University of Alberta

Effects of dietary fatty acids on reproductive function of lactating Holstein cows

by

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ABSTRACT

It was hypothesized that dietary inclusion of flaxseed will enhance embryonic development in dairy cows through reductions in intrafollicular IGF-I and estradiol, and endometrial oxytocin receptor populations. Thirty-nine lactating Holstein cows were assigned to 1 of 3 diets containing whole flaxseed (FLX; linolenic acid), sunflower seed (SUN; linoleic acid) or saturated fatty acids (SAT; palmitic and stearic acids). In Experiment 1, embryos were collected from 24 superovulated cows (n=8/diet) on Day 7 of pregnancy and total cell number determined. Mean cell number and plasma progesterone concentration were lower ($P<0.05$) in embryos of SAT (77.1±3.9) than in those of FLX (93.4±3.3) or SUN (97.2±3.5). In Experiment 2, samples of ovarian follicular fluid, blood, and endometrium were obtained from 15 cows (n=5/diet). Diets did not affect intrafollicular IGF-I, estradiol, or oxytocin receptor populations. Dietary unsaturated fatty acids enhanced embryonic development but not through reductions in intrafollicular IGF-I or estradiol, or uterine oxytocin receptors.

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DEDICATION

To my parents

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LIST OF ABBREVIATIONS

AI – Artificial insemination
ALA- Alpha linolenic acid
CL- Corpus luteum
CSFA- Calcium salt of fatty acids
DF- Dominant follicle
DHA- Docahexaneoic acid
DIM- Days in milk
E_2 . Estradiol
END- Enterodiol
ENL- Enterolcatone
EPH- Eicosapentaenoic acid
FA- Follicular aspiration
FLX- whole flaxseed
IGF-I- Insulin like growth factor-I
IFN-τ- Interferon –tau
OT- Oxytocin
OTR- Oxytocin receptors
$PGF_{2\alpha}$- Prostaglandin F2 alpha or 2 series
$PGF_{3\alpha}$- Prostaglandin of 3 series
PGFM -Prostaglandin metabolite (15-keto-13, 14-dihydro-prostaglandin $F_{2\alpha}$)
PUFA- Polyunsaturated fatty acids
SAT- Saturated fatty acid
SDG- Secoisolaricresinol diglycoside
SUN- whole sunflower seed

Chapter 1

General introduction

On a global basis, one-sixth of human food energy and one-third of human food protein are foods of animal origin (Murugavel, 2003), which reflects the importance of animal production. Dairy cattle make an important contribution to global food production. Dairy herd profitability is largely dependent on good reproductive efficiency.

An optimum calving interval of 12-13 months is suggested for a cow to achieve the maximum profitability. Early embryonic loss is the most common form of reproductive inefficiency in cattle (Ayalon. 1978). It has been reported that 40% of early embryonic loss occurred between 8 and 17 days of pregnancy (Thatcher et al., 1994).

Dietary fats (Staples et al., 1998) and fatty acids (Mattos et al., 2000) have been shown to improve reproduction in cattle. Dietary fats serve as the source for fatty acids that are the precursors for prostaglandin and increased cholesterol concentrations, with the latter being the precursor of progesterone. Progesterone and prostaglandin in turn have effect on utero-ovarian function and conception rates. Thus, dietary fats influence the reproductive performance of an animal. Linoleic acid and α-linolenic acid are two essential polyunsaturated fatty acids, shown to influence reproductive function in cattle.

Flaxseed is rich in α-linolenic acid, where as sunflower seed is an example of a good source of linoleic acid. Flaxseed based diets have been shown to improve conception rates and reduce pregnancy loss over sunflower based diet in dairy cattle, although the

underlying mechanisms are unclear. This thesis will discuss the literature related to the effects of dietary fatty acids on reproduction in cattle, and test the hypothesis that a flaxseed based diet will enhance embryonic development and reduce intrafollicular concentrations of insulin-like growth factor-I (IGF-I) and estradiol, leading to decreased estradiol and oxytocin receptors in the endometrium.

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Chapter 2

Review of the Literature

2.1. Introduction

Reproductive inefficiency is one of the costly problems facing the dairy industry today. It increases the costs related to feeding, breeding, labor, and the culling percentage, leading to increased economic losses. Efficient reproduction is the primary determinant of profitability in a dairy herd (Call, 1978). The productive life of a cow depends on its reproductive soundness since the lactation cycle is initiated and renewed by pregnancy. The rapid changes in dairy management practices and the genetics of the modern dairy cow have contributed substantially to reproductive inefficiency in modern dairy cows. First service conception rates have decreased over the years from over 60% in the 1940s and 1950s (Barret and Casida, 1946; Casida, 1961; Mares et al., 1961) to 40% or less in the 1990s (Schmitt et al., 1996; Pursley et al., 1997a; Butler, 1998; Washburn et al., 2002; Leblanc et al., 2005).

Milk production per cow has increased from 2600 kg/lactation in the 1970's (USDA, 1978) to over 9000 kg/lactation in the 2000's (USDA, 2004) which suggests an antagonistic relationship between milk production and reproduction (Hansen et al., 2000; Wiltbank et al., 2001; Lucy et al., 2001). Services per conception have increased from 1.62 to 2.91 between 1972 and 1996 (Silvia, 1998). As in the USA, UK dairy cattle fertility has declined considerably over the past three decades (Royal et al., 2000). During this period, pregnancy rates after first service decreased with an increase in calving

interval. Milk production increased during this time period as conception rates declined from approximately 55% to 35%. On the other hand, Peters and Pursley (2002) reported that lower producing cows had lower conception rates than higher producing cows following timed insemination after synchronization of ovulation. There is a negative correlation between days open and average milk production. Lower milk production is associated with increase in days open and vice versa. In dairy cows, peak milk yield will occur in less than 100 days of lactation and then decline gradually as lactation progresses (Nebel and Mcgilliard, 2003). In order to maintain consistent milk production in dairy herds, cows must calve at regular intervals. For example, in a Canadian study, the average milk yield per day declined for every 10 days that conception was delayed (Dohoo and Martin, 1984).

The calving interval is the period between consecutive calvings. Herd profitability can be maximized with a calving interval of 12 to 13 months. Some dairy farmers have recently justified longer calving intervals for high producing dairy cows (Lucy et al., 2001). The longer calving intervals result in fewer calves born during a cow's lifetime. Therefore, fewer calves are available either for sale or for use as replacement heifers. Increased culling percentage due to reproductive failure delays genetic progress, increases replacement cost and provides fewer replacement heifers for market. In modern dairy cows the length of interval to the first ovulation is longer than the optimum interval and the prevalence of anestrus condition is greater due to the negative energy balance (Lucy et al., 2001). High producing dairy cows have lower circulating progesterone concentrations due to high metabolic clearance rates and lower levels of progesterone,

which lead to infertility (Lucy et al., 2001). Modern dairy cows have a longer luteal phase (Wilson et al., 1998a, b) and a twinning rate of 5% (Fricke and Wiltbank, 1999). Beerepoot et al. (1992) reported twinning decreased milk production and fertility.

2.2. Estrous cycle

The domestic cow is a polyestrous species, that is, estrous cycles continue throughout the year, unless she becomes pregnant. The length of estrous cycle varies between 21±3 days in cows and 20±3 days in heifers (Ginther et al., 1989). The estrous cycle is commonly divided into four periods: proestrus, estrus, metestrus and diestrus. It can also be classified into two phases (i.e. a short estrogen-dominant follicular phase, and a long, progesterone-dominant luteal phase). Follicle stimulating hormone, released from the anterior pituitary, will cause the emergence of follicular growth in wave-like patterns (Adams, 1999).

Ovarian follicle development is a wave-like dynamic sequence of organized events under hormonal control (Pierson and Ginther, 1987). Follicular waves consist of recruitment, selection and dominance (Ginther et al., 1989). Cycling cows either have two or three follicular waves. Each wave consists of the recruitment of a group of follicles, one among them is selected for continued growth while the rest will undergo atresia (Ginther et al., 1989). The selected follicle known as the dominant follicle will continue to grow in a hormonal milieu that suppresses continued growth of the remaining follicles (Ginther et al., 1989). When there is a decline in FSH level, the dominant follicle will develop LH

receptors and become dependent on luteinizing hormone (Adams, 1999). LH surge takes place during the first 6 to 12 hours of estrus, initiating the processes leading to ovulation. After ovulation, a corpus luteum will be formed which secretes a hormone known as progesterone. In the midluteal phase of the estrous cycle, progesterone concentration will be increased due to the increase in the size of corpus luteum (McCracken et al., 1999). During the luteal phase, progesterone downregulates estrogen receptors by a negative feedback mechanism on gonadotropin releasing hormone (GnRH) in the hypothalamus that causes low frequency pulse secretion of FSH and LH (Niswender et al., 2000).

If conception does not occur, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterine endometrium will cause luteolysis between Day 15 and 17 of the cycle (Arosh et al., 2002). As a result of luteolysis, progesterone concentration will decline which leads to to the removal of negative feed back effect on gonadotropin releasing hormone (Adams, 1999) and subsequently, release of FSH and LH from pituitary (Adams, 1999).

Prostaglandin $F_{2\alpha}$ is the luteolytic substance produced by the uterine endometrium in cattle (McCracken et al., 1973). The release of oxytocin (OT) is involved in $PGF_{2\alpha}$ secretion from the uterus by a positive feedback loop. During the early-luteal phase of the estrous cycle, progesterone inhibits OT action by inhibiting E_2-induced OT receptor (OTR) formation and by a direct effect on OTR gene transcription (McCracken et al., 1999). Inhibition of luteolysis and the maintenance of the CL past the point of normal luteal regression are the two main factors for the pregnancy recognition (Binelli et al., 2001).

2.3. Early Embryonic Death

Fertilization is a process where union of haploid spermatozoa and oocyte occurs resulting in the formation of a single cell embryo with a diploid complement of chromosomes. Fertilization occurs in the oviduct (Gordon et al., 2001). For a single breeding, the fertilization rates of 82 to 100% can be expected in beef heifers (Diskin and Sreenan, 1980; Maurer and Chenault, 1983). Fertilization rates of 85 % to 89% were recorded in parous dairy cattle inseminated with fresh semen (Kidder et al., 1954; Boyd et al., 1969). Calving rates to a single insemination are 52 to 57% in dairy cattle (Roche et al., 1978) and approximately 55% in beef cattle (Diskin and Sreenan, 1980). The percentage of viable embryos was higher in non-lactating cows (82.3%) than in lactating cows (52.8%) despite similar fertilization rates (88-90%) (Wiltbank et al., 2002).

Among reproductive inefficiencies, early embryonic mortality is a major component occurring at a rate of 40% between 8 and 17 days of pregnancy (Thatcher et al., 1994). Early embryonic loss (< d 20 after mating) may account for as much as 80% of all early embryonic death (Sreenan and Diskin, 1983). A decrease in early embryonic death would lead to increased pregnancy rates, decreased inter-calving period and, subsequently, an increase in milk production with greater number of calves. The causes of embryonic mortality can be classified under two broad categories, namely, environmental and genetic factors (Ayalon, 1978; Kastelic et al., 1994). Environmental factors consist of both external and internal factors. The hormones secreted by the embryo, the uterine physiology, and the hormones secreted by the dam to maintain pregnancy or enhance embryo growth are classified as internal factors, whereas the roles of nutrition, disease,

temperature or environmental stressors are classified as external factors. The majority of embryonic loss in cattle is unnoticeable and it appears that the animal is in regular cycle and has not conceived (Ayalon, 1978). Maurer and Chenault (1983) reported that 67% of embryonic loss occurred by Day 8 in beef cows. Ayalon (1978) stated that embryonic mortality is the main reason for reproductive failure in heifers.

The fertilization rate after AI in beef cows is 90%, whereas embryonic survival rate is 93% by Day 8 and only 56% by Day 12 post AI (Diskin and Sreenan, 1980). In dairy cattle, only 48% of embryos were classified as normal on Day 7 after AI. Thus, substantial pregnancy loss probably occurs within two weeks post AI. A "repeat breeder" is generally defined as any cow that has not conceived after three or more services associated with true estrus (heat). Ayalon (1978) reported there was a high rate of early embryonic mortality in repeat breeder cows when compared to normal cows.

Despite high fertilization rates of 95%, conception rates on days 28 to 32 post AI were between 40 and 47% and 35 to 45% in cows (Pursley et al., 1997b; Fricke et al., 1998; Santos et al., 2004). After fertilization, the fertilized egg will undergo cell division and be free floating in the lower oviduct and cell divisions continue normally as the morula forms (32 or more cells) and subsequently reach the blastocyst by Day 7 or 8 (Mclaren et al., 1984). Day 7 and Day 15 or 16 after fertilization are critical stages of pregnancy period (Ayalon, 1978) and early embryonic death often occurs due to the pulsatile release of $PGF_{2\alpha}$. The length of the estrous cycle is unaffected if the embryonic death occurs before Day 15 in the cow (Northey and French, 1980). Around Day 15 or 16, embryos

are sufficiently developed and secrete interferon-tau to overcome the luteolytic effect of spontaneous uterine secretion of $PGF_{2\alpha}$. The failure to block the luteolytic effect of $PGF_{2\alpha}$ during the above critical stage would cause early embryonic loss and initiates new estrous cycle by regressing the corpus luteum (Northey and French, 1980).

Embryo loss ranged from 13.3% (Cartmill et al., 2001) to 45.9% (El Zarkouny et al., 2000) for lactating dairy cows in which Ovsynch protocol was used to synchronize estrus (Pursley et al., 1995) and bred by timed insemination. A range of 15 to 30% of early embryonic loss is not uncommon in timed inseminated lactating cows regardless of synchronization program or cycling status (Inskeep et al., 2002).

2.4. Estradiol

Estradiol secreted from a preovulatory dominant follicle is important for the initiation of luteolysis. The upregulation of endometrial oxytocin receptors plays an important role in the initiation of luteolysis in ruminants (McCracken et al., 1999). The interaction between oxytocin and its endometrial receptor initiates the pulsatile secretion of $PGF_{2\alpha}$, and results in luteal regression (Flint and Sheldrick, 1983). Estrogen increases the oxytocin receptor concentrations (Soloff et al., 1975) and progesterone decreases the oxytocin receptor concentrations (Vallet et al., 1990a).

Robinson et al. (1999) proposed that estradiol stimulates the expression of oxytocin receptors via estradiol receptors, which are present in the bovine luminal epithelium during the luteal phase of the estrous cycle. Administration of estradiol in mid-estrous

cycle initiated luteolysis after12 h, by increasing endometrial oxytocin receptor concentrations (Hixon and Flint, 1987). It has been shown that removal of estradiol results in a prolonged estrous cycle (Villa- Godoy et al., 1985).

Treatment with estradiol stimulated the uterine secretion of $PGF_{2\alpha}$ (Ford et al., 1975; Thatcher et al., 1984; Fincher et al., 1986). Estradiol also increased oxytocin -stimulated $PGF_{2\alpha}$ production in long-term-cultured bovine endometrial cells (Asselin et al., 1996). Moreover, estradiol stimulates both basal and oxytocin-regulated $PGF_{2\alpha}$ secretion in the mid luteal stage of the lactating cows (Thatcher et al., 1984) and progesterone-primed ovariectomized cows (Battye et al., 1996).

In both cattle and sheep, the ability of oxytocin to stimulate uterine secretion of $PGF_{2\alpha}$ was enhanced by estradiol treatment (McCracken, 1980; Homanics and Silvia, 1988; Lafrance and Goff, 1988). Thatcher et al. (1986) conducted a study with 10 Friesian heifers that were randomly assigned to receive treatments of either estradiol-17β or saline on Day 13 of the estrous cycle. Blood was collected for prostaglandin $F_{2\alpha}$ metabolite (PGFM) analysis from the jugular vein at 30 min interval for 2 h. The cows that received estradiol-17β injection had higher levels of PGFM compared to cows that received saline injection. The above studies showed that estradiol is indirectly responsible for luteolysis. High estradiol concentrations on the day of insemination were associated with increased embryonic mortality in cattle and estradiol concentrations on the day of insemination can be influenced by diet (Shore et al., 1998).

2.4.1. Estradiol in follicular fluid

The estradiol concentration in the follicular fluid of dominant follicle is higher than its subordinate follicles of the same wave. On Day 3 of the estrous cycle, estradiol levels in the follicular fluid of recruited follicles are low (Figure.2.1; Fortune, 1994). But, on Day 5 of the cycle, one follicle has emerged as morphologically dominant and has a very high follicular fluid concentration of estradiol, whereas subordinates have much lower levels (Figure 2.1; Badinga et al., 1992). Hence, a drastic increase in estradiol concentration is a key characteristic of the dominant follicle.

2.5. Progesterone

Progesterone is essential for the maintenance of pregnancy. Progesterone concentration is increased as early as Day 3 to 6 post-breeding in pregnant animals over open animals (Albihn et al., 1991). Concentrations of blood progesterone were influenced by nutrition and weight loss (Bael et al., 1978). In a study by Garrett et al. (1988), it was shown that Day 14 embryos recovered from cows, pretreated with progesterone during early pregnancy, had increased embryonic growth when compared to embryos recovered from control group animals.

2.6. Maternal recognition of pregnancy

The embryo must signal its presence to the dam for the establishment of pregnancy to occured and for its continued development. It has been proposed that embryos express bovine trophoblastic interferon namely Interferon-tau (IFN-τ) a signal, which inhibits

pulsatile release of $PGF_{2\alpha}$ during the critical period of pregnancy (Thatcher et al., 1997). Interferon-tau is produced by the mononuclear cells of the trophoblast of the developing bovine embryo (Thatcher et al., 1997). Not all embryos grow at the same pace, therefore slow growing embryos may fail to express sufficient IFN-τ at an appropriate time to inhibit pulsatile release of $PGF_{2\alpha}$, leading to lysis of corpus luteum and subsequently early embryonic loss (Mann et al., 1999).

Various theories have been proposed to illustrate the mechanism of IFN~τ induced suppression of $PGF_{2\alpha}$ secretion at the cellular level. First, IFN~τ may help in the synthesis of molecules such as kinases, phosphatases, and lipases, which could directly inhibit $PGF_{2\alpha}$ biosynthesis (Thatcher et al., 1997). In another possible mechanism, IFN~τ could alter gene expression to inhibit expression of molecules involved in $PGF_{2\alpha}$ biosynthesis. Locally, IFN~τ suppressed the development of uterine endometrial oxytocin receptors, which are responsible for $PGF_{2\alpha}$ secretion.

Mann et al. (1999) reported that cows with higher plasma progesterone on Day 15 to 17 after breeding had larger conceptuses that produced more IFN~τ. The amplification and sustained secretion of IFN~τ requires exposure to the uterine environment (Thatcher et al., 1997). Mann and Lamming (2001) studied the relationship between the maternal hormonal environment and embryonic development in non-lactating Holstein cows. Cows that had poorly developed embryos had the same estradiol profile but a delayed progesterone increase compared with cows that had well developed embryo.

2.7. Synthesis of Prostaglandin

Prostaglandins belong to a family called eicosanoids, which are derived from 20 carbon polyunsaturated fatty acids. They are produced from arachidonic acid, a 20-carbon polyunsaturated (5, 8, 11, 14-eicosatetraenoic acid) fatty acid (Greenspan and Gardner, 2001; Arosh et al., 2004).

The first step involves hydrolysis of arachidonic acid by phospholipases; the second step involves oxidation and reduction of arachidonic acid and the final step is the conversion of prostaglandin H_2 to biologically active end products by specific synthases (Smith et al., 2000; Arosh et al., 2002).

2.8. Insulin like growth factor-I

Insulin like growth factor-I (IGF-I) plays an important role in nutrition and reproduction of dairy cows (Zulu et al., 2002a). Insulin–like growth factors (IGFs) are single-chain polypeptide growth factors termed so due to their similarity to insulin in both functional and structural aspects. They are present in serum, follicular fluid and many tissues of the body. The IGF family has ligands (IGF-I and IGF-II), receptors (IGF-IR and IGF-IIR), binding proteins (IGFBP) and binding protein proteases (Clemmons et al., 1997; Daughaday et al., 1987; Hossner et al., 1997). IGF-I and IGF-II were purified and sequenced in 1978 (Rinderknecht et al., 1978 a, b).

The structure of IGF-I receptors and that of insulin receptors are very similar; both have a tetrameric receptor made up of two alpha and beta chains linked by disulfide bonds. The

structure of IGF-II receptor is different with a long extracellular domain and a short cytoplasmic domain. The binding affinity of insulin and IGF-I to their own receptors is high when compared to binding affinity of insulin to IGF- I receptor and vice versa (McGuire et al., 1992). The IGFBP- I concentration is positively correlated with insulin concentration in serum.

IGF- I is a 70-amino acid polypeptide, mainly secreted from liver. The amino acid sequence in IGF- I molecule is identical in the bovine, swine and human differing from ovine and murine at 66 position and murine (Wong et al., 1989). Circulating IGF-I was greatly influenced by nutrition (Adam et al., 1998) and it is a hormonal mediator of nutritional regulation of ovarian function in cattle (Chase et al., 1998).

The IGF-I concentration in serum is very high during dry period, with a subsequent decline around parturition due to change in energy balance (Rong et al., 1988; Zulu et al., 2002 a). Nutrition and body condition influenced IGF- I concentrations at circulating level (Vicini et al., 1991).

Underfed cows have been shown to have lower IGF- I and smaller corpus luteum when compared to well-fed cows and heifers (Yung et al., 1996). The concentration of IGF- I in plasma of dairy cows was influenced by negative energy balance (Spicer et al., 1993; Beam and Butler, 1998).

IGF- I concentration in plasma varies with age, breed and stage of lactation (Abribat et al., 1990). Serum IGF-I concentrations were highest early in the dry period and gradually decreased during parturition, reaching lowest levels at calving. After calving IGF-I concentrations started to increase with the number of days postpartum, and by 60 days it will attain prepartum levels. The concentration of IGF- I in blood may serve as an indicator for nutritional status of early postpartum (Zulu et al., 2002a). Beam and Butler (1997, 1998) reported that plasma IGF-I levels were higher in cows, which had ovulation during the first two weeks postpartum. Anestrus cows had lower plasma IGF-I than cows that started cycling during early postpartum (Thatcher et al., 1996).

In dairy cattle, increased concentrations of IGF-I in serum during early lactation were associated with increased luteal function and increased energy balance (Spicer et al., 1990). An antagonistic relationship between milk production and serum IGF-I seems to be exist. For example, increased milk yield was associated with a decrease in IGF-I concentration and energy balance, which lead to reduced ovarian activity (Spicer et al., 1990). Moreover, Rong et al. (1988) found a negative correlation ($r = -0.5$ to -0.7) between milk production and IGF-I secretion.

Hammond et al. (1982) first reported the presence of insulin and IGFs in the pig's ovary. Intraorgan IGF-I systems exist in the ovary, where IGF-I exhibits both paracrine and autocrine function (Hammond et al., 1991). Both thecal and granulosa cells of bovine follicle contain IGF-I mRNA (Spicer et al., 1995) and the levels will be increased in granulosa cells during the final stages of follicular development (Schams and Koll, 1999).

Hammond et al. (1988) reported that IGF-I concentrations in follicular fluid increased with follicular size and its concentrations in follicular fluid were positively correlated to progesterone concentrations across individual follicles. The action of IGF-I in the ovary was mediated by type-I IGF receptor. There was a positive corrrelation (r = 0.69) between the IGF concentrations in follicular fluid and serum. Small follicles contained significantly lower IGF-I concentrations than large follicles, which shows the local control of IGF-I levels.

Adahsi et al. (1992) reported that IGF-I stimulated the proliferation of follicular cells and enchanced gonadotrophin stimulated steroidogenesis in both luteal and follicular cells. Even though the liver is the major source for IGF-I in blood , Spicer et al. (1993) and Izadyar et al. (1997 b) reported IGF-I mRNA in ovarian tissue from cattle and mammalian species. Previous studies in rats have established the ovarian granulosa cell as a site of IGF-I action and secretion (Baranao and Hammond, 1984; Hammond et al., 1985).

There was a sharp increase in IGF-I concentration at the time of ovulation in normal cycling cows (Zulu et al., 2002a) and during estrus in goats (Hashizume et al., 2000). Echternkampe et al. (1990) showed a positive correlation between IGF concentrations and estradiol (E2) concentration in follicular fluid. Spicer et al.(1993) showed that the IGF-I concentrations were higher in the follicular fluid of large estrogen active follicles than in the follicular fluid from small to medium estrogen inactive follicles. Moreover, IGF-I stimulates bovine granulosa and luteal cell steroidogenesis *in vitro* (Schams et al.,

1988) and *in vivo* (Ginther et al., 2004). Intravenous infusion of insulin in lactating Holstein cows increased the level of circulating E2. This could be due to either an indirect effect of insulin in altering the IGF-I concentrations or increased ovarian responsiveness to IGF-I (Butler et al., 2004). Reduced IGF-I secretion caused by negative energy balance could suppress expression of estrus by altering the ovarian follicular E2 production (Spicer et al., 1990).

Some of the effects of IGF-I on the reproductive axis of dairy cattle are: proliferation and differentiation of granulosa cells; stimulation of the aromatase syste; induction of LH receptor; enchancement of GnRH secretion from hypothalamus (Zulu et al., 2002a); induction of luteinizing hormone receptors (Adashi, 1992); amplification of the effects of follicle stimulating hormone and luteinising hormone on the growth and differentiation of ovarian follicles (Spicer et al., 1993; Lucy, 2000); and promotion of ovarian sterioidogenesis by both the follicles and corpus luteum (Hammond et al., 1991).

Cows with low plasma level of IGF-I had reduced follicular growth and poor CL development (Chase et al., 1998). The receptors of IGF-I are abundant in the oviduct and endometrium, which suggests that IGF-I can influence the activity of the bovine reproductive tract (Kirby et al., 1996).

Concentrations of IGF-I in follicular fluid ranges between 100-500 ng/ml which is equal to or lower than the levels in plasma (Echternkamp et al., 1990; Spicer et al., 1995; Hammond et al., 1988). However, follicular fluid concentrations of IGF- I in heifers,

which were fasted for 2 days were higher than plasma concentrations of IGF-I (Spicer et al., 1992).

2.9. Dietary fats, fatty acids and reproduction

2.9.1. Fatty acids

Fatty acids are carboxylic acids with an aliphatic chain, either saturated or unsaturated. They mainly occur as esters in natural fats and oils, but also exist as free fatty acids in non-esterified form. Saturated fatty acids contain no double bonds in the aliphatic chain, whereas unsaturated fatty acids contain one (monounsaturated) or more (polyunsaturated) double bonds and eicosanoids (Abayasekara and Wathes, 1999). For example, coconut oil is highly saturated and fish oils have high polyunsaturated fatty acids. Fats of animal origin are high in saturated fatty acids, whereas fats of plant origin are high in unsaturated fatty acids. Stearic and palmitic acid are examples of saturated fatty acids, whereas linoleic and α-linolenic acid are examples of polyunsaturated fatty acids.

Animal tissues can synthesize the oleic acid family and they are non-essential fatty acids or dispensable fatty acids. Linoleic acid (18: ω -6-LA) and α-linolenic acid (18: ω -3-ALA) are termed as essential fatty acids or indispensable polyunsaturated fatty acids because the ruminal or mammalian microbes lack Δ12 and Δ15 desaturase enzymes to convert oleic acid into linoleic acid and α-linolenic acid. The ω-numbering system begins numbering carbons starting at the methyl end of the fatty acid (FA). In case of linoleic acid it is designated as C18:2, ω-6 because it has 18 carbons, two double bonds, and the first double bond is at the sixth carbon from the methyl end and linolenic acid is

designated as C18:3, ω-3. Linoleic acid produces arachidonic acid by elongation and desaturation process. Biologically active eicosanoids are derived from arachidonic acid (AA) (Kinsella et al., 1990). Flaxseed, hemp, fish oils and dark green forages are the main sources of α- linolenic acid (ALA). Sunflower, soyabean, nuts, pumpkin seeds, and sesame seeds are the main sources of linoleic acid (LA). ALA is also present in grass, but its concentrations are reduced during the silage making process. The metabolic transformations of the two major polyunsaturated FA are shown in Figure 2. 2.

2.9.2. Metabolism of Fats in the Rumen

The main difference between monogastric and ruminant animals, in regards to fat metabolism, is the extensive biohydrogenation of unsaturated fatty acids, which occurs in the rumen by the action of microorganisms (Ward et al., 1964). In ruminants, if the dietary FA is unsaturated the FA composition of blood and milk is highly saturated. The rate of release of the unsaturated fatty acids from feeds and their exposure to ruminal microbes determines the biohydrogenation. Ruminal microbes hydrogenate unsaturated fatty acids. Failure of the ruminal microbes to saturate fatty acids would cause the accumulation of unsaturated fatty acids and subsequently interfere with ruminal fermentation (Funston, 2004).

Ruminal microbes hydrolyze triglycerides and phospholipids of polyunsaturated fatty acids into their polyunsaturated fatty acid constituents and glycerol. Then, glycerol is fermented to propionic acid (Church, 1976; Noble, 1978). Staples et al. (1998) estimated the biohydrogenation efficiency of LA range to be from 70 to 90 %.

Burr and Burr (1929) reported that dietary fat was essential to growing rats. After 70d of dietary phase, rats without fat source in the diet experienced alopecia, cessation of growth, prolapsed penis and irregular ovulation. The same authors tried to identify the specific FA in a follow up study (Burr and Burr, 1930) by feeding lipid free diets to the growing rats. Thirteen out of twenty two female rats had irregular cycling or non cycling. When four of the non-ovulatory females were given five drops of either coconut (1%LA), olive (7%LA), corn (41%LA) or linseed (59%LA) oil daily, all resumed ovulation except for the rat supplemented with coconut oil. Elimination of FA from the diet caused cessation of synthesis of steroid hormones. The deficiency of FA in farm animals were studied in preweaned calves in 1954 (Lambert et al., 1954).

The total energy requirement for maintenance and milk production is generally more than total energy intake in early lactation phase (Staples et al., 1990). In order to meet this energy deficit, the cows must utilize the adipose stores in the form of nonesterified fatty acids (NEFA) to support lactation. Energy deficit in early lactation can be reduced by supplementing fats to the diet, but this leads to increase in milk yield resulting in not in the improvement of the energy status. Modern dairy cows may be deficient in the essential fatty acid, linoleic and α-linolenic acid . Dietary fats can provide fatty acid precursors for cholesterol and prostaglandin production. It has been shown by Staples et al. (1998) that dietary fats had beneficial effects on reproduction of lactating dairy cows.

It has been documented in many studies that dietary fat has improved reproductive performance in cows. A review by Staples et al. (1998) reported an improvement in the fertility rates of cows by dietary fats in 11 of the 20 articles referenced. He proposed

dietary fat enhanced reproduction by improving the energy status, increasing the production of progesterone and altering the synthesis of prostaglandins pathway. Staples et al. (1990) stated that dietary fats can provide FA precursor for cholesterol and eicosanoid (including prostaglandin) production which have effects on ovario-uterine function and conception rates. Progesterone, secreted by the corpus luteum is essential for the maintenance of pregnancy. It prepares the uterus for implantation of the embryo and induces the heterotrophic proteins from the endometrium for the nourishment of conceptus. Cows fed supplemental fat began to cycle sooner because of the enhanced follicular growth and development (Grummer and Caroll, 1991). They also reported that there was an increased concentration of circulating cholesterol, which is the precursor for progesterone.

Chylomicrons are formed during the process of absorption of fat from the small intestine. Cholesterol is an important compound in the formation of chylomicrons and thus more cholesterol is likely be needed. However, Hawkins et al. (1995) suggested there was a decrease in the clearance rate of progesterone rather than an increase in progesterone concentrations of cows fed fat.

Researchers at the University of Nebraska, (Son et al., 1996) found that cows fed at 2 % tallow vs. 0% of dietary DM had a greater level of blood cholesterol and peak plasma progesterone concentrations in the second ovulatory cycle. Higher concentrations of plasma cholesterol were associated with a shorter interval from calving to conception, and

with greater probabilities of conception and successful pregnancy by 150d of lactation (Kappel et al., 1984; Ruegg et al., 1992; Westwood et al., 2002).

Garcia-Bojalil et al. (1998) reported that pregnancy rates improved, plasma progesterone was greater until 50 DIM, and energy status did not change when cows were fed diets of 2.2% calcium salt of fatty acids (CSFA) compared to non fat-supplemented cows. A study (n = 443) conducted by (Scott et al., 1995) with CSFA at 0 or 450 g/d from 1 to 180 or 200d in five Holstein commercial herds reported the overall conception rate was increased from 93 to 98% and a tendency for fat supplemented cows to exhibit standing heat. The improvement in conception rates was confirmed by Ferguson et al. (1990) in 253 cows over four herds. The cows were fed 0 to 2% ruminally inert fat from 0 to 150 DIM and the lower content of linoleic acid in the oocyte membrane was responsible for a decline in the fertility of Holstein cows during summer season (Zeron et al., 2001).

Thatcher and Staples (2000) reported that omega -3 and omega-6 are the two main families of essential fatty acids that could improve fertility. Dietary LA (C18:2n-6) is the main source of omega-6 fatty acids and this is converted to arachidonic acid (C20: 4n-6), which is a precursor to the 2 series prostaglandins, such as $PGF_{2\alpha}$, by elongase and desaturase enzymes. The same enzymes also convert the dietary omega-3 fatty acids (C18: 3n-3) to eicosapentaenoic acid (EPA; C20: 5n-3), which is the precursor to 3 series prostaglandins example: $PGF_{3\alpha}$. (Abayasekara and Wathes, 1999). Since there is a competition between omega-3 and omega-6 precursors for desaturation and elongation, it is possible to decrease the production of 2 series prostaglandins by increasing the supply

of omega-3 FA (Barnouin and Chassagne, 1991). It has been found out that $PGF_{3\alpha}$ have lower biological activity than $PGF_{2\alpha}$ (Fly and Johnston, 1990). Embryonic mortality can be decreased by reducing the ovarian and endometrial synthesis of $PGF_{2\alpha}$ at the expense of $PGF_{3\alpha}$ (Mattos et al., 2000).

The high ALA and LA content in flaxseeds (57% LNA and 14% LA) may have been responsible for the improvement in conception rates (87.5 vs. 50.0%) of lactating dairy cows fed formaldehyde-treated whole flaxseed (17% of dietary DM) compared to those fed Ca salts of palm oil (5.6% of dietary DM) from 9 to 19 wk postpartum (Petit et al., 2001).

2.9.3. Follicular growth

Beyond its ability to improve conception rate, development of follicles is also improved by dietary fat. Reduced ovarian activity is expressed in cows with poor body condition and negative energy balance (Staples et al., 1990). Follicle development involves the stages of recruitment, selection and dominant (Thatcher et al., 1996). Low pulsatile secretion of LH was responsible for the reduction of ovarian activity during negative energy balance and early lactation in a study by Beam and Butler (1999).

Supplementation of fat in cattle increased the number of follicles in the medium-sized classification by 1.5- to 5-fold within 3 to7 wk (Ryan et al., 1992). Workers at Texas A&M University (Thomas et al., 1996) conducted a study with 27 non-mature lactating beef cows. Cows were randomly assigned to either one of the three fat supplemental

groups such as tallow, soybean, fishmeal or a control diet, which contains no fat source. Transrectal ultrasonography was used to study the ovarian morphology and cows that received fishmeal had a fourfold increase in medium sized follicles compared to cows that received control diets. Medium follicles in cows that received soybean and tallow had also increased in size after 7 weeks of dietary phase.

In another study, Simmental cows were fed either with CSFA (0.5% of BW) or an isocaloric control diet. Serum LH concentrations and total cholesterol for CSFA cows were greater than for control cows and follicular growth was also enhanced in CSFA cows (Hightshoe et al., 1991).

Eighteen lactating Holstein cows (60 to100 d in lactation) were fed CSFA at 2.2% of dietary DM or an isoenergetic diet in a cross over design. Even though animals were in similar energy balance, cows fed CSFA had a larger second wave dominant follicle (18.7 mm) than did cows fed the control diet (16.1 mm) (Lucy et al., 1993). Beneficial effects of dietary fatty acid on follicular growth could be due to increased serum insulin concentrations, which act either directly through its own receptors or indirectly modulating granulosa IGF-I production (Yoshimura et al., 1994).

2.9.4. Prevention of prostaglandin (PGF) synthesis

The two rate-limiting factors in $PGF_{2\alpha}$ secretion are the availability of arachidonic acid and the activity of endoperoxide synthases or cyclooxygenases (Thatcher et al., 1997; Figure 2.1). Cyclooxygenases exist in two isoforms, the constitutive COX-1 and

the inducible COX-2 (Vane and Botting, 1998). Arosh et al. (2002) indicated that the level of COX-2 expression was greater between Day 13 and 21 compared to between Day 1 and 12.

Stimulation (Burke et al., 1996; Filley et al., 1999) or inhibition (Baguma-Nibasheka et al., 1999; Cheng et al., 2001; Mattos et al., 2003) of prostanoid synthesis was regulated by the amount of particular fatty acids reaching the target tissues. Since the 1 and 3 series of prostaglandins are biologically less active than those of the 2 series, it is possible to decrease the biological activity of 2 series by supplementing diets rich in n-3 PUFA.

Polyunsaturated fatty acids inhibit prostaglandin synthesis by inhibiting cyclooxygenase activity and competing with arachidonic acid for processing. For example, diets rich in LA lead to increased levels of the 2 series of prostaglandins by inhibiting the enzymatic system (Barnouin et al., 1991). Researchers at University of Florida (Oldick et al., 1997) infused a) water b) 1kg/d of glucose c) 0.45 kg/d of tallow or d) 0.45 kg/d of yellow grease abomasally in lactating dairy cows in 4 x 4 Latin square design. On Day 15 of the synchronized estrous cycle, an injection of oxytocin was administered. Cows infused with yellow grease released less $PGF_{2\alpha}$ in response to oxytocin challenge. α-linolenic acid has been identified as an inhibitor of endometrial prostaglandin synthesis (Thatcher et al., 1994).

Eicosapentaenoic acid (EPA) and docosahexaneoic acid (DPA) are two other FA that inhibit prostaglandin release. They are converted into inactive prostaglandin 3 series by

phospholiapse A_2 and cyclooxygenase enzymes in the prostaglandin biosynthetic pathway (Thatcher et al., 2001a). Mattos et al. (2001) reported EPA and DPA inhibited $PGF_{2\alpha}$ secretion by bovine endometrial cells *in vitro*. Menhaden fishmeal had high concentrations of EPA and DPA (Binelli et al., 2001; Mattos et al., 2002).

Mattos et al. (2002) fed lactating dairy cows with diets containing 0, 2.6, 5.2, or 7.8% menhaden fishmeal for 50 d. After 30 to 34 d on the diets ovulations (D0) were synchronized and on Day 15 of the subsequent estrous cycle, estradiol and oxytocin were injected to induce $PGF_{2\alpha}$ release. Other than the control diet (fish meal 0), all other diets had reduced plasma PGFM concentration after estradiol and oxytocin challenge. Thatcher et al. (1997) also reported there was less PGFM release in lactating dairy cows, which were fed menhaden fish meal for 25d and challenged with estradiol and oxytocin injection on Day 15 of synchronized estrous cycle.

2.9.5. Diet rich in n-3 polyunsaturated fatty acids (PUFA)

Diets high in PUFA, of the n-3 family during the prepartum period cause delayed parturition in sheep (Baguma-Nibasheka et al., 1999) and increased placental retention in cattle (Barnouin and Chasssagne, 1991). On the other hand, supplement of diets high in PUFA of the n-3 family during the postpartum period enhanced conception rates in cattle (Petit et al., 2001; Ambrose and Kastelic, 2003) and reduced pregnancy losses (Ambrose et al., 2006) in dairy cows.

Ambrose et al. (2006) conducted a study in 121 lactating Holstein cows with rolled flaxseed as an experimental diet and rolled sunflower seed as control diet. Dietary phase started when cows were approximately 55 d postpartum and all cows were bred by timed artificial insemination (TAI). Pregnancy diagnosis was done by ultrasound 32 day after TAI and once pregnancy was confirmed, cows were removed from the experimental diets. Nonpregnant cows remained on the diets until the second TAI and pregnancy diagnosis occurred. Even though embryo survival rate was not different between the two diets on Days 24 and 32 of post TAI, the flax group of cows had a higher rate of early embryonic survival up to Day 24 post AI. The dominant follicle was also larger in cows fed flax diet. The plasma progesterone concentrations at AI were higher in cows fed flax than cows fed sunflower diet, suggesting delayed luteal regression of corpus luteum.

In another study, postpartum Holstein cows (n = 141) were assigned to one of the three diets containing whole flaxseed, Ca salts of palm oil, or micronized soybeans (Petit and Twagiramungu, 2006). The CL diameter is larger in cows fed flaxseed than that of cows fed soybeans (19.7 vs.16.9 mm), but not larger than that of cows fed Ca salts of palm oil (17.5 vs.26 mm). Even though diets did not affect conception rate ($P < 0.11$), embryonic loss from Day 30 to 50 after AI tended to be lower in cows fed flaxseed (0%) compared to Ca salts of palm oil (15.4%) or soybeans (13.6%).

Fish oil is a source of n-3 PUFA such as eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6). Pregnant Holstein cows (n = 17) and heifers (n = 9) were randomly assigned to the diets containing either olive oil or fish oil. Blood samples

were collected from Day 14 before the date of parturition to Day 21 postpartum for prostaglandin metabolite assays. Animals fed fish oil had a low level of PGFM when compared to the PGFM level of cows fed olive oil. Increased availability of EPA in membrane phospholipids as a result of feeding fish oil could displace arachidonic acid, leading to increased synthesis of less bioactive prostaglandin 3 series at the expense of the prostaglandin 2 series, such as $PGF_{2\alpha}$ (Mattos et al., 2004). The secretion of $PGF_{2\alpha}$ was reduced by Eicosapentaenoic acid and DHA in different animal cell culture systems (Achard et al., 1997). Polyunsaturated fatty acids such as linolenic acid, EPA and DHA attenuated secretion of $PGF_{2\alpha}$ in bovine endometrial cell lines induced by phorbol ester. Linolenic acid had less potential in reducing $PGF_{2\alpha}$ when compared to EPA and DHA (Mattos et al., 2003).

2.9.6. Flaxseed

Flaxseed belongs to a family called Linaceae. *Linum usitatissimum,* is the specific name for flax. Since 1994, Canada has been a world leader in the production and export of flaxseed. Canada exports 60% of its flax to the European Union, 30% to the United States and 4% to Japan (Anonymous, 2006).

α-linolenic acid is the main FA constituent of flaxseed at about 57% of the total fatty acids. Linoleic acid is another polyunsaturated fatty acid present in flaxseed, and it constitutes about 16% of the total fatty acids (Daun and DeClercq, 1994).

Flaxseed is the richest known source of secoisolaricresinol diglycoside (SDG). SDG is the precursor for mammalian lignan such as Enterodiol and Enterolcatone (Thompson et al., 1991). Enterodiol and Enterolcatone are diphenolic compounds (Figure. 2.3), formed by intestinal bacteria (Axelson and setchel, 1981; Borriello et al., 1985). Mammalian lignans undergo enterohepatic circulation and are excreted in the urine, mainly as glucuronide conjugates. Enterolactone (ENL) and Enterodiol (END)are produced *in vivo* and they are termed as "mammalian lignans" to differentiate them from plant lignans (Wang, 2002). Axelson et al. (1982) confirmed the dietary precursor SDG is responsible for the production of mammalian lignans *in vivo* by feeding semisynthetic diet to adult rats. Semisynthetic diets cause decreases in the excretion of mammalian lignan in urine, but when the diet is changed back to commercial ration, lignans reappeared in the urine.

The amount of the mammalian lignans excretion in urine is directly proportional to intake of plant lignans from foods such as flaxseed (Thompson et al., 1995). Borriello et al. (1985) studied *in vitro* production and metabolism of END and ENL by human fecal flora. He proposed SDG was metabolized to END, probably through hydrolysis, dehydroxylation and then demethylation by facultative bacteria, and then ENL was produced from END through oxidation by facultative bacteria. Two bacterial strains such as *Peptostreptococcus* sp strain SDG-1 and *Eubacterium* sp strain SDG-2 were isolated from fecal suspension and it was found that they are capable of demethylation and dehydroxylation respectively (Wang, 2002).

These mammalian lignans exhibited antiestrogenic properties in rats and causing irregular estrouss cycles (Orcheson et al., 1998). Flaxseed also reduced IGF-I in rats (Rickard et al., 2000). Supplementation of flaxseed at 5 % did not affect circulating IGF-I in steers (Dunn et al., 2003). Mechanisms involved in antiestrogenic properties of flaxseed have been explained and include: a) Enterolactone depresses estrogen stimulated uterine RNA synthesis in rat; b) Enterolactone inhibits steroid metabolizing enzymes such as aromatase; c) Enterolactone prevents binding of estrogen to the Type -2 nuclear estrogen receptor in cancer cells in culture (Mousavi and Adlercreutz, 1992). Moreover, Lignans interfere with estrogen-mediated tumorogenic processes by stimulating the hepatic synthesis of sex hormone-binding globulin, which increase the clearance of circulating estrogen and bind to estrogen receptors (Adlercreutz et al., 1986).

Crampton et al. (1951) in Canada and in the United States demonstrated the normal growth and reproduction in laboratory animals fed flaxseed oil. Flaxseed has also been added to the dietary rations of dairy cattle, swine and farm-raised fish with the aim of increasing the ALA fatty acid content of animal tissues and products derived from them (Vaisey-Genser and Morris, 1997). Diets rich in polyunsaturated fatty acids increased long chain fatty acids in milk rather than short chain fatty acids (Petit, 2003). Fat percentage in milk was lower in cows fed flax and sunflower diet when compared to cows fed megalac (Petit et al., 2002). Cows fed flaxseed had higher milk protein concentration than cows fed sunflower (Petit, 2003). Milk yield was higher in cows fed calcium salt of palm oil and flaxseed than milk yield of cows fed sunflower (Petit et al.,

2004). α- linolenic acid concentration was three times higher in the milk of cows fed rolled or whole flaxseed than the cows fed sunflower diet (Ambrose et al., 2006).

2.10. Nutritional strategies to prevent embryonic loss

Since a large percentage of early embryonic mortality occurs prior to or around the time of maternal recognition of pregnancy, many strategies have been tried to improve the pregnancy rates in cows. They include: a] increasing the growth rate of the CL to increase luteal phase progesterone concentrations (Binelli et al., 2001) (e.g. dietary omega-3 fatty acids increased the concentration of progesterone); b] decreasing the effect of dominant ovarian follicle at the critical period; c] increasing the antiluteolytic stimulation by the conceptus unit; d] decreasing the luteolytic response of the maternal unit (Binelli et al., 2001); e] increasing the biological inactive 3 series prostaglandins with decrease in active 2 series prostaglandins (e.g. diets supplemented with fish meal (Burke et al., 1997) and flaxseed (Petit et al., 2002) inhibited $PGF_{2\alpha}$, as well as increased the synthesis of the 3 series prostaglandins due to the competition for the same key enzymes); f] inhibition of cyclooxygenase activity (e.g. cyclooxygenase is the key enzyme responsible for the synthesis of $PGF_{2\alpha}$). It has been shown that the 20-carbon fatty acids such as EPA and dihomo-gamma-linolenic acid compete with arachidonic acid for active sites of prostaglandin – endoperoxide synthase complex, which causes a reduction in the conversion of arachidonic acid to the 3 series prostaglandins (Weber and Sellmayer, 1990).

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Table 2.1. Comparison of major fatty acids in edible oils (w/w % Fatty acid).

Oil	C18:0	C18:1	C18:2	C18:3
Peanut	2	47	32	0
Safflower	2	12	77	0
Canola	2	64	19	8
Cotton seed	25	21	50	0
Flaxseed	4	19	14	58
Corn	2	25	60	1
Tallow	15	41	8	1
Fishmeal	2	25	60	1
Palm	4	39	10	1
Sunflower	5	20	69	0
Megalac ®	3.5	32.3	7.8	0.3

C18:0- Stearic acid,

C18:1 – Oleic acid,

C18:2 – Linoleic acid,

C18:3 – Alpha linolenic acid

Adapted from Erasmus (1993).

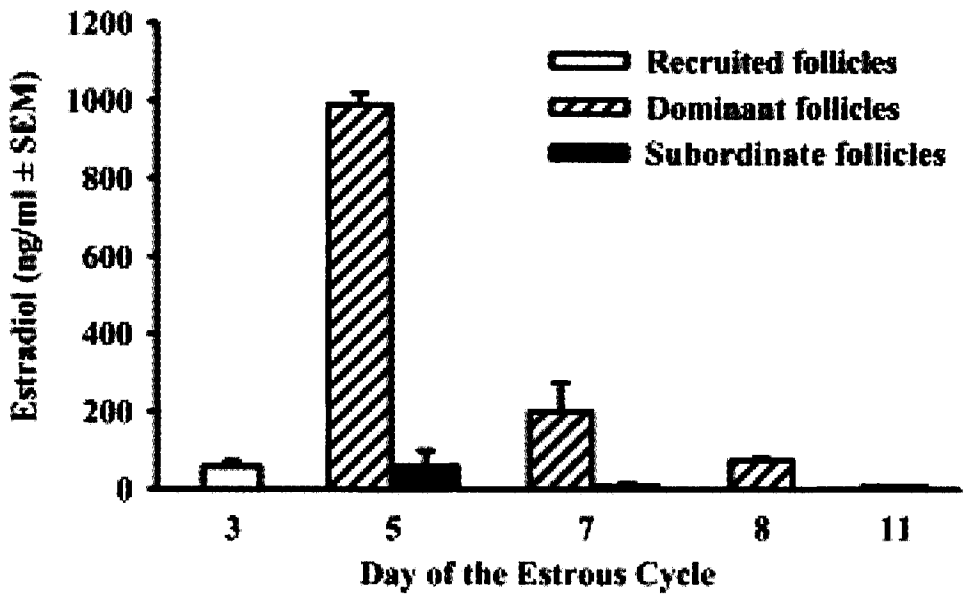


Figure 2.1. Concentrations of estradiol (ng/ml ± S.E.M.) in follicles collected during the first wave of follicular development of the bovine estrous cycle (Adapted: data taken from Fortune (1994) for days 3, 7 and 11, from Badinga et al. (1992) for day 5, and from Bridges and Fortune (2003) for day 8.

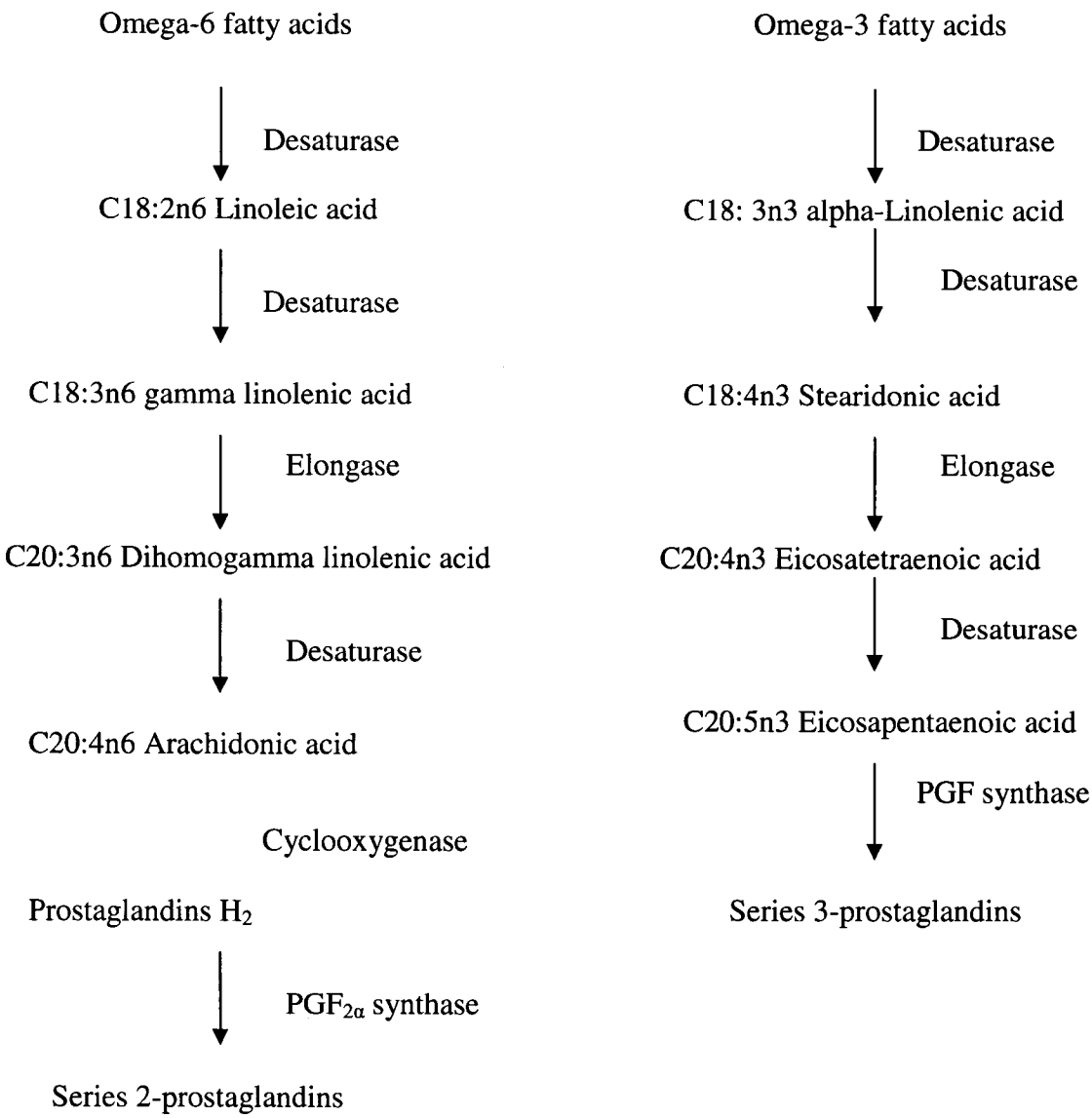
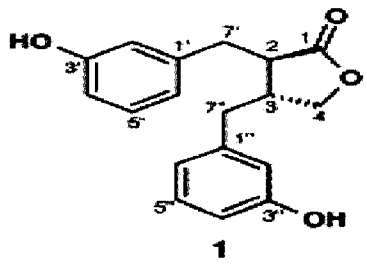
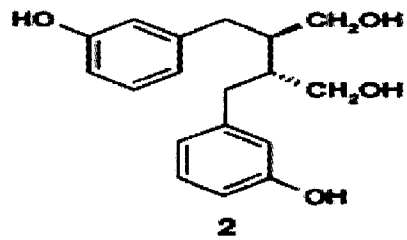


Figure 2.2. Metabolic transformations of the major polyunsaturated fatty acids families by desaturation and elongation.

Adapted from Abayasekara and Wathes (1999).





1- ENL

2-END

Figure 2.3. Structure of Enterolcatone (ENL) and Enterodiol (END)

Adapted from Wang, 2002.

Chapter 3

Early embryonic development in Holstein cows fed diets enriched in unsaturated or saturated fatty acids

3.1. Introduction

It has been documented in many studies that dietary fats (Staples et al., 1998) and fatty acids have beneficial effects on reproduction in dairy cows (Mattos et al., 2000). The inclusion of polyunsaturated fatty acids of the Omega-3 family (e.g. eicosapentaenoic acid, docosahexaenoic acid) of marine fish origin in rations of dairy cows has suppressed prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production (Thatcher et al., 1997), delayed luteal regression and improved conception rates (Burke et al., 1997).

Flaxseed (*Linum usitatissimum*) is rich in α- linolenic acid (ALA; C18: 3 n-3), which is also a polyunsaturated fatty acid of the Omega-3 family and a precursor to the fatty acids, eicosapentaenoic acid (C20: 5 n-3) and docosahexaenoic acid (C22: 6 n-3), that undergo limited biohydrogenation in the rumen (Ashes et al., 1992; Gulati et al., 1999). Dietary inclusion of flaxseed has been shown to improve conception rates in dairy cows (Petit et al., 2001; Ambrose et al., 2003) and decreased pregnancy losses (Ambrose et al., 2006; Petit and Twagiramungu, 2006). The mechanisms involved are not clear, but it has been proposed (Ambrose et al., 2006) that reduction in pregnancy losses occurs through enhancement of embryonic development prior to 24 d of gestation in cows fed a diet enriched in ALA.

Progesterone has positive effects on early embryonic growth (Garrett et al., 1988). We hypothesized that early embryonic development will be enhanced by dietary inclusion of ALA, and that such enhancement will occur through increases in plasma concentrations of progesterone. The objectives of the present study, therefore, were to compare the embryonic development (as determined by blastomere nuclei number) and plasma concentrations of progesterone in cows fed rations supplemented with the unsaturated fatty acids, ALA (flaxseed) or linoleic acid (sunflower seed) or saturated fatty acids.

3.2. Materials and Methods

3.2.1. Animals and diets

The experiment was conducted at the Dairy Research Unit of the University of Alberta, between September 2005 and February 2006, with all animal experimental procedures approved by the University of Alberta Animal Policy and Welfare Committee (2005-33c). A total of 24 cycling, lactating Holstein cows were randomly assigned to one of the three dietary groups. The experimental design is shown in Figure 3.1. At initiation of experiments, cows averaged 86 ± 22 d postpartum and 3.0 ± 0.4 lactations. Total mixed rations were formulated to meet or exceed the requirements of a 650 kg lactating cow as per NRC (2001) guidelines. Diets were isonitrogenous, and estimated energy intake was not similar across diets. Ingredients and diet composition are presented in Table 3.1 and 3.2. The concentrate mixture was formulated to provide 750 g of oil/cow/d from whole flaxseed (FLX), whole sunflower seed (SUN) or saturated fatty acids (SAT, Energy booster 100; JEFO Nutrition Inc., St-Hyacinthe, QC).

Cows were housed in tie-stalls and had unrestricted access to water. Cows were fed once daily at 0930 h, allowed 1 h of exercise each day (between 1030 and 1130 h), and milked twice daily between 0400 and 0600 h in the morning, and between 1530 and 1730 h in the afternoon. Diets were delivered individually, by a Data Ranger (American Calan Inc., Northwood, NH) and orts were weighed back next day prior to the commencement of feeding. Body condition score (BCS) and body weight were determined prior to the experiment. Average body weight of cows fed SAT, FLX or SUN was 627.7 ± 18.4, 641 ± 26.5, 618 ± 16.8 Kg respectively. Average BCS of cows fed SAT, FLX or SUN was 2.7 ± 0.1, 2.7 ± 0.2, 2.4 ± 0.1 respectively. The experimental period was 54 d.

3.2.2. Superovulation

After receiving the respective diets for 20 d, ovulation was synchronized in all cows using an established protocol (Ovsynch; Pursley et al., 1995). The experimental design is shown schematically in Fig. 3.1. The program involved administration of 100 μg of GnRH (gonadorelin acetate; Fertiline, Vetoquinol NA Inc., Lavaltrie, QC) followed by 25 mg of $PGF_{2\alpha}$ (dinoprost tromethamine; Lutalyse, Pfizer Animal Health, Kirkville, QC) 7 d later and a second administration of GnRH (gonadorelin acetate, 100 μg) 48 h after $PGF_{2\alpha}$. Transrectal ultrasonography (Aloka-500V scanner equipped with a 7.5 MHz linear transducer, Aloka Co., Tokyo, Japan) was performed in all animals, at first GnRH treatment and then once daily from the day of $PGF_{2\alpha}$ treatment until day of ovulation to determine ovarian dynamics and ovulation.

Five d after ovulation, the dominant follicle was ablated by a transvaginal ultrasound-guided procedure in all animals (Baracaldo et al., 2000; Guzeloglu et al., 2001). The subordinate follicle and any follicle greater than 9 mm diameter were also ablated at the same time. Ultrasonography was used in all animals to determine the ovarian follicle status at initiation of superovulation treatments.

Superovulation began on Day 7 (Day 0 = ovulation) with intramuscular injections of follicle stimulating hormone (FSH; Follotropin-V, 300 mg, Bioniche Animal Health Canada Inc., Belleville, ON) administered at descending doses of 60, 40, 30, and 20 mg, respectively, given twice daily over 4 consecutive days. Concurrent with the 7^{th} and 8^{th} FSH treatments, 25 mg of $PGF_{2\alpha}$ (Lutalyse) was given. Transrectal ultrasonography was performed to determine the number of follicles ≥ 9 mm on Day 11, and that of corpora lutea (CL) at embryo collection, respectively. Porcine luteinizing hormone (pLH; Lutropin-V, 25 mg, Bioniche Animal Health; Belleville, ON) was administrated between 1730 and 1800 h on Day 11. All animals were artificially inseminated (AI) twice with frozen-thawed semen from a single young sire on Day 12, am and pm.

3.2.3. Non-surgical Embryo Collection

Seven d after AI, embryos were collected non-surgically using a two-way Foley catheter (AB Technology, Pullman, WA), adopting a recto-vaginal technique (Rowe et al., 1980). Cows were restrained in a squeeze chute, the rectum was emptied of fecal matter, and the vulva and surroundings were cleaned and disinfected with povidone iodine (Prepodyne solution, West Penetone, Anjou, QC). Epidural anaesthesia was induced by administering

5 ml of a 2% Lidocaine solution (Bimeda-MTC Animal health Inc., Cambridge, ON) into the epidural space. A sterile metal stylet was inserted into the lumen of the catheter and the whole system was introduced into the vagina and subsequently passed through the cervix, and carefully diverted into one uterine horn. Upon entering the uterine horn, the catheter was advanced gently, until the tip of the catheter was palpable beyond the external bifurcation of the horns. At this step, the cuff of the catheter was inflated with air to prevent the escape of flushing fluid from the uterus with simultaneous palpation of the cuff per rectum, to avoid over- inflation. The amount of air used to inflate the balloon ranged from 10 to 20 cc, depending on the size of the uterus. As soon as the catheter was fixed, the metal stylet was withdrawn. The flushing media (Vigro^{TM} Complete Flush, AB Technology, Pullman, WA) was passed into the uterus by gravitational flow via silastic tubing that connected the flushing media container to the inflow tube of the Foley catheter. Once the uterus filled with an adequate quantity of flushing fluid, the inflow tube was closed and the outlet tube was opened which allowed the fluid to flow through the tube onto an embryo filter (Millipore Non-Vented Embryo Collection Filter, AB Technology, Pullman, WA) of 75 μm pore size, which was placed below the catheter level. The contents of the filter were emptied into a sterile petri dish to search for embryos under a stereomicroscope (MZ 7.5, Leica Microsystems Canada Inc., Richmond Hill, ON). Embryos were isolated, placed in a holding medium (Vigro^{TM} Holding plus, AB Technology, WA) and classified according to the International Embryo Transfer Society guidelines (Table 3.3).

3.2.4. Staining of embryos

The embryos were stained with propidium iodide for 20 sec and bisbenzamide overnight as described by Thouas et al. (2001). After staining, embryos were rinsed and placed on a glass slide individually, covered with a small drop of glycerol, and compressed gently under the weight of a coverslip. The edge of the coverslip was then sealed with nailpolish and slides stored at 4o C in a dark container, until counting of blastomeres occurred.

Three dimensional views of embryos were visualized using a 2–photon microscope (Axiovert 200M, Carl Zeiss, Jena, Germany) fitted with a HeNe543 laser and a Coherent Mira 900 femto-second laser pumped with a 5W Verdi laser (Coherent Inc., Santa Clara, CA). The 543 nm excitation laser line was used to excite propidium iodide and 760 nm was used to excite bisbenzamide. The three dimensional confocal image stacks were processed with Imaris software (Bitplane AG, Zurich, Switzerland), filtered with median filter, and blastomere nuclei counted using the spot-counting function of Imaris software.

3.2.5. Blood sampling

Blood samples were collected via the coccygeal vein daily from Day 0 (ovulation) to Day 8, and on Day 10 into evacuated tubes containing sodium heparin (Vacutainer, Beckton Dickinson and Co., Franklin Lakes, NJ). Sample tubes were immediately placed on ice and centrifuged at 4° C for 20 min at 3000 x g (ROTANTA 460 R, Hettich Zentrifugan., Tuttlingen, Germany) and stored at -20° C until analysis.

3.2.6. Determination of progesterone and insulin

Plasma concentrations of progesterone and insulin were determined using solid-phase radioimmunoassay kits (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA). Progesterone concentrations were determined on all samples from Days 0 to 10, whereas insulin concentrations were determined only from Day 5 samples of cows. Inter assay and intra assay coefficients of variation for plasma progesterone were 5.4 % and 9.9 % respectively. Sensitivity of the assay was 0.1 ng/ml. Intra assay coefficient of variation for plasma insulin was 7.9%.

3.2.7. Milk fatty acid

The fatty acid profile of milk was determined in a subset of cows (n=4/ dietary group) by gas chromatography as described by Ambrose et al. (2006).

3.2.8. Statistical Analyses

Data were analyzed by ANOVA for a completely randomized design. Repeated measures on plasma progesterone were analyzed using the MIXED procedure of SAS (1999) with the following model:

$$Y_{ijk} = \mu + D_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$$

Where μ is the population means, D_i is a population parameter corresponding to treatment i, β_j is the fixed effect of time j, $(\alpha\beta)_{ij}$ is the effect of treatment by time interaction, and e_{ijk} is the residual error. The covariance structure of the repeated measurements for each variable was modeled separately according to the lowest values of fit statistics for AIC (Akaike's Information Criteria), AICC (AIC Corrected), and BIC

(Bayesian Information Criteria) and an appropriate structure fitted (Littell et al., 2000). The PDIFF option was used in preplanned treatment comparisons and declared significant at $P < 0.05$. Proc GLM of SAS was used to analyze the plasma insulin and fatty acid profile data.

3.3. Results

3.3.1. Dry matter Intake (DMI), Milk yield, Fat and Protien

Milk yield, milk fat %, protein %, lactose % and DMI were not affected by diets ($P > 0.5$). Results of milk yield, milk fat %, protein % and lactose % are given in Table 3.4. Overall DMI (mean ± SEM) of cows fed SAT, FLX or SUN were 17.1 ± 0.4, 17.3 ± 0.4 and 17.7 ± 0.4 kg/d respectively.

3.3.2. Superovulation response and embryo recovery

The mean number of follicles, CL, total ova and embryos, transferable embryos, and fertilization rate did not differ among cows fed SAT, FLX or SUN (Table 3.5).

3.3.3. Blastomere number

Total blastomere number was affected ($P < 0.001$) by diet, stage of embryos, and their interactions ($P = 0.05$). The results are presented in Table 3.6. Embryos collected from cows fed SAT had fewer ($P < 0.003$) blastomeres than those from cows fed FLX or SUN. Cell numbers of morulae did not differ among treatment groups. However, blastocysts of cows fed SAT had fewer cells than those of cows fed SUN, but were not different ($P = 0.16$) from those of cows fed FLX. Differences were most evident in the expanded

blastocyst stage, with embryos of cows fed FLX and SUN having higher cell numbers than those fed SAT. Expanded blastocysts tended to differ (P = 0.08) between FLX and SUN cows. The picture of a stained embryo is shown in Figure 3.2.

3.3.4. Progesterone concentration

Mean plasma progesterone concentration of cows fed SAT was significantly lower (P < 0.04) than that of cows fed flax and sunflower (Table 3.7), but there was no difference in plasma progesterone concentration between SUN and FLX group cows (P = 0.92). The interaction between day and treatment was significant (P < 0.01). Plasma progesterone concentrations of cows fed SAT were lower (P < 0.02) than those of cows fed FLX and SUN from Day 6 to Day 8.

The temporal changes in plasma concentrations of progesterone are shown in Figure 3.3. There was no significant difference between the diets in plasma progesterone concentration on Day 0.

3.3.5. Plasma Insulin

Plasma concentrations of insulin on Day 5 were not different among diets (P > 0.05) (Table 3.7).

3.3.6. Milk fatty acid profile

Cows fed FLX had higher ALA concentrations in milk than those fed SUN or SAT (P < 0.01); there was no difference in ALA concentration between SUN and SAT dietary

group cows (Figure 3.4A). Linoleic acid (LA) concentrations were higher in milk of cows fed SUN than in those fed FLX or SAT ($P < 0.03$); however, we did not observe any difference in LA concentrations between FLX and SAT dietary group cows (Figure3.4B).

3.4. Discussion

3.4.1. Total blastomere number

We hypothesized that early embryonic development will be enhanced by dietary inclusion of ALA, and that such enhancement would occur through increases in plasma concentrations of progesterone and insulin. The total cell number of embryos recovered from cows fed SAT was lower than that of embryos recovered from cows fed FLX or SUN. However, we did not find any difference in cell numbers between the embryos recovered from cows fed SUN or FLX. In other words, unsaturated fatty acids of both flaxseed and sunflower seed-origin enhanced early embryonic development compared to a diet enriched in saturated fatty acids. The present finding agrees with results of Cerri et al. (2004) who found that embryos of cows fed calcium salt of linoleic and *trans* fatty acids tended to have higher total number of cells and were of better quality than embryos of cows fed calcium salt of palm oil. There is limited research in this area and the findings of the present study indicate that the type of dietary fatty acid would influence early embryonic development. Fatty acids act as an energy source for the developing embryos and there is evidence in the mouse, where sufficient supply of unsaturated fatty acids increased the development of preimplantation embryos from zygote over saturated fatty acids (Quinn and Whittingham, 1982).

Cows fed FLX and SUN had higher concentration of long chain fatty acids in milk and plasma (Ambrose et al., 2006). All polyunsaturated fatty acids can transfer across the placenta into the fetal blood circulation (Ruyle et al., 1990). In humans, it has been reported that fatty acids enter the fetal circulation from maternal circulation in a preferential order of docosahexaenoic acid, ALA, LA, oleic, and arachidonic acids (Haggarty et al., 1997). Even though our hypothesis that cows fed diets supplemented with flaxseed will have greater embryonic development was only partially supported, the present findings indicate that early embryonic development is enhanced in cows fed unsaturated fatty acids compared to those fed saturated fatty acids. The beneficial effect of unsaturated fatty acids on embryo development observed in the present study may be due to a direct effect of unsaturated fatty acids on blastocyst formation. Quinn and Whittingham (1982) reported that the mono unsaturated fatty acid, oleic acid, was better than the saturated fatty acid, palmitic acid, in promoting the formation and hatching of blastocysts from one cell stage of mouse embryo. Other studies reported polyunsaturated fatty acids, such as linoleic acid and ALA, have played important roles in embryonic development in humans and laboratory animals primarily by maintenance of the fluidity, permeability and conformation of membranes and also by acting as precursors of prostacyclins, prostaglandins, thromboxanes and leukotrienes (Herrera, 2002; Haggarty, 2002). In swine, gilts fed Menhaden fish oil (rich in ALA) had higher embryonic survival rate than those fed a starch-based control diet, and ALA was elevated in the blood of fetuses recovered from gilts that received the Menhaden oil-based diet (Perez Rigau et al., 1995). Another possibility for the enhancement of embryonic development was that cows fed unsaturated fatty acids had higher plasma progesterone concentrations than

cows fed saturated fatty acids from Days 6 to 8 post-insemination. Although we used a previously reported differential staining procedure to stain embryos in the current study, the propidium iodide stain was not clearly detectable and for this reason we have reported total cell numbers instead of reporting inner cell mass (ICM) and trophectoderm (TE) separately. Total cell numbers of embryos can also be used to determine embryonic development (Kubisch et al., 1998).

IFN-τ expressed by embryos is responsible for the maternal recognition of pregnancy (Thatcher et al., 1995). The size of the embryo is directly proportional to the amount of IFN-τ secreted (Hernandez-Ledezma et al., 1993). Therefore, an increase in size of the embryos will increase IFN-τ secretions (Leung et al., 2000; Geisert et al., 1988), thereby potentially allowing for timely suppression of endometrial $PGF_{2\alpha}$ release and increasing the chances for embryo survival. In the present study, the size of expanded blastocysts recovered from cows fed FLX or SUN was larger than from those recovered from cows fed SAT. Therefore, the size difference in embryos among the dietary groups will play an important role in embryonic development by more secretions of IFN-τ in large embryos.

3.4.2. Superovulation responses

There was no difference in the number of follicles or CL among diets of different fatty acid content in the present study. Consistent with our present findings, Ryan et al. (1992) reported there was no influence of dietary treatment (high lipid diet 5.4 % of added fat *versus* no lipid diet) either on FSH-stimulated recruitment of follicles or on the number of ovulations. However, cows fed saturated fatty acids (tallow) and polyunsaturated fatty

acids (soybean oil) had increased numbers of medium follicles compared to non-fatty acid control diet, yet there was no difference in number of ovulations among cows fed tallow, soybean oil and non-fatty acid control diet (Thomas et al., 1996). In general, many studies have reported that dietary fatty acid supplements would enhance follicle growth in cattle (Beam and Butler, 1997; Hightshoe et al., 1991). In the present study we did not have a control diet with no supplemental fat; therefore, it was not possible to make a direct comparison of any differences in ovarian follicular numbers between cows given diets supplemented with and without fatty acids.

We observed no differences in total embryos and transferable embryos among diets. Our result is consistent with the findings by Bader et al. (2005), in which they compared the superovulation response in prepartum beef cows fed with lipid and non-lipid dietary supplements. The fertilization rate was also not different in our study, which is consistent with the findings by Ryan et al. (1992) who showed there was no significant difference between high lipid and normal lipid diets. However, Cerri et al. (2004) reported that cows fed calcium salts of linoleic acid tended to have higher fertilization rates than cows fed calcium salt of palm oil. It has been proposed (Guilbault et al., 1991; Ryan et al., 1992) that the presence of a dominant follicle at the time of initiation of FSH treatment may have suppressive effects on follicular growth, averting any differences between the lipid and non-lipid diet. The ablation of the dominant follicle 48 h before the initiation of superovulation treatment promoted the follicular growth, increased ovulation and embryo production (Kim et al., 2001). Even though the dominant follicle present on Day 5 after

ovulation was ablated 48 h before the initiation of the superovulation regimen, we did not find any significant difference in superovulation responses among diets.

3.4.3. Plasma progesterone

In our study, cows fed SAT had low plasma progesterone concentrations compared to cows fed FLX and SUN. However, there was no significant difference between overall means of plasma progesterone concentrations of cows fed FLX and SUN. The latter result is consistent with the findings of Ambrose et al. (2006). An earlier study (Robinson et al., 2002) reported lower plasma progesterone concentrations in cows fed ALA and LA diets compared to cows fed a control diet with a lower content of ALA and LA. In their study blood samples were collected daily over four estrous cycles and the differences observed were likely due to the large number of samples evaluated. They proposed that the reductions in plasma progesterone may be due to a delay in ovulation and low levels of luteotrophic prostaglandin E_2. One of the factors that contribute to the failure in conception is the low plasma progesterone concentration during the early embryonic developmental phase (Mann and Lamming, 1999; Hommeida et al., 2004). The rise of postovulatory progesterone is essential for the initial embryonic development in cows (Demetrio et al., 2006). In the present study FLX and SUN group cows had higher levels of progesterone than SAT group cows from Days 6 to 8. Chagas de Silva et al. (2002) reported that peripheral progesterone concentrations on Days 5 to 7 were positively correlated with viable embryo recovery. Increase in progesterone concentration for cows fed polyunsaturated fatty acids over saturated fatty acids from Days 6 to 8 would have enhanced embryonic development or increased the embryonic cell numbers.

Developmental progress of the conceptus and maternal progesterone concentrations determine the capacity of the conceptus to secrete IFN-τ (Mann et al., 1999). Cows that had lower progesterone concentration 5 d after insemination had impaired blastocyst development and lower IFN-τ secretion (Mann et al., 1999). Even though there was no difference in plasma progesterone concentration among the dietary groups on Day 10, SAT group cows had numerically lower plasma progesterone concentrations. Lower maternal progesterone concentration is associated with reduced embryonic development, and both delayed post-ovulatory plasma progesterone rise and reduced progesterone concentrations lead to poor embryo development (Mann and Lamming, 2001).

Garrett et al. (1988) indicated that administration of progesterone (100 mg) on Days 1, 2, 3, and 4 of pregnancy accelerated embryonic development in beef cows. This provides additional evidence for the importance of maternal progesterone in early embryonic development in cows. Furthermore, Garrett and co-workers (1988) reported that cultured embryos recovered from beef cows pretreated with progesterone had increased length and secreted a greater range of proteins over a 24 h period compared to embryos recovered from control group cows. Pregnancy rate increased consistently with the administration of progesterone during the first wk of pregnancy in cows (Mann and Lamming, 1999). Progesterone inhibits luteolysis by binding to oxytocin receptors, which blocks the second messenger system by decreasing the sensitivity to oxytocin (Grazzini et al., 1998) and enhancing conceptus development (Mann and Lamming, 2001).

3.4.4. Plasma insulin

Embryonic development has been enhanced in cows fed a diet that increased insulin concentration (Mann et al., 2003). Feeding polyunsaturated fatty acids increases serum insulin and growth hormone concentrations in both dairy and beef cows (Williams and Stanko, 1999). Diets containing high amounts of unsaturated fatty acids increase the concentrations of propionate in the rumen, which is involved in hepatic gluconeogenesis (Elmes et al., 2004; Chalupa et al., 1986; Selner and Schultz, 1980; Keele et al., 1989) and pancreatic insulin release, whereas an increase in saturation of fatty acids decreased the percentage of propionate linearly (Elliot et al., 1997). Therefore, saturated fatty acid may not influence pancreatic insulin release as much as unsaturated fatty acids.

Thomas et al. (1997) reported that feeding of unsaturated fatty acids increased serum insulin concentration within 3 wk, whereas saturated fatty acids increased the insulin concentration only after 6 to 7 wk. Although we did not find any difference in insulin concentration between cows fed unsaturated and saturated fatty acids, mean insulin concentration was numerically higher in FLX cows. A negative relationship between the insulin concentration and embryonic development has also been reported (Velazquez et al., 2005; Green et al., 2005). Determining plasma insulin was not an objective of the present study. Therefore, we analyzed one sample only (Day 5) for insulin in contrast to the study by Thomas et al. (1997) in which daily samples were collected and serum insulin analyzed for the 7 wk duration of study. Determination of plasma insulin on more than one day may have allowed for the detection of differences.

3.4.5. Dry matter intake (DMI)

The inclusion of whole flaxseed at approximately 10% on a dry matter basis did not affect intake in the present study, which is in agreement with previous studies that have reported no difference in dry matter intake when rolled, whole, or formaldehyde-treated flaxseed was included in dairy cow rations at 9% (Ambrose et al., 2006), 5 to 15% (Kennelly and Khorasani, 1993), or at up to 17% levels (Petit et al., 2001). We found that whole flaxseed is as effective as rolled flaxseed at increasing ALA in milk (Thangavelu et al., 2006); hence, we used whole flaxseed in this study. Petit (2003) reported lower dry matter intake in cows fed sunflower seed *versus* either formaldehyde-treated or whole flaxseed. However, our findings indicate no such reduction in intake. We observed that in milk, ALA was higher in FLX, and LA was higher in SUN cows, which is consistent with the finding of Ambrose et al. (2006).

Milk yield, milk fat % did not differ between the dietary groups. This is consistent with Kennelly and Khorasani (1993) and Petit (2003). Moreover, milk protein % did not differ between the dietary groups which is in agreement with Kennelly and Khorasani (1993) and Ambrose et al. (2006). However, Petit (2003) reported a higher concentration of milk protein in cows fed flaxseed

In conclusion, diets enriched in unsaturated fatty acids enhanced early embryonic development in dairy cows as determined by differences in embryonic cell number. This increase in embryonic development was likely due to an increase in progesterone concentration between Days 6 and 8 post insemination. Although mean plasma insulin

concentration was numerically higher in cows fed flaxseed, it was not significantly different from that of other dietary groups. Further studies are required to understand the mechanisms involved in the enhancement of early embryonic development by unsaturated fatty acids.

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Table 3.1. Ingredients and composition of the experimental diets.

Item	SAT	Sunflower	Flax
Ingredients % of DM			
Alfalfa hay	12	12	12
Barley silage	27.5	27.5	27.5
Concentrate mix*	45.5	45.5	45.5
Flaxseed	0	0	10
Sunflower seed	0	10	0
Energy booster	6	0	0
Soybean meal	4	5	2.5
Beet pulp	5	0	2.5
Nutrient composition (% of DM)			
Organic matter	92.4	92.1	92.2
CP	18.1	18.1	18.1
NDF	32.5	32.9	32.6
ADF	19.7	18.9	19.5
Lipid	6.52	6.51	6.50
Estimated energy (Mcal/Kg)	1.81	1.69	1.70

*Concentrate mix contained rolled barley grain 62.15%, corn grain ground 15.1%, canola meal solv 2.2%, corn gluten meal 12.7%, dairy premix 2.7%, magnesium oxide 0.2%, limestone 1.8%, sodium bicarbonate 1.2%, molasses 1.4% and biophosphorus (ca 21%, P 17%) 0.5%.

Table 3.2. Ingredients and composition of the experimental diets.

Dairy premix

Calcium	0.10%	Zinc	5000 mg/kg
Phosphorus	0.60%	Manganese	3100 mg/kg
Sodium	0.04%	Copper	1170 mg/kg
Magnesium	0.30%	Vitamin A	800 KIU/kg
Fluorine	3.6 mg/kg	Vitamin D	200 KIU/kg
Cobalt	6.2 mg/kg	Vitamin E	11000 IU/kg
Iodine	80 mg/kg		
Iron	28 mg/kg		

This micro premix contains Niacin at 50 g/kg

Table 3.3. Standardized classification of bovine embryos based on stage of development as per manual of the International Embryo Transfer Society (IETS).

Name	IETS stage	Description
Ovum	1	Unfertilized
2-12 cell	2	Fertilized ovum but is less than 16 cells
Early morula	3	Contains 16 or more cells that are distinct individuals have not coalesced
Compact morula	4	Cells have coalesced to form a compact mass
Early blastocyst	5	Embryo has formed a small blastocoele up to a blastocoele that half-fills the embryonic mass
Blastocyst	6	Blastocoele is highly prominent but not enough to fill zona pellucida and begin stretching the zona
Expanded blastocyst	7	Overall the diameter of the embryo increases with a thinning the zona pellucida
Hatched blastocyst	8	Undergoing the process of hatching or may have completely shed the zona pellucida
Hatched expanding blastocyst	9	This is a stage 8 embryo that has re-expanded

Table 3.4. Milk yield and composition in cows fed SAT, flax and sunflower. Milk yields were recorded from 24 lactating Holstein cows 1 wk prior to the start of the experiment (before) and when cows were in the fourth wk (4^{th} wk) of diet. Milk composition (fat, protein, lactose) was determined during the above period in 12 cows that included six animals from Experiment 1 and six animals from Experiment 2.

	SAT		Flax		Sunflower		
	Before	4^{th} wk	Before	4^{th} wk	Before	4^{th} wk	*P* value (4^{th} wk)
Milk yield (kg /day)	39 ± 3.5	42 ± 3.3	31 ± 3.4	33 ± 3.3	37 ± 3.2	38 ± 3.1	0.17
Milk fat (%)	3.7 ± 0.7	4.2 ± 0.7	3.1 ± 0.7	3.2 ± 0.6	3.1 ± 0.7	3.3 ± 0.6	0.63
Milk protein (%)	2.9 ± 0.1	2.9 ± 0.2	2.9 ± 0.1	3.2 ± 0.1	2.9 ± 0.1	3.1 ± 0.2	0.49
Lactose (%)	4.5 ± 0.1	4.5 ± 0.1	4.7 ± 0.1	4.5 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	0.74

Table 3.5. Superovulation response and embryo recovery from cows fed saturated fatty acid (SAT), flaxseed (FLX) or sunflower seed (SUN (n = 8/diet). Superovulation was induced in all animals 7 d after ovulation by administration of FSH and porcine LH treatments and embryos were collected nonsurgically 7 d post insemination.

	Dietary Groups			
	SAT	Flax	Sunflower	*P* value
	Mean ± SEM			
No. of follicles	16.5 ± 2.5	19.4 ± 4.2	16.4 ± 3.4	0.49
No. of CL	5.5 ± 2	8.0 ± 2.9	7.1 ± 2.5	0.28
Total ova and embryos	3.6 ± 1.3	4.4 ± 1.6	4.7 ± 1.7	0.19
Recovery rate (%)	43	40	54	0.39
Fertilization rate (%)	93.3	84.4	75.5	0.17
Transferable embryos	2.5 ± 0.4	3.1 ± 0.9	3.8 ± 1.2	0.27

*Recovery rate is total number of ova and embryos collected/number of ovulations

Table 3.6. Mean total number of blastomere nuclei of embryos recovered from cows fed diets supplemented with saturated fatty acid (SAT), flaxseed (FLX) or sunflower seed (SUN). Embryos (n = 61) were collected nonsurgically 7 d after insemination.

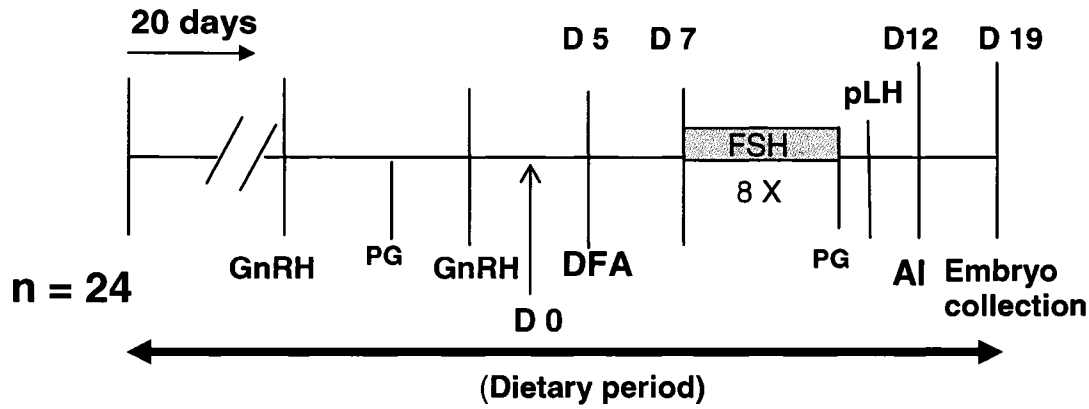
Stage of embryo	SAT	Flax	Sunflower	*P* value
Mean total cell number	77.1 ± 3.9[a]	93.4 ± 3.4[b]	97.1 ± 3.1[b]	0.01
Morula	64.4 ± 5.3	76.3 ± 5.7	65.6 ± 5.3	0.18
Blastocyst	77.5 ± 5.3[a]	88.6 ± 5.7[a,b]	93.7 ± 5.0[b]	0.04
Expanded blastocyst	89.3 ± 8.7[a]	115.4 ± 5.7[b]	132.3 ± 7.6[b]	0.01

*Total cell number include morula, blastocyst, expanded blastocyst

Table 3.7. Mean plasma progesterone concentrations (ng/ml; least square means ± SEM) in lactating Holstein cows from Days 0 to10, and mean insulin concentrations in plasma on Day 5 (Day 0 = ovulation).

	SAT	Flax	Sunflower	*P* value
[1]Progesterone (ng/ml)	1.42 ± 0.12[a]	1.81 ± 011[b]	1.79 ± 0.11[b]	0.04
[2]Insulin (μIU/ml)	7.4 ± 2.7	12.6 ± 2.5	7.7 ± 2.5	0.28

[1]Data represents 8 cows from each dietary group
[2]Data represents 8 cows for SAT, 8 cows for Flax, and 7 cows for sunflower.
[a, b] $P < 0.05$



DFA – Dominant follicle aspiration

Figure 3.1. Experimental design. Twenty-four lactating Holstein cows were assigned randomly but equally to 1 of 3 diets containing saturated fatty acids (SAT, high in palmitic acids), whole flaxseed (FLX, high in α-linolenic acid), or sunflower seed (SUN, high in linoleic acid).

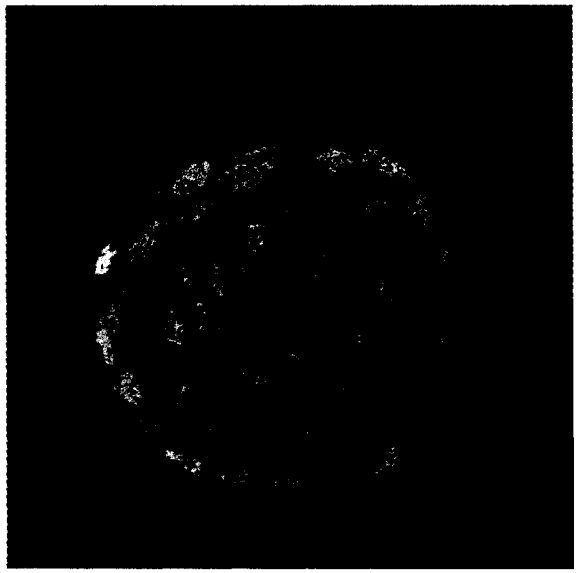


Figure 3.2. Stained embryo viewed under 2-photon microscope. Embryos were stained with propidium iodide and bisbenzimide. Embryos were visualized under 2-photon microscope and fluorescent cell nuclei were counted by Imaris software.

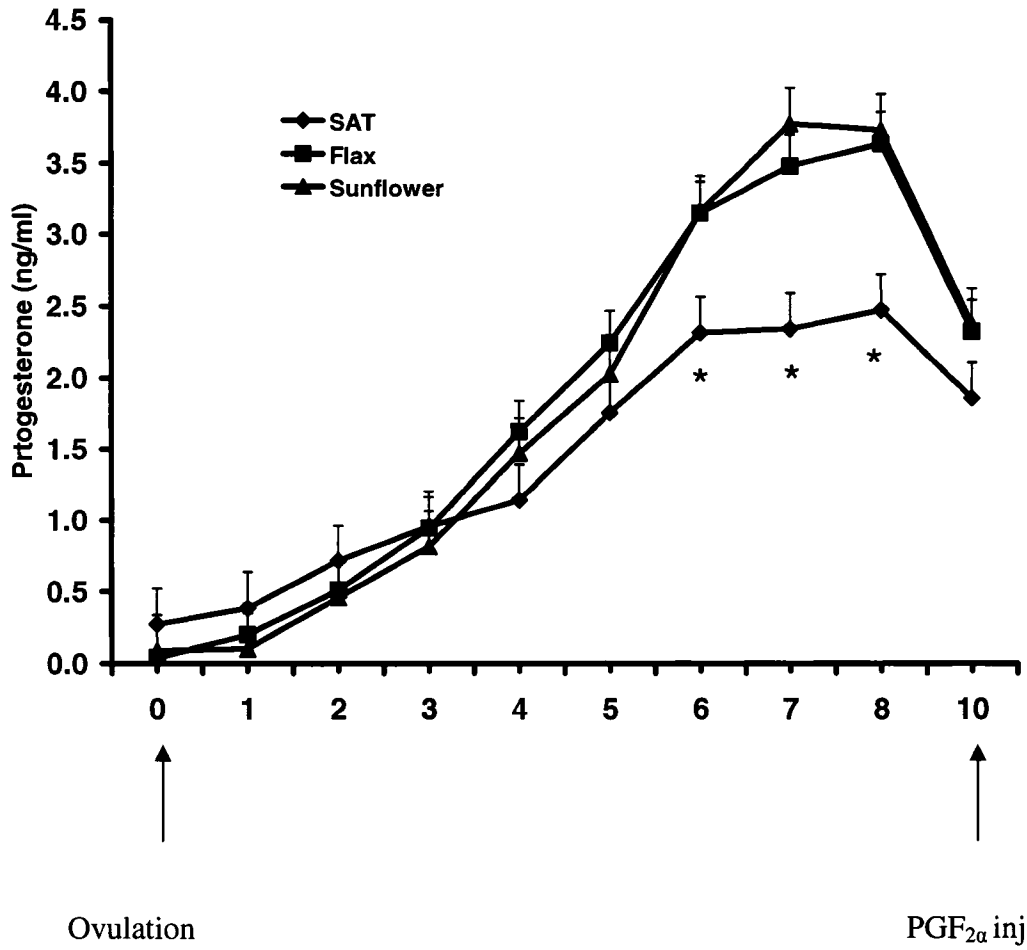


Figure 3.3. Mean progesterone concentrations from Day 0 to Day 10 for cows fed SAT, FLX and SUN (n = 24). *Progesterone concentrations were higher ($P < 0.02$) in cows fed unsaturated fatty acids, on Days 6, 7, and 8. Cows ovulated on Day 0, and received $PGF_{2\alpha}$ on Day 10 (8 h prior to sample collection). All cows were subjected to FSH-treatment starting on Day 7 and porcine LH on Day 11.

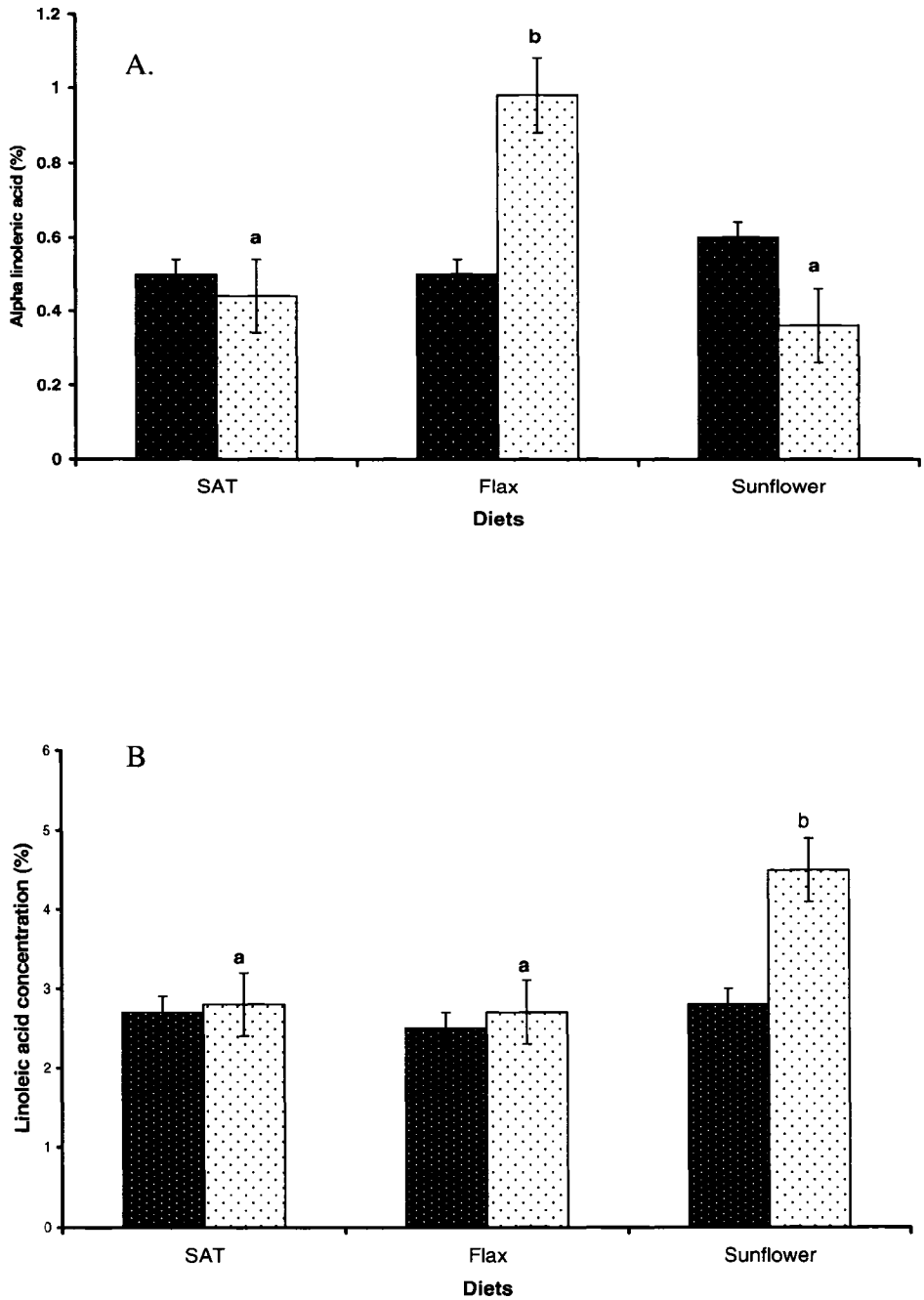


Figure 3.4. Alpha- linolenic acid (panel A) and linoleic acid (panel B) composition in milk of cows fed diets supplemented with saturated fatty acids, flax and sunflower (n = 4/diet). Milk samples were collected before the start of the diet (dark bars) and 4 wk into the dietary period (light bars). a,b = $P < 0.01$ in panel A and $P < 0.03$ in panel B.

Chapter 4

Influence of dietary fatty acids on concentrations of IGF-I and estradiol in ovarian follicular fluid, and oxytocin receptors in the endometrium of lactating dairy cows

4.1. Introduction

Early embryonic loss is a major contributor to reproductive inefficiency and about 40% of the losses occurs between 8 and 17 days of pregnancy (Thatcher et al., 1994). Estradiol secreted from the preovulatory follicle is important for the initiation of the luteolytic process (Thatcher et al., 1989) in nonpregnant cattle. Estradiol from the follicle acts on the endometrium to increase oxytocin receptor populations. Oxytocin released from the corpus luteum and posterior pituitary gland will interact with its receptors in the uterine endometrium resulting in the cascadic release of prostaglandin F_2 alpha (PG $F_{2\alpha}$) from the endometrium. This has been shown by several studies. For example, Hixon and Flint (1987) reported that the administration of estradiol in mid estrous cycle initiated luteolysis after 12 h with an increase in endometrial oxytocin receptor concentrations. Furthermore, the addition of estradiol to long-term cultured bovine endometrial cells enhanced oxytocin-induced $PGF_{2\alpha}$ production (Asselin et al., 1996) indicating that estradiol plays an important role in the luteolytic process in the bovine species.

Insulin like growth factor (IGF-I) is a polypeptide, secreted mainly from liver. Intraorgan IGF-I systems exist in the ovary, where IGF-I has a mitogenic role (Zulu et al., 2002) and exhibits both paracrine and autocrine functions (Hammond et al., 1991). Steroidogenesis

was stimulated by IGF-I *in vitro* in bovine granulosa and luteal cells (Schams and Koll, 1988). Moreover, Ginther et al. (2004) reported that administration of IGF-I directly into ovarian follicles of dairy cows stimulated the follicular fluid estradiol secretions. Therefore, an increase in intrafollicular concentrations of IGF-I should increase estradiol concentrations.

Flaxseed is the richest known dietary source of secoisolaricresinol diglycoside (SDG), a precursor for mammalian lignans such as enterolactone and enterodial (Thompson et al., 1991). Enterolactone and enterodial are diphenolic compounds, formed by colonic bacteria in humans and rodents (Borriello et al., 1985). These mammalian lignans have exhibited anti-IGF-I and antiestrogenic properties as rats fed a flaxseed based diet had reduced IGF-I (Rickard et al., 2000) and irregular estrous cycles (Orcheson et al., 1998).

Dietary inclusion of flaxseed has improved fertility in dairy cattle (Petit et al., 2001) largely through reduced early embryonic losses (Ambrose et al., 2006; Petit and Twagiramungu, 2006). However, the underlying mechanisms have not been elucidated. There is one school of thought that α-linolenic acid (ALA), a major component of flaxseed may be involved in the reduction of $PGF_{2\alpha}$. In cattle, maternal recognition of pregnancy occurs around Day 15 of gestation and is dependent on the timely secretion of interferon-tau (IFN-τ) by the embryo, which is essential for suppression of $PGF_{2\alpha}$ release, allowing pregnancy establishment. It has been proposed (Thatcher et al., 1989; Ambrose and Kastelic, 2003; Mann et al., 1999) that a considerable proportion of embryos may not express sufficient quantities of IFN-τ in a timely manner to be able to inhibit $PGF_{2\alpha}$

production. Even a slight delay in luteolysis may be adequate for slow-growing embryos to increase IFN-τ secretion and "rescue themselves" from the luteolytic action of PG $F_{2\alpha}$. Even though embryonic losses were reduced in cows fed diets enriched in ALA (flaxseed), *in vitro* studies do indicate $PGF_{2\alpha}$ suppression (Mattos et al., 2003).

A direct role for ALA in $PGF_{2\alpha}$ suppression has not been clearly demonstrated *in vivo* (Petit et al., 2001; 2004; Benefield et al., 2006). Measuring $PGF_{2\alpha}$ in peripheral circulation is difficult due to its unstable nature, therefore, only its metabolite 15-keto-13, 14-dihydro-prostaglandin $F_{2\alpha}$ (PGFM) is measured conventionally, in peripheral plasma. Due to the difficulties associated with measuring $PGF_{2\alpha}$ at its site of production (i.e. the uterus), an alternative approach is to determine IGF-I, estradiol, and oxytocin receptor populations, which have indirect roles in the luteolytic process. Therefore, our hypothesis for the present study was that the inclusion of flaxseed in the diets of dairy cows will reduce intrafollicular concentrations of IGF-I, leading to decreased estradiol synthesis, and subsequently reduce uterine oxytocin receptor populations.

The objectives were to determine intrafollicular concentrations of IGF-I and estradiol, and that of oxytocin receptors in the endometrium of cows fed diets enriched in unsaturated fatty acids (of flaxseed or sunflower seed origin) compared to that of cows fed saturated fatty acids. A secondary objective was to quantify estradiol receptor (α-sub unit; ERα) populations in the uterus.

4.2. Materials and Methods

4.2.1 Animals and diets

The study was carried out between October 2005 and February 2006 where lactating Holstein cows (n = 15) were randomly assigned to three different diets. At initiation of experimental diets, cows averaged 37 ± 3.1 d postpartum, and 2.0 ± 0.25 lactations, respectively. The schematic representation of the experimental design is shown in Figure 4.1. All cows received a base total mixed ration (TMR) with the principal forage components being barley silage and alfalfa hay. Base rations were supplemented with whole flaxseed (FLX), whole sunflower seed (SUN) or saturated fatty acids (SAT), respectively. Estimated energy was similar across the diets and rations were formulated to supply 750 g of oil per cow per day. Ingredients and nutrient composition of the diets are presented in Table 4.1and 4.2.

All experimental procedures were approved by the University of Alberta Animal Policy and Welfare Committee (2005-33c) and animals were cared for in accordance with the guidelines of the Canadian Council on Animal care (1993). The cows were housed in tie stalls with free access to water at all times. Cows were fed once daily (1030 h) milked twice (0500, 1600 h) and exercised once (0930-1030 h) daily. Body weights of cows were recorded using electronic scales. A single person recorded the body condition scores (BCS) of cows using a 1 to 5 scale (Edmonson et al., 1989). Average body weight of cows fed SAT, FLX or SUN was 608 ± 23.4, 630 ± 28.2, 604 ± 41.4 Kg respectively. Average BCS of cows fed SAT, FLX or SUN was 2.7 ± 0.1, 2.6 ± 0.04, 2.6 ± 0.1 respectively.

4.2.2. Synchronization of ovulation

Twenty days after initiation of diets, ovulation was synchronized in all cows by administration of 100 μg of GnRH (gonadorelin acetate; Fertiline, Vetoquinol NA Inc., Lavaltrie, QC) on day 0, followed by 25 mg of $PGF_{2\alpha}$ (dinoprost tromethamine, Lutalyse, Pfizer Animal Health; Kirkville, QC) 7 d later and a second administration of GnRH 48 h after $PGF_{2\alpha}$ (Pursley et al., 1995). Transrectal ultrasonography (Aloka-500V scanner, 7.5 MHz linear transducer, Aloka Co., Tokyo) was performed in all cows from day of $PGF_{2\alpha}$ administration to confirm the day of ovulation (Day 0). On Day 5, the first-wave dominant and co-dominant follicle (if any) including all follicles ≥ 9 mm diameter were ablated by an ultrasound-guided transvaginal procedure to induce the synchronized emergence of a new follicular wave (Baracaldo et al., 2000).

4. 2.3. Sample collection

4.2.3.1. Blood

Blood samples for plasma progesterone assays, were collected daily from Day 0 to Day 8 and on Day 10 to 12 in alternate days from the coccygeal veins of cows into evacuated 10 ml vacutainer tubes (Becton Dickinson, Franklin lakes, NJ) containing sodium heparin anticoagulant. The blood samples were collected on ice and centrifuged at 4° C for 20 min at 3000 ×g (ROTANTA 460 R, Hettich Zentrifugan, Tuttlingen, Germany) and stored at -20° C until subsequently assayed.

Follicular fluid samples were collected by transvaginal follicular aspiration. Animals were restrained in a chute and feces were manually removed from the rectum. The vulva

and perianal area were scrubbed and disinfected with povidone iodine (Prepodyne solution, West Penetone, Anjou, QC). Lidocaine (2%, 5 ml) (Bimeda-MTC Animal health Inc., Cambridge, ON) was administrated into the epidural space either in sacro-coccygeal joint or in between the 1st and 2nd coccygeal vertebrae for induction of epidural anaesthesia.

The transvaginal follicle aspiration apparatus consisted of an ultrasound transducer (5 MHz) and vaginal probe with a 17 G, 65 cm needle with an echogenic tip. A vinyl tube (0.14 cm I.D ×1.9 cm O.D, Science commodities Inc., Lake Havasu, AZ) connected the needle to a 15 ml collection tube and another tube connected the collection tube to a suction device (Gomco vacuum pump, Gomco Allied Healthcare Products, St.Louis, MO) through a receptacle.

An ultrasound machine (Aloka 500 V, Tokyo; Japan) equipped with 5.0 MHz convex-array transducer inside a vaginal probe with a dorsal-mounted needle guide was used to visualize the ovaries. Ovaries were positioned per rectum to align each follicle with the built-in puncture line on the ultrasound monitor representing the projected needle path. A 19 G, 3.81 cm needle tip fitted to a 17 G, 65 cm barrel was inserted into the needle guide of the transvaginal probe. After alignment of ovary and follicle, the needle was advanced until the tip of the needle became visible on the ultrasound monitor. The follicle was punctured by advancing the needle while monitoring needle movement on the monitor, and applying suction simultaneously. The dominant follicle and other follicles greater than 9 mm dia, if any, were aspirated on Day 5, whereas only dominant follicles were aspirated on Day 15. The collected follicular fluid was immediately centrifuged at 3000×

g for 20 min at 4°C and subsequently stored at -20°C. Where possible, a new needle and collection tube was used for each animal. If a needle or tubing was reused, they were rinsed thoroughly with a 0.9% saline solution (Invitrogen Canada Inc., ON, Canada) and cold sterilized with 70% alcohol, between uses. The aspiration took place under a constant vacuum pressure of < 60 mm Hg at a flow rate of 27 to 29 ml/min.

4.2.3.2. Endometrial tissue

Transcervical uterine biopsy was performed to collect uterine endometrial samples as described by Mann and Lamming (1994). Briefly, after emptying the rectum, the vulva and perianal area was cleaned and disinfected with povidone iodine (Prepodyne solution, West Penetone, Anjou, Quebec, Canada). Epidural anaesthesia was induced by administering 5 ml of 2 % lidocaine hydrochloride (Bimeda-MTC Animal health Inc., Cambridge, ON). A 25 cm long uterine biopsy forceps (Kevorkian-Younge, Fine Surgicals, Hempstead, NY) was adapted for use in cattle by lengthening it to 58 cm. The biopsy forceps was introduced into the uterus transcervically taking a rectovaginal approach, and diverted into the uterine horn ipsilateral to the side of the dominant follicle. When the forceps reached the uterus, an endometrial sample was collected from a location about 5 cm beyond the external bifurcation of uterus. The samples were placed in cryovials (Fisher Scientific, Ottawa, ON) and immediately stored in liquid nitrogen at -196°C. Each biopsy sample weighed approximately 250 to 300 mg.

4.2.4. Insulin-like Growth Factor–I assay

Concentrations of total IGF-I in plasma and follicular fluid were determined by homologous double antibody radioimmunoassay as described by Novak et al. (2002). The assay was validated by establishing parallel displacement curves between serially diluted, extracted follicular fluid and plasma samples with the antihuman IGF-I antiserum. Follicular fluid and plasma samples were extracted with acid-ethanol (12.5 % (v/v) of 2.2 M HCL in 87.5 (v/v) ethanol- Fisher HPLC grade reagent alcohol) and neutralized with Tris base (Sigma No. T-1503). Two hundred microlitres of assay buffer were used to dilute 100µl of samples and standards in replicates (n = 5). After dilution, 100 µl of first antibody antihuman IGF-I antiserum (product name AFP4892898, obtained from Dr. AF Parlow through the NIDDK's National Hormone and Pituitary Program, CA), diluted 1:360 with assay buffer, was added to all tubes and subsequently incubated at 4°C for 24 h. After 24 h, 100 µl of labeled IGF-I diluted with assay buffer (10,000 cpm/100µl) was added to all tubes and incubated at 4°C. After 24 h of incubation, the second antibody (goat anti-rabbit gamma globulin, diluted with 1:140 in 1:600 normal rabbit serum) was added to each sample (100 µl) followed by a 16 h incubation period at 4°C. After incubation, 1 ml of double distilled water was added and the samples were centrifuged for 30 min at 3000 ×g. The supernatant was decanted and the pellet was counted by gamma counter for 2 min. The inter and intra assay coefficients of variance were 17.7% and 5.56 % and the sensitivity of the assay was 16.4 ng/ml.

4.2.5. Follicular fluid estradiol assay

Follicular fluid samples were thawed at 37°C for 10 min and centrifuged at 3000×g, 4°C for 20 min. The centrifuged sample was diluted to 1:500 with zero calibrator. Estradiol concentrations in follicular fluid were analyzed by using a solid-phase radioimmunoassay kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA). All samples were run in one assay; the intra assay coefficient of variation was 9.8%.

4.2.6. Progesterone assay

Plasma concentrations of progesterone were determined using a solid-phase radioimmunoassay kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA). Inter assay and intra assay coefficients of variation were 5.4 % and 9.9 % respectively. Sensitivity of the assay was 0.1ng/ml.

4.2.7. Western Blotting for ERα and oxytocin receptors

ERα and oxytocin receptor populations in the endometrium of the uterus were determined by western blot procedures. Endometrial tissues were homogenized for 30 sec in 0.5 mL of homogenizing buffer (50 m*M* Tris, pH 8.0, 300 m*M* NaCl, 1 m*M* Na_3VO_4, 20 m*M* NaF, 1 m*M* $Na_4P_2O_7$, 1 m*M* EDTA, 1 m*M* ethylene glycol-bis [ß-aminoethyl ether]-N N N'N'-tetraacetic acid (EGTA), 0.5 m*M* phenylmethylsulfonyl fluoride, 10% v/v glycerol, 1% v/v Nonidet P-40, and 10 µg/mL each of aprotinin, leupeptin, and pepstatin) followed by centrifugation at 11,000 rpm (Beckman J2-21, rotor JA020.1) for 15 min at 4°C. The protein concentrations were determined in supernatants by using BCA protein assay reagent kit (Pierce, Rock ford, IL) as described by Roberts et al. (2002). Volumes of

homogenizing extract from each cow corresponding to 200 μg of protein were loaded onto 10% separating gel and 4 % stacking gel, subjected to SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes overnight.

Membranes were blocked for 3 h in 2% (wt/vol) of ECL advanced blocking agent (Amersham Biosciences, Piscataway, NJ) in Tris-buffered saline containing 0.1% Tween-20 (TBST), washed twice for 5 min in TBST, and incubated with mouse monoclonal ERα antibody (1:250; Santa Cruz Biotechnology, Santa Cruz, CA; Cat. No. Sc-787) or anti-rat OTR (1:500; Alpha Diagnostics Intl Inc, San Antonio, TX; Cat No. OTR 11A) overnight. The secondary antibodies were goat anti-mouse IgG_{2a}-HRP (1:4000 Santacruz biotechnology, Santa Cruz, CA; Cat. No.sc-2061) or goat anti-rabbit IgG (H+L) peroxidase purified (1:4000, Alpha diagnostics Intl Inc, San Antonio, TX; Cat No. 20320) for ERα and oxytocin receptors respectively. Proteins were detected using ECLA advance western blotting kit (Amersham Biosciences, Piscataway, NJ) and analyzed by comparision with background by Typhoon Trio+ scanner (Amersham Biosciences, Piscataway, NJ).

4.2.8. Milk fatty acid analysis

Fatty acid profile of milk was determined in a subset of cows (n=4/ dietary group) by gas chromatography as described by Ambrose et al. (2006).

4.2.9. Statistical analysis

Analysis of variance (ANOVA) using the generalized linear model procedures (PROC GLM) of the SAS (2001) was used to study the effects of diet on follicular hormonal concentration, plasma IGF-I, milk fatty acid profile and endometrial receptors. Linear regression analysis was used to determine the relationship of IGF-I between Day 5 plasma and follicular fluid.

Repeated measures on plasma progesterone were analyzed using the MIXED procedure of SAS (1999) with the following model:

$$Y_{ijk} = \mu + D_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$$

Where μ is the population mean, D_i is a population parameter corresponding to treatment i, β_j is the fixed effect of time j, $(\alpha\beta)_{ij}$ is the effect of treatment by time interaction, and e_{ijk} is the residual error. The covariance structure of the repeated measurements for each variable was modeled separately according to the lowest value values of fit statistics AIC = Akaike's Information Criteria, AICC = AIC Corrected, and BIC = Bayesian Information Criteria and an appropriate structure fitted (Littell et al., 2000). The PDIFF option was used in preplanned treatment comparisons and differences were declared significant at $P < 0.05$.

4.3. Results

4.3.1. Dry matter intake, milk yield, and milk composition

Milk yield, fat, protein, lactose and DMI were not affected by diets

($P > 0.5$). Results of milk yield, and milk composition are given in Table 4.3. Overall DMI (mean ± SEM) of cows fed SAT, FLX or SUN was 16.8 ± 0.5, 17 ± 0.5 and 17.4 ± 0.5 kg/d respectively.

4.3.2. IGF-I

Concentrations of total IGF-I were measured in Day 5 and Day 15 follicular fluid samples. Diets had no effect on IGF-I concentrations of either Day 5 ($P = 0.9$) or Day 15 follicular fluid ($P = 0.6$). To establish the relationship between IGF-I concentrations in peripheral plasma and follicular fluid, Day 5 plasma IGF-I concentrations were also determined. There was no difference in the concentrations of Day 5 plasma IGF-I between cows fed SAT, FLX or SUN. ($P = 0.9$). Overall means ± SEM for Day 5 plasma IGF-I, Day 5 follicular fluid IGF-I and Day 15 follicular fluid IGF-I are presented in Figure 4.2. Plasma IGF-I was positively correlated with follicular fluid IGF-I ($P < 0.01$) (Figure 4.3).

4.3.3. Estradiol

Follicular fluid estradiol concentrations were not different in cows fed SAT, FLX or SUN on Day 5 ($P = 0.2$) *or* Day 15 ($P = 0.6$). Overall means ± SEM of follicular fluid, and estradiol concentrations of cows fed SAT, FLX or SUN on Day 15 are presented in Table 4.4.

4.3.4. Progesterone

Overall mean plasma progesterone concentrations of cows were not affected by diets

(P = 0.4). Even though the main effect of diet was not significant, there was an interaction between day and diet (P < 0.01). Cows fed SAT had significantly lower plasma progesterone concentrations than cows fed SUN from Day 2 to Day 5 (P < 0.03), and cows fed SAT had lower progesterone concentrations than cows fed FLX only on Day 5 (P = 0.05). Cows fed FLX had significantly lower plasma progesterone concentrations than cows fed SUN from Day 2 to Day 4 (P < 0.04). The overall means ± SEM of plasma progesterone concentrations is shown in Table 4.4 and graph in Figure 4.4.

4.3.5. ERα and OT receptors

Diet had no significant effect on ERα and oxytocin receptors (P > 0.1). Overall mean (± SEM) of cows fed SAT, FLX or SUN for ERα were 28.5 ± 5.5, 21.9 ± 5.5 and 32.7 ± 5.5 OD and for oxytocin were 0.8 ± 0.08, 0.9 ± 0.08 and 0.6 ± 0.1 OD, respectively. We analyzed the receptor concentration in the endometrial samples of cows that had estrogenic dominant follicle (n = 9). One of the samples obtained from a cow fed sunflower seed did not express oxytocin receptors and hence we did statistical analysis with remaining cows. The western blot images are shown in Figure 4.5.

4.3.6. Milk fatty acids

Linoleic acid and α- linolenic acid concentration of milk fat in cows fed SAT, FLX or SUN are shown in figure 4.6A and B.

4.4. Discussion

4.4.1. Insulin like growth factor-I

We hypothesized that a diet enriched with flaxseed will decrease IGF-I and estradiol concentrations at the ovarian level, and subsequently result in down regulation of oxytocin receptor populations in the uterus. Findings of our research did not support our hypothesis. It has been reported that, at the ovarian level, IGF-I is involved in proliferation and differentiation of granulosa cells, stimulation of aromatase system (Zulu et al., 2002), and promotion of ovarian steriodogenesis (Hammond et al., 1991). Beam and Butler (1998) reported that lactating dairy cows fed a diet supplemented with prilled fat, high in long chain saturated fatty acids, had lower mean plasma IGF-I concentrations up to 3 wk of the postpartum period when compared to cows fed an isoenergic control diet (37.6 vs. 47.7 ng/ml). However, other studies reported no differences in plasma IGF-I concentrations between cows fed a control diet versus a diet supplemented with dietary fat source (Spicer et al., 1993; Salado et al., 2004).

Flaxseed is the richest dietary source of lignan precursor, secoisolariciresinol diglycoside (SDG) and rats supplemented with 5% of flaxseed or SDG at 1.5 mg/day had reduced plasma IGF-I compared to rats supplemented with a non flax diet (Rickard et al., 2000). In the present study, contrary to our expectations, there was no difference in Day 5 plasma IGF-I concentrations among the diets. This agrees with the previous findings by Dunn et al. (2003) in which beef steers were fed 5% ground flaxseed (on a dry matter basis) and given a trenbolone acetate and estradiol-17ß implant. Even though there were no detectable effects of flax on circulating concentrations of IGF-I, muscle IGF-I mRNA

expression was significantly lower in cows fed flaxseed. However, in that study muscle cells cultured with two different concentrations of ALA did not reduce IGF-I mRNA expression. Based on this finding they concluded that ALA may not be responsible for the reduction of IGF-I mRNA expression in cows fed dietary flaxseed. In the present study diets had no significant effects on follicular fluid IGF-I concentrations on Days 5 and 15. Earlier studies reported that SDG and mammalian lignans may be responsible for the downregulation of IGF-I (Rickard et al., 2000; Dunn et al., 2003). One of the limitations of the present study is that we did not determine the presence of the mammalian lignans, enterolactone and enterodiol in the peripheral circulation of our experimental animals. To our knowledge, there is no report on existence of mammalian lignans in ruminants. Without this information it is difficult to explain why the expected IGF-I decrease did not occur in cows supplemented with flaxseed. The failure of flaxseed to exert its anti-IGF-I properties may be due to several reasons. One possibility is that mammalian lignans may not be formed from SDG in cattle due to the fermentative digestion. Secondly, it is likely that the production of lignans was inadequate to exert its anti IGF-I properties. For instance, there is a report in humans that consumption of 25 g of SDG per day resulted in plasma lignan levels of only 500 ng/ml (about 1.7 μM; Morton et al., 1994). When compared to this result, in large animals like dairy cows, there may be negligible quantities of lignans produced, which may be inadequate to exert its anti-IGF-I property. Thirdly, liver is the major target organ for lignans in rats (Rickard et al., 2000), and in high producing dairy cows there is an increased liver metabolism due to the high blood flow to the liver (Sangsritavong et al., 2002; Vasconcelos et al., 2003), which may cause rapid clearance of lignans. As a result, there may be an increased

excretion of lignans in the urine, leading to inadequate concentrations in target tissues to exert its antagonisitic properties towards IGF-I. In this study, we determined total IGF-I in follicular fluid. It was suggested (de la Sota et al., 1996) that changes in the relative proportions of IGF binding proteins in follicular fluid may cause binding of more IGFs and it would lead to less availability of follicular IGF-I to act on granulosa cells for steroidogenesis.

Results from the present study indicated a positive correlation between intrafollicular and circulating IGF-I, which is consistent with the findings of Hammond et al. (1999). Even though there is an intra organ IGF-I system present in the ovary, the positive correlation between IGF-I in plasma and follicular fluid indicates that at least part of the follicular fluid IGF-I may be of hepatic origin.

4.4.2 Estradiol

It has been reported that IGF-I stimulates bovine granulosa and luteal cell steroidogenesis *in vitro* (Schams et al., 1988) and estradiol secretion *in vivo* (Ginther et al., 2004). To our knowledge, this is the first study that investigated the effects of flaxseed on estradiol concentrations at the ovarian follicular level. Out of 14 cows that were distributed among the three dietary treatments, two came into estrus prematurely on Day 12 of their cycle; therefore follicular aspiration was not performed on these cows as we were interested in obtaining follicular fluid closer to the time of initiation of luteolysis (i.e., Day 15). Three other animals had low estradiol concentrations on Day 15, possibly due to the aspiration of a regressing follicle that was no longer estrogenic. As a result, we used only data from

9 cows in the analysis. We found no difference in estradiol concentrations among the diets, which, again, did not support our hypothesis. Hightshoe et al. (1991) reported that calcium salt of palm oil fatty acid diet maintained lower serum estradiol concentration than control cows (1.41 vs.1.64 pg/ml). Ryan et al. (1992) also reported lower follicular estradiol concentrations in beef cows fed soybean oil at 5.4% of diet DM over a diet with no added fat. The present findings are not consistent with those of Robinson et al. (2002), who reported that dairy cows fed a diet rich in α- linolenic acid (LinPreme®) had higher estradiol concentration than cows fed diets rich in linoleic acid (SoyPreme®)) and control diet. Our study differs from Robinson et al. (2002) in the dietary source; we included flaxseed as a source of α- linolenic acid in the dietary supplement instead of lipid supplement (LinPreme®) and reports showed flaxseed has anti-estradiol properties in rats. Lucy et al. (1991) reported no difference in estradiol concentration between cows fed diet without fat and diet with long chain fatty acids.

Earlier studies reported that enhanced luteal function by dietary fatty acids over non-fat control diets may cause differences in estradiol concentrations. However, in the present study we did not have a non-fat control group and this may be one of the reasons for finding no differences among the diets, since luteal function was comparable in all three diets. In rats, flaxseed and SDG exerted antiestrogenic properties (Orcheson et al., 1998). There is no report to date showing the existence of mammalian lignans in ruminants. Mammalian lignans are formed in the anaerobic environment of the gut by colonic bacteria *Eubacterium* and *Peptostreptococcus* species. In cattle, it is possible that mammalian lignans are produced in rumen where various kinds of bacteria exist

including *Eubacterium* species. As mentioned earlier, even if lignans are produced in the rumen, they may be metabolized very rapidly due to the high metabolic nature of lactating dairy cows.

Future research is needed on the production, metabolism, absorption, and excretion of mammalian lignans in ruminants. Quantification of mammalian lignans in rumen fluid, blood, urine, feces, and *in vitro* fecal culture would be essential to fill the knowledge gaps on lignans in ruminants.

4.4.3. Progesterone

In this study diets had no significant effects on plasma progesterone concentrations. There was no difference in plasma progesterone concentrations between cows fed unsaturated and saturated fatty acids. This finding is consistent with the findings by Petit and Twagiramungu (2006), in which there was no difference in mean plasma progesterone concentration over cows fed megalac ® (calcium salt of palmitic acid) or micronized soybean. The result from the present study is in contrast to our previous findings (chapter-2), where cows fed unsaturated fatty acid had higher level of progesterone than cows fed saturated fatty acid. In our previous study, ovaries of cows were stimulated with FSH starting Day 7, and progesterone concentrations were analyzed from Day 0 to 8 and on Day 10. The main difference between our two experimental groups is that the present study had only 4 cows per dietary group for progesterone determination. Furthermore, in our previous experiment, cows received FSH treatment

starting Day 7 of their cycle, and the lack of difference in progesterone concentrations among diets may be explained by the above variations.

4.4.4. Oxytocin and ERα receptors

It has been reported in many studies there is an increase in oxytocin receptor populations around the time of luteolyis (Meyer et al., 1988; Fuchs et al., 1990; Robinson et al., 2001) and its up regulation plays a key role in the initiation of the luteolytic process in ruminants (McCracken, 1999). Administration of estradiol in cyclic cows will stimulate $PGF_{2\alpha}$ secretion (Knickerbocker et al., 1986) and there is an upregulation of ERα during luteolysis in sheep (Cherny et al., 1991; Wathes and Hammon, 1993). IFN-τ, which is responsible for the maternal recognition of pregnancy, has been shown to suppress oxytocin receptor populations (Farin et al., 1990).

Spencer and Bazer (1995) hypothesized that IFN-τ inhibits the oxytocin receptors by suppressing a preceding increase in ERα expression. Hence we planned to determine the endometrial receptor populations, which serve an indirect role in determining luteolysis. In the present study, we did not detect differences in ERα and oxytocin receptor populations among the diets. Follicular estradiol concentrations did not differ among the diets, which may be the reason for the absence of any differences in these receptor populations.

In conclusion, our hypothesis that dietary inclusion of flaxseed will reduce intrafollicular concentrations of IGF-I and estradiol, and estradiol and oxytocin receptors in the uterus,

was not supported. The manner by which flaxseed based diets reduce embryonic losses remains poorly understood. Therefore, further research is essential in this area, and it would be important to include a larger number of animals in each dietary group. Future studies should also determine if mammalian lignans are formed in ruminant species following ingestion of flaxseed. Such studies will help in the better understanding of effects of flaxseed on reproductive function in cattle.

4.5. Literature cited

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Table 4.1. Ingredients and composition of the experimental diets.

Item	SAT	Sunflower	Flax
Ingredients % of DM			
Alfalfa hay	12	12	12
Barley silage	27.5	27.5	27.5
Concentrate mix*	45.5	45.5	45.5
Flaxseed	0	0	10
Sunflower seed	0	10	0
Energy booster	6	0	0
Soybean meal	4	5	2.5
Beet pulp	5	0	2.5
Nutrient composition (% of DM)			
Organic matter	92.4	92.1	92.2
CP	18.1	18.1	18.1
NDF	32.5	32.9	32.6
ADF	19.7	18.9	19.5
Lipid	6.52	6.51	6.50
Estimated energy (Mcal/Kg)	1.81	1.69	1.70

*Concentrate mix contained rolled barley grain 62.15%, Corn grain ground 15.1%, Canola meal solv 2.2%, Corn gluten meal 12.7%, Dairy premix 2.7%, Magnesium oxide 0.2%, Limestone 1.8%, sodium bicarbonate 1.2%, molasses 1.4% and Biophosphorus (ca 21%, P 17%) 0.5%.

Table 4.2. Ingredients and composition of the experimental diets.

Dairy premix

Calcium	0.10%	Zinc	5000 mg/kg
Phosphorus	0.60%	Manganese	3100 mg/kg
Sodium	0.04%	Copper	1170 mg/kg
Magnesium	0.30%	Vitamin A	800 KIU/kg
Fluorine	3.6 mg/kg	Vitamin D	200 KIU/kg
Cobalt	6.2 mg/kg	Vitamin E	11000 IU/kg
Iodine	80 mg/kg		
Iron	28 mg/kg		

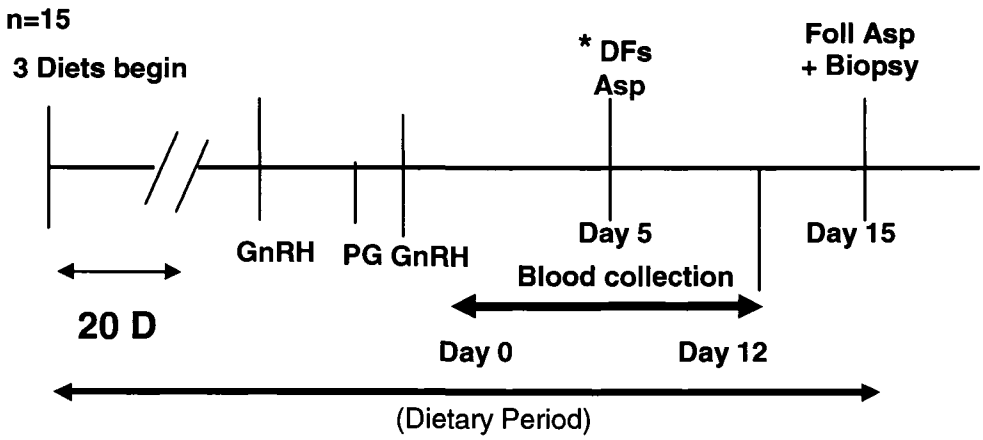
This micro premix contains Niacin at 50 g/kg

Table 4.3. Milk yield and composition in cows fed SAT, flax or sunflower. Milk yields were recorded from fifteen lactating Holstein cows before the start of the experiment and when cows were in the fourth wk of diet. Milk fat %, protein % and lactose % were determined during the above period in 12 cows that includes six animals from Experiment 1 and six animals from Experiment 2.

	SAT		Flax		Sunflower		
	Before	4th wk	Before	4th wk	Before	4th wk	*P* value (4th wk)
Milk yield (kg /day)	36 ± 4.4	39 ± 5.3	37 ± 4.4	40 ± 5.3	33 ± 4.4	35 ± 5.3	0.81
Milk fat (%)	3.7 ± 0.7	4.2 ± 0.7	3.1 ± 0.7	3.2 ± 0.6	3.1 ± 0.7	3.3 ± 0.6	0.63
Milk protein (%)	2.9 ± 0.1	2.9 ± 0.2	2.9 ± 0.1	3.2 ± 0.1	2.9 ± 0.1	3.1 ± 0.2	0.49
Lactose (%)	4.5 ± 0.1	4.5 ± 0.1	4.7 ± 0.1	4.5 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	0.74

Table 4.4. Least squares means (± SE) of plasma progesterone concentrations (ng/ml) from Days 0 to 12 in lactating Holstein cows fed saturated (SAT) or unsaturated fatty acids (FLX or SUN), and , and that of follicular estradiol concentrations (ng/ml) on Days 5 and 15. Day 0 = ovulation.

	SAT	Flax	Sunflower	*P* value
Progesterone	2.1 ± 0.3	2.4 ± 0.3	2.5 ± 0.3	0.42
Estradiol				
Day 5	328 ± 115	214 ± 115	452 ± 115	0.31
Day 15	398 ± 156	252 ± 156	486 ± 156	0.59



* DFs ASP = Dominant follicle aspiration

Figure 4.1. Experimental design. Fifteen lactating Holstein cows were assigned randomly, but equally, to 1 of 3 diets containing saturated fatty acids (SAT, high in palmitic acids), whole flaxseed (FLX, high in α-linolenic acid), or sunflower seed (SUN, high in linoleic acid). After 20 days of diet initiation, ovulation was synchronized in all animals.

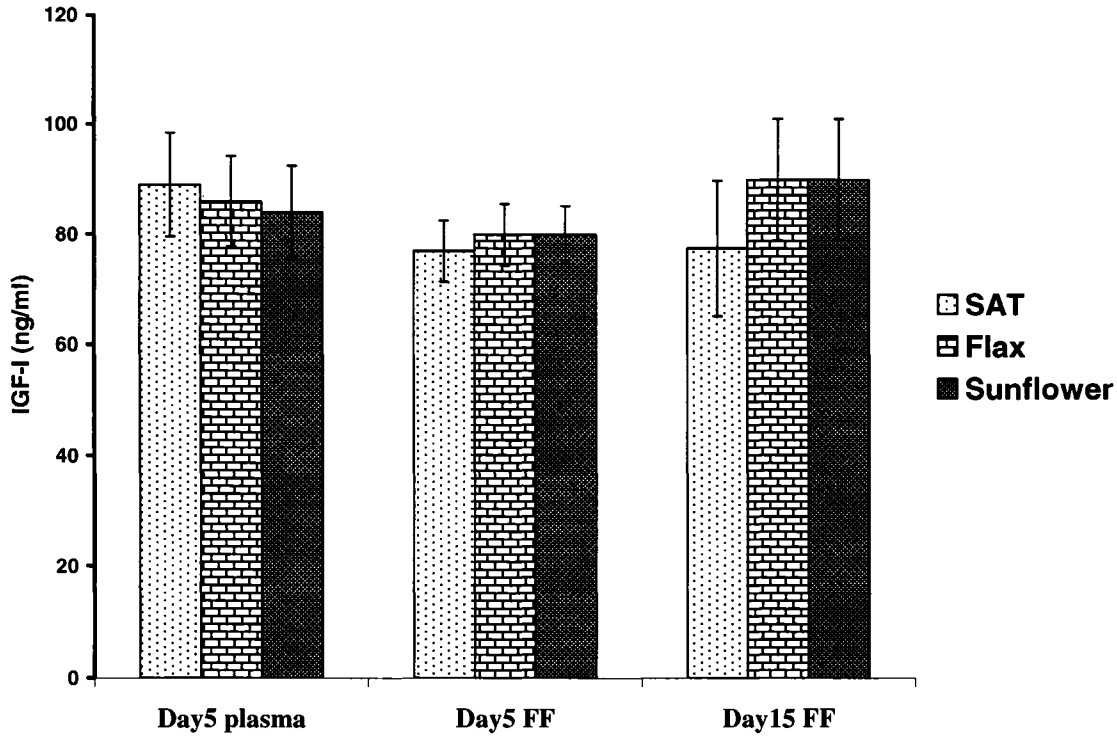


Figure 4.2. Mean concentrations of IGF-I on Day 5 plasma, Day 5 and Day 15 follicular fluid of cows fed SAT, FLX and SUN. Transvaginal follicular aspiration was performed in all animals on Days 5 and 15. There was no significant difference among diets.

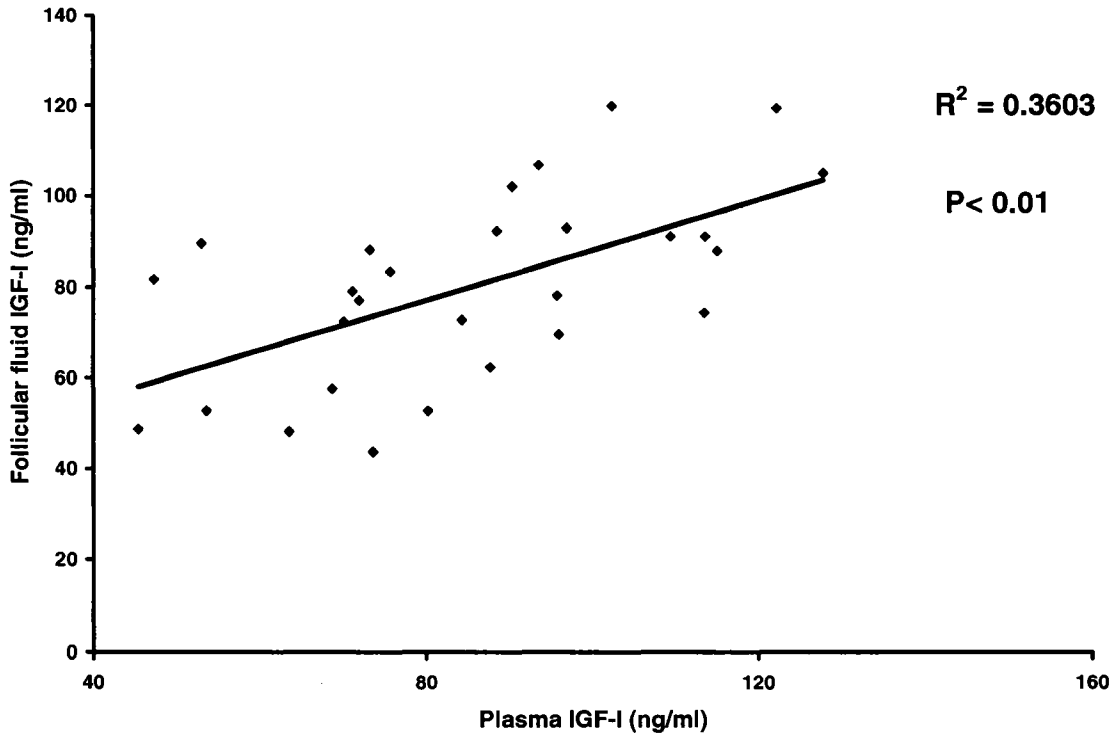


Figure 4.3. Correlation between Day 5 plasma and follicular fluid IGF-I concentrations (n=28). We determined the positive correlation ($P < 0.01$) between Day 5 plasma and follicular fluid IGF-I in cows fed SAT (saturated fatty acid), flaxseed or sunflower.

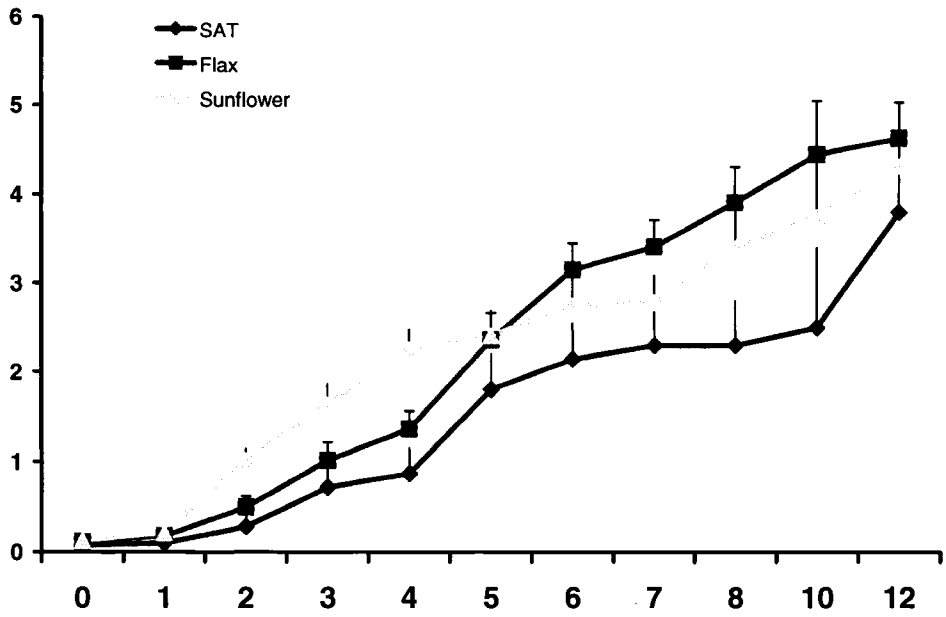
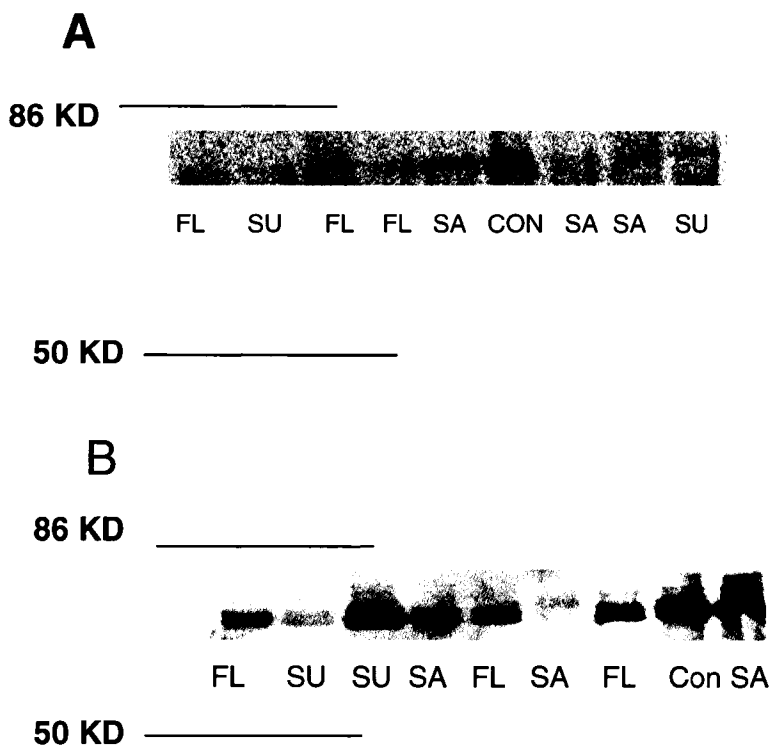


Figure 4.4. Mean plasma progesterone concentrations in dairy cows fed saturated fatty acids (SAT), flax (FLX) and sunflower (SUN) from Day 0 to Day 12 (n=14). Ovarian status of all animals was synchronized with an Ovsynch program and day of ovulation (Day 0) was confirmed by ultrasonography. Cows fed SAT had lower progesterone concentration when compared to cows fed sunflower from Day 2 to Day 5 ($P < 0.03$). Cows fed SAT had lower progesterone concentration than cows fed flax on Day 5 ($P = 0.05$). Cows fed flax had lower progesterone concentration when compared to cows fed sunflower from Day 2 to Day 4 ($P < 0.04$).



FL - Flax
SU- Sunflower
SA- Saturated fat
Con- Positive control

Figure 4.5. Distrubution of oxytocin receptors (A) and estrogen receptors (B) in endometrial samples of cows fed SAT, Flaxseed (FLX) or Sunflower (SUN)on Day 15 of the estrous cycle (n = 8). There were no differences in receptor populations among the treatments ($P > 0.05$).

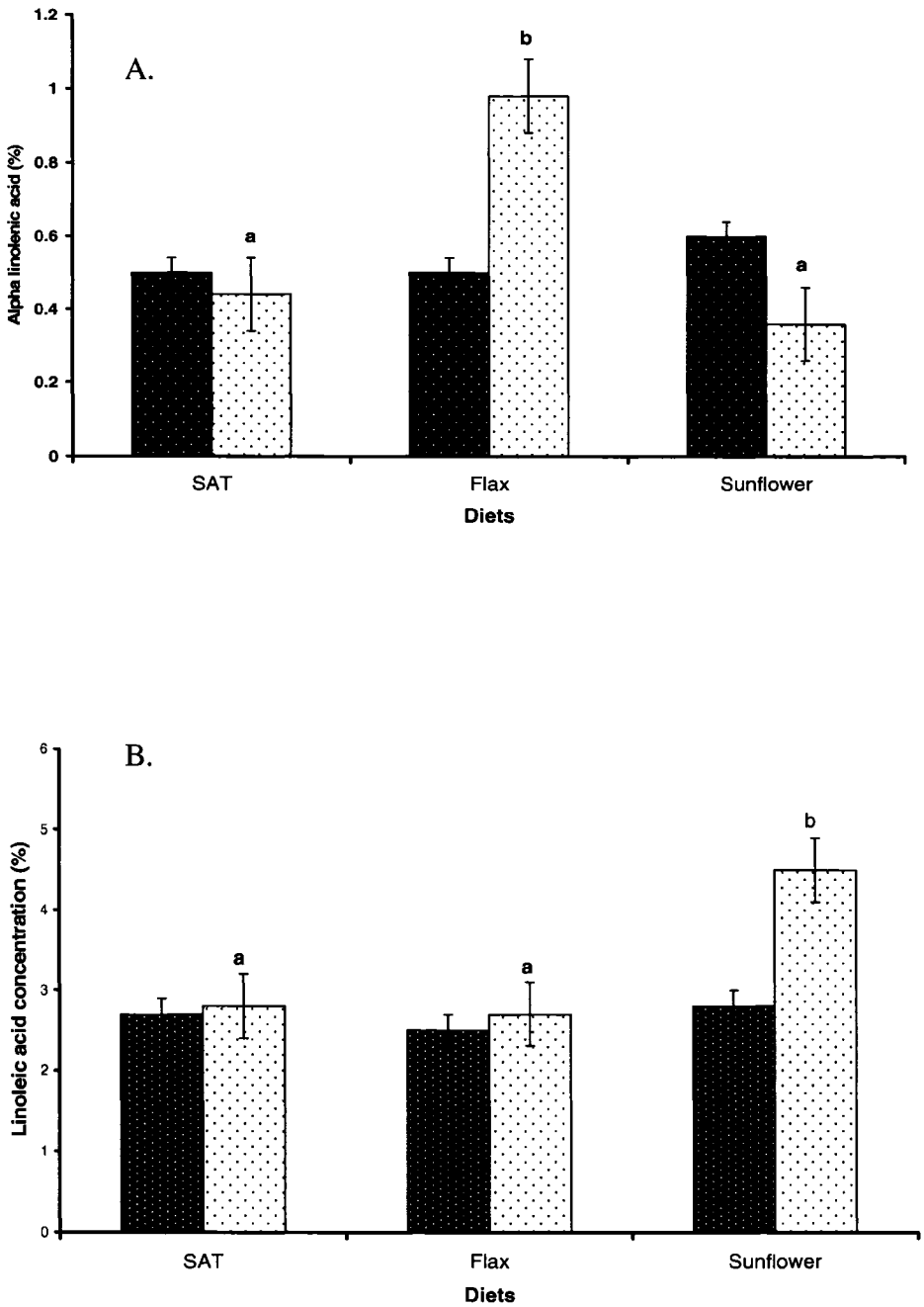


Figure 4.6. Alpha linolenic acid (A) and linoleic acid (B) composition in milk of cows fed diets supplemented with saturated fatty acids, flax and sunflower (n = 4/diet). Milk samples were collected before the start of the diet (dark bars) and 4 wk into the dietary period (light bars). a,b = $P < 0.01$ in panel A and $P < 0.03$ in panel B.

Chapter-5

General Discussion and Conclusions

5.1. General Discussion

Overall, this study provided information on the effects of unsaturated and saturated fatty acids on reproductive function in dairy cattle. In previous studies, cows fed a diet supplemented with flaxseed had reduced embryonic losses (Ambrose et al., 2006; Petit and Twarigamaru, 2006), but the underlying mechanisms had not been examined. In Chapter 2 of the present study, it was hypothesized that cows fed flaxseed (high in alpha linolenic acid) would increase embryonic development. Even though our hypothesis was not directly supported, it was found that diets based on unsaturated fatty acids enhanced embryonic development over that of saturated fatty acids.

The positive effects of polyunsaturated fatty acids on embryonic or fetal development have been reported in several species. In humans and rodents maternal diets deficient in omega-3 polyunsaturated fatty acids have been implied in visual impairment and other abnormalities of fetuses (Neuringer et al., 1984; Reisbick et al., 2006). In swine, embryo/fetal survival was improved after inclusion of dietary polyunsaturated fatty acids such as linoleic acids (Fengler, 1990). All polyunsaturated fatty acids can transfer across the placenta into the fetal blood circulation (Ruyle et al., 1990). Gilts fed Menhaden fish oil (rich in omega-3 fatty acid) had higher embryonic survival rate than those fed a control (starch) diet. Conceptuses of gilts fed Menhaden fish oil had higher plasma

omega-3 fatty acid composition than that of gilts fed coconut oil, control (starch-based) or soybean diets (Perez Rigau et al., 1995). This demonstrates that the transferring nature of fatty acids to fetal tissues and also implies the importance of polyunsaturated fatty acids in embryonic development.

Progesterone is essential for the maintenance of pregnancy. In the present study, cows fed saturated fatty acid diet had lower mean progesterone concentrations than cows fed diets enriched in unsaturated fatty acids, with the differences being clearly evident on Days 6 to 8 post ovulation. The rise of postovulatory progesterone concentration is essential for the early embryonic development (Demetrio et al., 2006) and the higher plasma progesterone concentrations in cows fed unsaturated fatty acids likely played a role in enhancing the embryonic development.

The relationship between insulin concentration and embryonic development in cattle has been studied previously, but the results have been inconclusive. Whereas Mann et al. (2003) reported positive effects of insulin on embryonic development; other studies have reported a negative relationship between embryonic development and insulin (Velazquez et al., 2005; Green et al., 2005). In the present study, we analyzed insulin concentrations only on Day 5 postovulation but no clear associations between insulin concentrations and embryonic development were established.

Insulin-like growth factor-I (IGF-I), estradiol and oxytocin receptors, all play an indirect role in luteolysis. Flaxseed is known to have anti-IGF-I (Rickard et al., 2000) and anti-

estradiol (Orcheson et al., 1998) properties in other species. In the second study (Chapter 3), we hypothesized that flaxseed would reduce IGF-I and estradiol at follicular level and subsequently reduce oxytocin receptor populations in the uterine endometrium.

The second hypothesis was based on reports in humans and rodents that flaxseed is the richest source of the mammalian lignan precursor SDG, which is converted into the mammalian lignans, enterodiol and enterolactone, by colonic bacteria. The presence of SDG in flaxseed is responsible for anti-IGF-I properties (Rickard et al., 2002; Dunn et al., 2003) and anti-estradiol properties of flaxseed (Orcheson et al., 1998). To our knowledge, there is no report on the existence of mammalian lignans in ruminants and no study has investigated the effects of flaxseed on IGF-I or estradiol concentrations at follicular level in ruminant species. We did not find differences in follicular estradiol, IGF-I or plasma IGF-I in cows fed flaxseed compared to cows fed sunflower or saturated fatty acids. One of the limitations of the present study is that mammalian lignans were not measured. The lack of expected differences in IGF-I and estradiol concentrations in cows fed flaxseed would have been easier to explain if mammalian lignans were quantified in blood, urine or feces of the experimental animals. Therefore future studies should attempt to quantify enterodiol and enterolactone in dairy cows after feeding flaxseed.

5.2. Conclusions

Based on the findings of the present study, it is concluded that dietary inclusion of unsaturated fatty acids (i.e., α- linolenic acid or linoleic acid) has a role in early embryonic development in dairy cattle, and that improvements in embryonic development potentially occurred through increased progesterone concentrations between Days 6 and 8, post ovulation. No clear associations existed between dietary fatty acids

and insulin concentrations. A flaxseed-based diet did not reduce intrafollicular concentrations of estradiol or IGF-I. Due to the lack of information relating to mammalian lignans in cows fed flaxseed, it is difficult to explain why the expected reductions in estradiol and IGF-I did not occur.

5.3. Future studies:

Future studies involving large number of animals, lipid analysis of embryos and early embryonic gene expression would be helpful in determining the mechanisms involved in the enhancement of reproduction by unsaturated fatty acids. *In vitro* studies on early embryonic development with serum collected from cows fed flaxseed, sunflower seed and saturated fatty acids may be performed to corroborate the effects of unsaturated fatty acids on embryonic development. Determination of the presence or absence of mammalian lignans in plasma, feces, and urine of cattle fed flaxseed will also be very helpful in further understanding the roles of dietary flaxseed and SDG in bovine reproductive function.

5.4. Literature cited

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Appendix

A.1. Progesterone assay

Kits: (Coat-a-count ®, DPC, CA, USA)
Polypropylene tubes coated with rabbit antibodies to progesterone.

Iodinated progesterone (^{125}I) – 105ml.

Progesterone calibrators: 0, 0.1, 0.5, 2, 10, 20 and 40 ng of progesterone/ml (A, B, C, D, E, F, G)

Procedure:

1] Label six plain (uncoated) 12×75mm polypropylene tubes for total counts (T) and non specific binding (NSB).

2] 100µL of zero standard were pipetted into NSB plain and coated tubes in triplicates.

3] 100µL of other standards were pipetted into coated tubes in triplicates namely from B to G

4] 100µL of samples were pipetted into coated tubes in duplicates.

5] Control samples were diluted into 100%, 50%, 25% and 12.5% and all the samples were pipetted into coated tubes in triplicates.

6] 1ml of ^{125}I progesterone was added in all tubes and vortexed.

7] Three hours incubation at room temperature.

8] Contents were removed by aspiration.

9] Radioactivity was counted for a minute in a gamma counter.

10] Assay was validated for parallelism previously

Calculations:

The resultant progesterone values (ng/tube) were calculated into ng/ml.

A.2. Insulin like growth factor I assay

Extraction:

Acid ethanol – 12.5% (V/V) of 2.2 M HCl (analytical reagent grade) in 87.5 (v/v) ethanol- Fisher HPLC grade reagent alcohol cat. no. A995-4).

Tris base - .0855 M Trizma base (Sigma No. T-1503) in IGF-I assay buffer

Neutralized acid ethanol: Tris base: Acid ethanol in the ratio of 4:1

Procedure:

1] Pipette 100μL of samples (follicular fluid and plasma) into 13mm×100 mm tubes. Add 3ml of acid ethanol to all the tubes, parafilm, vortex and incubate for 16-20 h time.

2] After incubation, centrifuge for 30 min at 3000rpm, 4°C. Pipette 200μL of the supernatant into 12mm×75mm tubes.

3] Add 800μL of Tris base to all tubes, vortex and incubated at 4°C for 2 days.

Control plasma and follicular fluid:

Serially dilute control samples with assay buffer to make four concentrations (100, 50, 25 and 12.5 μL).

Buffer

Sodium phosphate (monobasic 0.03M)	4.14g/L
EDTA-Na_2 (.01M)	3.72g/L
Protamine SO_4 (grade 2, 0.02%)	0.20g/L
Sodium azide (0.02)	0.20g/L
Dissolve and then add Tween 20	0.5ml

Make up to less than total volume with double distilled water and adjust to pH 7.5 with NaOH.

Recombinant human IGF-I for label; Bachem Cat. No.H3102

Antihuman IGF-I antiserum (product name AFP4892898, obtained from Dr. AF Parlow

Dr. Parlow: Harbor-UCLA Medical Center, 1000 West Carson Street, Torrance California, USA 90509)

Normal rabbit serum (NRS) # 71 available through animal services at the University of Alberta

Goat anti-rabbit gamma globulin (GARGG). Calbiochem catalogue number 539845, lot no.073890.

Standards:

Ten standards- 0.00097, 0.00195, 0.0039, 0.007825, 0.0156, 0.03125, 0.0625, 0.125, 0.25 and 0.5ng/tube.

Procedure:

1] Pipette 100µL of neutralized-extracted sample in duplicate into 12×75mm.

2] Pipette 100µL of control samples, standards, cold recoveries into 12×75mm.

3] Add 200µL of assay buffer to all tubes.

4] Dilute normal rabbit serum (NRS#71) 1:600; add 100µL to NSB only.

5] Then use remaining NRS solution to dilute first antibody.

6] Dilute first antibody (1/2616 bench diln. Stored at 1/50, Final tube dilution is 1.654, 000) and add 100µL to all tubes except Total and NSB's. Incubate all the tubes for 24 h at 4°C.

7] Add 100 µL of label (10,000cpm/100µL) to all tubes and subsequently incubate for 24 h at 4° C.

8] Add 100 µL of second antibody-goat anti-rabbit gamma globulin (GARGG-B12377) in dilution rate of 1:300 into all tubes except Totals.

9] Incubate for 16-24 h.

10] Add 1ml of cold deionized distilled water to all tubes except Totals.

11] Centrifuge at 2000 g, 4°c for 30min. Aspiration followed by counting the pellet for 2 min.

12] Serial dilutions of a standard plasma and follicle fluid showed parallelism to the standard curve.

13] Triplicate follicle fluid samples were spiked with 25 ng IGF-I or an equal volume of assay buffer for the blank tube, incubated overnight, extracted and assayed. %CR was 48.9% +/- 7.48. Data were not corrected for cold recovery.

14] Data were corrected to potency of the IGF-I standard vial Bachem lot #Z0103 (vial used at time of validation with current first antibody, in 2000).

Cold recovery:

$$\% \text{ CR} = \frac{\text{Amount of IGF-I recovered}}{\text{Amount added + blank}} \times 100$$

A.3. Estradiol Assay

Kits: (Coat-a-count ®, DPC, CA, USA).
Polypropylene tubes coated with rabbit antibodies to estradiol.

Iodinated estradiol (^{125}I) – 105 ml.

Estradiol calibrators: The calibrators contain, respectively, 0,20, 50, 150, 500, 1,800 and 3,600 picograms (A, B, C, D, E, F, G) of synthetic estradiol per milliliter (pg/mL) in processed human serum; equivalently: 0, 73, 184, 551,1,836, 6,608 and 13,216 picomoles per liter (pmol/L).

Procedure:

1] Label six plain (uncoated) 12×75mm polypropylene tubes for total counts (T) and non-specific binding (NSB).

2] 100µL of zero standard were pipetted into NSB plain and coated tubes in triplicates.

3] 100µL of other standards were pipetted into coated tubes in triplicates namely from B to G

4] Dilute samples with zero calibrator in the ratio of 1:500.

5] 100µL of samples were pipetted into coated tubes in duplicates

6] Control samples were diluted into 100%, 50%, 25% and 12.5% and all the samples were pipetted into coated tubes in triplicates.

7] 1ml of ^{125}I estradiol was added in all tubes and vortexed.

8] Three hour incubation at room temperature.

9] Contents were removed by aspiration.

10] Radioactivity was counted for a minute in a gamma counter.

Calculations:

The resultant estradiol values (pg/tube) were converted into pg/ml then into ng/ml.

A.4. Insulin Assay

Kits: (Coat-a-count ®, DPC, CA, USA).

Polypropylene tubes coated with antibodies to insulin.

Iodinated in insulin (^{125}I) – 105ml.

Insulin calibrators: The reconstituted calibrators have *lot-specific* insulin values representing *approximately* 0, 2.5, 5, 15, 50, 100, 200 and 350 (A, B, C, D, E, F, G) micro-International Units of insulin per milliliter (μIU/mL).

125 I Insulin (TIN2)

A concentrate, consisting of iodinated insulin. To each vial add a measured 100 mL of deionized water.

Procedure:

1] Label six plain (uncoated) 12×75mm polypropylene tubes for total counts (T) and non specific binding (NSB).

2] 100μL of zero standard were pipetted into NSB plain and coated tubes in triplicates.

3] 100μL of other standards were pipetted into coated tubes in triplicates namely from B to G.

4] 200 μL of samples were pipetted into coated tubes in triplicates.

5] Control samples were diluted into 100%, 50%, 25% and 12.5% and all the samples were pipetted into coated tubes in triplicates.

6] 1ml of ^{125}I insulin was added in all tubes and vortexed.

7] Overnight incubation at room temperature.

8] Contents were removed by aspiration.

9] Radioactivity was counted for a minute in a gamma counter.

Calculations:

The resultant insulin values (μIU/tube) were expressed in (μIU/mL) by multiplying the former value with five.

A.5. Effects of flaxseed processing on the recovery of α-linolenic acid in milk

Effects of flaxseed processing on the recovery of α-linolenic acid in milk were evaluated using ten primiparous Holstein cows (153 ± 30.7 DIM; mean±SD) in a crossover design with 14 d per period. We hypothesized that feeding unprocessed flaxseed is as effective as dry-rolled flaxseed at increasing α-linolenic acid concentration in milk fat. Experimental diets contained either whole (WH) or dry-rolled (DR) flaxseed at 10.1% of dietary DM. Dietary concentration of NDF, CP, ether extract, and α-linolenic acid were 39.1, 17.6, 7.0, and 2.7%, respectively (DM basis). Dry matter intake, milk yield, and concentrations of milk fat, protein, and lactose were not affected by treatments, and averaged 17.5 kg/d, 27.5 kg/d, 3.60%, 3.00%, 4.73%, respectively. Apparent total tract digestibility of ether extract was lower for WH compared with DR (48.6 vs. 62.4 %; $P < 0.01$). Moreover, excretion of α-linolenic acid in feces was greater for WH compared with DR treatments (259 vs. 129 g/d; $P < 0.001$). However, α-linolenic acid concentration in milk was not affected by treatments (0.83 and 0.86 % for WH and DR, respectively), and both treatments had three times as much α-linolenic acid concentration as the period prior to the experiment (0.26%), during which sunflower seed was fed in place of flaxseed. These data indicate that both WH and DR treatments increased the absorption of α-linolenic acid to a similar extent despite the lower digestibility for WH treatment, which can be attributed to less lipolysis or fatty acid biohydrogenation for WH compared with DR. This speculation is supported by that WH treatment decreased concentration of vaccenic acid, a fatty acid intermediate during biohydrogenation, in milk fat compared with DR (1.9 vs. 3.0 %; $P < 0.01$). Dry-rolling flaxseed does not necessarily

improve the absorption of α-linolenic acid probably because processing increases the extent of biohydrogenation in the rumen as well as digestibility.

A6.Western Blotting:

Reagents:

1. **1.5 M Tris-HCl, pH8.8**

   18.2g Tris base in 90ml ddH_2O
   adjust pH to 8.8 with 10 N HCl
   make up to 100ml with ddH_2O

2. **0.5 M Tris-HCl, pH 6.8**

   6.05g Tris base in 60ml sterile ddH_2O
   adjust pH to 6.8 with 10N HCl
   make up to 100ml with ddH_2O

3. **30% Acrylamide**

   8.76g acrylamide
   0.24 bisacrylamide
   make up to 30ml with ddH_2O
   store in cool, dark place for up to 1 week

4. **10% Ammonium persulfate (make fresh as required)**

   50mg ammonium persulfate
   make up to 500ml with ddH_2O

5. **Towbin's Transfer Buffer, pH8.3**

   12.1g Tris base
   57.6g glycine
   800ml methanol
   1ml 20% SDS
   make up to 4L with ddH_2O and store at 4° C

6. **5× Loading Dye**

   4ml glycerol
   1.7ml 1.5M Tris-HCl, pH 8.8
   0.8g SDS
   2ml ß-mercaptoethanol
   0.2 mg bromophenol blue

Store the mixture at -20° C

7. **5× Electrode running buffer (Stock solution)**

15.1g Tris base
72.0g glycine
5.0 g SDS
make up to 1 L with ddH_2O

8. **PBS, pH7.4**

32g NaCl
0.8g KCl
5.76 g Na_2HPO_4 make up to 1 L with ddH_2O
0.88 KH_2PO_4
make up to 4L with ddH_2O and adjust pH to 7.4 with concentrated HCl.

9. **TBS, pH7.4**

32g NaCl
0.8g KCl
12g Tris base
make up to 4L with ddH_2O and adjust pH to 7.4 with concentrated HCl.

10. **TBST**

0.01% Tween 20 in TBS

Procedure

Casting the separating and stacking gel:

Set up the casting apparatus for Mini-PROTEAN II gel unit (BioRad laboratories, Mississauaga, ON).

Before pouring the gel, ensure that there is a tight seal between the glass plates and rubber gasket on the casting apparatus.by checking with water.

Prepare 10% seperating gel.

5.9ml	ddH_2O
5.0ml	30% acrylamide
3.8ml	1.5M Tris-HCl, pH6.8
.15ml	10%SDS

.05ml 10% ammonium persulfate
.006ml TEMED

Before adding TEMED and ammonium persulfate degas on vacuum. Pour the separating gel between the glass plates and overlay the gel with saturated isobutanol.

Allow the gel to polymerize for approximately 45min

Prepare the 4% stacking gel
6.1ml ddH_2O
1.3ml 30% acrylamide
2.5ml 0.5M Tris-HCl, pH6.8
.10ml 10%SDS
.05ml 10% ammonium persulfate
.001ml TEMED

Pour the stacking gel and place a 10 well comb into the stacking gel.

Allow the gel to polymerize for 30min.

Sample preparation:

Dilute samples with homogenizing buffer and 5×loading to make the final concentration 5 μg/μl.

Heat the samples in a water bath followed by quenching and centrifugation.

Electrophoresis:

Place the gels in the gel unit.

Load the sample volume and marker

Fill the gel unit with 1×electrode running buffer and run the gel at 75V for 45 min and 100V for nearly 1.5h

Transfer of the protein to the nitrocellulose membrane:

Nitopure membrane (Micron sepertions, Inc., Westborough, MA), Whatman paper and fibre pad should be soaked in Towbins buffer to equilibrate for a duration of 30min.

Once electrophoresis is completed, remove the gel and place the gel down on the filter paper. Cover the membrane with another filter paper and place the fibre pads over the filter paper.

Close the gel holder and place the gel holder in the Mini Trans-Blot (Biorad Laboratories) so that the white side is in contact with the cathode and the black side is in contact with the anode.

Transfer of proteins takes place from the negative to the positive electrode and it performed at 40ma overnight.

Transfer can be confirmed by washing the nitro cellulose membrane in Ponceau solution for 5 min and subsequently washed with distilled water.

Blocking:

Wash the membrane in two changes of PBS (5 MIN each)

Incubate the membranes in 2%blocking agent (Amersham Biosciences, Piscataway, NJ) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 3h.

Wash the membrane for 5 min in TBST for two times.

Antibody:

Incubate the membrane with primary antibody (overnight)

Wash the membrane for 15 min in TBST, then in two more times for 15minutes each.

Incubate the membrane in secondary antibody, then follow the same washing procedure as described in primary antibody incubation above.

After washing, incubate the membrane for 1min in ECLA advance ECLA advance western blotting kit –equal proportions of Reagent A and B (Amersham Biosciences, Piscataway, NJ).

Primary antibodies of mouse monoclonal ERα antibody (1:250; Santacruz Biotechnology, Santa Cruz, CA; Cat. No. sc-787) or anti-rat OTR (1:500; Alpha Diagnostics Intl Inc, San Antonio, TX; Cat No. OTR 11A) were used for estradiol and oxytocin receptors respectively.

Secondary antibodies were goat anti-mouse IgG_{2a}-HRP (1:4000 Santacruz biotechnology, Santa Cruz, CA; Cat. No.sc-2061) or goat anti-rabbit IgG (H+L) peroxidase purified (1:4000, Alpha diagnostics Intl Inc, San Antonio, TX; Cat No. 20320) for estradiol and oxytocin receptors respectively.

Analyze by Typhoon Trio+ scanner (Amersham Biosciences, Piscataway, NJ).