high α-linolenic acid) seed (Colazo *et al.* 2009). The delay in resumption of cyclicity in canola fed cows was not associated with postpartum energy balance, health disorders or IGF-1 concentrations, but both pituitary LH pulsatility and pituitary responsiveness to GnRH were not evaluated. Moreover, a control diet without supplemental fat was not included in that study. Therefore, it is not known whether LH pulsatility and pituitary responsiveness to GnRH are altered by dietary fatty acids.

Maternal LCPUFA are the main source of fetal fatty acids essential for neonatal health and development (Duttaroy 2000). Several fatty acid transporters, such as fatty acid transport proteins (FATPs), fatty acid translocase (FAT/CD36), exist in placenta, involved in a preferential transportation of LCPUFA from maternal to neonatal blood (Campbell *et al.* 1998a, Duttaroy 2000). Given that expression of placental fatty acid transporters were influenced by fatty acid-activated transcription factors such as peroxisome proliferator-activated receptors (PPARs; Barak *et al.* 1999, Duttaroy 2004), maternal dietary fatty acids may affect their placental expression and consequently placental biology. Therefore, studies are warranted to investigate the effects of prepartum dietary fatty acids on the association between maternal and neonatal fatty acid profile, and cotyledonary long chain fatty acid transporters.

In other work from our research group (Ambrose *et al.* 2006) and elsewhere (Petit and Twagiramungu 2006), feeding cows a diet supplemented with flax seed during breeding period reduced pregnancy losses, likely through improved early embryonic development as later suggested by Thangavelu *et al.* (2007). Although embryos harvested from cows fed PUFA had a greater proportion of blastomeres relative to cows fed a saturated fat source (Thangavelu *et al.* 2007), there is no existing information about the effect of dietary fatty acids on the

transcriptomic profile of the embryos and also how fatty acids specifically influence post-fertilization embryo development.

These unanswered questions arising from the aforementioned reports and existing knowledge gaps formed the basis of my PhD research. Four separate studies were conducted.

Table 2. 1. List of fatty acids and their formula and saturation

Fatty acid	Formula	Saturation
Myristic acid	C14:0	Saturated fatty acid
Palmitic acid	C16:0	Saturated fatty acid
Stearic acid	C18:0	Saturated fatty acid
Oleic acid	C18:1 cis 9	Monounsaturated fatty acid
Nonadecanoic acid	C19:0	Saturated fatty acid
Linoleic acid	C18:2 n-6	Polyunsaturated fatty acid
γ-Linolenic acid	C18:3 n-6	Polyunsaturated fatty acid
α-Linolenic acid	C18:3 n-3	Polyunsaturated fatty acid
Dihomo-γ-linolenic acid	C20:3 n-6	Polyunsaturated fatty acid
Arachidonic acid	C20:4 n-6	Polyunsaturated fatty acid
Eicosapentaenoic acid	C20:5 n-3	Polyunsaturated fatty acid
Docosapentaenoic acid	C22:5 n-3	Polyunsaturated fatty acid
Docosahexaenoic acid	C22:6 n-3	Polyunsaturated fatty acid

Table 2.2. Effect of supplemental fat on the diameter of the dominant ovarian follicle of lactating dairy cows[1]

		Experimental diets	
Reference	Fat Source	Control (mm)	Fat (mm)
Ambrose et al. (2006)	Rolled flaxseeds	14.1	16.9
Beam and Butler (1997)	Tallow, yellow grease	11.0	13.5
Bilby et al. (2006a)	Ca-LCFA[2] or flaxseed oil	15.0	16.5
Lucy et al. (1991)	Ca-LCFA	12.4	18.2
Lucy et al. (1993)	Ca-LCFA	16.0	18.6
Oldick et al. (1997)	Yellow grease	16.9	20.9
Robinson et al. (2002)	Protected soybeans	13.3	16.9
Staples et al. (2000)	Soybean oil, fish oil	14.3	17.1
Zachut et al. (2008)	High proportion of UFAs[3]	11.8	15.4
Zachut et al. (2010)	Encapsulated-flaxseed oil or sunflower seed oil	11.7	11.9
Zachut et al. (2011)	Extruded flaxseed	14.7	15.8
Average		**14.1**	**17.3**

[1]The revised and updated table from review paper written by Staples and Thatcher (Staples and Thatcher 2005)

[2]Ca-LCFA: Calcium salt of long-chain fatty acids; [3]UFAs: unsaturated fatty acids

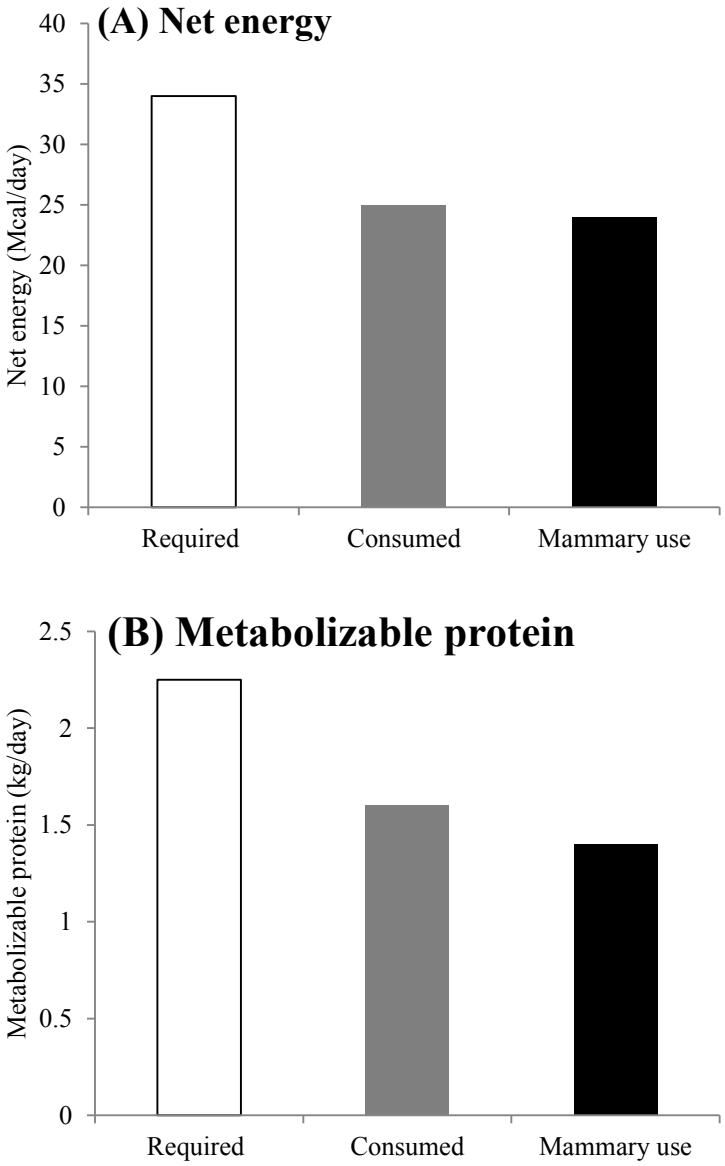

Figure 2.1. Calculations of amounts of net energy of lactation and metabolizable protein required, consumed, and utilized by lactating mammary gland of healthy dairy cows at 4 d postpartum.

Adapted from Drackley (1999).

Chapter 3. A prepartum diet supplemented with oilseeds high in oleic or linoleic acid reduced GnRH-induced LH release in dairy cows during second week postpartum

3.1. Abstract

Background: The objective was to determine the effect of prepartum diets supplemented with rolled canola seed (high in oleic acid) or sunflower seed (high in linoleic acid) on luteinizing hormone (LH) pulsatility and gonadotropin releasing hormone (GnRH)-induced LH release during early postpartum.

Methods: Thirty-one pregnant Holstein cows, blocked by body condition score, parity and expected calving date, were assigned to 1 of 3 prepartum diets supplemented with 8 % rolled canola or sunflower seed, or no oilseed (control) during the last 35 d of gestation. Blood samples were collected at Weeks (wk)-3, 0, +1 and +2, relative to calving, to determine non-esterified fatty acids (NEFA), β-hydroxy butyric acid (BHBA) and glucose. Additional blood samples were collected during wk1 (n = 5 per treatment) or wk2 (n = 5 or 6 per treatment), for 6 h, to measure LH pulsatility; thereafter, 100 mcg GnRH was administrated i.m., and blood was sampled for 4 h more, to measure GnRH-induced LH release.

Results: Dietary treatment did not affect prepartum energy balance, but cows fed the control diet were in a deeper state of negative energy balance during wk2, than those fed canola ($P = 0.03$) or sunflower ($P = 0.01$). Prepartum diets did not influence the mean plasma concentration of BHBA and glucose. However, NEFA concentration during wk2 was greater in control cows than those fed sunflower ($P = 0.03$) or canola ($P = 0.07$). Prepartum diets did not affect LH pulsatility (i.e. mean, minimum, maximum concentration, pulse frequency, and amplitude during wk1 and 2). GnRH-induced LH release did not differ among dietary treatments during wk1 but the mean GnRH-induced LH release during wk2 was either greater ($P = 0.02$)

and tended to be greater (P = 0.09) in control cows than in those fed canola and sunflower, respectively.

Conclusions: Prepartum diets did not affect LH pulsatility and GnRH-induced LH release during the first week postpartum, but cows fed a diet supplemented with oilseeds high in oleic or linoleic acid released less LH than control cows, in response to an exogenous GnRH challenge during the second week postpartum.

Keywords: GnRH, LH release, Canola, Sunflower, Long chain fatty acids

3.2. Background

Several studies have shown that dietary fat supplementation affects reproductive function in cattle (Gulliver *et al.* 2012). Effects of dietary fats on fertility (Burke *et al.* 1997), ovarian follicular development (Badiei *et al.* 2014) and steroidogenesis (Oldick *et al.* 1997) have all been reported. More recently, Colazo et al. (Colazo *et al.* 2009) reported that cows fed a prepartum diet supplemented with canola seed (high in oleic acid) had a longer interval from calving to first ovulation compared with those fed diets supplemented with either linola (high in linoleic) or flaxseed (high in linolenic). The ability of the first dominant follicle to ovulate during early postpartum is influenced by energy balance (Canfield & Butler 1990), postpartum health disorder (Beam & Butler 1997, Opsomer *et al.* 2000), Insulin-like growth factor I (IGF-1) concentration and LH pulsatility (Roche 2006). However, the delay in resumption of cyclicity observed in cows fed a prepartum diet supplemented with canola seed was not associated with energy balance, the incidence of health disorders or IGF-1 concentrations postpartum (Colazo *et al.* 2009). In addition, the diameter of the largest follicle at 7 ± 1 d after calving did not differ among dietary treatments but 25 % of cows fed a prepartum diet supplemented with canola

developed ovarian follicular cysts (Colazo *et al.* 2009) indicative of ovulatory dysfunction. It has been shown that feeding supplemental fat alters the growth dynamics of the ovarian follicle and that this effect is somewhat independent from energy (Mattos *et al.* 2000), but whether dietary fatty acids affect the secretion of LH in ruminants is unknown. Reports indicate that fatty acid signaling may affect the neuroendocrine control of reproduction acting directly at the brain level to regulate food intake and energy homeostasis in rats (Clement *et al.* 2002, Migrenne *et al.* 2007). Furthermore, Barb *et al.* (1995) showed that the addition of oleic acid to porcine pituitary cell culture resulted in significantly reduced GnRH-induced LH release compared to those cells cultured without added fat (Control). We hypothesized that the increased interval from calving to ovulation in dairy cows fed a diet supplemented with canola occurred through reduced pituitary responsiveness to hypothalamic GnRH. Therefore, the objective of this study was to determine the effects of prepartum diets supplemented with rolled canola or sunflower seed on LH pulsatility and GnRH-induced LH release during early postpartum period in lactating dairy cows.

3.3. Methods

3.3.1. Study design and experimental diets

This study was conducted at the Dairy Research Unit of the University of Alberta, Edmonton, Canada, from September to December 2012. All animal experimental procedures were approved by the University of Alberta's Animal Care and Use Committee for Livestock (protocol # 179/03/13, dated 16 April 2012). Animals were cared for in accordance with the Canadian Council of Animal Care Guidelines. Thirty-one non-lactating pregnant Holstein cows, parity 1 to 5 (8 primiparous, 23 multiparous), were used in the study. Approximately 35d before the expected calving date (wk-5), cows were blocked by body condition score (BCS), parity and

expected calving date, and assigned to 1 of 3 dietary treatments [Canola (high in oleic acid), sunflower (high in linoleic acid), or control (no oilseed)]. Diets were offered ad libitum as a total mixed ration containing forage (alfalfa hay and barley silage) and concentrates (**Table 3.1**). Cows were fed a diet supplemented with 8 % rolled oilseeds on a dry matter basis. Oilseeds were rolled as described previously (Ambrose *et al.* 2006) before incorporation in the diet. Upon calving, cows were placed on a common ration containing alfalfa hay, barely silage and concentrate balanced for a lactating dairy cow of 690 kg body weight (BW), producing 45 kg milk per day, according to NRC guidelines.

Cows were housed individually in tie-stalls during pre and postpartum periods, fed once daily at 0800 h and had unrestricted access to water. Postpartum, cows were allowed 1 to 2 h of exercise daily on week days, and milked twice daily in their stalls between 0400 and 0600 h and between 1530 and 1730 h. Milk production was automatically recorded at each milking and milk samples were collected only during second week postpartum from Tuesday P.M. to Friday A.M. to evaluate milk composition for energy balance calculation. Feed intake was recorded daily and weekly feed samples were taken from forages and concentrates to determine feed dry matter and diet composition (Sun & Oba 2014). Rations were adjusted weekly based on dry matter content.

Body weight (BW) and BCS were determined before dietary treatments began (wk-5), immediately after calving (wk0) and 5 weeks after calving (wk5). Prepartum BW measures were absolute, with no adjustments made for conceptus weight. The same technician assessed and assigned BCS to each cow, using a scale of 1 (emaciated) to 5 (overconditioned). Energy balance during pre and postpartum periods was calculated as described by Rabelo et al. (Rabelo *et al.* 2003) with a modification that instead of using a fixed calf weight of 40 kg, actual calf weights were used for calculating energy requirement of pregnancy. Fatty acid content of oilseeds was

determined at the Agricultural Experiment Station Chemical Laboratories (University of Missouri-Columbia, Columbia, MO) and presented in **Table 3.1**.

3.3.2. Blood sampling

Blood samples were collected 2 weeks after initiation of prepartum diets (wk-3), at calving (wk0), first (wk1) and second week postpartum (wk2) from all cows. Samples were collected at 8 h intervals over a 16 h period (2200, 0600 and 1400 h) in heparinized tubes (Vacutainer®, Beckton Dickinson, Franklin Lakes, NJ, USA), centrifuged and plasma harvested. Plasma samples from 2200 and 0600 h were kept at 4 °C until last sample collection at 1400. A pooled sample was prepared by mixing equal quantities of plasma from the 3 consecutive collections and stored at −20 °C until analyzed for non-esterified fatty acids (NEFA), β-hydroxy butyric acid (BHBA) and glucose. The blood sample collection schedule for LH determination is summarized in **Fig. 3.1**. Cows were assigned to two groups for blood sampling after parturition: wk1 (6 ± 1.0 d, n = 5 per treatment) or wk2 (9 ± 1.2 d, n = 5 or 6 per treatment). Cows sampled during wk1 for LH pulsatility and GnRH-induced LH were not used in wk2 sampling. Blood samples were collected (via indwelling jugular catheter) for 6 h, from 0700 to 1300 at 15 min intervals to assess LH pulsatility. Thereafter, GnRH (100 μg gonadorelin acetate, Fertiline®; Vétoquinol N.-A. Inc., Lavaltrie, QC, Canada) was administrated i.m. (1300 h) and blood was sampled for an additional 4 h, from 1300 to 1700 to determine GnRH-induced LH release. Blood samples were collected at 15 min intervals during the first hour (1300 to 1400) and then at 30 min intervals for the remaining 3 h. Samples were collected in sodium heparinized tubes (Vacutainer®, Beckton Dickinson), and immediately placed on ice until centrifugation (3000 × g for 20 min at 4 °C) within 3 h of collection. Plasma was harvested and stored at −20 °C until assayed.

3.3.3. Plasma LH and metabolites determination

Plasma LH concentrations were measured by radioimmunoassay using an anti-bovine LH monoclonal antibody (518B7; Quidel Corporation, San Diego, CA, USA). Plasma NEFA (NEFA-C kit, Wako Chemicals USA Inc., Richmond, VA), BHBA (Roche Diagnostics, Indianapolis, IN, USA) and glucose (P7119, Sigma-Aldrich, St. Louis, MO) were determined using commercially available kits. The intra-assay and inter-assay coefficients of variation for NEFA, BHBA and glucose were 1.49 and 3.32, 2.51 and 6.71, and 1.38 and 2.42 %, respectively.

3.3.4. Statistical analyses

Body weight, BCS, plasma NEFA, BHBA and glucose concentrations as well as energy balance were analyzed using the MIXED procedure of SAS (version 9.3, 2011; SAS Institute Inc., Cary, NC) for repeated measures with unstructured (UN) covariate structure. The final statistical model included dietary treatment, sampling time and parity as the main effects and dietary treatment by sampling time as interaction. Moreover, dry matter intake was analyzed with the same statistical model but First-order Ante Dependence [ANTE (1)] was used as covariance structure. All data are reported as mean ± SE; probabilities < 0.05 were considered significant, whereas those > 0.05 but < 0.10 were considered trends. The PC-Pulsar program (Merriam & Wachter 1982) was used to assess LH mean, maximum and minimum concentration (ng/mL), and pulse frequency/6 h and amplitude (ng/mL). Treatment differences were analyzed ($n = 5$ or 6 per treatment per week) using the MIXED procedure. The statistical model contained treatment, week and parity as the main effects and treatment by week as interaction. Plasma concentrations of GnRH-induced LH were analyzed ($n = 5$ or 6 per treatment per week) with the

MIXED procedure for repeated measures with Heterogeneous Autoregressive [(ARH(1)] as the covariate structure. Statistical model comprised treatment, week, sampling time and parity as the main effects and treatment by sampling time by week as interaction. The average of LH concentration in last 2 collected samples before GnRH administration was used as covariate in the model. Moreover, the peak concentration of LH and peak-time were chosen from each animal's raw dataset and then treatment difference was evaluated in created dataset with MIXED procedure. Model consisted of treatment, week and parity as the main effects and the treatment by week as interaction. Area under the curve (AUC) and incline rate were defined as below: AUC = $1/2\sum Xi\text{-}1\ (Yi\text{-}1 + Yi)$, where Xi and Yi are sampling time and LH concentration (ng/mL), respectively (Tai 1994). Incline rate = (Peak concentration– Basal concentration)/Time (min).

3.4. Results

3.4.1. Body condition score, body weight, dry matter intake and energy balance

There was no difference in BCS or BW among treatments at initiation of prepartum diets (BCS wk-5 and BW wk-5). Prepartum dietary treatments did not affect BCS (P = 0.17) and BW (P = 0.85) at calving (BCS wk0 and BW wk0) or 5 weeks after calving (BCS wk5 and BW wk5). Likewise, BCS between wk-5 and wk0 (P = 0.82), as well as between wk0 and wk5 (P = 0.36) did not differ (**Table 3.2**). Dry matter intake (DMI) significantly differed (P = 0.04) among dietary treatments (**Fig. 3.2**) during the entire experimental period; cows fed control diet consumed more (16.23 ± 0.49 kg) than those fed sunflower seed (14.58 ± 0.47 kg; P = 0.01) and tended to consume more than those fed canola seed (15.08 ± 0.64 kg; P = 0.08). Further analysis of pre and postpartum DMI as separate dataset indicated that during the prepartum period, cows

fed the control ration consumed more (15.30 ± 0.63 kg) than those fed sunflower (13.31 ± 0.57 kg; P = 0.01) or canola (13.54 ± 0.55 kg; P = 0.03). However, postpartum DMI did not differ among dietary treatments (mean, 17.50 kg; P = 0.37). Moreover, prepartum diets did not affect (P = 0.23) energy balance during the last 4 weeks of gestation (**Fig. 3.3a**), whereas postpartum energy balance (**Fig. 3.3b**) was affected (P = 0.009) by prepartum dietary treatments. Cows on a control diet (−8.39 ± 1.52 Mcal/day) were in a deeper state of negative energy balance than those fed diets supplemented with canola (−2.28 ± 1.60 Mcal/day; P = 0.03) or sunflower (−1.91 ± 1.63 Mcal/day; P = 0.01) during wk2 postpartum.

3.4.2. Plasma metabolites

Concentrations of plasma metabolites (NEFA, BHBA and glucose) are summarized in **Table 3.3**. Mean NEFA concentration tended (P = 0.07) to be higher in canola than control at wk-3 but it was not different from sunflower. During the postpartum period, cows fed control diet had greater NEFA concentration at second week compared with those fed sunflower (P = 0.03) and tended (P = 0.10) to be higher than those fed canola. Prepartum dietary treatments did not affect overall mean concentration of BHBA and glucose.

3.4.3. LH pulsatility

Dietary treatments did not affect mean, maximum and minimum concentrations of LH, LH pulse frequency or amplitude during wk1 and wk2 postpartum (**Table 3.4**). Likewise, LH pulsatility was not affected by postpartum week.

3.4.4. GnRH-induced LH release

Irrespective of prepartum dietary treatment, the mean LH (ng/ml) concentration after GnRH administration was increased ($P < 0.0001$) from wk1 (0.98 ± 0.09) to wk2 (1.97 ± 0.09) postpartum. Both at wk1 and wk2 postpartum, cows responded to exogenous GnRH administration by increasing LH release by 15 min post treatment ($P < 0.01$; **Fig. 3.4a and b**). In wk1, GnRH-induced LH concentrations (ng/mL) were higher only from 15 to 60 min post-GnRH (mean, 1.44 ± 0.20), relative to LH at time zero (0.58 ± 0.15). However, in wk2, GnRH-induced mean LH concentrations remained higher than at time zero (0.49 ± 0.15) from 15 min to the end of sampling at 240 min (2.27 ± 0.10). During wk1 postpartum, GnRH-induced LH release did not differ among dietary treatments, but during wk2 postpartum the mean LH released in cows fed a prepartum diet supplemented with canola (1.66 ± 0.23 ng/mL) was lower ($P = 0.02$) than that in those fed a control diet (2.42 ± 0.21 ng/mL). Cows fed a diet supplemented with sunflower tended ($P = 0.09$) to release less LH (1.83 ± 0.18 ng/mL) than those fed a control diet (**Table 3.5**). The interaction of dietary treatment by sampling time by wk postpartum indicated that there was no difference among dietary treatment by sampling time during wk1 (**Fig. 3.4a**). However, cows fed a control diet released more LH at 60, 90 and 120 min after GnRH administration than those fed either sunflower or canola during wk2 (**Fig. 3.4b**). Prepartum dietary treatments also affected AUC (ng/mL per 4 h) during wk2 postpartum, which was greater ($P = 0.01$) in control cows compared to those fed sunflower seed and tended ($P = 0.08$) to be greater than those fed canola. In addition, mean AUC of LH was higher ($P = 0.007$) in wk2 postpartum (143.33 ± 14.85) than in wk1 (58.23 ± 17.85). However, dietary treatments did not affect LH peak concentrations, LH peak-time, and incline rate during wk1 and wk2 postpartum (**Table 3.5**).

3.4.5. Ovarian follicle size

Prepartum diets did not affect the size of the largest ovarian follicle in either of the two postpartum weeks (**Table 3.6**).

3.5. Discussion

In dairy cows, energy balance is one of major factors affecting LH pulse frequency and resumption of ovarian activity during early postpartum (Bulman & Lamming 1978, Beam & Butler 1997, Opsomer *et al.* 2000, Wathes *et al.* 2007b). In the current study, lower net energy in the control diet resulted in higher dry matter intake compared to those fed diets supplemented with rolled sunflower or canola seed during the prepartum period. Cows fed a diet supplemented with canola seed tended to have higher NEFA at wk-3 than those fed control possibly due to lower feed intake during prepartum period. Although, prepartum dietary treatments did not affect postpartum DMI, cows fed a control diet prepartum were in a deeper state of negative energy balance and had higher NEFA concentrations during wk2 postpartum than those fed prepartum diets supplemented with sunflower or canola. In previous studies, restricting energy intake increased peak and total GnRH-induced LH release through reduced LH pulsatility (Beal *et al.* 1978, Randel 1990). Therefore, a more pronounced negative energy balance in control treatment during wk2 postpartum may have contributed to the increased GnRH-induced LH release in cows of that group compared to those fed oilseeds, albeit LH pulse frequency in the control group was not significantly lower than in cows fed oilseeds.

The possibility that cows on the control diet had larger ovarian follicles and higher estradiol concentrations in wk2, consequently resulting in higher GnRH-induced LH release was considered. However, as evident from **Table 3.6**, we found no significant influence of diets on follicle size in either of the two postpartum weeks. Although estradiol concentrations were not

measured in this study, it is highly unlikely that estradiol was the contributing factor for the increased release of GnRH-induced LH in the control group.

The mean basal (pre-GnRH treatment) LH concentration during wk1 postpartum (0.26 ng/mL) was lower than what was previously reported (1.5 ng/ml) in one study (Kesler *et al.* 1977), but agrees with another report in early postpartum dairy cows (0.32 ng/ml; (Fernandes *et al.* 1978)). Neither the first nor second postpartum week influenced LH pusatility in the present study irrespective of dietary treatment. Prepartum diets did not affect LH mean, maximum and minimum concentration, pulse frequency or pulse amplitude, which was likely due to low pituitary reserves of LH (Fernandes *et al.* 1978, Moss *et al.* 1985). To identify endocrine events that may influence the duration of postpartum anestrus, Moss et al. (Moss *et al.* 1985) slaughtered mature beef cows at 5, 10, 20 or 30 d after calving and another group at 12 to 14 d after their first postpartum estrus. They found that tissue concentrations of pituitary LH were low during early postpartum (at 5, 10 and 20 d) but significantly increased to levels comparable to luteal phase concentrations by 30 d postpartum. However, neither the GnRH receptor populations nor the affinity of anterior pituitary receptors for GnRH differed among the postpartum groups. In the same study, anterior pituitary cells from 5 d postpartum released significantly less LH in response to GnRH, when cultured *in vitro*, than those from luteal phase cows, but GnRH-induced LH release from pituitary cells *in vitro* in cows that were 10, 20 or 30 d postpartum did not differ from luteal phase cows. Thus, the replenishment of pituitary stores of LH may be one of the initial limitations to the reestablishment of reproductive competence after calving (Moss *et al.* 1985). Other studies have shown that pituitary LH content in dairy cattle increased during the postpartum period, but was not fully restored until approximately 19 d postpartum (Fernandes *et al.* 1978).

More recently, Garrel et al. (Garrel *et al.* 2011) infused a triglyceride emulsion (containing 61 % linoleic and linolenic acids) with heparin, through carotid catheter to rat brain for 24 h. Heparin was used to stimulate lipoprotein lipase activity and thus to release fatty acids from triglyceride. The infusion of the fatty acid emulsion did not affect serum LH concentrations in the above study. However, adding oleic and linoleic acid to rat (Garrel *et al.* 2011) and pig (Barb *et al.* 1995) pituitary cell culture increased LH concentrations by enhancing LHβ gene expression. The observed discrepancies between *in vivo* and *vitro* studies in LH secretion could be due to differences in concentrations and/or the mechanics of fatty acid delivery to gonadotropes, or due to species differences.

In the current study, independent of treatment effect, GnRH-induced LH release significantly increased from wk1 to wk2 postpartum, in agreement with previous studies (Kesler *et al.* 1977, Fernandes *et al.* 1978), but it was not affected by prepartum dietary treatments during wk1 postpartum. Moss et al. (Moss *et al.* 1985) indicated that the numbers of anterior pituitary receptors for GnRH at day 5 postpartum were the same as at 10, 20 or 30 d after calving. Therefore, the lower pituitary responsiveness to exogenous GnRH during wk1 postpartum (6 ± 1.0 d) was likely due to low pituitary LH content. Our finding that cows in wk2 postpartum released greater quantities of LH, which remained elevated for a longer duration, and had a higher AUC, support this notion. Furthermore, feeding a prepartum diet supplemented with canola seed (high oleic) resulted in lower LH release in response to exogenous GnRH in wk2 compared with those fed control diet, and cows fed a diet supplemented with sunflower seed (high linoleic) tended to release less GnRH-induced LH than those fed a control diet. Binding of GnRH to its receptor, a member of the G protein-coupled receptor family, initiates a wide array of signaling events among which one of them is GnRH mobilization of intracellular $Ca2^{+}$.

Intracellular $Ca2^{+}$ mobilization regulates acute gonadotropin release. Garrel et al. (Garrel *et al.* 2011) found that the addition of linoleic or oleic acid decreased GnRH signaling as evidenced by a significant reduction in GnRH-induced calcium mobilization in pituitary gonadotropes. It has also been reported (Boland & Drzewiecki 2008) that GnRH-induced increase in intracellular $Ca2^{+}$ is partly due to stimulation of voltage-gated calcium channels that can be inhibited by polyunsaturated fatty acids.

Cows fed canola and sunflower seed during the late gestation period in the present study, consumed 330 and 380 g of oleic and linoleic acids, respectively, and 485 and 450 g of total long chain fatty acids (oleic, linoleic and linolenic acids combined) daily. Long chain fatty acids consumed daily at these levels for up to 35 d preceding parturition could have had carryover effects interfering with GnRH-induced calcium mobilization in pituitary gonadotropes, thereby affecting LH release, although this remains a speculation at this time.

3.6. Conclusions

Current results show that prepartum diets did not affect pulsatile and GnRH-induced LH release during wk1 postpartum. Although prepartum diets also did not affect LH pulsatility during wk2 postpartum, cows that consumed either canola or sunflower seed prepartum had lower responsiveness to GnRH treatment, releasing less LH than cows fed a control diet. Greater negative energy balance and numerically lower LH pulse frequency in control treatment postpartum may have increased the releasable pool of LH to exogenous GnRH. Previous *in vitro* results (Barb *et al.* 1995, Garrel *et al.* 2011) and our present findings suggest that dietary long chain fatty acids (particularly oleic and linoleic) interrupt GnRH-induced LH release. While present findings lend support to our hypothesis that a longer interval from calving to ovulation in dairy cows fed canola seed (Colazo *et al.* 2009) occurred through suppression of pituitary

responsiveness to GnRH, the lack of differences in GnRH-induced LH release between canola (high oleic) and sunflower (high linoleic) diets, still leaves some questions unanswered, warranting further investigations.

3.7. Abbreviations

AUC: Area under curve; BCS: Body condition score; BHBA: β-hydroxy butyric acid; BW: Body weight; DMI: Dry matter intake; GnRH: Gonadotropin releasing hormone; LH: Luteinizing hormone; NEFA: Non-esterified fatty acids. Competing interests The authors declare that they have no competing interests.

3.8. Authors' contributions

DJA conceived the study, designed the experiment, assisted with sample collection and contributed to the writing and editing of the manuscript. MGC participated in experimental design, performed jugular catheterization, assisted with sample collection, data analysis and manuscript revisions. RS assisted with sample collection, managed and analyzed data, prepared tables and figures and drafted the manuscript. MO designed the rations and offered editorial suggestions. All authors read and approved the manuscript.

3.9. Acknowledgements

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Ana Ruiz-Sanchez and Mr. Amir Behrouzi during sample collection is also acknowledged and appreciated.

Table 3.1. Ingredient and nutrient composition of prepartum TMR diets and fatty acid content of canola and sunflower seed

	Control	Canola	Sunflower
Ingredient composition (% DM)			
Barley silage	60.0	60.0	60.0
Alfalfa hay	10.0	10.0	10.0
Ground barley	10.0	10.0	10.0
Soybean hulls	10.0	6.2	3.4
Canola meal	5.0	0.8	3.6
Canola seed[A]	0.0	8.0	0.0
Sunflower seed[B]	0.0	0.0	8.0
Vitamin / mineral supplements	5.0	5.0	5.0
Nutrient Composition (% DM)			
Crude protein	14.2	13.8	14.4
NDF	42.0	41.3	40.1
Crude fat	2.7	7.4	6.2
Net energy for lactation (Mcal/kg)	1.4	1.6	1.5

[A]Total fat: 45.2 %; Fatty acid content (% of total fat): 61.2 % oleic, 18.8 %, linoleic, 9.6 % linolenic, 10.4 % other; [B]Total fat, 43.3 %; Fatty acid content (% of total fat): 12.5 % oleic, 73.1 %, linoleic, 0.7 % linolenic, 13.7 % other

Table 3.2. Effects of prepartum diets on body condition score (BCS) and body weight (BW)

	Prepartum diets			P-value	
	Control (n =11)	Canola (n =10)	Sunflower (n =10)	Trt[A]	Trt*wk[B]
Body condition score				0.17	0.80
BCS (wk-5)	3.28±0.06	3.28±0.06	3.39±0.06		
BCS (wk0)	3.44±0.06	3.49±0.06	3.53±0.05		
BCS (wk5)	2.96±0.08	3.00±0.07	3.16±0.07		
BCS difference wk-5 & 0	0.15±0.09	0.20±0.09	0.15±0.08	0.82	-
BCS difference wk0 & 5	-0.49±0.09	-0.48±0.09	-0.33±0.08	0.36	-
Body weight				0.85	0.61
BW (wk-5)	642.5±24.7	621.1±22.9	634.4±23.5		
BW (wk0)	642.5±23.0	613.0±22.8	631.71±21.9		
BW (wk5)	557.8±23.4	562.0±21.1	564.6±21.7	0.99	-
BW difference wk0 & 5	-75.6±18.3	-44.1±19.3	-62.8±17.6	0.45	-

[A]Trt: Dietary treatment; [B]wk: Postpartum week

Table 3.3. Effects of prepartum diets on plasma metabolites concentration

	Prepartum diets			P-value	
	Control	Canola	Sunflower	Trt[A]	Trt*wk[B]
NEFA(mEq/dL)				0.40	0.05
wk-3	81±5[¥]	95±5[¥]	85±5		
wk0	507±54	397±61	441±54		
wk1	392±48	439±50	453±50		
wk2	584±64[§a]	428±68[§ab]	381±68[b]		
BHBA (mg/dL)				0.28	0.42
wk-3	11.3±0.5	9.9±0.5	9.7±0.5		
wk0	10.1±0.8	10.6±0.8	10.3±0.8		
wk1	12.0±1.0	12.9±1.0	11.2±1.0		
wk2	14.2±1.1	11.9±1.1	11.7±1.1		
Glucose (mg/dL)				0.50	0.78
wk-3	56.3±1.4	58.1±1.3	58.7±1.3		
wk0	77.9±5.3	75.9±5.5	80.6±5.5		
wk1	49.8±1.5	48.7±1.5	50.9±1.5		
wk2	46.0±1.5	47.7±1.5	50.4±1.5		

[a,b]: P = 0.03; [¥] Control tended (P = 0.07) to have lower NEFA concentration than canola;

[§]Control tended (P = 0.10) to have greater NEFA concentration than canola

[A]Trt: Dietary treatment; [B]wk: Postpartum week; wk-3: 2 wk after the initiation of prepartum diets; wk0: calving; wk1: first week postpartum; wk2: second week postpartum.

Table 3.4. Characteristics of LH pulsatilityA among prepartum dietary treatments

	Week 1[B]			Week 2[B]			P-value	
	Control	Canola	Sunflower	Control	Canola	Sunflower	Trt[C]	Trt*wk[D]
Cow (n)	5	5	5	6	5	5		
LH Mean	0.25±0.14	0.25±0.15	0.28±0.13	0.24±0.14	0.54±0.14	0.28±0.14	0.53	0.51
LH Min	0.11±0.06	0.10±0.06	0.09±0.05	0.11±0.07	0.16±0.07	0.10±0.07	0.83	0.92
LH Max	0.62±0.39	0.63±0.42	1.27±0.37	0.61±0.39	1.49±0.39	0.69±0.39	0.74	0.87
Frequency	3.91±0.65	4.15±0.69	4.75±0.60	3.45±0.63	4.95±0.63	4.25±0.63	0.30	0.55
Amplitude[E]	0.38±0.10	0.39±0.10	0.44±0.09	0.33±0.09	0.52±0.09	0.36±0.09	0.61	0.54

[A]Blood samples were collected every 15 min for 6 h; [B]Week 1: First week after calving; Week 2: Second week after calving; [C]Trt: Dietary treatment; [D]wk: Postpartum week; [E]Amplitude: The amplitude of hormone pulses is defined as the height from the preceding nadir to maximum height.

Table 3.5. Effects of prepartum diet on GnRH-induced LH release during early postpartum

	Week 1[A]			Week 2[A]			P-value	
	Control	Canola	Sunflower	Control	Canola	Sunflower	Trt[B]	Trt*wk[C]
Cows (n)	5	5	5	6	5	5		
Incline rate (ng/mL per min)[H]	1.14±0.14	0.84±0.23	0.96±0.21	2.42±0.21[¥a]	1.66±0.23[b]	1.83±0.18[¥ab]	0.03	0.05
AUC (ng/mL per 4 h)[F]	2.05±0.75	3.21±0.80	2.19±0.70	5.14±0.80	4.07±0.73	3.72±0.73	0.37	0.11
LH- Peak-time (min)[E]	32.50±13.26	48.00±13.78	22.50±10.27	81.37±21.53	101.63±21.53	63.37±21.53	0.48	0.74
LH- Peak (ng/mL)[D]	41.52±27.39	84.65±29.26	48.51±25.38	197.26±26.74[§c]	125.73±26.74[§cd]	106.99±26.74[d]	0.26	0.06
LH Mean (ng/mL)	0.09±0.02	0.09±0.02	0.08±0.02	0.06±0.02	0.07±0.02	0.07±0.02	0.98	0.97

[a,b]: P = 0.02; [c,d]: P = 0.01

[¥]Control tended (P = 0.09) to have greater LH mean concentration than sunflower at wk2.

[§]Control tended (P = 0.08) to have greaterAUC than canola at wk2.

[A]Week1: First week after calving (n = 5 cows per treatment); Week2: Second week after calving (n = 5 or 6 cows per treatment); [B]Trt: Dietary treatment; [C]wk: Postpartum week; [D]Peak: The highest concentration of LH after GnRH administration; [E]Peak-time (min): The interval in minutes from GnRH administration to LH peak; [F]AUC: Area under curve = $1/2\Sigma Xi\text{-}1\ (Yi\text{-}1 + Yi)$, where Xi and Yi are sampling time and LH concentration (ng/ml), respectively; [H]Incline rate = (peak concentration– basal concentration)/Time (min)

Table 3.6. The mean diameter of the largest ovarian follicle in postpartum weeks 1 and 2 in each of the three dietary groups

	Mean ± S.E. of follicle diameter (mm)	
Postpartum diet	Week 1[A]	Week 2 [A]
Follicular diameter		
Control	9.41±1.34	13.90±1.47
Canola	6.40±1.47	13.80±1.47
Sunflower	7.00±1.47	10.90±1.47

[A]Week1: First week after calving (n = 5 cows per treatment); Week2: Second week after calving (n = 5 or 6 cows per treatment). Follicle size was not influenced by treatment (P = 0.18) or treatment x week interaction (P = 0.45)

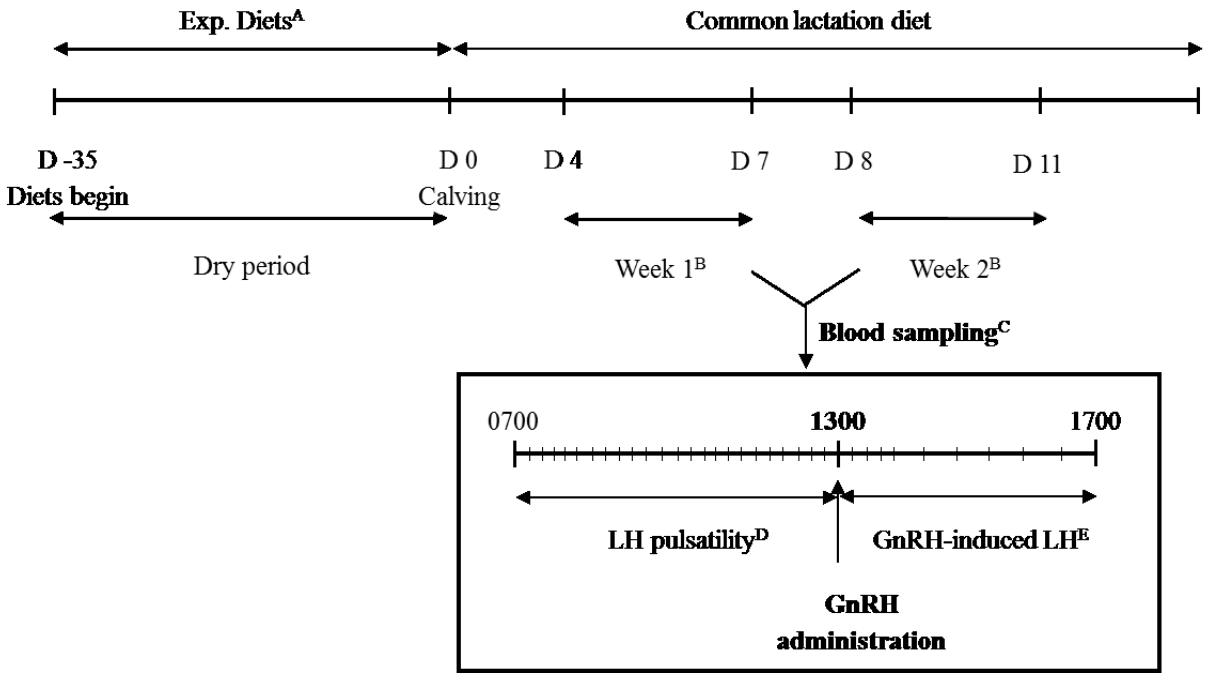

Figure 3.1. Experimental design and blood sample collection timelines.

A. During dry period cows were fed diets supplemented with rolled canola (8 %), or sunflower (8 %) or control (no oilseed). B. During first and second week after calving, blood sample collection was performed to evaluate metabolites, LH pulsatility and GnRH-induced LH release. Cows sampled during first week (n = 5 cows per treatment) for LH pulsatility and GnRH-induced LH were not used in second week sampling (n = 5 or 6 cows per treatment). Blood samples for metabolites were taken from all cows during both weeks. C. Blood sampling design to evaluate LH pulsatility and GnRH-induced LH during first and second week postpartum. D. Blood was sampled every 15 min for 6 h to assess LH pulsatility. E. Blood samples were taken for 4 h, at 15 min intervals during first hour and then at 30 min intervals to evaluate GnRH-induced LH.

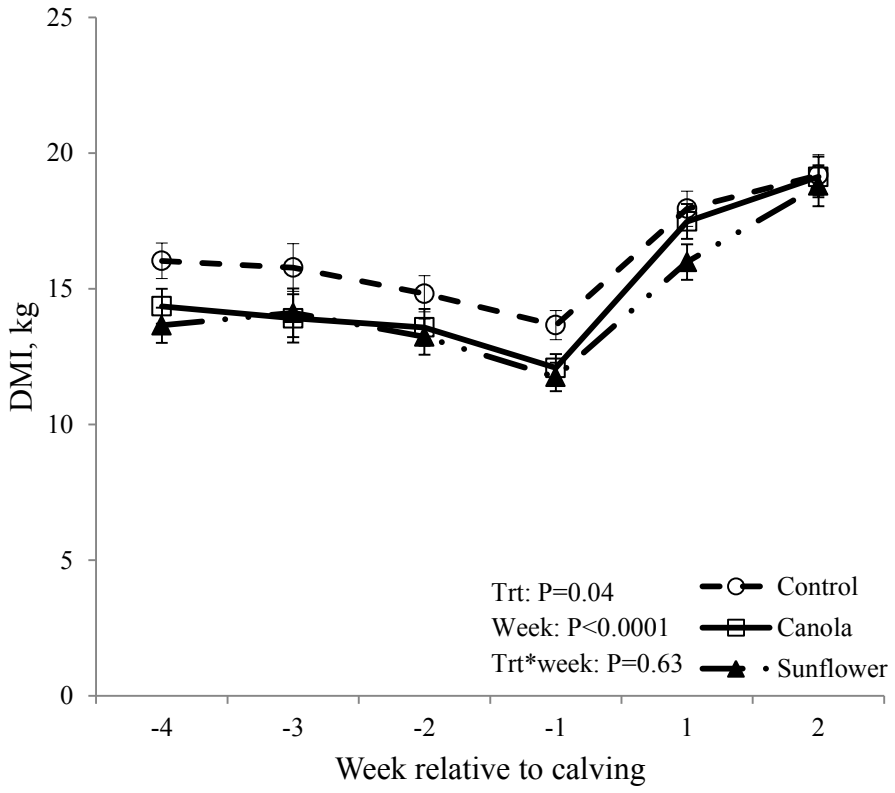

Figure 3.2. Effects of prepartum dietary treatments on dry matter intake (DMI).

Thirty-one cows (n = 10 or 11 per treatment) were fed experimental diets during the last five weeks of gestation. Cows fed control diet consumed more feed than those in sunflower and also tended to consume more feed than those in canola during entire experimental period.

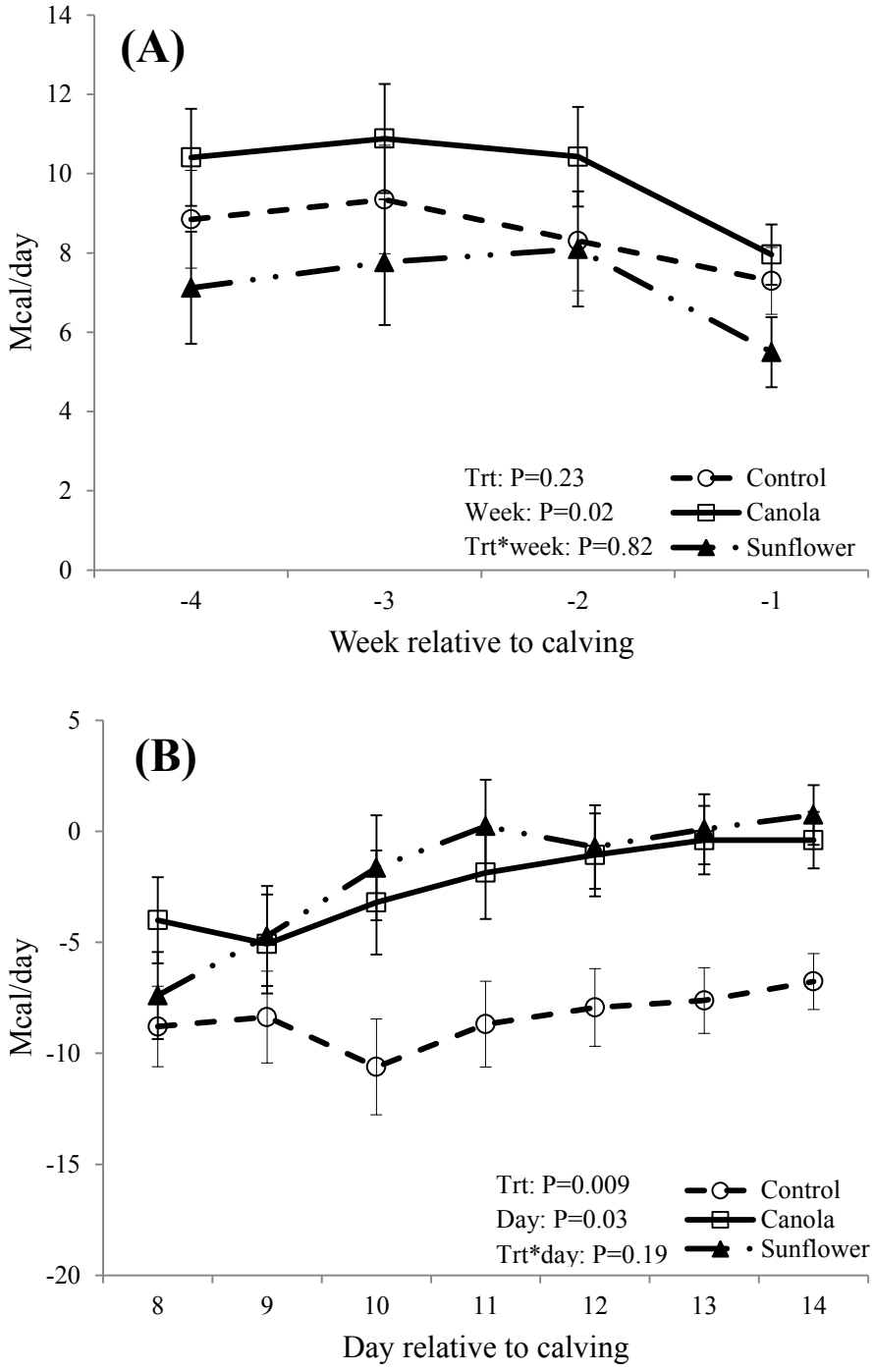

Figure 3.3. Effect of prepartum diets on energy balance (Mcal/day) during last 4 weeks of gestation and second week postpartum.

Thirty-one cows (n = 10 or 11 per treatment) were fed experimental diets during the last five weeks of gestation. Prepartum diets did not influence the energy balance during last 4 weeks of

gestation (A). The average of energy balance during last 4 weeks of gestation was 9.92 ± 1.05, 8.44 ± 1.08 and 7.11 ± 1.22 Mcal/day in canola, control and sunflower, respectively. Regardless of treatment effect, energy balance during last week of gestation was significantly reduced compared to wk–4, –3, and –2. Prepartum diets significantly affected postpartum energy balance during the second week (B). Cows fed control diet had a more pronounced negative energy balance than those fed diets supplemented with canola or sunflower. Energy balance was significantly higher on Day 14 than on Days 11, 10, 9 and 8.

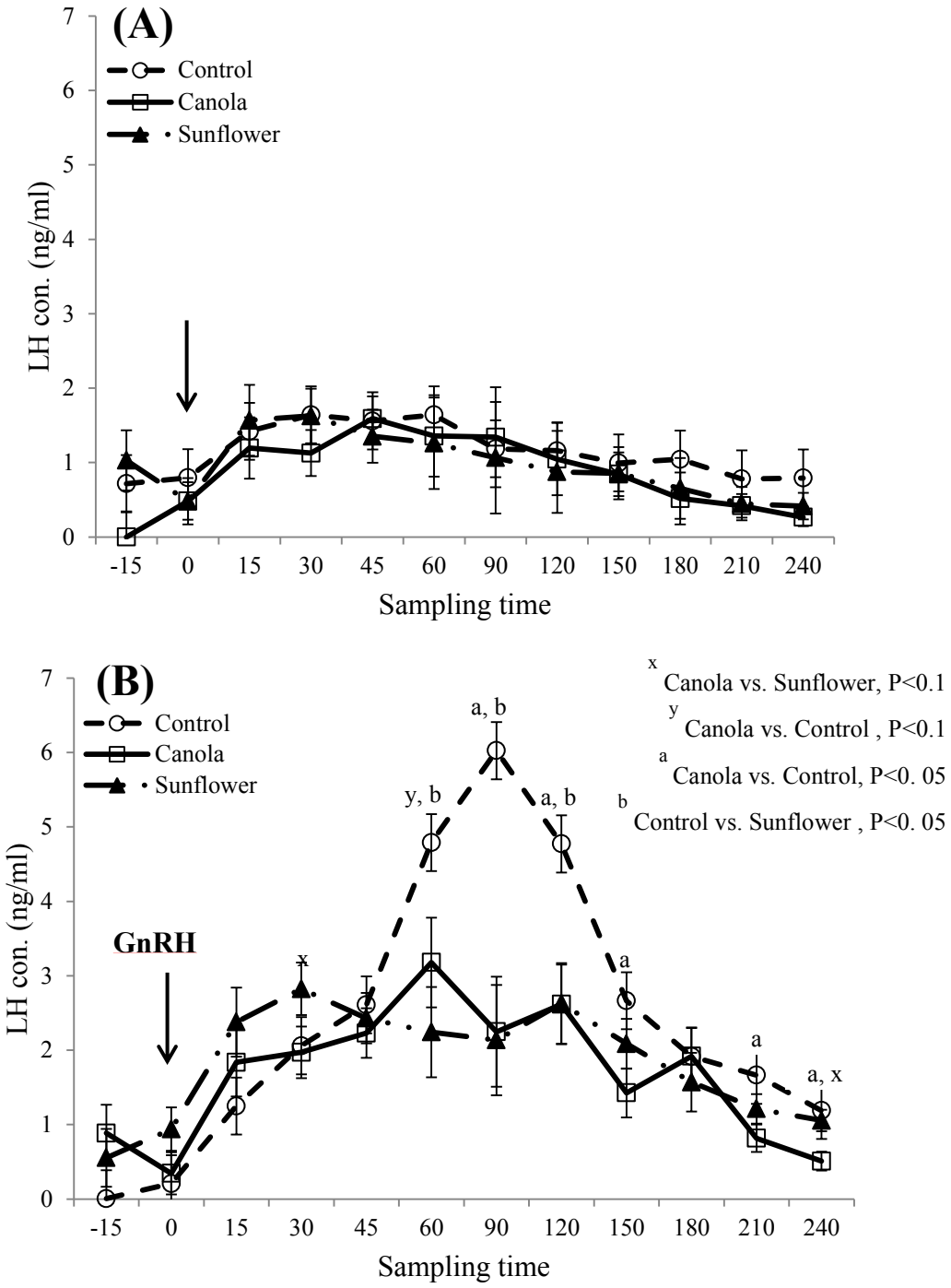

Figure 3.4. Effect of prepartum diets on GnRH-induced LH release during first and second week postpartum.

Cows were fed experimental diets during the last five weeks of gestation and assigned for GnRH-induced LH measurement in postpartum wk1 (n = 5 per treatment) or wk2 (n = 5 or 6 per

treatment). All cows responded to the GnRH treatment and had higher ($P < 0.05$) LH concentrations from 15 to 60 min post-treatment. However, prepartum dietary treatments did not affect GnRH-induced LH release pattern during wk1 postpartum (A). Prepartum diets significantly influenced GnRH-induced LH concentrations during wk2 postpartum (B). All cows responded to GnRH treatment, and LH concentrations remained elevated ($P < 0.05$) for up to 240 min post-treatment. Cows fed control (no oilseed) diet prepartum released more LH at 60, 90 and 120 min after GnRH administration than those fed sunflower or canola during wk2. The treatment by sampling time by week interaction was significant ($P < 0.03$)

Chapter 4. Effects of prepartum diets supplemented with rolled oilseeds on calf birth weight, postpartum health, feed intake, milk yield and reproductive performance of dairy cows

4.1. Abstract

The objectives were to determine the effects of supplemental fat (no-oilseed vs. oilseed) during late gestation and the source of fat (canola vs. sunflower seed), on dry matter intake (DMI), plasma metabolite concentrations, milk production and composition, calf birth weight, postpartum health disorders, ovarian function and reproductive performance in dairy cows. Pregnant Holstein cows, blocked by body condition and parity, were assigned to 1 of 3 diets containing rolled canola seed (high in oleic acid; n = 43) or sunflower (high in linoleic acid; n = 45) at 8% of dry matter, or no-oilseed (control; n = 43), for the last 35 ± 2 d of pregnancy. After calving, all cows received a common lactation diet. Blood samples were collected at wk -3 (i.e., 2 wk after initiation of prepartum diets) and at wks +1, +2, +3, +4 and +5 postpartum to determine the concentration of fatty acids (mEq/dL), β-hydroxybutyrate (BHB; mg/dL) and glucose (mg/dL). Ovarian ultrasonography was performed twice weekly to determine the first appearance of dominant (10 mm) and preovulatory-size (≥16 mm) follicles, and ovulation. Uterine inflammatory status based on the proportion of polymorphonuclear leukocytes (PMNL; subclinical endometritis: > 8% PMNL) was assessed at d 25 ± 1 postpartum. Significant parity by treatment interactions were observed for DMI and milk yield. Prepartum oilseed supplementation, more specifically sunflower seed, increased postpartum DMI in primiparous cows without affecting prepartum DMI and milk yield. Contrarily, in multiparous cows, prepartum oilseed supplementation decreased both prepartum and postpartum DMI, and milk yield during the first 2 wk. Regardless of parity, prepartum feeding of canola reduced postpartum

DMI compared to those fed sunflower. Mean fatty acids concentrations at wk -3 were greater in cows given supplemental oilseed than those fed no oilseeds. Gestation length and calf birth weight were increased in cows given supplemental oilseed prepartum compared to cows fed no oilseed, and a disproportionate increase in the birth weight of female calves was evident in cows fed oilseed. Total reproductive disorders tended to be greater in cows fed supplemental oilseed than those fed no-oilseed (42 vs. 23%). Furthermore, cows fed sunflower seed had a greater incidence of dystocia (35 vs. 18%) and total health disorders (52 vs. 32%) than those fed canola seed. Added oilseed and type of oilseed did not affect uterine inflammation at 25 ± 1 d postpartum. Oilseed supplementation neither altered the intervals from calving to establishment of the first dominant follicle, preovulatory-size follicle, and ovulation, nor fertility (conception rate to first AI and proportion of pregnant cows by 150 d after calving). In summary, prepartum oilseed supplementation (6.2 to 7.4% ether extracted, % of dietary DM) decreased DMI during entire experimental period (pre- and postpartum) and decreased milk yield during early lactation in multiparous cows, increased calf birth weight and postpartum health disorders, with no significant improvement in ovarian function and reproductive performance.

Key words: prepartum diets, oilseeds, calf birth weight, reproductive performance

4.2. Introduction

Plasma fatty acids concentrations usually increase early postpartum due to mobilization of adipose tissue as high-producing cows often cannot consume enough energy to meet requirements for milk production and maintenance (Bell 1995, Drackley 1999). An elevated fatty acids concentration in the periparturient period is the major factor contributing to postpartum accumulation of lipids in the liver (Bell 1981). Cow performance is negatively affected when

lipid accumulation in the liver is excessive (Herdt 1988). Therefore, management strategies that minimize adipose tissue mobilization postpartum may be beneficial to improve cow performance. Whereas postpartum fat supplementation is a common practice in the dairy industry (reviewed by (Santos *et al.* 2008)), prepartum fat supplementation is less common. Although plasma fatty acids concentrations increase when fat is supplemented to the diet of lactating dairy cows (Grummer & Carroll 1991), this response is inconsistent. Grum *et al.* (1996) reported that feeding fat (6.5% of dietary DM; Qual-Fat® mainly high in palmitic and oleic acid) to dairy cows during the dry period (from 60 up to 7 d before expected calving date) essentially abolished accumulation of triacylglycerol in the liver during the postpartum period immediately after calving. Drackley (1999) has proposed that alterations in liver metabolism through fatty acid supplementation might adapt the ruminant liver to contend with the large peripartal increases in blood fatty acids and minimize the risk for the development of fatty liver. Importantly, the type of fatty acid supplements may affect plasma concentrations of fatty acids. Cows fed linseed oil (high in linolenic acid; 1.35% of dietary DM prepartum and 1.5% of dietary DM postpartum) had greater plasma concentrations of fatty acids at 2 and 5 wk postpartum and greater hepatic concentrations of fat at 2 wk postpartum compared with cows fed a high linoleic acid fat formulated at the same fatty acid concentrations (Amaral 2008). These results collectively indicate that, in dairy cattle, the effect of prepartum fat supplementation on plasma metabolite concentration during calving transition period is not consistent.

In a previous study, Colazo *et al.* (2009) reported that dry cows fed diets supplemented with linola (high in linoleic acid) or flax seed (high in linolenic acid) had a numerically lower incidence of ovarian cysts and ovulated sooner after calving compared to those cows fed diets supplemented with canola (high in oleic acid). Prepartum dietary treatments did not affect calf

birth weight, postpartum health disorders, energy balance or fertility (Colazo *et al.* 2009); however, a control diet without supplemental fat was not included in that study. As canola seed is a common ingredient in dairy cattle rations, we wanted to confirm our previous observations and further investigate the effect of dietary canola seed on reproductive function. We hypothesized that cows given a prepartum diet supplemented with canola seed will have a longer interval from calving to first ovulation, as observed previously, compared to those fed diets supplemented with no oilseed or sunflower seed. Our objectives were to determine the effects of supplemental fat (no oilseed vs. oilseed) during late gestation and the source of fat (canola vs. sunflower seed), on DMI, plasma metabolite concentrations, milk production and composition, calf birth weight, postpartum health disorders, ovarian function and reproductive performance in dairy cows.

4.3. Materials and methods

4.3.1. Animals and diets

One-hundred-thirty one dry pregnant Holstein cows, parity 1 to 5 (46 primiparous and 85 multiparous), were used in the study. Thirty-five days (35 ± 2 d) before the expected calving date (wk -5), cows were blocked by Body condition score (BCS) and parity, and assigned to 1 of 3 dietary treatments [sunflower seed (high in linoleic acid), canola seed (high in oleic acid) or no-oilseed (control)], with rolled oilseeds added at 8% on a DM basis. Oilseeds were rolled as described previously (Ambrose *et al.* 2006) before incorporation in the diet. Diets were offered ad libitum as a TMR containing barley silage, alfalfa hay, and concentrates. Upon calving, cows were placed on a common lactation diet. The ingredients and nutrient composition of both pre and postpartum diets are presented in **Table 4.1**. The crude fat content and fatty acid profile of

oilseeds are presented in **Table 4.2**. The study was conducted at the Dairy Research Unit of the University of Alberta, with all animal experimental procedures approved by the University of Alberta's Animal Care and Use Committee for Livestock (Protocol # AUP00000131), and animals were cared for according to the Canadian Council of Animal Care Guidelines (Olfert *et al.* 1993).

Cows were housed individually in tie-stalls during pre and postpartum periods, fed once daily at 0800 h and had unrestricted access to water. Postpartum cows were allowed 1 to 2 h of exercise on week days, and milked twice daily in their stalls between 0400 and 0600 h (AM) and between 1530 and 1730 h (PM). Milk production was automatically recorded at each milking. Feed intake was recorded daily and weekly feed samples were taken from forages and concentrates to determine feed DM and diet composition (Sun & Oba 2014). Ratios of feed ingredients (as-fed basis) for rations were adjusted weekly based on each feed's DM values. Body condition scoring was performed on all cows at wk -5 to block the cows for treatment assignment. Body condition score (BCS) and body weight (BW) also were recorded for a subset of cows (n = 22 cows per treatment including 12 cows per treatment used for milk and blood sampling) at wk 0 (calving time) and wk +5. The same technician assigned BCS to each cow on a scale of 1 (emaciated) to 5 (overconditioned) (Edmonson *et al.* 1989).

Cow health status was monitored and recorded daily. Dystocia was defined as calving with assistance (easy pull), hard pull or surgery (Sewalem *et al.* 2008). Retained fetal membrane was defined as the lack of detachment of fetal membranes within 24 h after calving (Roberts 1986). Reproductive tracts of all cows were palpated per rectum at day 14 ± 2 postpartum for diagnosis of metritis. Presence of watery, fetid vaginal discharge of uterine origin with elevated rectal temperature (> 39.5°C) was diagnosed as puerperal metritis (Sheldon *et al.* 2006). An

endometrial cytological sample was obtained on d 25 ± 1 postpartum from 95 cows using a cytobrush (Medscand Medical, Malmö, Sweden) as described by Dourey et al. (Dourey *et al.* 2011) to evaluate uterine inflammatory status. The cows with more than 8% PMNL in the cytological sample were defined as having subclinical endometritis and those with less than or equal to 8% PMNL were defined as having a healthy uterus. Mastitis was defined as inflammation of the mammary gland with heat, pain, and induration detectable by manual palpation and confirmed by the California Mastitis Test (Schalm & Noorlander 1957). Clinical ketosis was determined when animals had decreased appetite with evidence of elevated urine ketones (> 2.0 mmol/L; Ketostix® reagent strips, Bayer Inc., Mississauga, ON, Canada) after exclusion of other clinical disease.

4.3.2. Milk and blood sample collection

Based on previous publications and a priori power analysis for sample size determination, we obtained milk and blood samples from a subset of 12 cows, assigned randomly, per treatment. Samples for milk composition were obtained from 6 consecutive milking sessions (Tuesday P.M. to Friday A.M.) during wk +2 and +5 postpartum.

Blood samples were collected 2 wk after initiation of prepartum diets (wk -3), first (wk +1), second (wk +2), third (wk +3), fourth (wk +4) and fifth (wk +5) wk postpartum. Samples were collected at 8 h intervals over a 16-h period (2200, 0600 and 1400 h) in heparinized tubes (Vacutainer®, Beckton Dickinson and Co., Franklin Lakes, NJ), centrifuged, and plasma harvested. Plasma samples from 2200 and 0600 h were kept at 4°C until last sample collection at 1400 h. A pooled sample was prepared by mixing equal quantities of plasma from the 3

consecutive collections and stored at -20^{o}C until analyzed for fatty acid profile (wk -3 and +1), fatty acids, BHB and glucose.

4.3.3. Reproductive management

The ovaries and uterus of 95 cows (sunflower: n = 33, canola: n = 31 and control: n = 31) were examined by transrectal ultrasonography (MicroMAXX, color Doppler scanner equipped with a multifrequency 5 to 10 MHz linear transducer, SonoSite Inc., Bothell, WA, USA), twice weekly, from 7 ± 1 until 35 ± 1 d after calving. We recorded the intervals from calving to first appearance of a 10 mm follicle (referred to as dominant follicle in this manuscript), and that of a 16 mm follicle (referred to as preovulatory-size follicle in this manuscript). Ovulation was confirmed by the absence of a dominant (diameter ≥ 10mm) follicle that had been detected at the previous examination, and subsequent CL formation (Colazo *et al.* 2009). Anovulatory follicles ≥ 25 mm in diameter that persisted for at least 10 d in the absence of a CL were defined as cysts (Garverick 1997). Cows were subjected to a Presynch/Ovsynch protocol and timed-AI (**TAI**; (Moreira *et al.* 2001)) at 75 ± 3 d postpartum. Cows were inseminated by one technician with frozen-thawed semen, and pregnancy diagnosis performed by ultrasonography 32 d after TAI.

4.3.4. Plasma fatty acid profile and metabolites

Plasma fatty acids (NEFA-C kit, Wako Chemicals USA Inc., Richmond, VA), BHB (Roche Diagnostics, Indianapolis, IN, USA) and glucose (P7119, Sigma-Aldrich, St. Louis, MO) were determined using commercially available kits in triplicate. The intra- and inter-assay coefficients of variation for fatty acids, BHB and glucose were 1.49 and 3.32, 2.51 and 6.71, and 1.38 and 2.42%, respectively. Fatty acids in plasma samples were extracted as described by

Folch et al. (Folch *et al.* 1957). The fatty acid profile of extracted lipids was assessed by gas chromatography as previously described by Cruz-Hernandez et al. (Cruz-Hernandez *et al.* 2007).

4.3.5. Milk composition

Milk samples were analyzed for fat, protein, lactose, and MUN by infrared spectroscopy (AOAC International, 2002; method 972.16; MilkoScan 605, Foss North America, Brampton, ON, Canada) at the Alberta Central Milk Testing Laboratory (Edmonton, AB, Canada).

4.3.6. Statistical analyses

The plasma concentrations of fatty acids, BHB, glucose, BCS, BW, plasma fatty acid profile, milk yield and composition, DMI, interval to dominant, preovulatory-size follicle, ovulation, gestation length and calf birth weight were analyzed using the MIXED procedure of SAS (version 9.3, 2011; SAS Institute Inc., Cary, NC). The repeated measures data (fatty acids, BHB and glucose) were analyzed for each week separately. Preplanned contrast analysis was used to compare means for oilseed vs. no-oilseed and sunflower vs. canola fed cows. The final statistical model included prepartum dietary treatment and parity as the main effects for metabolites, BCS, BW, gestation length, and intervals to dominant follicle, preovulatory-size follicle, and ovulation. The same approach was used for analyzing data of milk yield, milk composition, DMI and calf birth weight with interaction of dietary treatment by parity or calf sex. Calf sex was used as covariate for interval to dominant, preovulatory-size follicle, and ovulation. The interval from calving to first ovulation was also evaluated with survival analysis (PROC LIFETEST). Dietary treatment, time (d) and status (with the value 0 indicating censoring) were included in the model. Total reproductive disorders were defined as the sum of retained fetal membranes, metritis, vaginal discharge and ovarian cyst incidence. Total health

disorders were defined as the sum of retained fetal membranes, metritis, vaginal discharge, ovarian cyst, clinical ketosis, mastitis and dystocia. The statistical model used to evaluate the effect of prepartum dietary treatment by uterine inflammatory status interaction [healthy (≤ 8% PMNL) or subclinical endometritis (>8% PMNL)] on reproductive performance included prepartum dietary treatment, uterine inflammatory status and parity as the main effects and prepartum dietary treatment by PMNL category as interaction. The proportion of cows that ovulated within 35 d after calving (yes or no), conceived (yes or no) at the first TAI and by 150, 250 days in milk (DIM) or had any type of disorders (dystocia, retained fetal membranes, metritis, vaginal discharge, subclinical endometritis, ovarian cyst, mastitis, clinical ketosis, total reproductive disorders or total health disorders) or death were analyzed using the PROC GENMOD procedure of SAS. Model specifications included a binomial distribution and logit link function and calf sex as covariate.

The Pearson's test was used to calculate the associations of pre- and postpartum DMI and PMNL percentage at 25 ± 1 DIM with intervals to formation a dominant follicle, preovulatory-size follicle and first ovulation (n = 95 cows in total). The association between prepartum DMI and calf birth weight was evaluated first in all cows (n = 131) regardless of parity and diet, then within prepartum dietary treatments (control, n = 43; canola, n =43; sunflower, n = 45) and finally within parity groups, primiparous (n = 46) or multiparous (n = 85) cows. All data are reported as mean ± SE with $P < 0.05$ considered significant, and $P > 0.05$ but < 0.10 considered trends.

4.4. Results

4.4.1. Body condition score, body weight and feed intake

Prepartum dietary supplementation of oilseed and type of oilseed did not affect BCS and BW at wk -5 (BW: 623.6 ± 18.38 kg, BCS: 3.33 ± 0.04), 0 (BW: 610.6 ± 22.88 kg, BCS: 3.49 ± 0.04) and wk +5 (BW: 556.3 ± 15.22 kg, BCS: 3.06 ± 0.05). Significant parity by treatment interactions were observed for DMI ($P < 0.05$), and results are shown separately for primiparous and multiparous cows (**Figure 4.1**). Oilseed supplementation during the last 5 wk of gestation did not affect prepartum DMI in primiparous cows vs. no-oilseed, but increased postpartum DMI from wk +2 to +5 (**Figure 4.1A**). More specifically, primiparous cows fed sunflower seed prepartum had the greatest postpartum DMI. Contrarily, prepartum oilseed supplementation decreased both pre and postpartum DMI in multiparous cows, and postpartum DMI reduction was greater in cows fed canola seed before calving (**Figure 4.1B**).

4.4.2. Plasma fatty acid profile and metabolites

In wk -3, C18:1 and C18:2 were the highest in cows fed diets supplemented with canola and sunflower seed, respectively. In wk +1, plasma C18:2 remained greater in cows fed sunflower seed prepartum compared to canola fed cows. Plasma concentration of C18:3 were also significantly increased in canola fed cows both during wk -3 and +1(**Table 4.3**).

The plasma concentration of fatty acids was greater ($P = 0.004$) at wk -3 in cows given supplemental oilseeds compared to those given no-oilseed (**Table 4.4**); additionally, cows fed canola had greater fatty acids concentration than those fed sunflower at wk +4. Prepartum dietary treatments did not affect concentrations of plasma BHB and glucose during pre and postpartum periods (**Table 4.4**).

4.4.3. Milk yield and composition

A significant parity by treatment interaction was observed for milk yield ($P < 0.05$); hence, results are shown separately for primiparous and multiparous cows (**Figure 4.2**). In primiparous cows, prepartum oilseed supplementation and type of supplemental oilseed did not affect milk yield compared to no-oilseed. In multiparous cows, milk yield was lower in prepartum oilseed fed cows during wk +1, +2 and +4 postpartum than those fed no-oilseed. There were no differences among treatments at wk +3 and +5.

Prepartum oilseed supplementation decreased milk protein yield (kg/d) in wks +2 and +5; a significant parity by treatment interaction was observed for milk protein production ($P < 0.05$) in wk +2 (**Table 4.5**). Although, prepartum oilseed supplementation did not affect milk protein yield in primiparous cows, it reduced milk protein yield in multiparous cows in wk +2 by 0.19 kg/d. Prepartum oilseed supplementation also reduced MUN (mg/dL) in wk +2 and tended to reduce milk fat production (kg/d) in wk +5. Additionally, prepartum oilseed supplementation increased lactose (%) in wk +5. Type of supplemental oilseed (canola vs sunflower) did not influence milk composition during wk +2 and +5.

4.4.4. Gestation length and calf birth weight

Prepartum oilseed supplementation increased gestation length (**Table 4.6**), but diet by parity ($P = 0.44$) or diet by fetal sex ($P = 0.32$) interactions on gestation length were not significant. Cows that were fed supplemental oilseed prepartum delivered heavier calves than those fed no supplemental oilseed (**Table 4.6**). Moreover, calf birth weight was significantly influenced ($P = 0.02$) by the interaction between dietary treatment and calf sex, with female calf birth weight being greater in cows that received supplemental oilseed during the prepartum period. More specifically, mean birth weight of female calves tended to be greater in cows that

received sunflower seed prepartum than in those that received canola. A diet by parity interaction did not influence ($P = 0.93$) calf birth weight.

4.4.5. Ovarian function and pregnancy

Prepartum dietary treatments affected neither the intervals from calving to formation of a dominant follicle, preovulatory-size follicle, and first ovulation, nor the proportion of cows that ovulated by 35 DIM (**Table 4.7**). Furthermore, there was no significant difference amongst dietary treatments in the interval from calving to first ovulation, based on survival analysis. Although, prepartum oilseed supplementation and type of supplemental oilseeds did not influence pregnancy at first TAI and the proportion of pregnant cows by 150 DIM, the proportion of pregnant cows by 250 DIM tended to be greater in oilseed supplemented groups (**Table 4.7**).

4.4.6. Postpartum health and reproductive disorders

The effects of prepartum diets on the incidence of early postpartum reproductive and health disorders are summarized in **Table 4.8**. The overall incidence of retained fetal membranes, metritis, vaginal discharge and ovarian cysts, collectively referred to as total reproductive disorders, tended to be greater in cows fed supplemental oilseed during the prepartum period, but there was no difference between canola and sunflower treatments . Cows fed sunflower seed had a significantly greater incidence of dystocia and total health disorders than those fed canola. Oilseed supplementation and type of oilseed added to the diet did not affect the proportion of cows with subclinical endometritis at 25 DIM (**Table 4.8**).

The interactions between diets and uterine health based on PMNL category on the intervals to the establishment of the first dominant follicle, formation of a preovulatory-size

follicle, and first ovulation are presented in **Table 4.9**. Postpartum reproductive performance was not influenced by uterine inflammatory status (healthy vs. subclinical endometritis) and prepartum dietary treatments.

4.4.7. Associations among DMI, PMNL, ovarian function and calf birth weight

The interval from calving to formation of first dominant follicle was positively correlated with PMNL percentage (r = 0.30, *P* = 0.003). The interval from calving to the establishment of a preovulatory-size follicle also was correlated PMNL percentage (r = 0.38, *P* = 0.002) and negatively correlated with mean postpartum DMI (r = -0.29, *P* = 0.004). However, none of factors evaluated during pre and postpartum periods was associated with the interval to first ovulation. Moreover, the interval from calving to first ovulation had a positive association with that of dominant follicle (r = 0.23, *P* = 0.04) and preovulatory-size follicle (r = 0.45, *P* = <.0001). Prepartum DMI was associated (r = 0.20; P = 0.02) with calf birth weight when all cows were considered regardless of parity and dietary treatment. When analyzed by dietary treatment, prepartum DMI influenced calf birth weight in control (r = 0.40; P = 0.01) and canola (r = 0.33; P = 0.03) fed cows but not in cows fed sunflower seed (r = 0.07; P = 0.62). When analyzed by parity, prepartum DMI and calf birth weight were not associated; primiparous (r = -0.06; P = 0.68) vs multiparous cows (r = -0.18; P = 0.11).

4.5. Discussion

4.5.1. Calf birth weight, postpartum health and reproductive performance

In previous work from our laboratory, Colazo *et al.* (2009) found that the type of oilseed supplemented during the prepartum period influenced the interval from calving to first ovulation

postpartum. Cows fed diets supplemented with either linola or flax seed had a shorter interval from calving to the first ovulation than those fed a diet supplemented with canola seed. However, prepartum dietary treatment did not affect the diameter of the largest follicle at 7 d postpartum (Colazo *et al.* 2009). In the present study, prepartum oilseed supplementation and type of oilseed did not affect the intervals from calving to formation a dominant follicle, to that of a preovulatory-size follicle, and ovulation, or the proportion of cows that ovulated by 35 d postpartum. Due to the scarcity of reports on the effects of prepartum dietary fatty acids on interval from calving to first ovulation, we compared our results with some studies that fed dietary fatty acids during both pre and postpartum periods. Juchem et al. (Juchem *et al.* 2010) fed one of two diets consisting of calcium salt (2% of DM) of either palm oil (high in palmitic acid, n = 246) or transoctadecenoic acid (n = 255) from 25 d prepartum to 80 d of lactation. Ovaries were examined weekly by ultrasonography until the sixth week of lactation to determine the first postpartum ovulation based on the presence of a CL. In another study (Caldari-Torres *et al.* 2011), cows were fed diets containing fat supplements enriched in saturated fatty acids (1.5% of DM, n = 10), calcium salts of transoctadecenoic fatty acids (1.8% of DM, n = 10) or calcium salts of safflower oil fatty acids (high in linoleic acid, 1.8% of DM, n = 9). Diets were fed from 4 wk before until 7 wk after calving and ovulation was determined by elevated (> 1 ng/mL) plasma progesterone concentrations for 2 sampling days. In both studies, dietary treatments did not affect the interval from calving to first ovulation. The mean interval to first ovulation in the current study, regardless of dietary treatments, was shorter than what has been reported in previous studies: 31 d (Juchem *et al.* 2010), 33 d (Caldari-Torres *et al.* 2011), 32 d (Gumen *et al.* 2005) and 35 d (Ambrose *et al.* 2007). Differences in the physiological status of animals and environmental influence associated with management practices could be an explanation for

discrepancies among experiments. Regardless of dietary treatment, in the current study, the overall interval to ovulation was 21.4 ± 1.5 d, which is similar to that reported by Colazo et al. (Colazo *et al.* 2009) for cows fed prepartum diets supplemented with flax (21.0 ± 3.1 d) or linola (23.7 ± 3.2 d). However, in the study by Colazo et al. (Colazo *et al.* 2009) the interval from calving to first ovulation in canola fed cows was 34.7 ± 3.1 d, which is considerably longer than the 22.9±1.5 d observed in our study. Notably, the study by Colazo et al. (Colazo *et al.* 2009) and the current study were both performed in the same herd with no substantial differences in herd management practices in the years between the two study periods. One reason for discrepancies in the intervals to ovulation between the two experiments could be the greater incidence of ovarian follicular cyst in canola treatment in the previous study (Colazo *et al.* 2009) compared to our current study (25 vs. 14%, respectively).

Sartori et al. (Sartori *et al.* 2013) reported that DMI (energy intake) can alter ovarian physiology and hormone concentrations. They found that cows with greater DMI had larger ovulatory follicles and CL volume than those with lower DMI (Sartori *et al.* 2013). In the current study, there was no association between DMI and interval from calving to first dominant follicle, but postpartum DMI was correlated with the interval to preovulatory-size follicle; the greater the postpartum DMI, the shorter the interval from calving to preovulatory-size follicle. However, there was no association between pre and postpartum DMI with the interval to first ovulation.

One of the factors affecting resumption of ovulation is postpartum health (Beam & Butler 1997, Opsomer *et al.* 2000, Wathes *et al.* 2007a). The most common risk factor associated with dystocia in dairy cattle is oversized calves (Mee 2012). There is an association between calf birth weight and dystocia, which is dependent on pelvic area, breed, parity and dystocia case definition (Mee 2012). For Holstein cows the range is between 42 and 45 kg of birth weight,

above which dystocia rate increases significantly (Menissier & Foulley 1979). Although mean calf birth weight for cows fed no-oilseed was lower than the threshold reported by Menissier and Foulley (Menissier & Foulley 1979), mean calf birth weights in cows fed canola and sunflower were in the middle and the highest level of the range, respectively. Additionally, in the present study, there was an interaction between dietary treatment and calf sex, with oilseed fed cows, particularly those fed sunflower seed delivering heavier female calves than those fed control diet. It has been shown that male calves are approximately 9% heavier than female calves (Kertz *et al.* 1997). Johanson and Berger (Johanson & Berger 2003) indicated that the majority of the increase in dystocia rate associated with male calves is attributable to their greater body weight. Whereas male calves in control and canola treatments were 8.17 and 6.76% heavier than female calves, male calves in sunflower treatment were marginally lighter (-0.66%) than female calves in the same treatment group. Therefore, we could conclude that the increase of dystocia incidence in sunflower-fed cows resulted from both a greater male-to-female ratio and an increased female calf birth weight, which may have consequently increased the total health disorders during the postpartum period. Although the increased birth weight of calves was associated with prepartum DMI when all cows were considered regardless of dietary treatments, a similar association was found in the control- and canola-fed cows when treatment groups were considered separately. However, prepartum DMI and calf birth weight were not associated in sunflower-fed cows. In view of the latter finding, it is rather intriguing how dietary inclusion of sunflower seed differentially affected birth weight in a sex-specific manner, favoring weight gain in female fetuses. As neither prepartum energy intake nor energy balance differed among the oilseed groups, we speculate that the high linoleic acid content in the sunflower seed may have played a role in the increased birth weight of female calves. In rodents, estrogen plays a key role in fatty

acid unsaturation (Ostwald *et al.* 1966) allowing somatic tissues of female rats to accumulate greater quantities of unsaturated fatty acids than that of male rats (Pudelkewicz *et al.* 1968) without any differences in body weight. It is not clear whether such reports exist for ruminants or whether a preferential accumulation of unsaturated fatty acids in female fetuses, if provable, could cause increased body weight. Nevertheless, future studies in cattle should investigate if our finding of a disproportionate increase in female calf birth weight in cows fed sunflower seed is repeatable.

Calf birth weight is affected by gestation length, which in turn is influenced by parity, fetal sex, maternal nutrition, sire (Mee 2012) and heat stress (Tao & Dahl 2013). It is noteworthy that short and long gestation lengths, which are associated with increased risk of dystocia, were defined as < 265 d or > 285 d, respectively (Philipsson 1976). Regardless of dietary treatment, the average gestation length in the present study was 275.3 d, which is well within the normal gestation length reported by Norman et al. (Norman *et al.* 2009). Moreover, gestation length was not affected by either parity or calf sex. A diet high in linoleic acid (e.g. a diet supplemented with sunflower) is generally thought to be associated with an increased risk of preterm delivery (Wathes *et al.* 2007a). In this regard, sheep fed a diet rich in linoleic acid during late gestation had greater circulating concentrations of 2-series prostaglandins, produced more prostaglandins in uterine and placental tissues, and appeared to be more susceptible to preterm parturition (Elmes *et al.* 2004, Cheng *et al.* 2005). Similar observations have been reported in women, where those who delivered prematurely had greater n-6 (i.e linolenic acid) polyunsaturated fatty acid concentrations in erythrocytes (Reece *et al.* 1996, Reece *et al.* 1997). In dairy cows, feeding a diet supplemented with safflower oil (high in linoleic acid) for 30 d before expected date of calving did not affect gestation length compared to those fed palm oil (Silvestre *et al.* 2011b).

However, our results contradict previous findings in cows, ewes and women. Although the underlying mechanisms are not known, oilseed supplementation increased the gestation length by 3 to 4 days in our study. As we had no control over sire selection, semen from 39 different sires were randomly used in current study negating the possibility for any meaningful statistical evaluation of sire effect on gestation length. Fetal growth rate averages 0.3-0.4 kg/d during the last month of gestation (Meijering 1984). Thus, it could be expected that a 3 to 4 d longer gestation length would result in approximately 0.9-1.6 kg increase in calf birth weight, whereas, feeding sunflower increased birth weight by 5 kg in female calves. Therefore, it could be speculated that calf birth weight was mainly affected by prepartum maternal nutrition (oilseed supplementation) rather than gestation length. We infer that feeding oilseeds, particularly sunflower seed, during the prepartum period increased the incidence of dystocia, most likely through increased calf birth weight, and subsequently greater health disorders than in cows fed no-oilseed diet.

Juchem et al. (Juchem *et al.* 2010) reported that feeding calcium salts of linoleic and transoctadecenoic acids from 25 d pre to 80 d postpartum enhanced fertility though reduced the incidence of puerperal metritis than those fed palm oil. Feeding a diet supplemented with safflower oil during transition period induced a proinflammatory state during first week postpartum evidenced by the increased neutrophil expression of adhesion molecules, production of cytokines, enhanced bactericidal activity, and increased circulating acute phase proteins compared to palm oil (Silvestre *et al.* 2011a). However, in the present study, prepartum oilseed and type of oilseeds supplementation did not affect the proportion of cows with subclinical endometritis. Our results are in agreement with the findings of Amaral (Amaral 2008) wherein cows were fed a diet supplemented with calcium salts of safflower oil, a mix of palm and fish oil,

or a control diet (no fat supplement) from 34 d pre until 49 d post calving. The concentration of neutrophils in the uterine flushing, neutrophil as a proportion of total cells or as a proportion of white blood cells also did not differ among treatments at 37 ± 3 d postpartum (Amaral 2008). Current results on the association between PMNL percentage and interval from calving to the establishment of dominant and preovulatory-size follicles partially support those reported by Dourey et al. (Dourey *et al.* 2011). They found that cows with > 8% PMNL on d 25 had a longer interval from calving to first ovulation (45 vs. 32 days).

Prepartum oilseed supplementation and type of supplemental oilseeds did not affect pregnancy at first AI and the proportion of pregnant cows by 150 DIM in the current study, but oilseed supplementation tended to improve the proportion of pregnant cows by 250 DIM. Similarly, feeding of oilseed (Colazo *et al.* 2009) or n-3 fatty acid (Optomega, Optivite Co., Worksop, UK; (Badiei *et al.* 2014)) prepartum did not influence postpartum reproductive performance. However, in studies where feeding dietary fatty acids prepartum was continued to either 80 (transoctadecenoic fatty acids; (Juchem *et al.* 2010)) or even further to 160 d postpartum (fish oil; (Silvestre *et al.* 2011b)), pregnancy rate at first or second AI, respectively, was improved. Thus, it seems that benefits to altering the fatty acid profile in the diet of dairy cows may depend on both the length of the dietary period during which they consume the respective fatty acids and the type of fat supplemented. The moderate increase in the proportion of pregnant cows by 250 DIM observed in the present study might not be related to prepartum oilseed supplementation.

4.5.2. Feed intake, plasma metabolites, milk yield and composition

In the current study, oilseed supplementation had different effects on the DMI of primiparous and multiparous cows. In primiparous cows, prepartum oilseed supplementation did not affect prepartum DMI compared with control, but increased postpartum DMI. In multiparous cows, prepartum oilseed supplementation decreased both prepartum and postpartum DMI. Primiparous and multiparous cows used in the current study had moderate BCS (3.36 ± 0.14 and 3.29 ± 0.21, respectively; mean ± SD), and it is noteworthy that high fat diets decreased DMI of multiparous cows that were not over-conditioned. Our DMI results are contrary to that of Garcia et al. (Garcia *et al.* 2014) who fed cows three diets during last 8 wk of gestation: no fat supplementation (Control), enriched in saturated fatty acids (mainly stearic acid, 1.7% of DM; Energy Booster 100, Milk Specialties, Dundee, IL), or enriched in essential fatty acids (1.7% of DM; Megalac R, Church and Dwight, Princeton, NJ). They found that neither prepartum fat supplementation nor the type of fatty acid fed affected mean DMI during prepartum period, although cows fed essential fatty acids consumed 0.9 kg/d less DM compared with those fed diet enriched in saturated fatty acid. Colazo et al. (Colazo *et al.* 2009) also found feeding different types of oilseeds did not affect pre and postpartum DMI. However, in both studies (Colazo *et al.* 2009, Garcia *et al.* 2014), the interaction of treatment by parity for DMI was not considered. Therefore, we conclude that DMI responses to prepartum fat supplementation were affected by parity.

Firkins and Eastridge (Firkins & Eastridge 1994) evaluated treatment means from 11 experiments and reported that DMI decreased as degree of unsaturation of long chain fatty acid increased. In the present study, the type of supplemental oilseed (canola vs. sunflower) did not affect prepartum DMI, but cows fed canola prepartum had lower DMI during postpartum period than those fed sunflower, regardless of parity. However, the type of supplemental oilseed (canola

vs. sunflower) did not affect fatty acids concentration during pre and postpartum periods. Canola seed contained more oleic and linolenic acids and less linoleic acid than sunflower seed, which were reflected in plasma fatty acid profile. In a previous *in vitro* hepatocyte metabolism study, Mashek et al. (Mashek *et al.* 2002) found that palmitic acid metabolism was greater when incubated with oleic or linolenic acid compared with linoleic acid, increasing oxidative metabolism and triglyceride synthesis in hepatocytes. Therefore, in the current study, feeding canola seed prepartum might have increased triglyceride accumulation in the liver, which is known to decrease postpartum DMI (Grummer 1993). Moreover, linolenic acid can activate peroxisome proliferator-activated receptors, and induce hepatic fatty acid oxidation (Jump *et al.* 2005). Feeding canola seed, prepartum, which contained more linolenic acid than sunflower seed, might have enhanced fatty acid oxidation to a greater extent in the liver after calving, and greater hepatic oxidation may lead to lower postpartum DMI (Allen *et al.* 2009). However, a specific mechanism cannot be identified from the data obtained from the current study warranting further investigation.

Grum et al. (Grum *et al.* 1996) showed that prepartum fat supplementation increased fatty acids without affecting BHB and glucose. Similarly, in our study, prepartum dietary treatments did not affect plasma BHB and glucose concentration during pre and postpartum periods. The data of Grummer and Carroll (Grummer & Carroll 1991) indicated that the effects of fat supplementation on plasma BHB were inconsistent. The elevation in plasma BHB concentrations in response to fat supplementation seems to depend on the metabolic status of the cow. During the transition period when plasma fatty acids concentrations increase, there is a greater probability of a coincidental increase in plasma BHB concentrations than later in lactation (Grummer & Carroll 1991).

Previous studies have shown inconsistent results of milk production and composition in response to fat supplementation. Douglas et al. (Douglas *et al.* 2004) and Ballou et al. (Ballou *et al.* 2009) indicated that feeding multiparous cows a diet supplemented with fat during prepartum period did not affect milk yield and composition. However, prepartum oilseed supplementation reduced milk yield in multiparous cows during early postpartum and milk protein yield in wk +2 in the present study. It also increased lactose (%) and tended to decrease milk fat yield in wk +5. Duske et al. (Duske *et al.* 2009) reported that feeding cows diets supplemented with fat prepartum decreased milk yield and lactose (%), but increased fat (%) during first 4 wk postpartum. A recent meta-analysis and meta-regression by Rabiee et al. (Rabiee *et al.* 2012) indicated that feeding oilseeds have no effect on milk yield, whereas, reduced milk fat (%) and protein (%). Additionally, they (Rabiee *et al.* 2012) indicated that milk yield is positively correlated with DMI. We infer that feeding high fat diets during prepartum period reduced DMI resulting in reduced milk yield in multiparous cows.

4.6. Conclusions

Oilseed supplementation during the prepartum period reduced DMI and milk yield in multiparous cows, and increased female calf birth weight. Dystocia and total health disorders were increased in cows fed supplemental sunflower seed prepartum than in those fed canola. However, neither prepartum oilseed supplementation nor the type of oilseed affected pregnancy at first service or the proportion of pregnant cows by 150 DIM. Although our earlier finding of a longer interval from calving to first ovulation in cows fed canola in the prepartum period was a basis for the present study, we were unable to find any evidence of prepartum diets influencing postpartum ovarian function through alterations in the interval from calving to first ovulation.

4.7. Acknowledgments

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Table 4.1. Ingredients and nutrient composition of pre and postpartum diets

	Prepartum dietary treatment			Postpartum diet
	Control	Canola seed	Sunflower seed	
Ingredients, %DM				
Barley silage	60.0	60.0	60.0	24.1
Alfalfa silage	-	-	-	20.9
Alfalfa hay	10.0	10.0	10.0	7.0
Ground barley grain	10.0	10.0	10.0	7.1
Barley, rolled	-	-	-	12.0
Corn, rolled	-	-	-	7.2
Wheat DDGS	-	-	-	4.8
Soybean hulls	10.0	6.2	3.4	-
Canola meal	5.0	0.8	3.6	7.4
Soybean meal	-	-	-	0.4
Canola seed, rolled	0.0	8.0	0.0	-
Sunflower seed, rolled	0.0	0.0	8.0	-
Poultry-tallow[1]	-	-	-	1.3
Megalac®[2]	-	-	-	1.1
Molasses	-	-	-	0.8
Vitamins/minerals[3]	5.0	5.0	5.0	1.9
Nutrient composition, %DM				
CP	14.2	13.8	14.4	16.0
NDF	42.0	41.3	40.1	36.0

Starch	16.0	14.2	14.2	21.5
Ether extracts	2.7	7.4	6.2	5.0
NE_L, Mcal/kg[4]	1.40	1.61	1.55	1.63

[1] Poultry-tallow: Northern Alberta Processing; [2] MegaLac®: Church and Dwight

[3] Contained 13.0% Ca, 3.32% P, 13.0% Na, 5.59% Cl, 5.20% Mg, 0.47% S, 0.05% K, 3295.2 mg/kg of Fe, 3473.6 mg/kg of Cu, 6305.8 mg/kg of Mn, 72.0 mg/kg of Co, 5222.3 mg/kg of Zn, 34.03 mg/kg of Se, 158.6 mg/ kg of I, 764.2 kIU/kg of vitamin A, 129.2 kIU/kg of vitamin D, and 2906.5 kIU/kg of vitamin E (DSM, Nova Scotia, Canada).

[4] NE_L: ¸ 0.0245 × TDN (%) - 0.12

Table 4.2. Crude fat content and fatty acid profile of oilseeds added to prepartum experimental diets

	Canola seed	Sunflower seed
Crude Fat (%)	45.2	43.3
Fatty acid profile of oilseeds (g/100g of total fatty acids)		
Palmitic (C16:0)	4.0	6.8
Stearic (C18:0)	1.8	5.2
Oleic (C18:1-c9)	61.2	12.5
Linoleic (C18:2n6)	18.8	73.1
Alpha linolenic (C18:3n3)	9.6	0.7
Arachidic (C20:0)	0.6	0.3
Other fatty acids	4.0	1.4

Table 4.3. Effects of prepartum dietary treatment on fatty acid profile of plasma (g / 100g of total fatty acids) in dairy cows at Week -3 and +1 relative to calving

	Prepartum dietary treatment			P-values	
	Control	Canola seed	Sunflower seed	Con vs. Oilseeds[1]	Canola vs. Sunflower[2]
No. of cows (n)	12	12	12		
wk -3					
C16:0	16.4 ± 0.54	13.9 ± 0.54	14.9 ± 0.54	< 0.01	0.21
C18:0	24.0 ± 0.84	25.2 ± 0.84	24.5 ± 0.84	0.42	0.58
C18:1	11.4 ± 0.57	14.2 ± 0.57	10.4 ± 0.57	0.21	< 0.0001
C18:2	35.3 ± 1.09	32.6 ± 1.09	40.1 ± 1.09	0.43	< 0.0001
C18:3	4.0 ± 0.69	6.3 ± 0.69	2.9 ± 0.69	0.48	0.001
Unidentified fatty acids	8.9 ± 1.37	7.8 ± 1.37	7.2 ± 1.37	0.42	0.75
wk +1					
C16:0	18.6 ± 0.58	17.4 ± 0.55	17.6 ± 0.58	0.14	0.88
C18:0	18.0 ± 0.60	18.9 ± 0.58	17.8± 0.60	0.64	0.16
C18:1	15.5 ± 0.75	14.6 ± 0.72	13.8 ± 0.75	0.18	0.47
C18:2	34.9 ± 1.12	36.8 ± 1.07	41.4 ± 1.12	< 0.01	< 0.01
C18:3	5.4 ± 0.75	6.6 ± 0.72	3.7 ± 0.75	0.78	0.01
Unidentified fatty acids	7.7 ± 1.25	5.6 ± 1.20	5.7 ± 1.25	0.19	0.96

[1]Con vs. Fat: Oilseed supplementation: oilseed vs. no-oilseed

[2]Canola vs. Sunflower: type of oilseed supplementation

Table 4.4. Effect of prepartum dietary treatments on plasma metabolites

	Prepartum dietary treatment			P-values	
	Control	Canola seed	Sunflower seed	Con vs. Oilseeds[1]	Canola vs. sunflower[2]
No. of cows (n)	12	12	12		
wk -3					
Fatty acids (mEq/dL)	70 ± 9.9	114 ± 10.3	103 ± 10.8	0.004	0.46
BHB[3] (mg/dL)	10.7 ± 0.30	10.9 ± 0.40	10.3 ± 0.43	0.81	0.33
Glucose (mg/dL)	62.0 ± 2.38	56.8 ± 2.48	58.0 ± 2.59	0.12	0.73
wk +1					
Fatty acids (mEq/dL)	271 ± 58.7	398 ± 56.2	379 ± 58.7	0.10	0.81
BHB (mg/dL)	11.2 ± 0.82	10.4 ± 0.79	11.4 ± 0.82	0.74	0.37
Glucose (mg/dL)	52.5 ± 2.73	56.0 ± 2.61	53.8 ± 2.73	0.47	0.55
wk +2					
Fatty acids (mEq/dL)	259 ± 52.9	353 ± 52.9	320 ± 52.9	0.24	0.66
BHB (mg/dL)	12.2 ± 1.39	13.1 ± 1.39	11.7 ± 1.39	0.90	0.50
Glucose (mg/dL)	52.2 ± 3.28	54.3 ± 3.28	51.5 ± 3.28	0.85	0.55

wk +3					
Fatty acids (mEq/dL)	178 ± 43.8	301 ± 43.8	221 ± 43.9	0.13	0.20
BHB (mg/dL)	11.4 ± 1.83	14.8 ± 1.83	10.8 ± 1.83	0.53	0.13
Glucose (mg/dL)	51.9 ± 3.08	56.1 ± 3.08	52.6 ± 3.08	0.51	0.42
wk +4					
Fatty acids (mEq/dL)	163 ± 36.7	295 ± 36.7	188 ± 36.6	0.08	0.04
BHB (mg/dL)	11.7 ± 2.07	15.3 ± 2.07	10.8 ± 2.07	0.60	0.14
Glucose (mg/dL)	51.9 ± 2.36	52.9 ± 2.36	53.0 ± 2.36	0.71	0.96
wk +5					
Fatty acids (mEq/dL)	177 ± 33.7	206 ± 32.2	199 ± 32.2	0.53	0.87
BHB (mg/dL)	12.1 ± 3.35	16.6 ± 3.21	12.4 ± 3.21	0.56	0.36
Glucose (mg/dL)	53.7 ± 3.63	57.7 ± 3.48	53.5 ± 3.48	0.66	0.40

[1]Con vs. Fat: Oilseed supplementation: oilseed vs. no-oilseed

[2]Canola vs. Sunflower: type of oilseed supplementation

[3]Beta-hydroxybutyric acid

Table 4.5. Effect of prepartum dietary treatment on milk composition

	Prepartum dietary treatment				
				P-values	
	Control	Canola seed	Sunflower seed	Con vs. Oilseeds[1]	Canola vs. Sunflower[2]
No. of cows (n)	12	12	12		
wk +2					
Fat, kg/d	1.78 ± 0.10	1.58 ± 0.10	1.60 ± 0.10	0.15	0.90
Protein, kg/d *					
Primiparous cows	0.84 ± 0.09	1.00 ± 0.07	0.97 ± 0.07	0.17	0.78
Multiparous cows	1.67 ± 0.05	1.49 ± 0.06	1.47 ± 0.06	0.01	0.82
Lactose, kg/d	1.60 ± 0.08	1.54 ± 0.08	1.51 ± 0.08	0.48	0.86
Fat, %	4.88 ± 0.18	4.57 ± 0.18	4.64 ± 0.18	0.21	0.77
Protein, %	3.86 ± 0.08	3.70 ± 0.08	3.65 ± 0.08	0.07	0.69
Lactose, %	4.34 ± 0.03	4.40 ± 0.03	4.39 ± 0.03	0.17	0.72
MUN, mg/dL	6.87 ± 0.46	5.48 ± 0.46	5.70 ± 0.46	0.03	0.74
wk +5					
Fat, kg/d	1.60 ± 0.09	1.37 ± 0.08	1.41 ± 0.09	0.06	0.76
Protein, kg/d	1.31 ± 0.06	1.12 ± 0.06	1.12 ± 0.06	0.01	0.91
Lactose, kg/d	1.97 ± 0.09	1.83 ± 0.09	1.81 ± 0.08	0.17	0.87
Fat, %	3.85 ± 0.20	3.59 ± 0.20	3.67 ± 0.19	0.36	0.78
Protein, %	3.07 ± 0.09	2.89 ± 0.09	2.93 ± 0.09	0.15	0.74
Lactose, %	4.59 ± 0.04	4.70 ± 0.04	4.68 ± 0.04	0.04	0.70
MUN, mg/dL	7.37 ± 0.43	6.60 ± 0.42	6.58 ± 0.41	0.14	0.97

[1]Con vs. Fat: Oilseed supplementation: oilseed vs. no-oilseed

[2]Canola vs. Sunflower: type of oilseed supplementation

* Treatment by parity interaction was significant ($P < 0.05$)

Table 4.6. Effect of prepartum dietary treatment on gestation length and calf birth weight

	Prepartum dietary treatment			P-values	
	Control	Canola seed	Sunflower seed	Con vs. Oilseeds[1]	Canola vs. sunflower[2]
Calf sex, Male/Female (ratio)	19/23 (0.82)	21/21 (1.00)	26/19 (1.36)	-	-
Gestation length, d	273 ± 0.9	275 ± 0.9	277 ± 0.9	0.008	0.32
Calf birth weight, kg[3]	41.0 ± 0.84	42.9 ± 0.83	44.4 ± 0.83	0.01	0.15
Male	43.7 ± 1.27	44.7 ± 1.12	45.1 ± 1.09	0.41	0.78
Female	40.4 ± 1.22	42.3 ± 1.28	45.4 ± 1.35	0.02	0.08

[1]Con vs. Fat: Oilseed supplementation: oilseed vs. no-oilseed

[2]Canola vs. Sunflower: type of oilseed supplementation

[3]Calf birth weight was not influenced (P = 0.93) by diet x parity interaction.

Table 4.7. Effect of prepartum dietary treatments on interval from calving to formation of dominant follicle, preovulatory-size follicle and first ovulation

	Prepartum dietary treatment			P-values	
	Sunflower seed	Canola seed	Control	Canola vs. sunflower[2]	Con vs. Oilseeds[1]
No. of cows (n)	31	31	33	-	-
Interval to dominant follicle[3], d	8.9 ± 0.52	9.2 ± 0.47	10.0 ± 0.44	0.25	0.27
Interval to preovulatory-size follicle[4], d	13.5 ± 0.87	15.0 ± 0.47	15.3 ± 0.75	0.12	0.81
Interval to first ovulation[5], d	20.7 ± 1.59	22.9 ± 1.50	20.6 ± 1.41	0.47	0.17
Proportion of cows ovulated by 35 DIM, n (%)	22 (71.0)	24 (77.4)	28 (84.8)	0.21	0.39
Pregnancy at first AI, n (%)	7 (22.6)	8 (25.8)	11 (33.3)	0.53	0.52
Proportion of pregnant cows by 150 DIM	13 (41.9)	16 (51.6)	14 (42.4)	0.78	0.27
Proportion of pregnant cows by 250 DIM	18(58.1)	23 (74.2)	24 (72.7)	0.06	0.62

[1]Con vs. Fat: Oilseed supplementation: oilseed vs. no-oilseed; [2]Canola vs. Sunflower: type of oilseed supplementation; [3]Dominant follicle: follicle of ≥ 10 mm diameter; [4]Preovulatory-size follicle: follicle of ≥ 16 mm diameter; [5]Ovaries were monitored by ultrasonography from d 7±1 to 35±1 postpartum.

Table 4.8. Effect of prepartum dietary treatment on incidence of early postpartum reproductive and health disorders

	Prepartum dietary treatment			P-values	
	Control	Canola seed	Sunflower seed	Con vs. Oilseed[1]	Canola vs. Sunflower[2]
No. of cows (n)	43	43	45	-	-
Dystocia, n (%)	7/43(16)	8/43(19)	16/45(36)	0.15	0.02
Calf survival[3], n (%)	41/43(95)	38/43(88)	44/45(98)	0.64	0.20
Retained fetal membranes, n (%)	2/43(5)	2/43(5)	4/45(9)	0.77	0.42
Metritis, n (%)	4/43(9)	9/43(21)	9/45(20)	0.21	0.98
Vaginal discharge, n (%)	4/43(9)	8/43(19)	8/45(18)	0.29	0.74
Subclinical endometritis, n (%)	16/43(37)	14/43(33)	15/45(33)	0.84	0.90
Ovarian cyst, n (%)	4/43(9)	6/43(14)	10/45(22)	0.23	0.26
Mastitis, n (%)	2/43(5)	0/43(0)	1/45(2)	0.12	0.23
Clinical Ketosis, n (%)	1/43(2)	0/43(0)	1/45(2)	0.33	0.24
Death, n (%)	1/43(2)	1/43(2)	2/45(4)	0.44	0.09
Total reproductive disorders[4], n (%)	10/43(23)	14/43(33)	22/45(49)	0.06	0.52
Total health disorders[5], n (%)	12/43(28)	14/43(33)	24/45(53)	0.25	0.03

[1]Con vs. Fat: Oilseed supplementation: oilseed vs. no-oilseed

[2]Canola vs. Sunflower: type of oilseed supplementation

[3]Calf survival: calf delivered alive.

[4]Total reproductive disorders: The sum of retained fetal membranes, metritis, vaginal discharge and ovarian cyst incidence

[5]Total health disorders = the sum of retained fetal membranes, metritis, vaginal discharge and ovarian cyst, clinical ketosis, mastitis and dystocia

Table 4.9. Effect of prepartum dietary treatments by PMNL category interaction on interval to dominant, preovulatory-size follicle formation and first ovulation

	Prepartum dietary treatment				
	Sunflower seed	Canola seed	Control	Con vs. Oilseeds[1]	Canola vs. sunflower[2]
No. of cows (n)	31	31	33	-	-
≤ 8% PMNL[3]					
Interval to dominant follicle[4], d	8.9 ± 0.66	8.9 ± 0.52	9.9 ± 0.58	0.54	0.21
Interval to preovulatory-size follicle[5], d	13.6 ± 1.10	14.3 ± 0.87	15.6 ± 0.97	0.29	0.31
Interval to first ovulation, d	20.9 ± 2.18	23.2 ± 1.73	21.0 ± 1.91	0.63	0.39
Pregnancy at first AI, n (%)	5/16(38.5)	6/18(33.3)	8/20(47.0)	0.85	0.32
Proportion of pregnant cows by 150 DIM	8/16(50.0)	12/18(66.7)	9/20(45.0)	0.83	0.94
Proportion of pregnant cows by 250 DIM	11/16(68.7)	16/18(88.9)	13/20(65.0)	0.42	0.07
> 8% PMNL[3]					
Interval to dominant follicle[4], d	8.9 ± 0.73	11.0 ± 1.04	10.0 ± 0.66	0.10	0.43
Interval to preovulatory-size follicle[5], d	14.1 ± 1.23	17.8 ± 1.73	15.1 ± 1.10	0.15	0.20
Interval to first ovulation, d	20.5 ± 2.44	21.8 ± 3.43	20.1 ± 2.18	0.89	0.68
Pregnancy at first AI, n (%)	2/15(14.3)	2/13(16.7)	3/13(23.0)	0.60	0.96
Proportion of pregnant cows by 150 DIM	5/15(33.3)	4/13(30.8)	5/13(38.5)	0.58	0.13
Proportion of pregnant cows by 250 DIM	7/15(46.7)	7/13(53. 8)	10/13(76.9)	0.14	0.70

[1]Con vs. Fat: Oilseed supplementation: oilseed vs. no-oilseed; [2]Canola vs. Sunflower: type of oilseed supplementation; [3]PMNL: Polymorphonuclear leukocytes cells; [4]Dominant follicle: follicle of ≥ 10 mm diameter; [5]Preovulatory-size follicle: follicle of ≥ 16 mm diameter

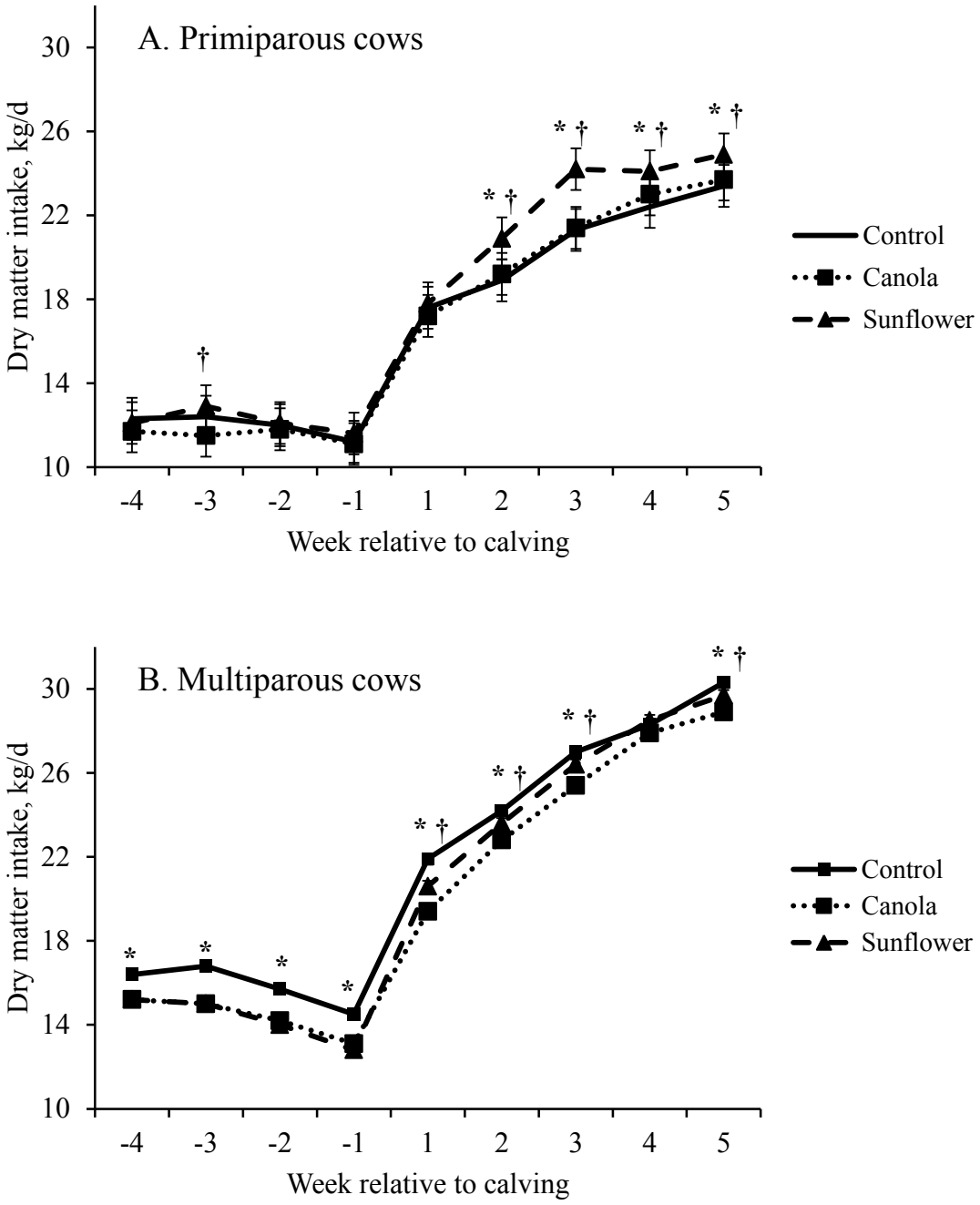

Figure 4.1. Effects of prepartum dietary treatment on dry matter intake (DMI) during calving transition for primiparous cows (A) and multiparous cows (B).

* Significant effect of prepartum oilseed supplementation ($P < 0.05$); † Significant effects of type of oilseed supplementation ($P < 0.05$). The mean (±SEM) DMI prepartum in primiparous cows

were 11.9 ± 0.1, 11.5 ± 0.1 and 12.1 ± 0.1 and postpartum DMI were 20.9 ± 0.1, 20.7 ± 0.1 and 22.3 ± 0.1 kg/d, in control, canola and sunflower groups, respectively. In multiparous cows, mean prepartum DMI were 15.8 ± 0.1, 14.3 ± 0.1 and 14.2 ± 0.1 and postpartum DMI were 26.3 ± 0.1, 24.8 ± 0.1 and 25.7 ± 0.1 kg/d, for control, canola and sunflower, respectively.

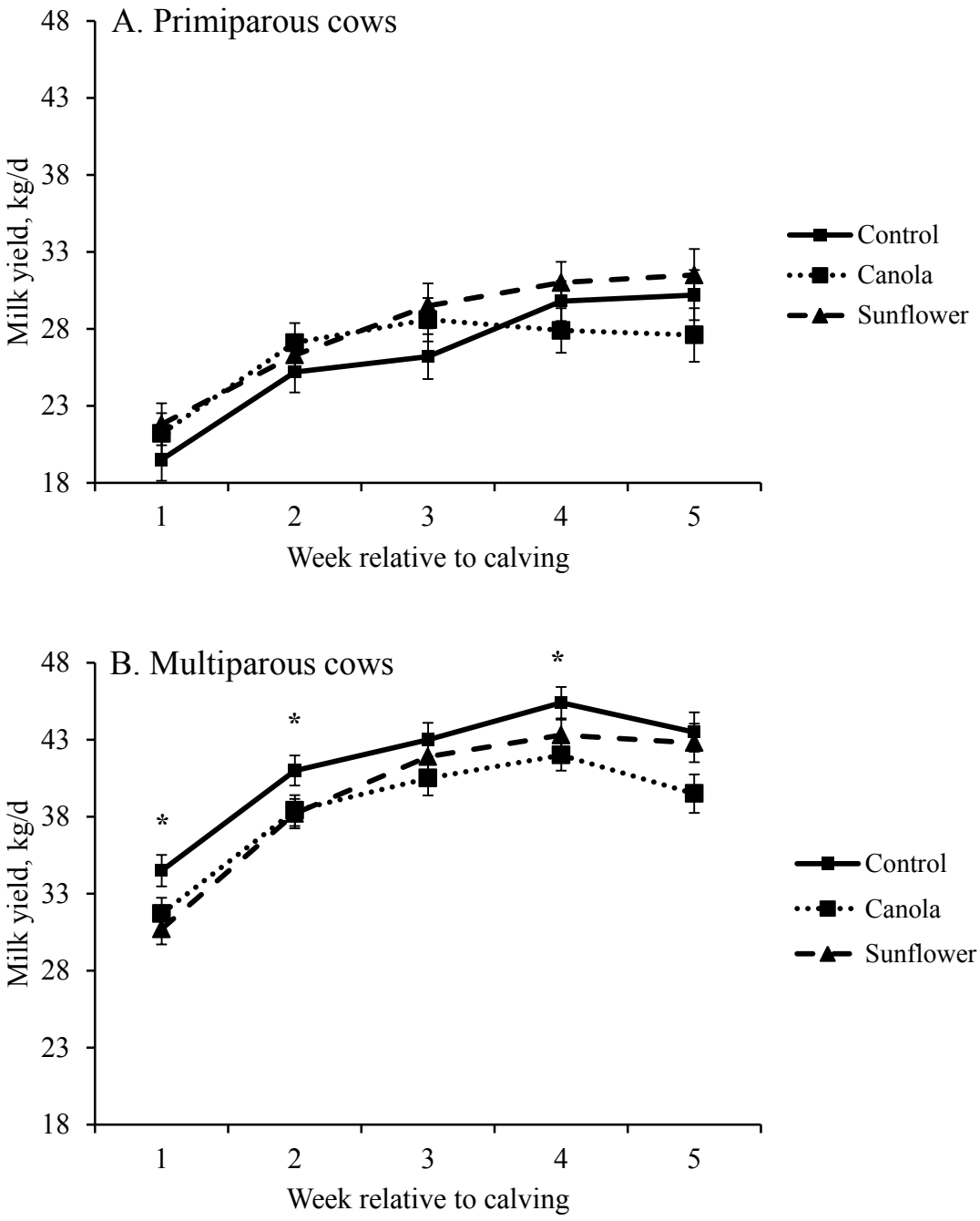

Figure 4.2. Effects of prepartum dietary treatment on mean (±SEM) milk yield for primiparous cows (A) and multiparous cows (B) across weeks.

***** Significant effect of prepartum oilseed supplementation ($P < 0.05$); † Significant effects of type of oilseed supplementation ($P < 0.05$). The mean milk yields (kg/d ± SEM) of primiparous cows

were 25.2 ± 0.6 (control), 27.3 ± 0.6 (canola) and 28.0 ± 0.6 (sunflower) and that of multiparous cows were 41.4 ± 0.4 (control), 38.4 ± 0.4 (canola) and 39.3 ± 0.4 (sunflower).

Chapter 5. Prepartum maternal diets supplemented with oilseeds altered fatty acid profile in bovine neonatal plasma possibly through reduced placental expression of fatty acid transporter protein 4 (*FATP4*) and fatty acid translocase (*FAT/CD36*)

5.1. Abstract

Maternal long-chain polyunsaturated fatty acids (LCPUFA) are the main source of fetal fatty acids essential for neonatal health and development. Whereas transfer of fatty acids from mother to the fetus is required to meet fetal demands in some species, and a preferential transportation of essential fatty acids over nonessential fatty acids exists in the human placenta, related information is scarce in the bovine species. Therefore, we determined the effects of dietary fat (oilseeds) and the type of fat supplementation during late gestation in cattle on maternal and neonatal plasma fatty acids, and expression of placental fatty acid transporter genes. In Experiment 1, Holstein cows in the last 35 d of gestation were assigned to diets containing either sunflower seed (n = 8; high in linoleic acid, LA) or canola seed (n=7; high in oleic acid, CAN), or no oilseed (n=7; control). Blood was sampled for fatty acid determination within 3 h of parturition from dam and neonate, before feeding colostrum. In Experiment 2, placental cotyledon samples were collected from cows (SUN: n=4; CAN: n=4; control: n=5) soon after parturition to quantify gene expression. Prepartum fat supplementation reduced maternal LCPUFA, neonatal total n-3 fatty acids and eicosapentaenoic acid (EPA), and also tended to reduce docosahexaenoic acid (DHA) and total fat compared to those fed control diet. Feeding SUN prepartum vs. CAN tended to increase peroxisome proliferator-activated receptor-α (*PPARA*) expression, whereas the expression of *PPARD* and *PPARG* tended to be higher in CAN fed cows than SUN. Fatty acid protein transporter 4 (*FATP4*) and fatty acid translocase (*FAT*/*CD36*) expression was lower in placental tissue of cows fed supplemental fat than control.

We concluded that reduced total n-3 fatty acids, EPA and DHA in neonates born of dams fed fat prepartum is likely due to alteration in *PPARs* and reduced expression of placental *FATP4* and *FAT/CD36*.

5.1. Introduction

Essential fatty acids (EFA) especially long-chain polyunsaturated fatty acids (LCPUFA), such as eicosapentaenoic acid (EPA; C20:5 n-3) and docosahexaenoic acid (DHA; C22:6 n-3) are important for fetal growth and development in rodents and humans (Neuringer *et al.* 1986); Wainwright 1992; (Duttaroy 2000). Maternal or fetal deficiencies in LCPUFA, particularly arachidonic acid (C20:4 n-6), and DHA, during pregnancy result in cognitive impairment in infants (Connor & Neuringer 1988, Weisinger *et al.* 2001). Fetus depends on both a rich maternal fatty acid reservoir, and an efficient placental transfer mechanism to meet its high demands for LCPUFA, as its own capacity for LCPUFA synthesis is insufficient to meet its developmental requirements (Chambaz *et al.* 1985, Szitanyi *et al.* 1999).

Passive diffusion of free fatty acids through placental membranes is one mechanism by which fatty acids are transferred to the fetus (Hamilton 1998, Hamilton & Kamp 1999) although passive diffusion may not fully meet the fatty acid demand of developing fetus. Previous research in the human placenta and in BeWo cells, a human choriocarcinoma cell line (Campbell *et al.* 1997, Campbell *et al.* 1998b, Duttaroy 2000), revealed a selective transportation of LCPUFA over nonessential fatty acids to satisfy fetal demands. It has been reported that several fatty acid transporters such as fatty acid transport proteins (FATPs), fatty acid translocase (FAT/CD36) and intracellular fatty acid binding proteins (FABPs) facilitate fatty acid transport in human placenta (Campbell *et al.* 1998a, Duttaroy 2000). Given that expression of fatty acid

transporters and placental trophoblast differentiation are influenced by fatty acid-activated transcription factors such as peroxisome proliferator-activated receptors (PPARs; Barak *et al.* 1999, Duttaroy 2004), maternal dietary fatty acids may affect their placental expression and consequently placental biology.

Although, in bovine and ovine species with epitheliochorial placentas, less fatty acids get transferred to the fetus compared with human with hemochorial placenta (Battaglia & Meschia 1988), it has been shown that ovine fetuses mainly depend on maternal circulation to meet their fatty acid requirements (Campbell *et al.* 1994). However, limited information is available regarding transfer of specific LCPUFA from dam to fetus and the expression of placental fatty acid transporters in bovine. We hypothesized that dietary supplementation of oilseeds prepartum will alter maternal and neonatal fatty acid profile based on type of supplemented oilseed. Furthermore, based on results of our first experiment, our second hypothesis was that oilseed supplementation prepartum will reduce placental fatty acid transporters. Therefore, the present study was designed to determine the effects of maternal diet (supplemental fat vs. control) and further investigate the type of fat [high linoleic (C18:2 n-6; SUN) vs. high oleic acid (C18:1 cis 9; CAN)] supplementation during last trimester of gestation on (1) the association between maternal and neonatal fatty acid profile [Experiment 1], and (2) cotyledonary mRNA expression of fatty acid protein transporters (FATPs), fatty acid translocase (FAT/CD36), fatty acid binding proteins (FABPs), fatty acid synthase (FASN) and peroxisome proliferator-activated receptors (PPARs) [Experiment 2].

5.2. Materials and methods

5.2.1. Animals and diets

Both experiments were conducted with subsets of Holstein cows used in a larger study (Salehi *et al.,* 2016), housed and fed individually in a tie-stall research farm. Therefore, animals in Experiments 1 and 2 were under identical diets and management. Approximately 35 days before the expected parturition date, cows were blocked by body condition score, parity and expected parturition date, and assigned to 1 of 3 dietary treatments containing either rolled sunflower seed (high in linoleic acid; SUN) or rolled canola seed (high in oleic acid; CAN) at 8% on a dry matter basis, or no oilseed (control). Crude fat content and fatty acid profile of oilseeds (sunflower and canola) added to prepartum experimental diets are shown in **Table 5.1**. Diets were offered *ad libitum* as a total mixed ration containing forage (alfalfa hay and barley silage) and concentrates (Salehi *et al.* 2015) and cows had access to clean drinking water at all times. Supplemented oilseeds were rolled as described previously (Ambrose *et al.* 2006) before inclusion. Feed intake was recorded daily. Gestation length was calculated based on the interval in days from successful insemination to calving (parturition). Calf birth weight was recorded soon after parturition, but prior to the first feeding of colostrum. The study was conducted at the Dairy Research and Technology Center of the University of Alberta, Edmonton, Canada, with all animal experimental procedures approved by the University of Alberta Animal Care and Use Committee (Protocol # AUP00000131), and animals were cared for according to the Canadian Council of Animal Care (1993) guidelines.

5.2.2. Experiment 1

Non-lactating pregnant Holstein cows (10 primiparous, 12 multiparous) were assigned to one of three diets (SUN: n = 8, CAN: n = 7 or control: n = 7). Blood samples were collected 2 weeks after initiation of prepartum diets (d -21) and within 3 hours after parturition (d 0) from dam and neonate, before feeding colostrum, in sodium heparinized tubes (Vacutainer®, Beckton Dickinson and Co., Franklin Lakes, NJ), centrifuged (3000 × *g* for 20 min at 4°C) and plasma harvested. Plasma samples were kept at -20°C until further analysis. To evaluate maternal metabolic status and examine the effects of prepartum dietary treatments on blood metabolites, plasma samples from d -21 and 0 were used to evaluate non-esterified fatty acids (NEFA), β-Hydroxybutyric acid (BHBA) and glucose. Maternal and neonatal d 0 plasma samples were used for fatty acid profile analysis.

5.2.3. Plasma fatty acid profile and metabolites

Plasma NEFA (NEFA-C kit, Wako Chemicals USA Inc., Richmond, VA), BHBA (Roche Diagnostics, Indianapolis, IN, USA) and glucose (P7119, Sigma-Aldrich, St. Louis, MO) were determined using commercially available kits in triplicate. The intra- and inter-assay coefficients of variation for NEFA, BHBA and glucose were 1.49 and 3.32, 2.51 and 6.71, and 1.38 and 2.42%, respectively. Fatty acids in plasma were extracted (Folch *et al.* 1957) and the fatty acid profile of extracted lipids was assessed by gas chromatography as previously described (Cruz-Hernandez *et al.* 2007).

5.2.4. Experiment 2

Approximately similar sized placental cotyledons were obtained from each cow (SUN: n = 4; CAN: n = 4; control: n = 5) that received identical dietary treatments as in Experiment 1. Placental cotyledons were collected within 2 h following parturition but before spontaneous placental detachment. Cotyledonary samples were removed using a sterile pair of scissors, snap frozen in liquid nitrogen and stored at -80°C until processed for RNA extraction.

Total RNA was extracted from cotyledonary tissues with Trizol reagent (Life Technologies, Burlington, ON, Canada) according to the manufacturer's instructions. Thereafter, total RNA was treated with DNAse (QIAGEN®), cleaned up with Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, CA, USA) and then evaluated with a spectrophotometer (ND-2000, Nanodrop Technologies, Wilmington, DE) for RNA concentration. The quality and integrity of the total RNA extracted were verified by TapeStation (Agilent Technologies, Waldbronn, Germany). Then equal amounts of RNA (1µg) were reverse-transcribed with multiscribe reverse transcription (Life Technologies) and random primers (Life Technologies) in a volume of 20 µL.

Real-time RT-PCR assays were performed with a StepOnePlus Real-Time PCR System (Life Technologies) using Fast SYBR® Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. Primers were designed (**Table 5.2)** using Primer Express v.3.0 (Life Technologies) and analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to verify primer specificity. Melting curve analyses were performed at the end of each run to ensure the specificity of the amplification. Each essay included negative controls with no template, and each sample was analyzed in duplicate. The standard curve (with at least five serial dilutions) method was used to evaluate expression of each gene. The mRNA abundance of target genes was

normalized based on the geometric mean of the three reference genes (*RPL19*, *ACTB*, and *H2AFZ*) as previously described (Vandesompele *et al.* 2002, Laarman *et al.* 2012, Schlau *et al.* 2012).

5.2.5. Statistical analyses

The plasma concentrations of NEFA, BHBA, glucose and fatty acids, feed intake, gestation length, birth weight and the abundance of mRNA expression were analyzed using the MIXED procedure of SAS (version 9.3, 2011; SAS Institute Inc., Cary, NC). We defined LCPUFA as the sum of all polyunsaturated fatty acids with 20 or more carbons. Preplanned contrast analyses were used to compare means for fat vs. control and SUN vs. CAN fed cows. The final statistical model included prepartum dietary treatment (control, CAN, SUN) or source (maternal or neonatal) as the main effects. The effect of dietary treatment by parity interaction was also evaluated for feed intake, gestation length and calf birth weight. The Pearson's test was used to calculate the associations between maternal and neonatal fatty acids as well as with gestation length and birth weight. All data are reported as mean ± SEM with $P \leq 0.05$ considered significant, and $P > 0.05$ but ≤ 0.10 considered trends.

5.3. Results and discussion

5.3.1. Experiment 1: Effect of prepartum diet on cow performance and maternal and neonatal fatty acid profile

The duration of prepartum diets did not differ ($P = 0.53$) among dietary treatments (SUN: 36.33±1.29; CAN: 35.48±1.53; control: 35.48±1.43 d). There was an interaction between dietary treatment and parity for feed intake prepartum (**Table 5.3**). Although prepartum fat

supplementation, specifically SUN, increased feed intake in primiparous cows, inclusion of fats (both SUN and CAN) reduced feed intake in multiparous cows during prepartum period. Glucose, BHBA and NEFA concentration in plasma did not differ among treatments at either time points (d -21 and 0; **Table 5.3**). Prepartum fat supplementation tended to increase birth weight (**Table 5.3**) with primiparous cows fed supplemented fat having approximately 5 d longer gestation period.

Prepartum fat supplementation, more specifically SUN, increased linoleic acid, total n-6 fatty acids, reduced LCPUFA, n-3/n-6 ratio and also tended to reduce docosahexaenoic acid (DHA, C22:6 n-3) in maternal plasma compared to those fed control (**Table 5.4**). Furthermore, higher maternal linoleic acid was associated with lower EPA, total n-3 fatty acids and n-3/n-6 ratio and also tended to lower DHA and total fat in plasma of calves born of cows fed fat than of control cows. Similarly, Garcia *et al.* (2014) found that feeding cows during the last 8 weeks of gestation, a diet supplemented with EFA (27.4% linoleic acid and 4.5% stearic acid) resulted in a numerically greater neonatal linoleic acid, with lower EPA, DHA and total n-3 fatty acids than those of saturated fatty acid (49.9% stearic acid with no linoleic acid). It has been shown that high levels of linoleic acid in the diet and blood could down-regulate production of the desaturase enzymes, required to convert shorter-chain forms of both n-3 and n-6 to long-chain derivatives (Harnack *et al.* 2009, Friesen & Innis 2010, Novak *et al.* 2012). Decreased availability of desaturase enzymes limits production of all LCPUFA. Hence, reduced LCPUFA may be associated with increased linoleic acid in fat fed cows compared to control. To our knowledge, no study has evaluated the effects of reduced neonatal EPA, DHA or total n-3 fatty acids on calf growth and life time production and/or how feeding colostrum may overcome this reduction; therefore further investigations are needed.

Feeding CAN during prepartum period also increased oleic, α-linolenic acid, total monounsaturated fatty acids (TMUFA) and total n-3 fatty acids compared to those fed SUN (**Table 5.4**). Unexpectedly, feeding CAN, which contains about 10% α-linolenic acid, did not affect the percentage of DHA. The DHA can be synthesized from α-linolenic acid by elongation and desaturation, compared with those fed SUN. It has been reported that inclusion of flaxseed oil in prepartum diets increased maternal α-linolenic acid without affecting neonatal α-linolenic acid or maternal and neonatal DHA (Moallem & Zachut 2012). Others (Blank *et al.* 2002) found that incorporation of the n-3 LCPUFA such as EPA and DPA into erythrocytes, plasma, liver, and brain tissues was linearly related to dietary α-linolenic acid. Conversely, the incorporation of DHA into all tissues was related to dietary α-linolenic acid in a curvilinear manner, with the maximum incorporation of DHA appearing to be between the linoleic/α-linolenic acid ratios of 4:1 and 2:1. Therefore, it could be inferred that the linoleic/α-linolenic acid ratio is more important for synthesis of DHA from α-linolenic acid rather than only the amount of α-linolenic acid supplementation, although this remains to be confirmed.

Previous research in human and other species such as ovine (Shand & Noble 1981) and bovine (Moallem & Zachut 2012) indicated several differences between maternal and neonatal fatty acid profile. Our results (**Table 5.4**) confirm the previous findings; regardless of prepartum dietary treatment, maternal plasma had higher concentration of linoleic acid, total n-6, total n-3 fatty acids, TPUFA than neonatal fatty acids, whereas neonatal plasma had greater concentration of oleic acid and TMUFA compared to maternal fatty acid. Prepartum fat supplementation also affected maternal and neonatal plasma fatty acid associations. Neonatal plasma had greater DHA and lower EPA and total fat compared to maternal plasma in fat fed treatments (both SUN and CAN), whereas there was no difference between maternal and neonatal plasma for those fatty

acids in control. Moreover, we found that α-linolenic acid was not detectable in neonatal plasma, whereas it constituted approximately 5% of the total fat in maternal plasma. Previous findings in sheep (Shand & Noble 1981), cow (Moallem & Zachut 2012) and human (Rump *et al.* 2001) also indicated that the proportion of α-linolenic acid is markedly decreased, whereas the proportion of arachidonic acid is much higher in the lamb, calf and umbilical cord plasma compared to that in maternal circulation. In vitro studies with human placenta also confirmed (Kuhn & Crawford 1986, Kuhn *et al.* 1988) a greater portion of radiolabelled arachidonic acid compared to α-linolenic acid was transferred from the maternal to fetal circulation. Collectively, results support a selective materno-fetal placental transport of physiologically important LCPUFA, such as DHA, and also indicate that prepartum dietary fatty acids affect maternal and neonatal fatty acid associations.

In the current study, there was a positive correlation between maternal and neonatal plasma linoleic acid (r: 0.48, P: 0.02), EPA (r: 0.44, P: 0.03), and n-6/n-3 ratio (r: 0.45, P: 0.03). Similarly, a positive correlation has been found between maternal and neonatal profile of DHA in bovine (Moallem & Zachut 2012). These results are in agreement with previous findings in sheep (syndesmochorial), pig (epitheliochorial), and cat (endotheliochorial placenta) and confirm that the materno-fetal fatty acid transfer is small (Elphick *et al.* 1979, Leat & Harrison 1980, Hull & Stammers 1985) in species with placenta having both maternal and fetal layers.

The association between maternal and neonatal fatty acid profile with gestation length and birth weight is presented in **Figure 5.1**. Maternal arachidonic acid and total saturated fatty acids had a negative association, but maternal linoleic acid and total n-6 fatty acids as well as maternal and neonatal n-6/n-3 ratio were positively correlated with gestation length. Arachidonic acid is the precursor of prostaglandin F2α (Calder & Grimble 2002) which is mainly responsible

for pregnancy termination. Although, correlation analysis has been adjusted for prepartum dietary treatments, these results should be interpreted with caution as dams received diet supplemented with oilseed. Therefore, further studies required to confirm these results. Furthermore, in the current study, we found that only neonatal arachidonic acid tended to have positive correlation with birth weight which agrees with a previous human study (Elias & Innis 2001), wherein infant triacylglycerol and cholesteryl ester arachidonic acid content were positively related to birth weight.

5.3.2. Experiment 2: Effect of prepartum diet on cotyledonary fatty acid transporters expression

A previous study (Moallem & Zachut 2012) and our present results indicate a selective transportation of LCPUFA in the bovine species. However, whether prepartum dietary fatty acids affect placental fatty acid transporters' expression in the bovine placenta is largely unknown. Therefore, in Experiment 2, we investigated the effect of prepartum fat and type of fat supplementation in maternal diets on mRNA expression of placental fatty acid transporters.

Prepartum fat supplementation did not affect the mRNA expression of *PPARA*, *PPARD* or *PPARG* in cotyledonary tissues; however, *PPARA* expression tended to be higher in placenta of cows fed SUN than in placenta of cows fed CAN, whereas the expression of *PPARD* and *PPARG* tended to decrease in SUN fed cows compared to those fed CAN (**Figure 5.2**). Daoud and coworkers evaluated the expression of *PPAR* subtypes in human trophoblast cells treated with linoleic acid and found that linoleic acid treatment resulted in a downregulation of *PPARG* expression and tended to upregulate of *PPARA* expression (Daoud *et al.* 2005). Similar results were also obtained with preadipocyte cells treated with α-lipoic acid and conjugated linoleic acid showing an inhibition of *PPARG* expression (Cabrero *et al.* 2001, Cho *et al.* 2003). About the

physiological role of *PPARA* in placenta, it has been found that maternal diabetes increased placental concentrations of triglycerides and cholesteryl esters, and fetal concentrations of phospholipids (Martinez *et al.* 2008). However, *PPARA* agonists reduced fetal and placental lipid concentrations and synthesis in non-diabetic (control) and diabetic rats (Martinez *et al.* 2008). Wang *et al.* (2003) found that targeted activation of *PPARD* in adipose tissue specifically induces expression of genes required for fatty acid oxidation and energy dissipation, which in turn leads to reduced adiposity. Additionally, acute treatment of genetically obese mouse with a *PPARD* agonist depletes lipid accumulation (Wang *et al.* 2003). In the current study prepartum fat supplementation also reduced the expression of *FATP4* and *FAT/CD36* in cotyledonary samples compared to those fed the control diet. The type of supplemented fatty acid also tended to affect *FATP4* expression, with a tendency for reduced *FATP4* expression in placenta of cows fed SUN than in those fed CAN. However, fat supplementation and type of supplemented fat did not influence placental expression of *FASN*, *FATP1*, *FABP3*, *FABP4* and *FABP5* (**Figure 5.2**). Larque *et al.* (2006b) indicated that among FATPs only *FATP4* expression was significantly correlated with DHA in cord blood phospholipids. Hence, a tendency for lower plasma DHA content in neonates born of fat-fed dams could be related to the reduction of *FATP4* expression. Thus, it is likely that prepartum diet supplemented with fat (SUN and CAN) reduced neonatal total n-3 fatty acids and EPA, and also tended to decrease DHA and total fat most probably through altered placental PPARs and subsequently reduced *FATP4* and *CD36* expression.

In conclusion, essential fatty acids (e. g. DHA and EPA) needed for neonatal health and development were reduced in plasma of calves born of dairy cows fed oilseeds during the late gestational period, most likely through altered placental PPARs and reduced fatty acid transporters expression.

5.4. Conflict of interest statement

Authors confirm that there are no conflicts of interest associated with this study and both authors have seen and approved the final version of the manuscript.

5.5. Acknowledgements

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Table 5.1. Crude fat content and fatty acid profile of oilseed added to prepartum experimental diets.

	Sunflower	Canola
Crude Fat (%)	43.3	45.2
Fatty acid profile of oilseeds (g/100 g FAMEs)		
Palmitic acid	6.8	4.0
Stearic acid	5.2	1.8
Oleic acid	12.5	61.2
Linoleic acid	73.1	18.8
Linolenic acid	0.7	9.6
Arachidonic acid	0.3	0.6
Other fatty acids	1.4	4.0

Table 5.2. Primer sequences for quantitative real-time polymerase chain reaction (qRT-PCR) amplification of mRNA of different genes.

Gene name	Primer	Primer sequence (5'-3')
PPARA	Forward	TTGTGGCTGCTATCATTTGC
	Reverse	AGAGGAAGACGTCGTCAGGA
PPARD	Forward	CACTCTCACTGCTGGACCAA
	Reverse	GCAGATCCGCTCACATTTCT
PPARG	Forward	GTGAAGCCCATTGAGGACAT
	Reverse	AGCTGCACGTGTTCTGTCAC
FASN	Forward	CTCTCCCTCAGCCGTTCG
	Reverse	GCCTGTCATCATCTGTCACC
CD36/FAT	Forward	TCATTGCTGGTGCTGTCATTGG
	Reverse	AACTGTCACTTCATCTGGATTCTGC
FATP1	Forward	GCGGCATTTGGGCACTACT
	Reverse	ACCGTCAGCCCATAGATGAGA
FATP4	Forward	GTGGTGCACAGCAGGTATTACC
	Reverse	CCTATCCCCACGATGTTTCCT
FABP3	Forward	TACGCGTTCTCTGTCGTCTTTC
	Reverse	AACCGACACCGAGTGACTTCA
FABP4	Forward	GGTGCTGGAATGTGTCATGAA
	Reverse	GGACAACGTATCCAGCAGAAAGT
FABP5	Forward	GCGAGGATGAACAAGCTCAGT
	Reverse	ATGACCCTAATTAAACCAACACTTTGA

RPL19	Forward	TCGATGCCGGAAAAACAC
	Reverse	ATTCTCATCCTCCTCATCCAG
ACTB	Forward	GAGAAGCTCTGCTACGTGG
	Reverse	CCGGACAGCACCGTGTTGG
H2AFZ	Forward	AGAGCCGGTTTGCAGTTCCCG
	Reverse	TACTCCAGGATGGCTGCGCTGT

Table 5.3. Feed intake, gestation length, calf birth weight and maternal plasma metabolite concentrations.

	Prepartum dietary fatty acid			P-value	
	Control (n = 7)	CAN (n = 7)	SUN (n = 8)	Control vs. fat	CAN vs. SUN
Feed intake (kg/d ± SEM)	13.12 ± 0.17	12.74 ± 0.19	13.49 ± 0.15	0.99	0.002
Primi	11.36 ± 0.24	11.27 ± 0.30	13.07 ± 0.24	0.01	<.0001
Multi	14.88 ± 0.24	14.21 ± 0.24	13.92 ± 0.19	0.005	0.35
Gestation length (d ± SEM)	270.83 ± 3.17	275.63 ± 2.84	279.00 ± 3.55	0.12	0.47
Primi	265.75 ± 3.49	275.00 ± 4.03	280.00 ± 4.94	0.02	0.44
Multi	281.00 ± 4.94	276.50 ± 3.49	278.25 ± 3.49	0.52	0.72
Calf birth weight (kg ± SEM)	39.39 ± 2.30	44.62± 2.30	43.83± 2.20	0.10	0.80
Maternal plasma metabolites					
NEFA[1] (mEq/dL)					
d -21	135 ± 21	126 ± 21	104 ± 20	0.46	0.47
d 0	405 ± 75	543 ± 75	472 ± 72	0.28	0.51
BHBA[2] (mg/dL)					
d -21	9.50 ± 0.86	11.01 ± 0.86	10.37 ± 0.83	0.27	0.60
d 0	8.82 ± 0.78	10.12 ± 0.78	9.31 ± 0.75	0.36	0.47
Glucose (mg/dL)					

d -21	68.71 ± 4.71	59.89 ± 4.71	61.55 ± 4.51	0.18	0.80
d 0	99.81 ± 8.43	90.44 ± 8.43	86.41 ± 8.06	0.28	0.73

[1]non-esterified fatty acid

[2] β-Hydroxybutyric acid

Table 5.4. Effect of prepartum dietary treatments on maternal and neonatal plasma fatty acid profile (g/100g of total fatty acids).

	Maternal plasma fatty acid profile[1]			P-value		Neonatal plasma fatty acid profile[2]			P-value	
	Control	CAN	SUN	Control vs. fat	CAN vs. SUN	Control	CAN	SUN	Control vs. fat	CAN vs. SUN
Myristic acid (C14:0)	0.99±0.19	0.60±0.19	0.67±0.18[a]	0.14	0.78	0.70±0.15	0.23±0.15	0.27±0.14[b]	0.02	0.84
Palmitic acid (C16:0)	18.63±0.57[a]	16.09±0.57[a]	15.01±0.53[a]	0.0003	0.18	29.64±1.37[b]	31.19±1.37[b]	30.24±1.31[b]	0.52	0.62
Stearic acid (C18:0)	15.36±0.66[a]	16.66±0.66[a]	15.72±0.61[a]	0.31	0.31	13.23±0.72[b]	11.68±0.72[b]	12.54±0.67[b]	0.18	0.49
Oleic acid (C18:1 cis 9)	16.32±1.12[a]	17.92±1.12[a]	14.77±1.04[a]	0.98	0.05	28.57±2.09[b]	33.76±2.09[b]	33.05±1.96[b]	0.07	0.80
Nonadecanoic acid (C19:0)	0.47±0.33	0.33±0.33	0.25±0.31	0.66	0.85	1.45±0.70	1.00±0.70	0.45±0.66	0.40	0.57
Linoleic acid (C18:2 n-6)	28.55±1.82[a]	30.15±1.82[a]	39.81±1.70[a]	0.009	0.001	2.25±0.33[b]	2.48±0.33[b]	3.61±0.31[b]	0.28	0.007
γ-Linolenic acid (C18:3 n-6)	0.27±0.12	0.33±0.12	0.57±0.11	0.25	0.16	ND[3]	ND	ND	.	.
α-Linolenic acid (C18:3 n-3)	6.02±0.54	6.87±0.54	4.46±0.51	0.59	0.004	ND	ND	ND	.	.
DGLA* (C20:3 n-6)	1.46±0.26	1.26±0.26	1.24±0.24[a]	0.50	0.94	1.85±0.31	1.29±0.31	2.09±0.29[b]	0.68	0.08
Arachidonic acid (C20:4 n-6)	2.70±0.17[a]	3.00±0.17	2.63±0.16[a]	0.12	0.77	4.14±0.81[b]	3.92±0.81	5.00±0.76[b]	0.51	0.45

	Maternal plasma fatty acid profile[1]			P-value		Neonatal plasma fatty acid profile[2]			P-value	
	Control	CAN	SUN	Control vs. fat	CAN vs. SUN	Control	CAN	SUN	Control vs. fat	CAN vs. SUN
EPA* (C20:5 n-3)	1.66±0.18	1.49±0.18[a]	1.19±0.17[a]	0.17	0.26	1.19±0.16	0.52±0.16[b]	0.61±0.15[b]	0.006	0.71
DPA* (C22:5 n-3)	0.92±0.22[a]	0.61±0.22	0.29±0.20	0.10	0.30	0.23±0.18[b]	0.25±0.18	0.34±0.17	0.76	0.72
DHA* (C22:6 n-3)	0.57±0.25	0.09±0.25[a]	0.01±0.24[a]	0.10	0.82	2.90±0.86	1.16±0.86[b]	1.31±0.81[b]	0.10	0.90
Unidentified fatty acids	5.71±1.04	4.83±1.04	3.33±0.97	0.21	0.31	12.86±1.18	11.16±1.09	10.39±1.02	0.15	0.61
TSFA*	36.76±1.12[a]	34.63±1.12[a]	32.25±1.05[a]	0.02	0.13	45.11±1.24[b]	47.22±1.24[b]	43.89±1.16[b]	0.77	0.06
TMUFA*	20.09±1.43[a]	21.52±1.43[a]	17.50±1.34[a]	0.74	0.05	40.40±2.39[b]	43.03±2.39[b]	43.11±2.23[b]	0.36	0.98
TPUFA*	43.14±1.88[a]	43.83±1.88[a]	50.23±1.76[a]	0.10	0.02	14.47±1.96[b]	11.36±2.12[b]	12.98±1.84[b]	0.35	0.57
TPUFA/TSFA	1.18±0.09[a]	1.29±0.09[a]	1.56±0.08[a]	0.03	0.03	0.32±0.04[b]	0.24±0.04[b]	0.29±0.04[b]	0.36	0.44
LCPUFA[4]*	7.63 ± 0.65	6.18 ± 0.65	5.38 ± 0.61[a]	0.03	0.38	10.10 ± 1.72	7.38 ± 1.72	9.37 ± 1.61[b]	0.42	0.41
Total n-3	8.26±0.67[a]	8.46±0.67[a]	5.67±0.63[a]	0.16	0.007	3.83±0.83[b]	1.60±0.83[b]	1.70±0.80[b]	0.04	0.92

	Maternal plasma fatty acid profile[1]			P-value		Neonatal plasma fatty acid profile[2]			P-value	
	Control	CAN	SUN	Control vs. fat	CAN vs. SUN	Control	CAN	SUN	Control vs. fat	CAN vs. SUN
Total n-6	34.83±1.66[a]	35.32±1.66[a]	44.55±1.55[a]	0.02	0.0007	9.96±1.41[b]	7.61±1.41[b]	10.67±1.35[b]	0.64	0.13
n-6/n-3 ratio	4.46±0.43	4.42±0.43	7.93±0.41	0.004	<.0001	4.23±2.01	5.20±2.01	10.58±1.92	0.15	0.07
n-3/n-6 ratio	0.24±0.01	0.23±0.01	0.12±0.01	0.02	0.0006	0.37±0.06	0.18±0.06	0.14±0.06	0.02	0.68
Total fat (ng)	418.39±73.85	480.94±73.85[a]	570.48±69.08[a]	0.24	0.38	317.06±39.05	205.61±39.05[b]	277.48±36.53[b]	0.10	0.19

[a,b] $P < 0.05$ indicates significant difference between maternal and neonatal fatty acid within same treatment group (Maternal vs. neonatal for control, SUN and CAN).

[1]Maternal blood samples were collected within 3 hr after parturition to evaluate fatty acid profile.

[2]Neonatal blood samples were collected before feeding first colostrum to evaluate fatty acid profile.

[3]ND: not detectable; α-linolenic acid, precursor of EPA and DHA, was not detectable in the GC analysis.

[4]LCPUFA: sum of all polyunsaturated fatty acids with 20 or more carbons.

*DGLA: Dihomo-γ-linolenic acid; EPA: Eicosapentaenoic acid; DPA: Docosapentaenoic acid; DHA: Docosahexaenoic acid; TSFA: Total saturated fatty acid; TMUFA: Total mono-unsaturated fatty acid; TPUFA: Total poly-unsaturated fatty acid; LCPUFA: long-chain polyunsaturated fatty acids.

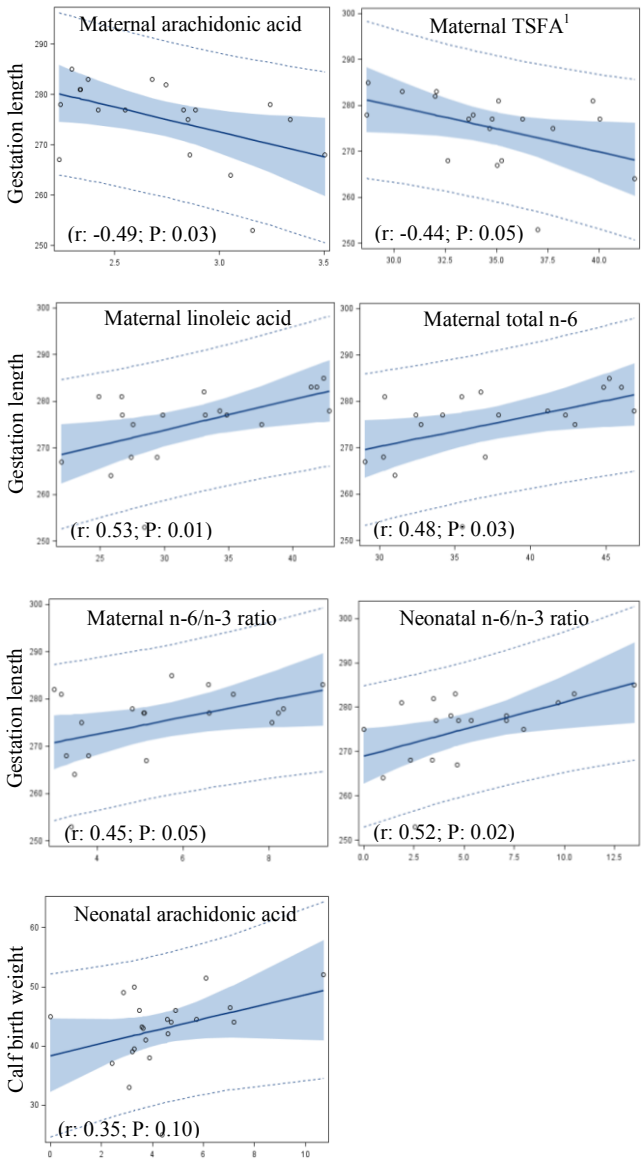

Figure 5.1. Correlation between gestation length and calf birth weight with maternal and neonatal fatty acid profile

[1]TSFA: Total saturated fatty acid

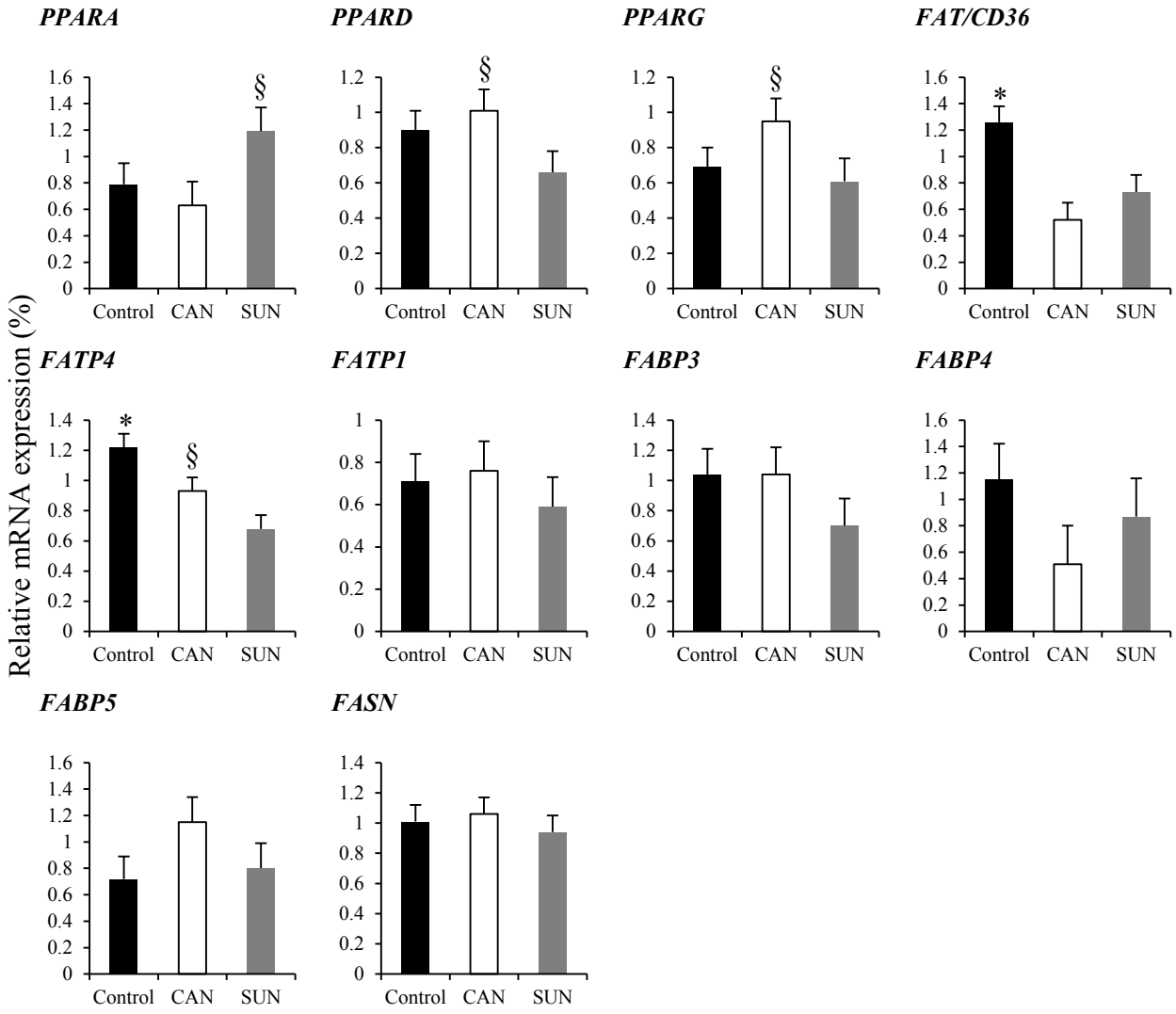

Figure 5.2. The relative mRNA expression of peroxisome proliferator-activated receptor (PPAR)-A, D and G, fatty acid translocase (*FAT/CD36*), fatty acid transporter protein 4 (*FATP4*), fatty acid transporter protein 1 (*FATP1*), fatty acid synthesis (*FASN*), and fatty acid binding protein (*FABP*) 3, 4 and 5 and in bovine placental cotyledonary samples among treatments.

*Control vs. fat: $P < 0.05$; § CAN vs. SUN: $P < 0.1$

Chapter 6. Morphologic and transcriptomic assessment of bovine embryos exposed to long chain fatty acids

6.1. Abstract

The main objectives were to determine: (1) the influence of diets enriched in α-linolenic, linoleic or oleic acid on the development and transcriptomic profile of embryos collected from dairy cattle; and (2) the effects of adding serum collected from cows fed diets enriched in α-linolenic or linoleic acid to the *in vitro* embryo culture medium on embryonic development and expression of selected genes. In Experiment 1, non-lactating Holstein cows received one of three diets supplemented with 8% rolled oilseeds: flax (FLX, n=8), sunflower (SUN, n=7) or canola (CAN, n=8). After a minimum 35-day diet adaptation, cows were superovulated, artificially inseminated and ova/embryos recovered non-surgically 7.5 days later. Cows fed FLX had less degenerated embryos and more viable embryos than those fed CAN or SUN. One-hundred-seventy-five genes were differentially expressed in blastocysts from cows fed FLX than in cows fed CAN or SUN. The differentially expressed genes were mainly involved in cellular growth and proliferation, cellular development, and cell survival and viability. In Experiment 2, sera harvested from cows (FLX or SUN) at estrus in Experiment 1, were added to the post-IVF embryo culture medium. Adding 5% serum from cows fed FLX compared to SUN increased the expression of genes responsible for cell proliferation and differentiation (*ANXA1*) and maternal pregnancy recognition (*IFNT*) in Day 8 embryos without affecting morphological development. In conclusion, n-3 polyunsaturated fatty acids reduced early embryonic degeneration possibly through enhancing the oocyte competence and improving cell survival and viability.

6.2. Introduction

The establishment of pregnancy in cattle requires ovulation of a competent oocyte, insemination at the optimum time and production of sufficient embryonic interferon τ to inhibit prostaglandin $F_2\alpha$ ($PGF_2\alpha$) release (Hunter 1989, Robinson *et al.* 2006). In this regard, it has been shown that feeding dairy cows diets supplemented with n-3 polyunsaturated fatty acids such as eicosapentaenoic and docosahexaenoic acids suppressed $PGF_2\alpha$ production, delayed luteal regression, and improved conception rate (Burke *et al.* 1997). Although it is widely accepted that dietary fats (Thatcher 2010) and fatty acids (Mattos *et al.* 2000) enhanced reproductive performance in dairy cows, the effect of fatty acids on the transcriptome profile of early embryos remains unknown.

Bovine oocytes contain 63 μg of fat (McEvoy *et al.* 2000) that is used as an energy source during oocyte maturation and the extended period of embryo development before implantation (Paczkowski *et al.* 2013, Paczkowski *et al.* 2014). Inhibition of fatty acid oxidation in bovine oocytes reduced their capacity to form blastocysts after fertilization (Ferguson & Leese 2006). However, *in vitro* culture (IVC) models indicated that high-NEFA concentrations, found in the follicular fluid of the dominant follicle during early postpartum period (Leroy *et al.* 2004b), were detrimental to the oocyte's nuclear maturation and developmental competence and to granulosa cell viability and steroidogenic capacity (Leroy *et al.* 2005, Vanholder *et al.* 2005). It has been demonstrated that palmitic and stearic acid predominate the follicular fluid of lactating dairy cows and adding these fatty acids to *in vitro* maturation medium of bovine cumulus–oocyte complexes (COC) inhibited cumulus expansion, reduced progression to metaphase II and blastocyst rate (Leroy *et al.* 2005). However, adding oleic acid to *in vitro* maturation medium reversed the detrimental effects of palmitic and stearic acids on subsequent

embryo development (Aardema *et al.* 2011). Canola seed has high amount of oleic acid (61% of total fat) and is a common ingredient in dairy cattle rations in Western Canada, however, its influence on *in vivo* early embryonic development has not been investigated.

Previously, our group (Thangavelu *et al.* 2007) found that feeding a diet enriched in α-linolenic (flax seed) or linoleic acid (sunflower seed) to lactating dairy cows enhanced embryonic development through a significant increase in blastomere number compared to feeding a diet enriched in saturated fatty acid (high in stearic and palmitic acids). Moreover, cows that were fed a diet supplemented with a partially rumen protected n-3 polyunsaturated fatty acids (PUFA; high in eicosapentaenoic and docosahexaenoic acids) had a lower proportion of degenerated embryos than those fed a diet supplemented with palmitic acid (Childs *et al.* 2008). Nevertheless, using an *in vivo* model does not determine whether the effect of PUFA on embryo quality is exerted at the follicular (oocyte) and/or at the oviduct-uterine (embryo) level. A combination of *in vivo* and *in vitro* approaches has been used to examine the effect of PUFA on oocyte developmental competence (Moallem *et al.* 2013). In this regard, feeding a diet enriched in α-linolenic acid (flaxseed oil) increased the numbers of follicles, oocytes collected by transvaginal ultrasonography, enhanced the cleavage rate of *in vitro* fertilized oocytes and tended to improve blastocyst rate compared with a diet enriched in saturated fatty acid (Moallem *et al.* 2013). In another study, adding α-linolenic acid to *in vitro* maturation medium enhanced oocyte maturation and subsequent embryo development, whereas linoleic acid significantly inhibited cumulus cell expansion, delayed development of the oocytes to the metaphase II stage and reduced cleavage and blastocyst rate (Marei *et al.* 2009, Marei *et al.* 2010). These results suggest that α-linolenic acid enhanced embryo development most probably through improved oocyte competence. Although these studies have provided significant information regarding the

effect of fatty acids on *in vivo* embryo development or on oocyte competence, very limited information is available about the transcriptome profile of embryos exposed to different fatty acids during their development as well as how fatty acids specifically influenced post-fertilization embryo development. We hypothesized that (1) feeding cows a diet enriched in α-linolenic acid will improve early embryonic development compared to those fed linoleic or oleic acid, and (2) adding serum from cows fed a diet enriched in α-linolenic acid to *in vitro* embryo culture medium will enhance early embryonic development compared to those collected from linoleic or oleic acid. Therefore, the main objectives of this study were to examine: (1) the influence of diets enriched in α-linolenic, linoleic or oleic acid on the development and transcriptomic profile of embryos collected from dairy cattle (Experiment 1); and (2) the effects of supplementing *in vitro* embryo culture medium with serum from cows fed diets enriched in α-linolenic or linoleic acid on *in vitro* embryonic development and the expression of selected genes (Experiment 2).

6.3. Materials and methods

6.3.1. Experiment 1: *in vivo* embryo production

The study was conducted at the Metabolism Research Unit of the University of Alberta, Edmonton, Canada, with all animal experimental procedures approved (Protocol # AUP00000131) by the University of Alberta Animal Care and Use Committee and animals cared in accordance with the Guidelines of the Canadian Council of Animal Care (1993). Non-lactating Holstein cows (726 ± 1.14 kg) were blocked by parity and body weight, and assigned to one of three diets supplemented with flax (FLX; high in α-linolenic acid, n=8), sunflower (SUN; high in linoleic acid, n=7) or canola (CAN; high in oleic acid, n=8) seeds. Cows were individually fed a

diet containing hay (8.8 kg/d on a dry matter basis) and concentrate mix (3.8 kg/day on a dry matter basis). The concentrate portion of diet contained 0.99 kg/d dry matter basis of rolled canola, sunflower or flax seed. The protein, fat and fatty acid composition of canola, sunflower or flax seed are presented in **Table 6.1.** After a minimum 35-day diet adaptation, ovarian status was synchronized and super-stimulated as described previously (Colazo *et al.* 2005) with purified porcine follicle stimulating hormone (FSH). Cows were then artificially inseminated twice, 12 h apart, with frozen-thawed semen from the same sire and ova/embryos recovered non-surgically 7.5 days post-insemination. A total of 35 embryo collections (uterine flushing) were performed (FLX: 12, SUN: 11 and CAN: 12). Once started on a diet, cows continued to receive the same diet until the end of the experiment. Before embryo collection, ovarian status was determined by transrectal ultrasonography and cows with less than two corpus lutea were not used for embryo collection. Collected embryos were classified according to the Manual of the International Embryo Transfer Society (3rd ed., Savoy, IL) as Stage 1 (unfertilized), Stage 2 (2 to 8-cell), Stage 3 (early morula), Stage 4 (morula), Stage 5 (early blastocyst), Stage 6 (blastocyst) and Stage 7 (expanding or expanded blastocyst). In the present study, embryos of Stage 6 and 7 were considered blastocysts. We defined transferable embryos and the proportion of viable embryos as total number of grade 1 and 2 blastocysts and the proportion of grade 1 and 2 blastocysts over total collected embryos, respectively.

6.3.2. Experiment 2: *in vitro* embryo production

To investigate the effect of sera from cows fed FLX or SUN on *in vitro* embryo development, blood samples were collected at estrus from the same cows used in Experiment 1, before starting superovulatory treatments. Blood samples were collected in non-heparinized, silicone-coated tubes (Vacutainer, Beckton Dickinson, Franklin Lakes, NJ, USA), and kept 4 h at

room temperature for clotting. Blood samples were then centrifuged (3000 × *g*, 20 min, 4°C) and serum harvested. The FLX and SUN sera collected from different cows were pooled, heat-inactivated (56°C, 30 min), passed through a 0.22 µm filter, and stored in 2 mL aliquots (−80°C) until used. The same batch of FLX and SUN sera were used throughout the experiment.

6.3.3. In vitro embryo production

All chemicals used for *in vitro* maturation, fertilization and embryo culture were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise indicated.

6.3.4. Oocyte collection, *in vitro* maturation and fertilization

Bovine ovaries from an abattoir were delivered to the laboratory within 10h of slaughter. Cumulus–oocyte complexes (COC) were aspirated from 2–6 mm follicles using a 10 ml syringe and 18-gauge needle. Cumulus–oocyte complexes were evaluated morphologically and only those with more than five layers of compact unexpanded cumulus cells were used for in *vitro* embryo production as described previously (Oba *et al.* 2013). Briefly, COC were washed four times in Dulbecco's phosphate-buffered saline (D-PBS; Gibco® laboratories, Grand Island, NY, USA) supplemented with bovine serum (5%; New Zealand origin; Gibco® laboratories, Grand Island, NY, USA) and gentamicin sulfate (5 µg/ml). Thereafter, COC were washed twice, cultured in TCM-199 supplemented with bovine serum (5%), FSH (0.02 mg/ml) and gentamicin sulfate (5 µg/ml), and placed (groups of 20-25 COC in 100 µL droplets) in *in vitro* maturation medium covered with mineral oil (22–24 h, 38.5°C, 5% CO_2 in air, maximum humidity).

For *in vitro* fertilization (Day 0), motile spermatozoa obtained by discontinuous Percoll density gradient centrifugation (45% and 90%; 700 × *g*, 20 min) of frozen-thawed semen (37°C,

30 sec) were co-incubated with matured COC (20 COC per drop; 38.5°C; humidified 5% CO_2 in air, 16 h) at a final concentration of 5×10^6 sperm/ml fertilization medium.

6.3.5. *In vitro* culture

The excess sperm and cumulus cells were removed by gentle pipetting in D-PBS supplemented with 5% of treatment serum (FLX or SUN serum) and 5µg/ml gentamicin sulfate. Denuded presumptive zygotes were washed with D-PBS (2X), with culture medium (CR1aa, 1X) and then transferred to 100-µL droplets (20 to 25 zygotes per drop) of CR1aa supplemented with related serum (5%) and cultured for 8 days (38.5°C, 5% CO_2 in air, maximum humidity). Cleavage rate and embryonic development were assessed on Days 2, 7 and 8 (Day 0 = fertilization), respectively. Embryo development on Days 7 and 8 was classified according to the Manual of the International Embryo Transfer Society (3rd ed., Savoy, IL) as explained in Experiment 1. Embryos of Stage 5 were considered early blastocysts, whereas embryos of Stage 6 and 7 were considered blastocysts.

6.3.6. Total RNA isolation

In Experiment 1, only grade 1 and 2 blastocysts recovered from each cow were pooled to generate four replicates per cow assayed. Total RNA was extracted from pools of 1 to 5 blastocysts.

In Experiment 2, total RNA was extracted from four biological replicates with a pool of 5 grade 1 blastocysts (Day 8) per replicate. Total RNA was extracted using Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, CA, USA) in both experiments. The RNA quality and integrity of each total RNA sample was evaluated by Bioanalyzer RNA 6000 Pico LabChip

(Agilent Technologies, ON, Canada) and all extracted samples showed RNA integrity number greater than 7.5 in both experiments.

6.3.7. RNA amplification and labelling for microarray analysis

Extracted RNA from Experiment 1 samples were amplified using RiboAmp HSPlus kit (Applied Biosystems, CA, USA) following the manufacturer's instructions and generated amplified RNA (aRNA) targets for microarray reactions. Thereafter, the quantity and quality of the aRNA products from RNA amplification reactions were evaluated by the Nanodrop (ND-2000, Nanodrop Technologies, Wilmington, DE, USA) and TypeStation (2200 TapeStation, Agilent Technologies, UK), respectively. Two μg of aRNA were used in each labelling reaction. All labelling reactions were performed using the ULS Fluorescent Labelling Kit (Kreatech Diagnostics, Amsterdam, Netherlands) as per manufacturer's instructions. The labelling of aRNA targets was processed under an ozone-free environment. Labelling efficiency of each labelled sample was evaluated using Nanodrop.

6.3.8. Microarray experimental procedure

Amplified RNA (825 ng per replicate) was hybridized on Agilent-manufactured EmbryoGENE slides in a two-color dye swap design (Robert *et al.* 2011). The EmbryoGENE Bovine Microarray is composed of 42,242 probes, including 21,139 known reference genes; 9,322 probes for novel transcribed regions (NTRs); 3,677 alternatively spliced exons; 3,353 3′-tiling probes; and 3,723 controls. After 17 hours at 65 °C, microarray slides were washed in Gene Expression Wash Buffer 1 (room temperature; 1 min), in Gene Expression Wash Buffer 2 (42 °C, 3 min), in 100% acetonitrile (room temperature, 10 sec), and in Stabilization and Drying Solution (30 sec, Agilent Technologies, Wilmington, DE, USA) and scanned with Axon

4200AL scanner (Molecular Device, Sunnyvale, USA). Intensity files were analyzed with FlexArray (Blazejczyk *et al.* 2007).

Intensity raw data were corrected by background subtraction and normalized within (green or red) and between each array (Loess and quantile, respectively) (Tsoi *et al.* 2012). The dataset of the microarray results has been deposited in NCBI's Gene Expression Omnibus and is accessible through Gene Expression Omnibus series accession number: GSE67686. To identify differentially expressed genes, the normalized microarray data was analyzed using the "limma" package (Smyth 2005) of Bio-conductor through FlexArray (Robert *et al.* 2011). For any particular comparison, only genes with *P*-value $\leq$ 0.05 and a fold change (FC) $\geq$ 2.0 were considered significantly up- or down-regulated.

6.3.9. Functional analysis of differential gene expression profile

Expression data obtained from the comparative transcriptomic analysis were analysed using the Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com).

6.3.10. Biological functions analysis

The biological functions were performed under BH-FDR multiple testing correction conditions. Only the biological functions with a BH-FDR corrected *P*-value (B-H *P*-value) < 0.05 and with a –log (B-H *P*-value) > 2.0 were considered significant.

6.3.11. Network generation

Differentially expressed genes (*P*-value $\leq$ 0.05 and a fold change (FC) $\geq$ 2.0), called network-eligible molecules, were overlaid onto a global molecular network developed from information contained in Ingenuity's Knowledge Base.

6.3.12. IPA upstream regulator analysis

Upstream regulator analysis predicted the activation status of the upstream regulators by calculating a regulation Z-score and an overlap *P*-value, which were based on the number of known regulation target genes from the dataset of interest, expression changes of these target genes, and their agreement with literature findings. Upstream regulators with an overlap *P*-value ≤ 0.05 and an IPA activation Z-score ≥ 2.0 (or ≤ -2.0) were considered significantly activated (or inhibited). Overlap *P*-value measures overlap of observed and predicted regulated gene set, and Z-score assesses the match of observed and predicted up/down regulation patterns based on IPA data basis.

Calculation of the IPA regulation Z-score and overlap *P*-value was as described in IPA white papers "A Novel Approach to Predicting Upstream Regulators" and a full description is available on the IPA website (http://www.ingenuity.com) under "Upstream Regulator Analysis", "Biological Functions Analysis", and "Ingenuity Canonical Pathways Analysis".

6.3.13. Validation microarray results (Experiment 1)

Total extracted RNA from independent samples (three replicates for each treatment) was used to confirm the expression of six genes (*PTGS2*, *LGALS3*, *ANXA1*, *IL1RN*, *KRT19* and *SRXN1*) based on microarray data analysis by Real-time Quantitative PCR (q-PCR). Each sample contained a pool of 3 to 5 *in vivo*-produced grade 1 and 2 blastocysts.

6.3.14. Experiment 2 real-time q-PCR

Total extracted RNA from *in vitro*-produced embryos (Experiment 2) was used to evaluate the expression of 10 selected genes based on microarray results (*PTGS2*, *LGALS3*,

ANXA1, *IL1RN*, *KRT19*, *ID2*, *PLAC8*, *SLC7A7* and *TDGF1, NANOG*) and 6 other genes of interest (*IFNT*, *KRT18*, *GATA2*, *GATA3*, *MYL6* and *PLIN2*).

6.3.15. Real-time q-PCR

Primers were designed (**Supplementary Table 6.1**) using Integrated DNA Technologies (https://www.idtdna.com/scitools/Applications/RealTimePCR) and analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to verify primer specificity. A total of 1 ng of total RNA extracted from each pool of embryos was reverse transcribed into cDNA using a high capacity reverse transcriptase (SuperScript VILO cDNA Synthesis Kit, Invitrogen) in a 20 µl reverse transcription (RT) reaction volume, as per manufacturer's instructions. The cDNA products were then diluted 1 time, and 1 µl of the diluted cDNA was used as the template with the StepOnePlus Real-Time PCR System (Applied Biosystems) and Fast SYBR Green Master Mix (Applied Biosystems). Melting curve analyses were performed at the end of each run to ensure the specificity of the amplification. Each assay included negative controls with no template, and each sample was analyzed in duplicate. The standard curve (with at least five serial dilutions) method was used to evaluate expression of each gene and determine the primer efficiency. The mRNA abundance of target genes was normalized to the expression level of a reference gene [ribosomal protein L19 (*RPL19*)], according to the ΔΔCt method with correction for amplification efficiency of each primer pair (Pfaffl 2001, Abedini *et al.* 2015).

6.3.16. Serum metabolites and fatty acid composition

Serum NEFA (NEFA-C kit, Wako Chemicals USA Inc., Richmond, VA), β-hydroxybutyric acid (BHBA; Roche Diagnostics, Indianapolis, IN, USA) and glucose (P7119, Sigma-Aldrich, St. Louis, MO) were determined using commercially available kits in triplicate.

The intra- and inter-assay coefficients of variation for NEFA, BHBA and glucose were 1.49 and 3.32, 2.51 and 6.71, and 1.38 and 2.42%, respectively. Fatty acids in serum samples were extracted as per (Folch *et al.* 1957) and fatty acid composition was assessed by gas chromatography (Cruz-Hernandez *et al.* 2007).

6.3.17. Statistical analysis

The abundance of mRNA, serum metabolites and fatty acid profile as well as Experiment 1 results including total number of corpora lutea, anovulated follicles, embryos, blastocyst, unfertilized ova and degenerated embryos were analyzed using the MIXED procedure of SAS (version 9.3, 2011; SAS Institute Inc., Cary, NC). The statistical model included dietary treatment as the main effects.

In vitro embryo results (Experiment 2; proportion of cleaved embryo, blastocyst formation and early blastocyst) as well as fertilization rate and proportion of viable embryos (Experiment 1) were analyzed using the GENMOD procedure of SAS. The statistical model included type of serum (FLX or SUN) or dietary treatment (FLX or SUN) as main effect. Model specifications included a binomial distribution and logit link function. Results are reported as proportion or as mean ± SE. For all results probabilities ≤ 0.05 were considered significant, whereas $P > 0.05$ but < 0.1 was considered trends.

6.4. Results and discussion

Superovulatory response did not differ among dietary treatments based on number of CL, anovulated follicles and total ova/embryos collected (**Table 6.2**). Cows fed SUN tended ($P = 0.06$) to produce more transferable embryos (total number of grade 1 and 2 embryos) than those fed CAN, but it did not differ from those fed FLX. Cows fed SUN had fewer unfertilized ova

and, hence, greater fertilization rate than those fed either CAN or FLX. Previous findings indicated that a diet enriched in linoleic acid had either a positive (Cerri *et al.* 2009) or no (Thangavelu *et al.* 2007) effect on the fertilization rate in lactating dairy cows. Conversely, using an *in vitro* model, it was demonstrated that linoleic acid negatively influences oocyte maturation and subsequent blastocyst development (Marei *et al.* 2010), a response attenuated by adding antioxidants during the *in vitro* maturation (Khalil *et al.* 2013). Although these contradictory results could be associated with the status of animals (lactating vs. non-lactating) or the model used (*in vitro* vs. *in vivo*), the effect linoleic acid on bovine oocyte competence and fertilization rate remains controversial.

In Experiment 1, cows fed FLX had fewer degenerated embryos compared to cows fed either CAN or SUN. Feeding FLX also increased ($P < 0.05$) or tended to increase ($P < 0.10$) the proportion of viable embryos (the proportion of grade 1 and 2 embryo over total collected embryos) compared with CAN or SUN, respectively. Similarly, cows fed a diet supplemented with a partially rumen protected n-3 PUFA (high in eicosapentaenoic and docosahexaenoic acids) had a lower proportion of degenerated embryos compared to cows fed a diet supplemented with palmitic acid (Childs *et al.* 2008). It has also been reported that feeding cows a diet enriched in α-linolenic acid (flax seed oil) increased the presence of α-linolenic acid in collected oocytes and subsequently those oocytes had greater cleavage rate and tended to have higher blastocyst rate compared to those fed a diet enriched in saturated fatty acid (Moallem *et al.* 2013). Therefore, it is plausible that the reduced number of degenerated embryos in cows fed FLX resulted from enhanced oocyte developmental competence and embryo development.

Evaluation of the transcriptome profile of *in vivo* produced embryos collected from cows in Experiment 1 revealed that 175 genes were differentially expressed in blastocysts from cows fed

FLX compared with those from cows fed either SUN or CAN (**Figure 6.1**; FC > 2.0 and *P*-value < 0.05; **full list of genes provided in supplementary Table 6.2**). The functional analysis of IPA was used in order to have a better understanding of the functions of those differentially expressed genes. The list of biological functions influenced [BH-FDR corrected *P*-value (B-H *P*-value) < 0.05 with a –log (B-H *P*-value) > 2.0] by FLX vs. SUN, which includes cellular growth and proliferation, cellular development, lipid metabolism and molecular transport, is summarized in **Table 6.3.** Moreover, cell viability and survival functions were activated functions having Z-score > 2.0 (**Figure 6.2**). IPA analysis also identified 13 activated pathways based on differentially expressed genes (**Supplementary Table 6.3**).

Blastocysts collected from cows fed FLX had greater mRNA expression of claudin 4 (*CLDN4*) and solute carrier family 9, subfamily A, member 3 (*SLC9A3*) compared with those blastocysts collected from cows fed LA. It has been reported that *Cldn4* and *Cldn6* are essential for tight junctions and blastocyst development (Moriwaki *et al.* 2007). In this regard, when embryos were cultured with an inhibitor of *Cldn4* and *Cldn6* the development of normal blastocysts was remarkably reduced (Moriwaki *et al.* 2007). The solute carrier family 9 (SLC9A) [previously known Na^+/H^+ exchangers (NHEs)] consists of 5 members (SLC9A1, 2, 3, 4 and 5), which are located in the plasma membrane and involved in intracellular pH maintenance, cell volume regulation and transduction of signals that promote cell proliferation (Yun *et al.* 1995). It was found that the addition of specific SLC9A3 inhibitor to embryo cultures did not affect the embryonic development from 2-cell to morula stage but inhibited the formation of blastocyst in a concentration-dependent manner (Kawagishi *et al.* 2004).

Our results also indicated that blastocysts collected from cows fed FLX had higher mRNA expression of Keratin 19 (*KRT19*) and Lectin galactoside-binding soluble 3 (*LGALS3*) compared

to SUN. A previous study has shown that *Krt19* is essential for the integrity of trophoblast cells and embryo survival in mouse (Hesse *et al.* 2000). Several studies have also reported that LGALS3 is involved in cell proliferation, growth, adhesion and polarization (Liu *et al.* 2002, Friedrichs *et al.* 2007, Nangia-Makker *et al.* 2007) and, more importantly, in cell cycle progression and cell survival (Liu *et al.* 2002). Furthermore, it was 2.8 times more abundant at blastocyst stage compared to morula stage in bovine (Demant 2012) suggesting that LGALS3 is important for blastocyst development. Collectively, greater expression of genes involved in blastocyst development (*CLDN4* and *SLC9A3*), trophectoderm integrity (*KRT19*) and cell proliferation, growth and anti-apoptosis (*LGALS3*) in embryos collected from FLX fed cows compared to those fed SUN, likely enhanced and supported development of embryos which otherwise may have undergone degeneration.

Compared to those from cows fed with SUN, embryos from FLX-fed cows exhibited higher expression of the T-box 3 (*TBX3*) and Nanog homeobox (*NANOG*) genes, which are responsible for early embryonic cell differentiation and lineage segregation. The lack of proper cell differentiation during blastocyst development would result in embryonic death. Several transcription factors have been found being responsible for proper embryonic cell differentiation; for instance, CDX2 is considered as trophectoderm-specific marker at the blastocyst stage (Dietrich & Hiiragi 2007), whereas the pluripotency marker NANOG is specifically expressed in inner cell mass in bovine embryos (Kuijk *et al.* 2008). *Nanog*-null mice embryos fail to establish epiblast identity because of inner cell mass degeneration (Mitsui *et al.* 2003, Silva *et al.* 2009). Additionally, TBX3, a pluripotency-related transcription factor of the T-box gene family, is necessary for both self-renewal of mouse embryonic stem cells and for their differentiation into embryonic endoderm (Chapman *et al.* 1996). Among T-box family members, *Tbx3* is the earliest

expressed gene in mouse inner cell mass cells (Chapman *et al.* 1996) and its deletion results in embryonic lethality or deficiencies in the mammary gland, limbs, and yolk sac (Davenport *et al.* 2003). Therefore, it is suggested that the reduction in the proportion of degenerated embryos reported in cows fed a diet enriched in FLX is the result of a higher embryonic expression of *TBX3* and *NANOG* genes.

Feeding a diet containing FLX increased the blastocyst expression of transmembrane receptor Beta-2-microglobulin (*B2M*) and placenta-specific 8 (*PLAC8*) involved in cell survival. Recent studies have shown that B2M expression increased in embryos at blastocyst stage (Tanaka *et al.* 2005) and in endometrial tissue around the time of maternal recognition of pregnancy (Forde *et al.* 2011). The increased expression of B2M gene is positively correlated with the expression of blastocyst MHC class Ia which protects embryonic trophoblast cells from maternal NK cells (Tanaka *et al.* 2005), thus avoiding maternal rejection of the embryo (Lanier 1998). Interestingly, Ghanem *et al.* (2011) found that B2M expression was down-regulated in *in vivo* derived bovine embryos that resulted in no pregnancy or greater pregnancy loss relative to those embryos that resulted in pregnancies carried to term (Ghanem *et al.* 2011). Similarly, the expression of *PLAC8* gene was up-regulated (26-fold) in blastocyst biopsies obtained from embryos that resulted in full-term calves compared to those associated with pregnancy loss (El-Sayed *et al.* 2006), as well as in the endometrium of pregnant cows compared to non-pregnant ones (Galvez *et al.* 2003, Klein *et al.* 2006), suggesting a possible role of PLAC8 in placenta development and fetus maternal interface. Hence, increased expression of genes involved in maternal immunity modulation (*B2M*) and placenta development (*PLAC8*) in blastocysts collected from cows fed FLX compared to blastocysts obtained from cows fed SUN support previous findings regarding

reduced pregnancy loss in lactating cows fed a diet supplemented with flax seed (Ambrose *et al.* 2006, Petit & Twagiramungu 2006).

The IPA upstream regulator analysis predicts that cAMP responsive element binding protein 1 (CREB1) and transforming growth factor beta 1 (TGF-β1) are the upstream regulators of activated (or inhibited) genes by FLX vs. SUN (**Figure 6.3**). The CREB family consists of a family of transcription factors which support somatic cell survival and this has been most fully investigated in neuronal signalling (Walton & Dragunow 2000). FBJ murine osteosarcoma viral oncogene homolog (*FOS*) and prostaglandin-endoperoxide synthase 2 (*PTGS2*) are a part of CREB1 and TGF-β1 target genes, respectively. In Experiment 1, *FOS* and *PTGS2* mRNA expression was greater by 2.2- and 5.0-fold in blastocysts collected from cows fed FLX compared with SUN. FOS mainly regulates cell proliferation, differentiation, and survival (Hess *et al.* 2004) and is present in pre-implantation embryos of several species such as the cow, sheep, pig and mouse (Muller *et al.* 1982, Whyte & Stewart 1989, Pal *et al.* 1993, Xavier *et al.* 1997, Tetens *et al.* 2000). In knock-out experiments, *c-fos* deficient mice died just before birth, indicating the importance of FOS in fetal survival (Johnson *et al.* 1992). It has been suggested that in sheep and cows, conceptus-secreted prostaglandins modulate endometrial gene expression which subsequently lead to enhanced embryo elongation and maternal recognition of pregnancy (Dorniak *et al.* 2012, Spencer *et al.* 2013). Moreover, greater expression of *PTGS2* was detected in biopsies derived from blastocysts resulting in successful pregnancy and calf delivery (El-Sayed *et al.* 2006). *Ptgs2*-null mice have multiple reproductive impairments characterized by poor ovulation, reduced fertilization rates, and failure of implantation and decidualization, which are responsive to prostaglandin replacement (Dinchuk *et al.* 1995, Lim *et al.* 1997).

Our results indicate that sulfiredoxin 1 (*SRXN1*) and interleukin 1 receptor antagonist (*IL1RN*) are expressed more in blastocyst collected from cows fed FLX than SUN. In addition, the IPA upstream regulator analysis predicted that *SRXN1* and *IL1RN* are CREB1 and TGF-β1 target genes, respectively. SRXN1 eliminates reactive oxygen species (e. g. H_2O_2 and NO) and protects cells from apoptosis (Baek *et al.* 2012). Three members of interleukin-1 (IL-1) family, IL-1β, IL1RN, and IL-1 receptor type I, are present in oocytes and in all stages of human embryo (De los Santos *et al.* 1996). Exposure of bovine oocytes to a mixture of stearic, palmitic and oleic acid during *in vitro* maturation reduced the expression of *IL1RN* in formed blastocysts (Van Hoeck *et al.* 2015). Interestingly, in our study, *IL1RN* expression increased in embryos collected from cows fed a diet enriched in FLX. Collectively, these findings indicate that *IL1RN* expression in bovine embryos is affected by different type of fatty acids. However, further investigations are required to elucidate the exact function of *IL1RN* genes during bovine embryonic development.

Based on microarray analysis, six genes were validated by q-PCR in 3 biological replicates from *in vivo*-produced embryos in Experiment 1 (**Figure 6.4**). They confirmed the up-regulation of *LGALS3* in FLX compared to SUN embryos. Moreover, there was a tendency for a higher expression of *PTGS2*, *ANXA1* and *IL1RN* genes in embryos collected from cows fed FLX than SUN. Although greater expression of *KRT19* and *SRXN1* was apparent, the difference was not significant between FLX and SUN.

The addition of serum collected from animals of varying physiological states or different nutritional management to the *in vitro* culture medium could be an efficient approach to study effects of maternal conditions or nutritional status on early stage embryo development (Leroy *et al.* 2010, Oba *et al.* 2013). Using an *in vitro* model allowed us to evaluate how serum enriched in

specific fatty acids may affect embryo development, specifically during the post-fertilization period (Experiment 2). The analysis of serum composition indicated that dietary treatments altered the serum fatty acid profile; increasing the concentrations of α-linolenic and linoleic acids in the serum of cows fed FLX (high in α-linolenic acid) and SUN (high in linoleic acid), respectively (**Table 6.4)**. Moreover, the concentration of other primary serum metabolites did not differ between treatment groups (**Table 6.4)**.

Adding serum collected from cows fed FLX to the *in vitro* embryo culture medium did not affect cleavage rate (FLX: 78.74% vs. SUN: 77.95%; $P = 0.92$), total blastocyst rate, or developmental stage on Day 7 and 8 (**Table 6.5**). However, the *in vitro* produced blastocysts exposed to serum from cows fed FLX had greater mRNA expression of *ANXA1* (**Figure 6.5**). These results are in agreement with results from Experiment 1 where feeding FLX also increased the mRNA expression of *ANXA1* in *in-vivo* embryos by 2.5-fold compared to embryos collected from cows fed SUN. In addition, all annexin family members participate in various signalling pathways that lead to cell differentiation, migration, proliferation and maintenance of cellular calcium homeostasis (Gerke *et al.* 2005, Mussunoor & Murray 2008, Grewal & Enrich 2009, Grewal *et al.* 2010). Additionally, *ANXA1* had higher expression in bovine *in vivo*-produced 16-cell embryos compared to *in vitro*-produced 16-cell embryos (Gad *et al.* 2012). Adding serum from cows fed FLX in the culture media tended to increase the mRNA expression of *IFNT* in *in vitro* produced embryos. Interferon-tau is a type I interferon synthesized and secreted by the trophectoderm of the blastocysts and is required for maternal recognition of pregnancy in ruminant species (Roberts *et al.* 1992, Bazer *et al.* 1997). In summary, adding serum collected from cows fed FLX compared to SUN increased the expression of genes responsible for cell

proliferation and differentiation (*ANXA1*) and maternal pregnancy recognition (*IFNT*) in Day-8 embryos *in vitro* without affecting their morphological development.

Our results provide novel information on how feeding a diet enriched in α-linolenic acid (flaxseed) reduces the number of degenerated embryos. The addition to *in vitro* embryo culture of serum collected from cows fed a diet enriched in α-linolenic (flax seed) or linoleic (sunflower seed) acid did not affect embryo development. Therefore, we conclude that the reduced number of degenerated embryos seen in cows fed a diet enriched in α-linolenic acid most probably resulted from either enhanced oocyte competence or improved maternal-embryo crosstalk at the oviductal/uterine level. It is therefore plausible that reduced pregnancy losses previously reported (Ambrose *et al.* 2006, Petit & Twagiramungu 2006) in cows fed diets enriched in n-3 PUFA occurred through improved embryo survival.

6.5. Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

6.6. Funding

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6.7. Acknowledgments

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Table 6.1. Crude protein, fat content and fatty acid profile of oilseeds added to experimental diets.

	Oilseeds		
	Flax seed	Sunflower seed	Canola seed
Crude protein (%)	24.01	18.10	22.66
Crude fat (%)	42.04	43.32	45.19
Fatty acid profile of oilseeds (g/100g of total fatty acids)			
Palmitic acid	5.41	6.80	4.08
Stearic acid	3.72	5.29	1.80
Oleic acid	18.04	12.47	61.18
Linoleic acid	15.37	73.13	18.84
α-Linolenic acid	56.52	0.71	9.62
n-6:n-3 ratio	0.27	10.3	1.95
Total SFA*	9.57	13.55	7.175
Total MUFA*	18.11	12.63	63.79
Total PUFA*	71.91	73.84	28.50
PUFA:SFA ratio	7.51	5.45	3.97

*Abbreviations: SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Table 6.2. Superovulatory response and embryo production1 in cows fed diets supplemented with flax (FLX), sunflower (SUN) or canola (CAN) seed.

	Dietary treatment		
	FLX	SUN	CAN
Corpora lutea (n)	13.1 ± 1.5	14.5 ± 1.2	12.8 ± 2.6
Anovulated follicles (n)	2.6 ± 0.9	2.0 ± 1.1	3.0 ± 1.4
Total ova/embryos (n)	7.3 ± 1.2	8.6 ± 1.7	7.5 ± 2.0
Transferable embryo (n)	4.8 ± 1.3^{cd}	6.1 ± 1.7^{c}	4.3 ± 1.5^{d}
Unfertilized (n)	1.8 ± 0.9^{b}	0.6 ± 0.5^{a}	1.3 ± 0.6^{b}
Degenerated (n)	0.7 ± 0.4^{a}	1.9 ± 0.5^{b}	1.9 ± 0.6^{b}
Fertilization rate (%)	75.3^{b}	93.0^{a}	82.7^{b}
Proportion of viable embryos (%)	$87.3^{a,c}$	$76.3^{ab,d}$	69.4^{b}

Within a row [a, b] *P*-value ≤ 0.05; [c, d] *P*-value < 0.10

[1]Embryos was collected from a total of 35 embryo collections (FLX, n= 12; SUN, n= 11; and CAN, n=12).

Table 6.3. List of biological functions based on differentially expressed genes in Day 7.5 embryos collected from cows fed diets supplemented with flax seed (FLX) vs. sunflower seed (SUN).

Category	B-H p-value	Molecules
Cellular Growth and Proliferation	2.33E-03-3.65E-02	B2M, ID2, LGALS3, GADD45B, EMILIN2, XDH, HMGN1, NR3C1, XRCC2, CLDN4, TIMP1, ANXA1, CXCL14, IFI30, MGLL, IKZF1, ABCG2, ULBP1, TDGF1, PLCL2, EFNA1, FOS, IL1RN, KRT19, BTG2, ZAP70, EDN3, PTGS2, GLDC, SFN
Cellular Development	2.33E-03-3.65E-02	B2M, ID2, GADD45B, LGALS3, XDH, HMGN1, CD8B, NR3C1, CLDN4, TIMP1, ANXA1, CXCL14, GIMAP4, PLAC8, TBX3, IKZF1, ABCG2, ULBP1, TDGF1, SCIN, PLCL2, EFNA1, UBD, FOS, IL1RN, ZAP70, BTG2, EDN3, PTGS2, SFN, GLDC
Lipid Metabolism	3.45E-03-3.65E-02	SCIMP, LIPH, LTA4H, LGALS3, XDH, ABCG2, HMGN1, PLA2R1, SLC34A2, NR3C1, FOS, MGAT4A, MGST2, IL1RN, TIMP1, ANXA1, CXCL14, MGLL, EDN3, PTGS2
Molecular Transport	3.45E-03-3.65E-02	SCIMP, LTA4H, LGALS3, XDH, ABCG2, HMGN1, PLA2R1, SLC34A2, NR3C1,

		GSTO1, FOS, SLC23A1, MGAT4A, IL1RN, TIMP1, ANXA1, CXCL14, MGLL, EDN3, PTGS2
Small Molecule Biochemistry	3.45E-03-3.65E-02	SCIMP, LTA4H, DPYS, LGALS3, XDH, PLA2R1, HMGN1, HSD17B11, PFKL, NR3C1, GFPT2, SLC23A1, TIMP1, ANXA1, CXCL14, MGLL, LIPH, ABCG2, SLC34A2, GSTO1, FOS, MGAT4A, MGST2, IL1RN, GCSH, PTGS2, EDN3, GLDC
Amino Acid Metabolism	3.88E-03-3.02E-02	GCSH, GLDC
Post-Translational Modification	3.88E-03-3.16E-02	GCSH, BTG2, AS3MT, GLDC
Cell Death and Survival	4.82E-03-3.65E-02	B2M, ID2, EMILIN2, GADD45B, LGALS3, XDH, HMGN1, AS3MT, PTPN5, CAMK2N2, NR3C1, SCARB2, XRCC2, CLDN4, TIMP1, PRSS8, ANXA1, ZNF280B, MAP1LC3A, CALB1, GIMAP4, HEBP2, PLAC8, CCBL1, TBX3, IKZF1, ABCG2, ULBP1, TDGF1, ANXA4, SCIN, SRXN1, EFNA1, UBD, FOS, IL1RN, KRT19, ZAP70, BTG2, PTGS2, SFN
Cell Cycle	5.76E-03-3.65E-02	ID2, GADD45B, LGALS3, TBX3, ABCG2, IKZF1, HMGN1, ORC6, CAMK2N2, NR3C1,

		UBD, XRCC2, FOS, TACC2, TIMP1, KRT19, ZAP70, BTG2, EDN3, PTGS2, SFN, PLAC8
Nucleic Acid Metabolism	6.03E-03-3.65E-02	DPYS, XDH, ABCG2, GLDC
Cellular Assembly and Organization	9E-03-3.65E-02	UBD, LGALS3, CLDN4, IKZF1, KRT19, XDH, HMGN1, SFN, NR3C1
Cellular Function and Maintenance	9E-03-3.65E-02	B2M, ID2, LGALS3, GADD45B, XDH, HMGN1, CD8B, NR3C1, CLDN4, ANXA1, IFI30, MAP1LC3A, CALB1, DSC2, GIMAP4, PLAC8, IKZF1, ABCG2, SLC34A2, PLCL2, SRXN1, EFNA1, UBD, FOS, IL1RN, ZAP70, EDN3, PTGS2
DNA Replication, Recombination, and Repair	9E-03-3.65E-02	UBD, XRCC2, IKZF1, SFN, GLDC, NR3C1

Table 6.4. The concentrations of metabolites and fatty acid profile of serum added to bovine in vitro embryo culture media.

	Treatments		
	FLX[1]	SUN[2]	*P*-value
NEFA*	143.90 ± 18.62	105.66 ± 18.62	0.18
BHBA*	11.51 ± 1.32	10.12 ± 1.32	0.48
Glucose	60.65 ± 1.78	61.86 ± 1.78	0.64
Fatty acid profile of serum (g/100g of total fatty acids)			
Palmitic acid	11.86 ± 0.80	11.76 ± 0.80	0.93
Stearic acid	22.88 ± 1.06	22.19 ± 1.06	0.65
Oleic acid	9.85 ± 1.02	9.79 ± 0.91	0.96
Linoleic acid	32.13 ± 2.75	41.63 ± 2.46	0.04
α-linolenic acid	17.69 ± 1.57	6.08 ± 1.41	0.002
Total n-6 fatty acids	33.17 ± 2.33	44.14 ± 2.09	0.02
Total n-3 fatty acids	20.37 ± 2.17	10.89 ± 1.94	0.01
n-6:n-3 ratio	1.65 ± 1.34	5.35 ± 1.20	0.04
Total SFA*	34.87 ± 1.56	34.69 ± 1.39	0.92
Total MUFA*	11.46 ± 1.65	9.79 ± 1.47	0.55
Total PUFA*	53.55 ± 1.89	55.03 ± 1.69	0.68
PUFA:SFA ratio	0.86 ± 0.22	0.85 ± 0.20	0.96

[1]Serum collected from cows fed a diet supplemented with flax seed.

[2]Serum collected from cows fed a diet supplemented with sunflower seed.

*Abbreviations: NEFA: non-esterified fatty acid; BHBA: β-hydroxybutyric acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Table 6.5. Effect of serum collected from cows fed a diet supplemented with flaxseed (FLX) or sunflower (SUN) on *in vitro* embryo development by Day 7 and 8.

	Day 7[1]			Day 8[1]		
	FLX	SUN	P-value	FLX	SUN	P-value
Total zygotes, n[2]	271	239	-	271	239	-
Total blastocyst from cleaved zygotes, n (%)	42 (15.49)	37 (15.48)	0.99	63 (23.24)	60 (25.10)	0.62
Early blastocyst from cleaved zygotes, n (%)	6 (14.28)	2 (5.40)	0.20	4 (6.34)	0 (0.00)	0.44
Blastocyst from cleaved zygotes, n (%)	36 (85.71)	35 (94.59)	0.92	59 (93.65)	60 (100.00)	0.44

[1]Day 0 = fertilization; [2]These data relate to 5 different batches of ovaries collected from slaughterhouse.

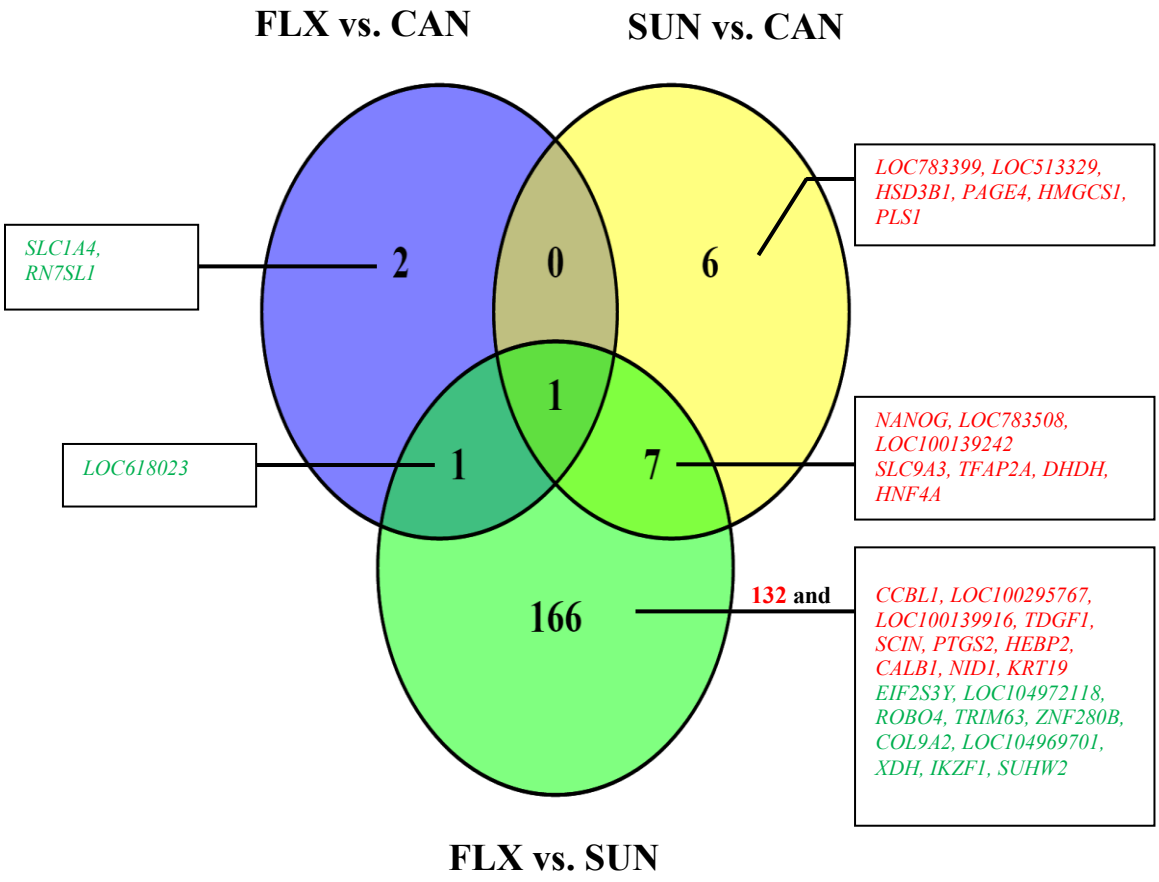

Figure 6.1. Number of differentially expressed genes (≥ 2.0 fold changes with a P-value ≤ 0.05) in embryo (Day 7.5) collected from cows fed a diet supplemented with flax (FLX), sunflower (SUN) or canola (CAN) seed (Experiment 1).

The 175 differentially expressed genes used for further functional analysis with Ingenuity Pathway Analysis. Gene names appearing in red and green indicate up- and down-regulated genes, respectively.

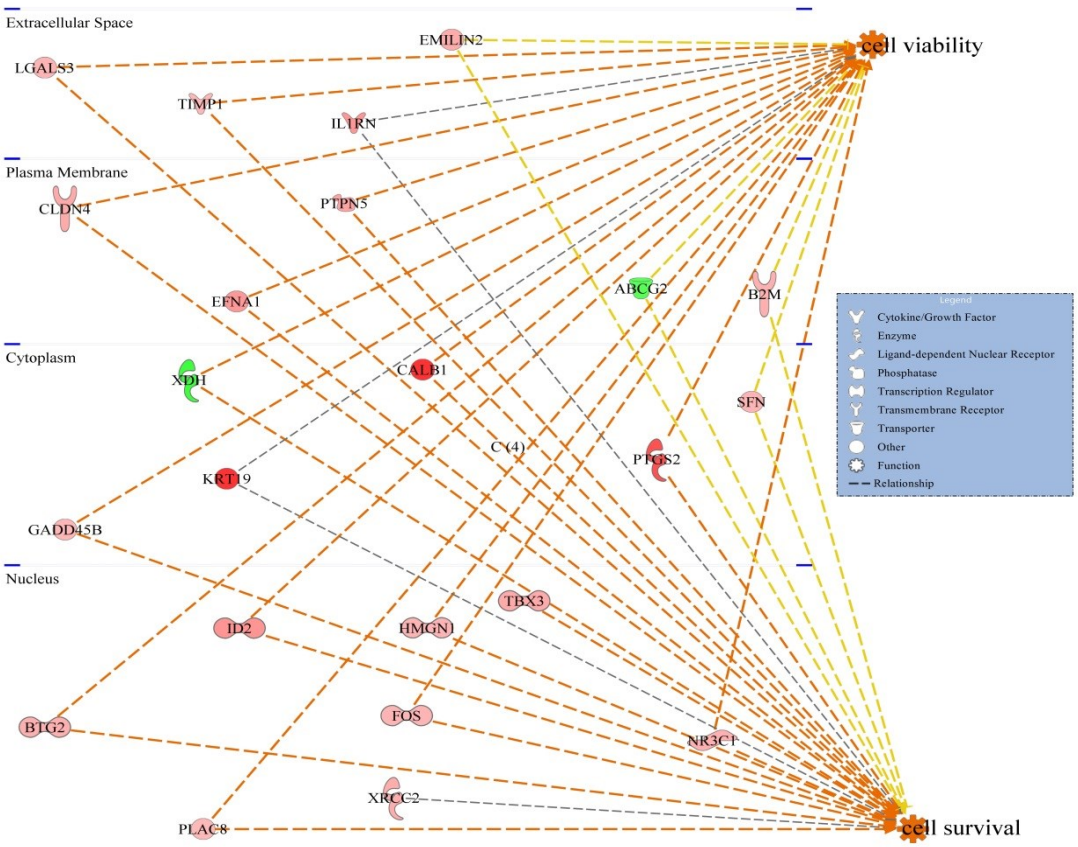

Figure 6.2. Biological function analysis using Ingenuity Pathway Analysis software[1]. Genes involved in cell viability and survival (Z-score > 2.0) functions that are differentially expressed in embryos (Day 7.5) collected from cows fed a diet supplemented with flax (FLX) compared with those collected from cows fed sunflower (SUN) seed (Experiment 1). Up- or down-regulated genes involved in these functions are indicated with red and green symbols, respectively.

[1]The functional analyses were generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity).

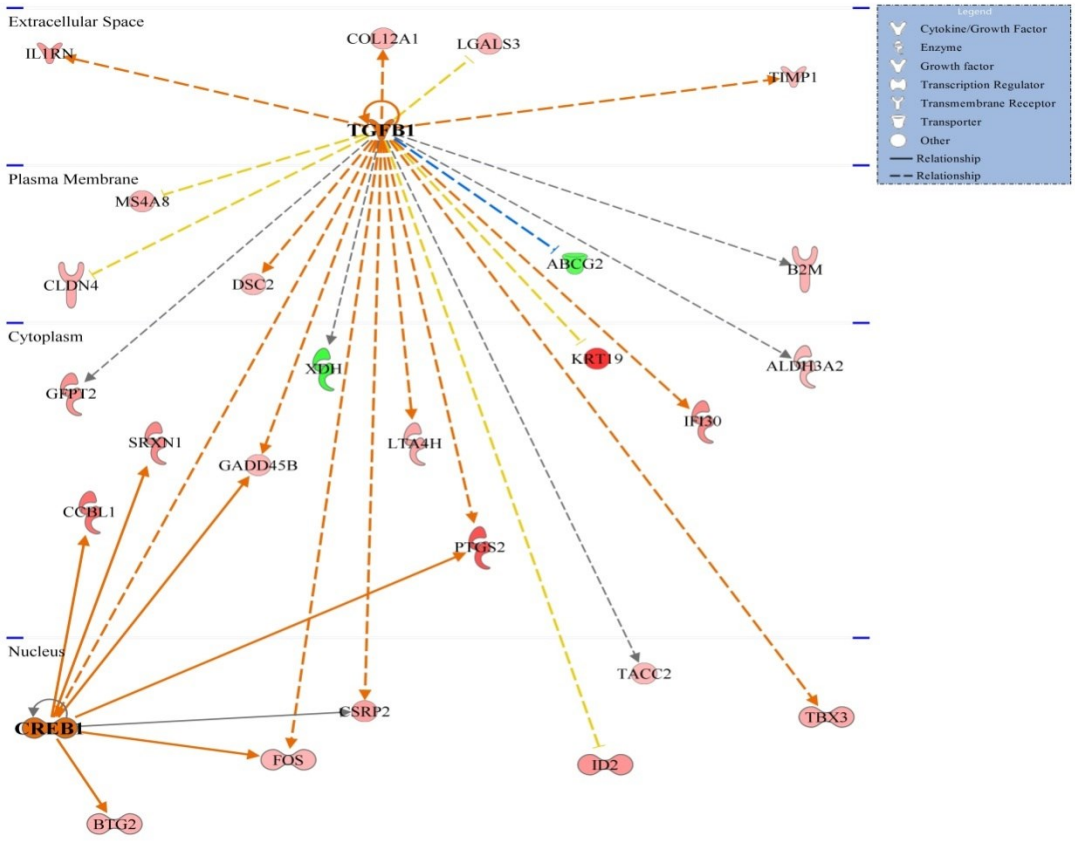

Figure 6.3. Upstream regulator of differentially expressed genes[1].

cAMP responsive element binding protein 1 (CREB1; transcription factor) and transforming growth factor beta 1 (TGF-β1; growth factors) are the upstream regulator of differentially expressed genes in embryos (Day 7.5) collected from cows fed a diet supplemented with flax (FLX) compared with those collected from sunflower (SUN) seed (Experiment 1). Up- or down-regulated genes are indicated with red and green symbols, respectively. Direct or indirect relationships between molecules are indicated by solid or dashed connecting lines, respectively.

[1]The upstream regulator network was generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity).

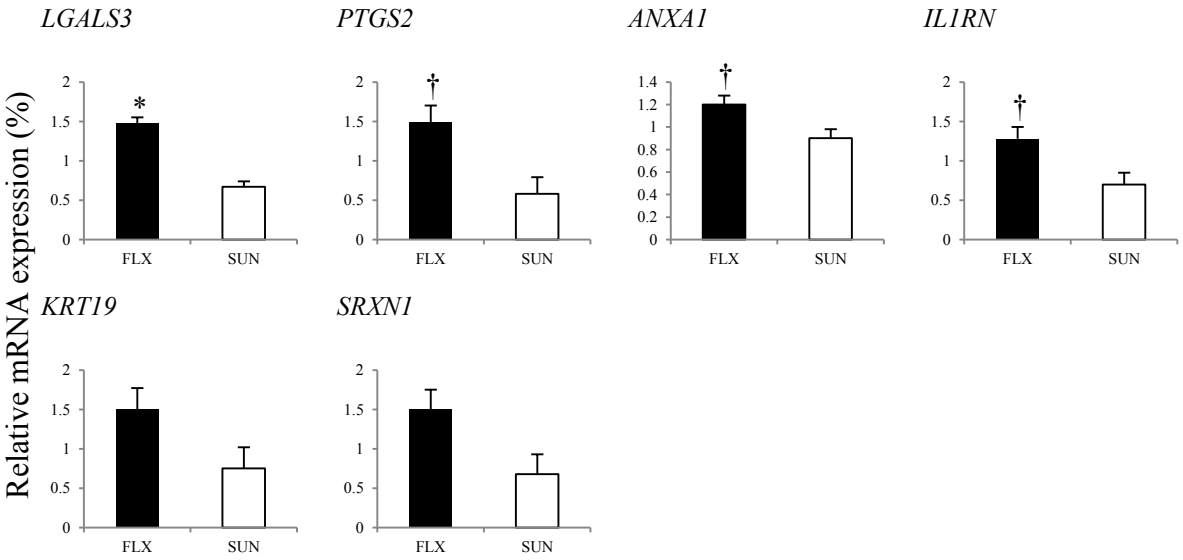

Figure 6.4. QPCR confirmation of microarray results in embryos (Day 7.5) collected from cows fed a diet supplemented with flax (FLX) or sunflower (SUN) seed (Experiment 1).

$^{*}P < 0.05$; $^{\dagger}P < 0.10$.

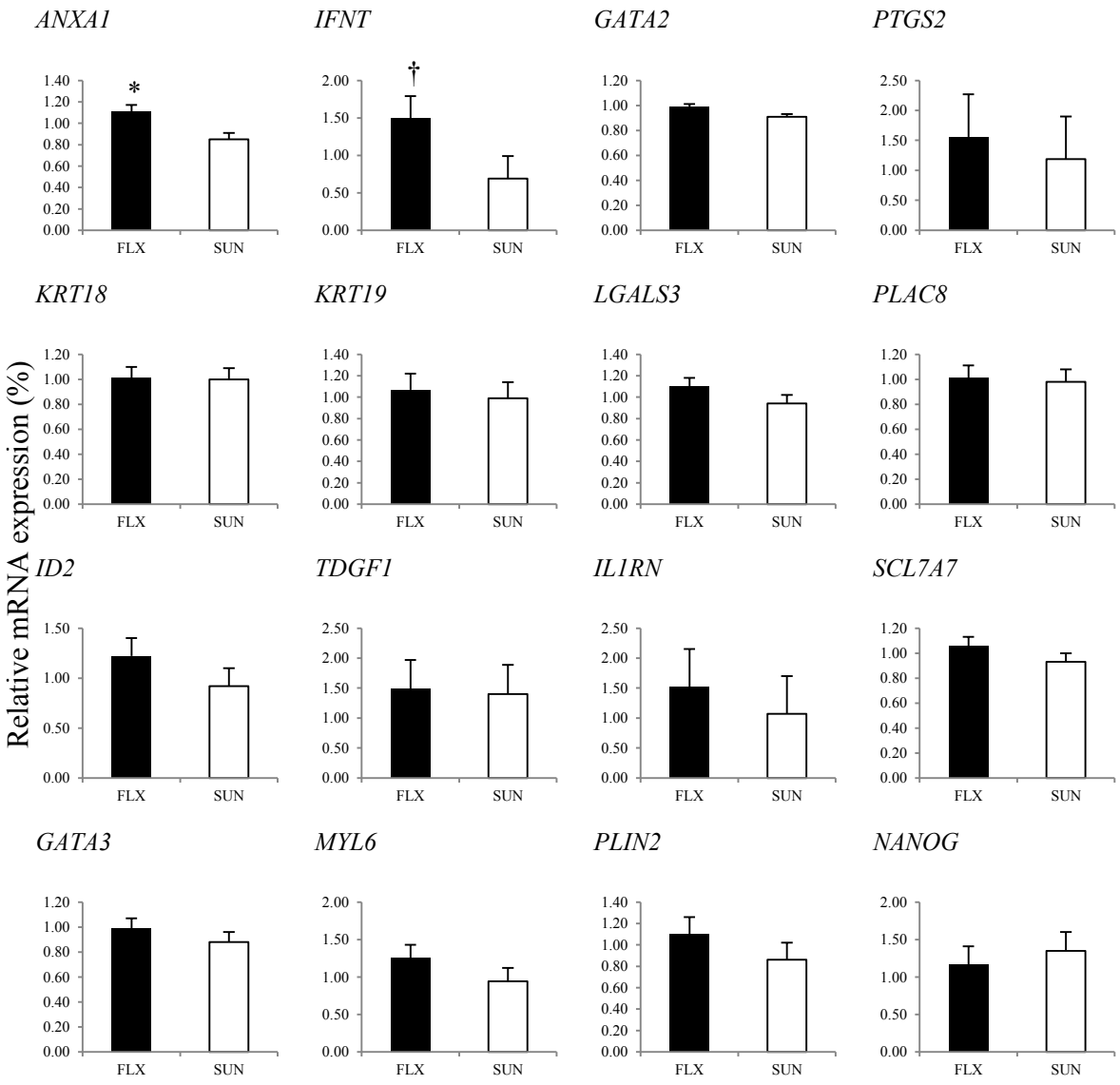

Figure 6.5. Effect of serum collected from cows fed a diet supplemented with flaxseed (FLX) or sunflower (SUN) on mRNA expression of selected genes in Day 8 *in vitro*-produced embryos (Experiment 2).

$^{*}P < 0.05$; $^{\dagger}P < 0.10$.

Supplementary Table 6.1. Primer sequences for quantitative real-time polymerase chain reaction.

Gene name	Primer	Primer sequence (5'-3')
PTGS2	Forward	CTCTGTCTTACTGGAACATGGTC
	Reverse	GATACTTTCTCTACTGCGACTGG
LGALS3	Forward	TTATGACCTGCCTTTTCCTGG
	Reverse	AGCGTGGGTTAAAGTGGAAG
ANXA1	Forward	AGGTGACATCGAGAAATGCC
	Reverse	TCAGAGCGGGAAACCATAATC
IL1RN	Forward	CCAATTAGTTGCTGGATACTTGC
	Reverse	TCTCCAGATTTTACGCAGGC
PLIN2	Forward	ACAACACACCCCTCAACTGG
	Reverse	CTGCCTGCCTACTTCAGACC
KRT18	Forward	GCAGACCGCTGAGATAGGA
	Reverse	GCATATCGGGCCTCCACTT
MYL6	Forward	CTGAGATCCGGCACGTTCTC
	Reverse	GATATGCCTCACAAACGCTTCA
SLC7A7	Forward	GCAGGCATTGTTAGACTTGG
	Reverse	TTCAGTGACATAGTTGAGGGTG
PLAC8	Forward	TTCACAGCCAGGTTACAGC
	Reverse	TGACATGAAAGGCACAGGG
TDGF1	Forward	ACCAGCCTTCCCAATTTGTAG
	Reverse	AGTTGCGTCCATAGAAAGAGG

IFNT	Forward	GATCCTTCTGGAGCTGGCTG
	Reverse	GCCCGAATGAACAGACTCCC
GATA2	Forward	CACCTGTTGCGCAAACTGTC
	Reverse	CGGGTCTGGATGCCTTCCTT
GATA3	Forward	CTACCACAAGATGAACGGACAG
	Reverse	AGGGTCTCCATTGGCATTTC
NANOG	Forward	CTCGCAGACCCAGCTGTGTG
	Reverse	CCCTGAGGCATGCCATTGCT
ID2	Forward	AGGCTTCTGAATTCCCTTCTG
	Reverse	GTTCAGCGCCTTAAAAGAGC
KRT19	Forward	CGACTACAGCCACTACTTCAAG
	Reverse	TGCTCCGTCTCAAACTTGG
RPL19	Forward	TCGATGCCGGAAAAACAC
	Reverse	ATTCTCATCCTCCTCATCCAG

Supplementary Table 6.2. Probe ID, gene symbol, gene description and fold change of differentially expressed genes with ≥2-fold difference and P-value ≤ 0.05 in embryo (Day 7.5) collected from cows fed a diet supplemented with flax (FLX), sunflower (SUN) or canola (CAN) seed (Experiment 1).

Probe ID	Gene_Symbol	Description	Fold change
EMBV3_09735	A2ML1	alpha-2-macroglobulin-like 1"	2.4
EMBV3_41955	ABCG2	ATP-binding cassette"	-2.2
EMBV3_35035	ABHD14B	abhydrolase domain containing 14B"	2.6
EMBV3_29073	AGPAT5	1-acylglycerol-3-phosphate O-acyltransferase 5 (lysophosphatidic acid acyltransferase"	-2.3
EMBV3_16614	AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9"	2.3
EMBV3_13650	AGXT2L1	alanine-glyoxylate aminotransferase 2-like 1"	-2.3
EMBV3_15390	AIM1L	absent in melanoma 1-like"	2.0
EMBV3_01265	AKR1B1	aldo-keto reductase family 1"	2.6
EMBV3_19607	ALDH3A2	aldehyde dehydrogenase 3 family"	2.1
EMBV3_13791	ANXA1	annexin A1"	2.5
EMBV3_26384	ANXA3	annexin A3"	3.4
EMBV3_37951	ANXA4	annexin A4"	2.4
EMBV3_04716	AS3MT	arsenic (+3 oxidation state)	2.3

		methyltransferase"	
EMBV3_10132	ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1"	2.0
EMBV3_02360	AUTS2	autism susceptibility candidate 2"	-2.5
EMBV3_03050	B2M	beta-2-microglobulin"	2.3
EMBV3_41900	BDH2	MGC127950 3-hydroxybutyrate dehydrogenase"	3.0
EMBV3_16838	BTG2	BTG family"	2.3
EMBV3_39486	CALB1	calbindin 1"	6.0
EMBV3_40130	CALML5	calmodulin-like 5"	2.2
EMBV3_03657	CAMK2N2	calcium/calmodulin-dependent protein kinase II inhibitor 2"	2.5
EMBV3_03790	CCBL1	cysteine conjugate-beta lyase"	4.1
EMBV3_33454	CD8B	CD8b molecule"	3.1
EMBV3_29550	CDH24	cadherin 24"	2.2
EMBV3_22731	CHPT1	choline phosphotransferase 1"	2.8
EMBV3_03807	CLDN23	claudin 23"	3.3
EMBV3_33550	CLDN4	claudin 4"	2.4
EMBV3_19796	COL12A1	collagen"	2.2
EMBV3_23715	COL9A2	collagen"	-2.9
EMBV3_06120	CSRP2	cysteine and glycine-rich protein 2"	2.7
EMBV3_08113	CXCL14	chemokine (C-X-C motif) ligand 14"	-2.2

EMBV3_21301	DENND5A	DENN/MADD domain containing 5A"	-2.2
EMBV3_17175	DNAJC15	DnaJ (Hsp40) homolog"	-2.3
EMBV3_08476	DPYS	dihydropyrimidinase"	3.3
EMBV3_15542	DSC2	PREDICTED: Bos taurus desmocollin 2 (DSC2), transcript variant X1, mRNA	2.1
EMBV3_21599	EDN3	endothelin 3"	3.8
EMBV3_16476	EFNA1	ephrin-A1"	3.0
EMBV3_39949	EIF2S3Y	PREDICTED: Bos taurus eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked (EIF2S3Y), mRNA	-9.3
EMBV3_19918	EMILIN2	elastin microfibril interfacer 2"	2.4
EMBV3_25213	ENPP3	ectonucleotide pyrophosphatase/phosphodiesterase 3"	3.5
EMBV3_22974	EPS8L1	EPS8-like 1"	2.2
EMBV3_34432	FAM127A	family with sequence similarity 127"	2.1
EMBV3_11265	FOLH1	folate hydrolase (prostate-specific membrane antigen) 1"	2.1
EMBV3_35481	FOS	FBJ murine osteosarcoma viral	2.2

		oncogene homolog"	
EMBV3_36326	FOXRED2	PREDICTED: Bos taurus FAD-dependent oxidoreductase domain containing 2 (FOXRED2), transcript variant X1, mRNA	2.1
EMBV3_15729	FSIP2	PREDICTED: Bos taurus fibrous sheath interacting protein 2 (FSIP2), transcript variant X3, mRNA	2.4
EMBV3_09789	GADD45B	growth arrest and DNA-damage-inducible"	2.1
EMBV3_15661	GCSH	glycine cleavage system protein H (aminomethyl carrier)"	-2.6
EMBV3_22338	GFPT2	glutamine-fructose-6-phosphate transaminase 2"	3.2
EMBV3_18090	GIMAP4	GTPase"	3.2
EMBV3_31163	GIPC2	GIPC PDZ domain containing family"	3.0
EMBV3_31391	GLDC	glycine dehydrogenase (decarboxylating)"	-2.6
EMBV3_15659	GSTO1	glutathione S-transferase omega 1"	2.1
EMBV3_30615	HEBP2	heme binding protein 2"	5.7
EMBV3_41588	HERC5	hect domain and RLD 5"	2.6
EMBV3_15385	HMGN1	high-mobility group nucleosome	2.1

		binding domain 1"	
EMBV3_43318	HSD17B11	hydroxysteroid (17-beta) dehydrogenase 11"	2.3
EMBV3_35871	ID2	inhibitor of DNA binding 2"	3.1
EMBV3_12009	IFI30	gamma-inducible protein 30"	2.5
EMBV3_36905	IKZF1	PREDICTED: Bos taurus IKAROS family zinc finger 1 (Ikaros) (IKZF1), transcript variant X5, mRNA	-2.6
EMBV3_25244	IL1RN	interleukin 1 receptor antagonist"	3.0
EMBV3_08592	KCTD1	potassium channel tetramerisation domain containing 1"	-2.1
EMBV3_14322	KIAA0930	PREDICTED: Bos taurus KIAA0930 ortholog (KIAA0930), transcript variant X1, mRNA	2.2
EMBV3_34552	KLK7	kallikrein-related peptidase 7"	2.5
EMBV3_20772	KRT19	keratin 19"	8.8
EMBV3_36484	LGALS3	lectin"	2.1
EMBV3_07513	LIPH	lipase"	2.3
EMBV3_27821	LOC100139916	similar to interleukin 32"	4.3
EMBV3_26631	LOC100141258	similar to Interleukin-32 precursor (IL-32) (Natural killer cells protein 4) (Tumor necrosis factor alpha-	2.2

		inducing factor)"	
EMBV3_41455	LOC100295767	hypothetical protein LOC100295767"	4.2
EMBV3_05935	LOC100296035	similar to defensin"	-2.1
EMBV3_24500	LOC100301020	similar to cytochrome c oxidase subunit I"	2.1
EMBV3_41706	LOC100301215	similar to tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)"	2.3
EMBV3_30027	LOC100336840	hypothetical protein LOC100336840"	2.4
EMBV3_10009	LOC100336976	glycine cleavage system H protein"	-2.4
EMBV3_18054	LOC100337018	hypothetical protein LOC100337018"	2.5
EMBV3_07711	LOC100851524	PREDICTED: Bos taurus netrin receptor UNC5B-like (LOC100851524), partial mRNA	2.1
EMBV3_20097	LOC101904472	PREDICTED: Bos taurus uncharacterized LOC101904472 (LOC101904472), ncRNA	2.8
EMBV3_16273	LOC101906690	PREDICTED: Bos taurus uncharacterized LOC101906690 (LOC101906690), transcript variant	3.9

		X1, ncRNA	
EMBV3_23559	LOC104969701	PREDICTED: Bos taurus zinc finger protein 280B-like (LOC104969701), mRNA	-2.7
EMBV3_14404	LOC104972086	PREDICTED: Bos taurus uncharacterized LOC104972086 (LOC104972086), ncRNA	3.1
EMBV3_37702	LOC104972118	PREDICTED: Bos taurus uncharacterized LOC104972118 (LOC104972118), transcript variant X1, ncRNA	-3.5
EMBV3_22394	LOC504800	similar to Apolipoprotein B48 receptor"	-2.1
EMBV3_38575	LOC504995	similar to growth differentiation factor 3"	3.0
EMBV3_23990	LOC505800	similar to Interleukin-32 precursor (IL-32) (Natural killer cells protein 4) (Tumor necrosis factor alpha-inducing factor)"	3.4
EMBV3_38874	LOC511937	PREDICTED: Bos taurus annexin A1 pseudogene (LOC511937), misc_RNA	2.2
EMBV3_14504	LOC514078	similar to Myosin-Vb"	2.1

EMBV3_20544	LOC515333	PREDICTED: Bos taurus multidrug resistance-associated protein 4 (LOC515333), transcript variant X1, mRNA	2.2
EMBV3_05008	LOC539596	similar to Transforming growth factor-beta induced protein IG-H3 precursor (Beta IG-H3) (Kerato-epithelin) (RGD-containing collagen associated protein) (RGD-CAP)"	-2.4
EMBV3_02462	LOC613274	hypothetical protein LOC613274"	2.1
EMBV3_08783	LOC616199	similar to Aldose reductase (AR) (Aldehyde reductase) (20-alpha-hydroxysteroid dehydrogenase) (20-alpha-HSD)"	2.6
EMBV3_11029	LOC616956	similar to Ras-related protein Rab-17"	2.5
EMBV3_08932	LOC781039	similar to pericardine"	2.6
EMBV3_42370	LOC783343	similar to ULBP27"	2.6
EMBV3_05432	LOC783680	PREDICTED: Bos taurus beta-2-microglobulin (LOC783680), mRNA	2.2
EMBV3_11190	LOC783730	similar to family with sequence	2.2

		similarity 127"	
EMBV3_22442	LOC784769	similar to MGC127725 protein"	-2.0
EMBV3_39144	LOC786521	similar to growth differentiation factor 3"	2.7
EMBV3_32072	LOC787129	similar to Glycine cleavage system H protein"	-2.6
EMBV3_18132	LPLUNC1	MGC142624 von Ebner minor salivary gland protein"	2.1
EMBV3_31650	LTA4H	leukotriene A4 hydrolase"	2.6
EMBV3_34525	LY6G6C	lymphocyte antigen 6 complex"	2.9
EMBV3_38619	MAP1LC3A	microtubule-associated protein 1 light chain 3 alpha"	2.4
EMBV3_02885	MFF	C2orf33 MGC127725 mitochondrial fission factor"	-2.2
EMBV3_35590	MGAT4A	mannosyl (alpha-1"	2.5
EMBV3_26024	MGLL	monoglyceride lipase"	2.0
EMBV3_39533	MGST2	microsomal glutathione S-transferase 2"	2.1
EMBV3_15047	MPV17L2	Bos taurus MPV17 mitochondrial membrane protein-like 2 (MPV17L2), mRNA	2.2
EMBV3_23885	MS4A8B	membrane-spanning 4-domains"	2.3
EMBV3_21955	NID1	nidogen 1"	6.3

EMBV3_25297	NIPSNAP3A	nipsnap homolog 3A (C. elegans)"	2.1
EMBV3_33627	NME7	non-metastatic cells 7"	2.1
EMBV3_40204	NR3C1	nuclear receptor subfamily 3"	2.3
EMBV3_15333	ORC6L	origin recognition complex"	2.0
EMBV3_03901	OSBPL6	oxysterol binding protein-like 6"	2.1
EMBV3_27275	PFKL	phosphofructokinase"	2.0
EMBV3_05556	PLA2R1	phospholipase A2 receptor 1"	2.1
EMBV3_41904	PLAC8	placenta-specific 8"	2.0
EMBV3_27122	PLCL2	phospholipase C-like 2"	-2.1
EMBV3_00706	PLLP	plasma membrane proteolipid (plasmolipin)"	2.3
EMBV3_22986	PPM1K	protein phosphatase 1K (PP2C domain containing)"	-2.3
EMBV3_03766	PQLC3	PQ loop repeat containing 3"	3.0
EMBV3_33907	PRSS8	protease"	3.1
EMBV3_40343	PSAT1	phosphoserine aminotransferase 1"	2.6
EMBV3_34931	PTGR1	MGC127457 prostaglandin reductase 1"	2.1
EMBV3_16391	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)"	5.0
EMBV3_40038	PTPN5	protein tyrosine phosphatase"	2.6
EMBV3_10555	ROBO4	MGC160058 roundabout homolog	-3.5

		4"	
EMBV3_22627	SCARB2	scavenger receptor class B"	4.0
EMBV3_20733	SCIMP	PREDICTED: Bos taurus SLP adaptor and CSK interacting membrane protein (SCIMP), transcript variant X3, mRNA	-2.0
EMBV3_29816	SCIN	scinderin"	4.6
EMBV3_10917	septin 10	septin 10"	2.2
EMBV3_34532	SFN	stratifin"	2.1
EMBV3_19159	SH3BGRL	SH3 domain binding glutamic acid-rich protein like"	2.6
EMBV3_00793	SLC23A1	similar to solute carrier family 23 (nucleobase transporters)"	2.9
EMBV3_39345	SLC25A12	solute carrier family 25 (mitochondrial carrier"	2.8
EMBV3_38150	SLC34A2	solute carrier family 34 (sodium phosphate)"	2.1
EMBV3_25352	SLC7A7	solute carrier family 7 (cationic amino acid transporter"	2.5
EMBV3_07643	SMPDL3A	sphingomyelin phosphodiesterase"	2.0
EMBV3_24134	SORL1	sortilin-related receptor"	2.0
EMBV3_30354	SRXN1	sulfiredoxin 1 homolog (S. cerevisiae)"	3.4

EMBV3_36703	SUHW2	suppressor of hairy wing homolog 2"	-2.6
EMBV3_17695	SUSD2	sushi domain containing 2"	2.6
EMBV3_03801	TACC2	transforming"	2.0
EMBV3_18954	TBX3	T-box 3"	2.4
EMBV3_18825	TDGF1	teratocarcinoma-derived growth factor 1"	4.6
EMBV3_17854	THEM4	thioesterase superfamily member 4"	2.8
EMBV3_28952	TIMP1	TIMP metallopeptidase inhibitor 1"	2.0
EMBV3_40419	TMEM125	transmembrane protein 125"	2.2
EMBV3_13397	TMEM45B	transmembrane protein 45B"	2.1
EMBV3_13588	TMEM5	transmembrane protein 5"	2.7
EMBV3_15487	TMEM50B	transmembrane protein 50B"	2.3
EMBV3_20420	TMEM52	transmembrane protein 52"	2.1
EMBV3_10091	TPI1	triosephosphate isomerase 1"	3.0
EMBV3_07741	TRIM63	tripartite motif-containing 63"	-3.1
EMBV3_03231	TRPM6	transient receptor potential cation channel"	-2.1
EMBV3_30084	TSPAN12	tetraspanin 12"	2.2
EMBV3_25141	UBD	ubiquitin D"	2.4
EMBV3_04934	ULBP1	UL16 binding protein 1"	3.0
EMBV3_04813	URGCP	upregulator of cell proliferation"	-2.4
EMBV3_01943	VPS36	vacuolar protein sorting 36	2.1

		homolog (S. cerevisiae)"	
EMBV3_26850	XDH	xanthine dehydrogenase"	-2.7
EMBV3_41858	XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2"	2.4
EMBV3_29144	ZAP70	zeta-chain (TCR) associated protein kinase 70kDa"	-2.1
EMBV3_07313	ZDHHC2	PREDICTED: Bos taurus zinc finger, DHHC-type containing 2 (ZDHHC2), mRNA	-2.3
EMBV3_15239	ZNF280B	zinc finger protein 280B"	-3.0
Seven common differentially expressed genes between "FLX vs. SUN" and "SUN vs. CAN" comparison			
EMBV3_07344	NANOG	Nanog homeobox"	12.4
EMBV3_39028	LOC783508	similar to ULBP27"	24.3
EMBV3_30571	LOC100139242	similar to Protein BOP-1"	4.9
EMBV3_17294	SLC9A3	Bos taurus solute carrier family 9, subfamily A (NHE3, cation proton antiporter 3), member 3 (SLC9A3), mRNA	7.0
EMBV3_18537	TFAP2A	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)"	3.3

EMBV3_41727	DHDH	dihydrodiol dehydrogenase (dimeric)"	3.0
EMBV3_09561	HNF4A	hepatocyte nuclear factor 4"	4.1
One common differentially expressed gene between "FLX vs. SUN" and "FLX vs. CAN" comparison			
EMBV3_38850	LOC618023	PREDICTED: Bos taurus EKC/KEOPS complex subunit LAGE3 (LOC618023), mRNA	-2.7
Two common differentially expressed genes based on "FLX vs. CAN" comparison			
EMBV3_07887	SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter)"	-2.2
EMBV3_10046	RN7SL1	Bos taurus RNA, 7SL, cytoplasmic 1 (RN7SL1), SRP RNA	-2.6
Six common differentially expressed genes based on "SUN vs. CAN" comparison			
EMBV3_26059	LOC783399	similar to Equ c1"	3.7
EMBV3_38933	LOC513329	similar to Equ c1"	2.9
EMBV3_35958	HSD3B1	hydroxy-delta-5-steroid dehydrogenase"	2.1
EMBV3_35870	PAGE4	P antigen family"	2.1
EMBV3_14732	HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)"	2.1
EMBV3_36692	PLS1	novel gene; evidence: embryonic	2.0

ESTs"

Supplementary Table 6.3. Differentially expressed genes involved in activated pathways1 in embryos (Day 7.5) collected from cows fed a diet supplemented with either flax (FLX) or sunflower (SUN) seed (Experiment 1). Red and green gene symbols indicate up- and down-regulated genes, respectively.

Network	Top diseases and functions	Molecules in network
1	Amino Acid Metabolism, Post-Translational Modification, Small Molecule Biochemistry	**ABCG2**, Akt, **BTG2**, **CALB1**, CD3, **CLDN4**, **CLDN23**, **COL12A1**, **COL9A2**, **CXCL14**, **DSC2**, **EPS8L1**, estrogen receptor, F Actin, **GCSH**, **GLDC**, Gm-csf, histone deacetylase, Histone h4, **HMGN1**, Hsp27, **ID2**, Mek, **MGLL**, N-cor, **PFKL**, **PLAC8**, Ppp2c, **PRSS8**, STAT5a/b, **TACC2**, Tnf (family), **TRIM63**, **ULBP1**
2	DNA Replication, Recombination, and Repair, Nucleic Acid Metabolism, RNA Post-Transcriptional Modification	**ABHD14B**, ACOT1, **AIM1L**, APMAP, DARS2, DCAF13, EMG1, **ETNPPL**, **FAM127A**, **FSIP2**, **GIMAP4**, **HEBP2**, KBTBD6, KRT2, **LIPH**, **MFF**, **MPV17L2**, NEDD8, **NIPSNAP3A**, NSUN5, NUCB1, NUDT19, **PSAT1**, QTRT1, QTRTD1, RNPEP, SCFD2, **SH3BGRL**, **SLC34A2**, STAT5B, **TMEM45B**, UBC, **URGCP**, **ZDHHC2**, ZYG11B
3	Cellular Growth and Proliferation, Cell Death	**ALDH3A2**, Alpha catenin, Calcineurin protein(s), **CALML5**, caspase, Cg, **EDN3**, **ENPP3**, FSH,

	and Survival, Gastrointestinal Disease	**GADD45B**, **GFPT2**,GOT, Hdac, **IFI30**, Ifn, IL12 (complex), **IL1RN**, Immunoglobulin, **KIAA0930**, **KRT19**, LDL, Lh, MEF2, NFkB (complex), Nos, **PTGS2**, SAA, **SFN**, Sod, **SRXN1**, **TBX3**, TCF, Tgf beta, **TIMP1**, **XDH**
4	Cell Morphology, Hematological System Development and Function, Cell-mediated Immune Response	**ANXA1**, **ANXA4**, **B2M**, BCR (complex), calpain, **CAMK2N2**, CD8, **CD8B**, Cyclin A, **EFNA1**, ERK1/2, **FOLH1**, Ige, Igm, Laminin, **LGALS3**, MHC Class I (complex), Mucin, Nfat (family), **NID1**, Pak, PI3K (family), PLA2, **PLA2R1**, **PLCL2**, **PTPN5**, Rac, Rap1, **SCIN**, Sos, SYK/ZAP, **TDGF1**, **TRPM6**, TSH, **ZAP70**
5	Cellular Development, Lipid Metabolism, Small Molecule Biochemistry	**A2ML1**, Acot1, AGPAT1, APOBEC1, AQP11, BCL9, beta-estradiol, C8orf4, **CCBL1**, cholesterol, **CHPT1**, ELAVL1, FAM107A, FAM134C, GFM1, **GIPC2**, HCST, **HSD17B11**, **IFI30**, IFNG, **LTA4H**, **MS4A8**, PDCD7, **PPM1K**, PTGDR, RT1-EC2, RTP4, **SCIMP**, **SMPDL3A**, **SUSD2**, TGFB1, **TMEM50B**, WWOX, ZNF638, **ZNF280B**
6	Endocrine System	Ap1, **AS3MT**, **AUTS2**, Collagen type IV,

	Development and Function, Cell Death and Survival, Neurological Disease	Collagen(s), **CSRP2**, ERK, **FOS**, GNRH, GST, **GSTO1**, IL1, Interferon alpha, Mapk, **MGAT4A**, **MGST2**, Mmp, NADPH oxidase, NMDA Receptor, **NR3C1**, P38 MAPK, p85 (pik3r), Pdgf (complex), PDGF BB, Pka, Pkc(s), PLC, **PSAT1**, Ras, **SCARB2**, **SLC7A7**, TCR, Tlr, trypsin, Vegf
7	Inflammatory Response, Cell-To-Cell Signaling and Interaction, Nervous System Development and Function	**ANXA2**, **ANXA3**, APP, **CDH24**, CLEC2B, **DPYS**, GC, GRB2, Hmgn2 (includes others), IFI35, KRT2, **LY6G6C**, MDK, NCL, neuroprotectin D1, NFAM1, NMT2, **ORC6**, OSM, Pde4d, PDK3, **PLLP**, **PQLC3**, PTGDR, RNASEL, **ROBO4**, SERPING1, **SLC23A1**, TDO2, TMC4, TMCO4, TMEM245, TNF, TP53, **TSPAN12**
8	Cell Morphology, Inflammatory Response, Cell Cycle	26s Proteasome, Actin, ALAD, Atf, CCDC71, Endothelin, FAM3B, FBXO45, FFAR4, **FOXRED2**, Gamma tubulin, GPN3, Gsk3, H2-L, Histone h3, **HSD17B11**, IgG, **IKZF1**, IL12 (family), Insulin, Jnk, **LARP1B**, **MAP1LC3A**, MTCH2, **NME7**, PADI3, PI3K (complex), RFTN1, RNA polymerase II, **SLC25A12**, TNFAIP8L2, **TPI1**, **UBD**, Ubiquitin, **VPS36**

9	Cell Death and Survival, Cellular Growth and Proliferation, Cellular Development	ADM, **ANP32A**, AQP1, ATF3, BLM, CASP3, CASP10, **CYR61**, DNMT3B, EIF2AK3, ELK1, **EMILIN2**, EPHA7, GCG, HBEGF, HFE, **HK2**, KLF4, LMNB1, NDRG1, NFATC3, **NGFR**, NUPR1, **OSBPL6**, PEA15, PIM1, PTGES3, PTMA, RAD51, SLC2A1, TFAP2A, TNFRSF10A, TNFRSF10B, UNC5B, **XRCC2**
10	Developmental Disorder, Hereditary Disorder, Metabolic Disease	GPR3, **TMEM5**

[1]The pathway analysis was generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity).

Chapter 7. General discussion, future directions and conclusions

7.1. General discussion

Postpartum follicular growth generally resumes within 7–10 days after calving in most cows, associated with a transient rise in follicle-stimulating hormone (FSH) that occurs within 3–5 days after parturition; however, only a small proportion of dairy cows ovulate the first dominant follicle (Crowe 2008). The lack of ovulation of dominant follicles during the early postpartum period is associated with infrequent LH pulses. In the first study, I hypothesized that the increased interval from calving to ovulation in dairy cows fed a diet supplemented with canola (Colazo *et al.* 2009) occurred through reduced LH pulsatility and pituitary responsiveness to GnRH. The LH pulsatility and GnRH-induced LH release were evaluated during first and second weeks postpartum. During the first week postpartum, GnRH administration increased LH concentrations by 1 to 2 ng/ml in all treatment groups, whereas in the second week, GnRH-induced LH was increased in canola and sunflower by about 2 ng/ml and in control by over 4 ng/ml. These results clearly indicated that although the pituitary responded to GnRH administration, pituitary LH reserves were lower during first week than during the second week postpartum. According to previous report (Fernandes *et al.* 1978), pituitary LH content might not have been fully restored until approximately 19 days postpartum.

In agreement with previous study (Fernandes *et al.* 1978), GnRH-induced LH release increased from first to second week postpartum. During second week postpartum, cows fed no-oilseed diet (control) had a greater mean concentration of GnRH-induced LH compared to those fed a diet supplemented with canola or sunflower. One of the main factors affecting LH pulsatility during the postpartum period is energy balance, with negative energy balance

reducing LH pulsatility in postpartum dairy cows. Therefore, increased GnRH-induced LH release in cows fed no-oilseed diet was possibly a consequence of more pronounced negative energy balance during the second week postpartum in control cows that numerically reduced LH pulse frequency. Such a reduction in LH pulse frequency may have increased the available pool of pituitary LH leading to greater LH release following GnRH administration. In one study (Garrel *et al.* 2011), GnRH signalling was interfered with when linoleic or oleic acid was added to rat pituitary cell culture. Therefore, I speculated that GnRH-induced LH release was hindered with prepartum supplementation of sunflower (high in linoleic acid) or canola (high in oleic acid) compared to those fed a control diet in this study. However, this needs further confirmation in the bovine model by adding specific fatty acids of interest to bovine pituitary cell culture, since oilseeds contain a mixture of fatty acids. In conclusion, findings did not support my hypothesis as prepartum supplementation of canola or sunflower did not affect LH pulsatility during first and second weeks postpartum, and GnRH-induced LH release did not differ between canola and sunflower fed cows during second week postpartum.

Postpartum fat supplementation is a common practice in the dairy industry to improve energy balance, but few studies have investigated the possible carryover effects of prepartum fat supplementation on postpartum health, milk yield and reproductive function. I found that DMI and milk yield responses to prepartum fat supplementation were affected by parity. Prepartum oilseed supplementation did not affect BCS in primiparous cows at calving time; however, it increased that of multiparous cows. Increased BCS in multiparous cows at calving time may be associated with their lower energy requirement (maintenance and fetal development) during prepartum period compared to the higher energy needs of primiparous cows (maintenance, growth, and fetal development). Consequently, I speculated that increased body fat in

multiparous cows during prepartum period resulted in a greater fat mobilization and NEFA concentration resulting in reduced DMI during the postpartum period. A recent meta-analysis and meta-regression study reported that oilseed supplementation had no effect on milk yield, whereas milk yield was positively correlated with DMI. Therefore, I inferred that feeding high fat diets during prepartum period reduced DMI resulting in reduced milk yield in multiparous cows. Unfortunately, very few studies were available to make direct comparisons. In most studies, fat was supplemented either continuously pre and postpartum or only postpartum. Further studies are required to confirm my findings and determine if DMI and milk yield responses to prepartum fat supplementation were affected consistently by parity or it only happened at certain level of fat supplementation.

Calf birth weight is affected by gestation length, which in turn is influenced by parity, fetal gender, maternal nutrition, and sire. In the present study, oilseed supplementation increased the gestation length by 3 to 4 days. Considering that fetal growth rate averages 0.3-0.4 kg/d during the last month of gestation, a 3 to 4 d increase in gestation length should have resulted in 0.9-1.6 kg increase in calf birth weight. Contrary to this expectation, feeding sunflower increased birth weight by 5 kg in female calves in this study. Male calves are approximately 9% heavier than female calves and the majority of the increase in the incidence of dystocia is associated with male calves and attributable to their greater body weight. Whereas male calves in control and canola treatments were 8.2 and 6.8% heavier than female calves, male calves in sunflower treatment were lighter (-0.7%) than female calves in the same treatment group. Therefore, calf birth weight was mainly affected by prepartum maternal nutrition (oilseed supplementation) rather than gestation length. Based on these observations, I inferred that feeding oilseeds, particularly sunflower seed, during the prepartum period increased the incidence of dystocia

most likely through increased female calf birth weight. It is noteworthy that sire selection could not be controlled, and semen from 39 different sires was randomly used removing the possibility of any meaningful statistical evaluation of sire effect on gestation length. Therefore, more studies are required to confirm the effect of prepartum fat supplementation on gestation length and calf birth weight in dams inseminated with semen sourced from the same sire.

Correlation analysis indicated that the birth weight of calves was associated with prepartum DMI when all cows were considered regardless of dietary treatments. However, when their correlation was considered within treatments, DMI was not associated with calf birth weight in sunflower-fed cows. It is rather intriguing how dietary inclusion of sunflower seed differentially affected birth weight in a gender-specific manner, favouring weight gain in female fetuses. Several studies evaluated gender effects on fat metabolism in human, and they found that while estrogen (female sex hormone) stimulated, testosterone (male sex hormone) had an inhibitory effect on the conversion of essential fatty acids into their longer-chain forms (Decsi & Kennedy 2011). Additionally, neonatal plasma concentration of arachidonic acid, the elongated form of linoleic acid, correlated with birth weight in dairy cattle (Salehi et al. unpublished) and human (Elias & Innis 2001). Collectively, I surmised that the high linoleic acid content in the sunflower seed may have played a role in the increased birth weight of female calves, which needs to be confirmed. Further research is required to study mechanisms involved in the regulation of PUFA transfer through the placenta and whether such transfer differs between fetal genders in the bovine species.

Studies have shown that, when feeding dietary fatty acids prepartum was continued through 80 (trans-octadecenoic fatty acids; Juchem *et al.,* 2010) or 160 (fish oil; Silvestre *et al.,* 2011b) d postpartum, conception rates both at first and second AI were improved, respectively.

However, in the current study, oilseed supplementation neither altered ovarian function nor fertility. Hence, it seems that benefits to altering the fatty acid profile in the diet of dairy cows depend on the length of the dietary period during which they consume the respective fatty acids. In the Chapter 4, although I hypothesized that cows given a prepartum diet supplemented with canola seed will have a longer interval from calving to first ovulation compared to those fed diets supplemented with no-oilseed or sunflower seed, my results did not support the hypothesis and prepartum oilseed supplementation and the type of supplemented oilseed did not affect the interval from calving to first ovulation.

Maternal LCPUFA, neonatal total n-3 fatty acids, EPA and DHA were reduced in cows fed oilseed prepartum compared to those fed a no-fat control diet. A reduced activity of desaturase enzymes has been reported in women with high levels of linoleic acid in the diet and blood (Harnack *et al.* 2009, Friesen & Innis 2010, Novak *et al.* 2012). Therefore, it is plausible that a higher concentration of linoleic acid in oilseed group decreased the activity of desaturase enzymes, limiting production of all LCPUFA. Unexpectedly, feeding cows a diet supplemented with canola (61% oleic, 19% linoleic and 10% α-linolenic acid) during prepartum period did not affect the percentage of DHA, which can be synthesized from α-linolenic acid by elongation and desaturation, compared with those fed sunflower. Blank *et al.* (2002) found that the incorporation of n-3 LCPUFA such as EPA and DPA into all tissues was linearly related to dietary α-linolenic acid, whereas DHA incorporation was associated to dietary linoleic/α-linolenic acid ratios in a curvilinear manner (Blank *et al.* 2002). Therefore, I inferred that the linoleic/α-linolenic acid ratio is more important for synthesis of DHA from α-linolenic acid rather than only the amount of α-linolenic acid supplementation, although this remains to be confirmed.

The expression of *PPARA* tended to be higher in cotyledonary tissues of cows fed sunflower than those fed canola, whereas the expression of *PPARD* and *PPARG* tended to decrease in sunflower fed cows compared to those fed canola. It is suggested that the expression of PPARs was regulated differentially based on type of supplemented fatty acids rather than fat supplementation *per se*. Previous research has shown *PPARA* and *PPARD* as transcription factors were mainly involved in the activation of genes responsible for reducing lipid synthesis (Martinez *et al.* 2008) and fatty acid oxidation (Wang *et al.* 2003), respectively. In the present study, although, the expression of FABPs was not affected by dietary treatments, the expression of fatty acid protein transporter 4 (*FATP4*) and fatty acid translocase (*FAT*/*CD36*) was lower in cotyledonary tissues of cows fed supplemental fat than control. My results suggested that the modulation of PPARs was associated with the expression of *FATP4* and *FAT/CD36*, but future research is warranted to confirm these findings. Collectively, reduced total n-3 fatty acids, EPA and DHA concentration in neonate's plasma born of dams fed fat prepartum is likely associated with reduced expression of placental *FATP4* and *FAT/CD36*.

To evaluate the effect of long chain fatty acids on morphology and transcriptomic profile of in *vivo* embryos, I used non-lactating cows to obtain embryos, as milk production and negative energy balance in lactating cows could affect embryo development and quality. A significant finding of this work was that cows fed flax seed had a fewer degenerated embryos and a greater proportion of viable embryos compared to cows fed either canola or sunflower. Flax seed contains high amount of α-linolenic acid, an n-3 PUFA, which reportedly improved oocyte competence, early embryonic development, and reduced pregnancy loss. It was found feeding cows a diet enriched in n-3 PUFA reduced pregnancy loss in average %9.6 (Ambrose *et al.,* 2016). For instance, in a dairy herd with 200 cows, feeding a diet enriched in n-3 PUFA

would reduce pregnancy loss around 19 pregnancies. If the average cost of a pregnancy loss (abortion) was $555 (De Vries 2006), total profit would be $10545. However, very limited information is available about the effect of n-3 PUFA on early embryonic gene expression on genome-wide basis. Microarray technology allowed me to quantitatively measure the gene expression profile of transcripts on a genome-wide basis. Evaluation of the transcriptome profile of in *vivo* produced embryos revealed that 175 genes were differentially expressed in blastocysts from cows fed flax compared with those from cows fed either sunflower or canola. Cellular growth and proliferation, cellular development, lipid metabolism, and cell viability and survival were the main functions of differentially expressed genes. For the first time, my results provided novel information on possible mechanisms by which feeding a diet enriched in α-linolenic acid (flax seed) reduced the proportion of degenerated embryos. In this study, in *vivo* embryos were collected from superovulated cows to attain sufficient number of embryos for microarray analysis. Although the superovulatory responses (i.e., total numbers of corpora lutea and embryos/ova) did not differ among dietary treatments, the embryo quality and gene expression profile of embryos could have been affected by superovulatory treatment. Hence, the developmental ability and transcriptome profile of embryos collected from non-superovulated cows fed diets comparable to that of the present study must be evaluated in future studies.

Using an in *vivo* model does not allow the determination of whether the effect of n-3 PUFA on embryo quality is exerted at the follicular (oocyte) level and/or at the oviduct-uterine (embryo) level. Therefore, I designed an in *vitro* study to examine if PUFA could affect embryo development specifically during the post-fertilization period. Adding serum collected from cows fed flax seed or sunflower seed to in *vitro* embryo culture media did not affect embryo development during post fertilization period. Based on my findings from the *in vivo* and *in vitro*

studies, I concluded that the reduced number of degenerated embryos seen in cows fed a diet enriched in α-linolenic acid most probably resulted from either enhanced oocyte competence or improved maternal-embryo crosstalk at the oviductal/uterine level

7.2. Future research

Cows in the present study were fed a diet supplemented with canola or sunflower seed or a control diet with no added fat. Oilseeds or other dietary fat sources such as calcium soap of fatty acids are a mixture of different fatty acids making it very difficult to formulate a diet to be enriched in a specific fatty acid of interest. Therefore, to examine if and how PUFA such as linoleic or α-linolenic acid affect bovine pituitary function, specific fatty acids need to be added to bovine pituitary cell culture, and LH secretion and GnRH-induced LH release evaluated in *vitro*.

Recently, Rodney *et al.* (2015) evaluated the effect of diets supplemented with fat on reproductive performance by using meta-analysis approach. They revealed that out of over 5000 research papers reporting the effect of fat on reproductive performance in dairy cattle, only 17 studies were suitable for inclusion in the meta-analysis. Moreover, many published studies do not have adequate number of animals where the outcome of interest is a binomial variable such as ovulation, conception, or pregnancy loss. For instance, to evaluate the effect of PUFA on conception rate in dairy cows, power analysis determined that about 800 cows are needed to show a 5% difference at $P < 0.05$. Consequently, many of the published studies reporting fertility outcomes have been underpowered. Clearly, more appropriate studies (such as having control treatment, individual feeding and recording DMI) are required with adequate numbers of animals in each group to evaluate the effect of diets supplemented with fat on fertility.

Prepartum oilseed supplementation (8% DM) resulted in reduced DMI in multiparous cows and subsequently milk production in the present study. Reduced DMI could be associated with the amount of supplemented oilseed. Moreover, a recent meta-analysis study (Rodney *et al.* 2015) indicated that increasing dietary intake of slower fermenting carbohydrates (NDF) during transition period in diet containing fat improved the proportion of pregnant cows. On the other hand, similar to previous studies (Colazo *et al.* 2009), prepartum oilseed supplementation did not affect pregnancy at first AI and the proportion of pregnant cows by 150 DIM in current study. However, in studies where feeding dietary fatty acids prepartum was continued during the postpartum period, pregnancy rate was improved. Collectively, it seems that the benefits of altering the fatty acid profile in the diet of dairy cows may depend on both the length of the dietary period during which they consume the respective fatty acids and the type of fat supplemented. Therefore, further research is required to investigate the optimal quantity and duration of oilseed supplementation which could increase the amount of unsaturated fatty acids reaching small intestine and modify fatty acids profile of reproductive tissues to enhance productive and reproductive function without affecting DMI and incurring huge costs.

My results indicated that prepartum oilseed supplementation reduced neonatal plasma concentration of total n-3 fatty acids, EPA and DHA concentration. More studies are required to evaluate the effect of prepartum dietary fatty acids on colostrum quality and whether feeding colostrum may overcome this reduction. In second and third studies, my results showed that prepartum oilseed supplementation increased calf birth weight (Chapter 4), while reducing neonatal essential fatty acids (Chapter 5). Therefore, future research should investigate the effects of prepartum dietary fatty acids on calf growth, health and life time production.

Feeding cows diets enriched in n-3 PUFA increased progesterone production and reduced the synthesis and release of prostaglandin F2α from the uterus which is necessary for pregnancy establishment. To determine the effect of flax seed based diets on early embryo development and uterine environment, embryos collected from cows fed a diet supplemented with flax seed or control (no-oilseed) may be transferred to recipients fed either flax or control diet. Conception rate and pregnancy losses need to be recorded and compared between treatments. It would be very interesting to slaughter some cows at day 18-20 of pregnancy, collect embryos and caruncular and inter-caruncular tissues to investigate the effect of flax seed on embryo-maternal cross talk during early implantation period.

In the last study, feeding flax seed reduced the number of degenerated embryos possibly through alterations in the expression of genes involved in embryonic cell survival and viability. Although adding specific fatty acids to in *vitro* media may not reflect the complex biology of the whole body, in future studies, α-linolenic acid need to be added to in *vitro* media to confirm whether the effect of flax seed on early embryonic development resulted from α-linolenic acid. Moreover, to understand in which stage of embryo development (oocyte or embryo level) fatty acid supplementation could be more effective, α-linolenic acid should be added to in *vitro* maturation medium, embryo culture medium, or both, and subsequent embryonic development compared. To further investigate the effect of α-linolenic acid on embryonic development and subsequently pregnancy establishment, in *vitro* produced embryos in the presence of α-linolenic acid need to be transferred to recipients fed a control diet, and conception rate and pregnancy losses compared.

Preimplantation human embryos take up individual fatty acids at different rates at different stages of development (Haggarty *et al.* 2006). Moreover, embryos that developed

beyond the 4-cell stage contained higher concentrations of the unsaturated and a lower concentration of total saturated fatty acid. The higher unsaturated concentration at later stages of development could be explained by preferential uptake of unsaturated fatty acids such as linoleic acid from 8 cells to blastocyst stage compared to saturated fatty acids (i.e. palmitic acid) (Haggarty *et al.* 2006). Hence, it is important to investigate how feeding dietary fatty acids could affect the uptake of fatty acids by oocytes and embryos collected from cows fed different type of fatty acids and evaluate their subsequent developmental capacity.

7.3. Conclusions and implications

In contrast to what was previously reported by Colazo *et al.* (2009), prepartum supplementation of canola seed, a common ingredient in dairy cattle rations in western Canada, did not have any detrimental carryover effect on the onset of postpartum cyclicity (i.e., interval from calving to first ovulation) and reproductive function. However, prepartum oilseed supplementation at 8% of dry matter is not recommended for dairy cows as it had negative effects on DMI and milk yield, increased calf birth weights with no beneficial carryover effects on the reproductive function. Feeding dairy cows a diet enriched in n-3 PUFA during breeding period reduced early embryonic degeneration. Although feeding diets enriched in n-3 PUFA in the breeding season may improve embryo quality in dairy cows, feeding cows a diet enriched in n-3 PUFA such as flax seed is an expensive option and may not be readily adopted by dairy producers.

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