EFFECT OF OMEGA-3 FATTY ACIDS ON THE OVARIES OF LACTATING DAIRY COWS

by

Mallikarjun Bidarimath

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia

in co-operation with

Nova Scotia Agricultural College
Truro, Nova Scotia

December 2011

DALHOUSIE UNIVERSITY

NOVA SCOTIA AGRICULTURAL COLLEGE

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "EFFECT OF OMEGA-3 FATTY ACIDS ON THE OVARIES OF LACTATING DAIRY COWS" by Mallikarjun Bidarimath in partial fulfillment of the requirements for the degree of Master of Science.

Dated: December 6, 2011

External Examiner: _____

Co-Supervisors: _____

_____

Readers: _____

_____

DALHOUSIE UNIVERSITY

AND

NOVA SCOTIA AGRICULTURAL COLLEGE

DATE: December 6, 2011

AUTHOR: Mallikarjun Bidarimath

TITLE: **EFFECT OF OMEGA-3 FATTY ACIDS ON THE OVARIES OF LACTATING DAIRY COWS**

DEPARTMENT OR SCHOOL: Department of Plant and Animal Sciences

DEGREE: MSc   CONVOCATION: October   YEAR: 2012

Permission is herewith granted to Dalhousie University to circulate and to have copied for non-commercial purposes, at its discretion, the above title upon the request of individuals or institutions. I understand that my thesis will be electronically available to the public.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

The author attests that permission has been obtained for the use of any copyrighted material appearing in the thesis (other than the brief excerpts requiring only proper acknowledgement in scholarly writing), and that all such use is clearly acknowledged.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The objectives of this study were to evaluate the effect of rumen-protected fish oil (RPFO) and rumen-protected marine algae (RPMA) supplements on ovarian function of lactating dairy cows on pasture or in confinement during the estrus and ovulation synchronization period. Thirty-six Holstein cows were assigned to one of the two feeding systems and fed with lipid supplements from 30d before to 100d after calving. The resumption of cyclicity and onset of estrus were not influenced by LS. Mean daily number of the large follicles was similar across the treatments. During the Ovsynch period, RPFO treated cows had larger follicles ($\geq$10mm; $P<0.05$). Ovulation was delayed in RPFO and RPMA group but the number of ovulation was not altered. The number and diameter of CL were greater in the RPMA group. Progesterone concentrations were greater in the RPMA group on pasture ($P<0.05$). These findings indicate that RPMA supplementation improves the ovarian function.

LIST OF ABBREVIATIONS AND SYMBOLS USED

AA	Arachidonic Acid
ABTS	2, 2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid
ADF	Acid detergent fiber
ALA	Alpha linolenic acid
BW	Body weight
Ca-LCFA	Calcium salts of long chain fatty acid
Ca-LCPUFA	Calcium salts of long chain polyunsaturated fatty acid
CL	Corpus luteum
CLs	Corpora lutea
CP	Crude protein
CV	Coefficient of variation
DCAD	Dietary cation-anion difference
DHA	Docosahexaenoic acid
DM	Dry matter
DMI	Dry matter intake
E_2	Estradiol
EE	Ether extract
EPA	Eicosapentaenoic acid
FS	Feeding system
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
HRP	Horseradish peroxidase
i.m.	Intramuscular
LA	Linoleic acid
LC	Long chain
LH	Luteinizing hormone
LS	Lipid supplement
ME	Metabolizable energy
MUFA	Monounsaturated fatty acid

n-3 PUFA	Omega-3 polyunsaturated fatty acid
n-6 PUFA	Omega-6 polyunsaturated fatty acid
NDF	Neutral detergent fiber
NE_g	Net energy of gestation
NE_L	Net energy of lactation
NE_m	Net energy of maintenance
NFC	Non-fibre carbohydrate
NSB	Non-specific binding
P_4	Progesterone
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGHS	Prostaglandin H synthase
PGI_2	Prostaglandin I_2
PP	Postpartum
PUFA	Polyunsaturated fatty acid
RDP (%CP)	Rumen degradable protein
RPFO	Rumen-protected fish oil
RPMA	Rumen-protected microalgae
RUP	Rumen un-degradable protein
SEM	Standard error of mean
SFA	Saturated fatty acid
TAG	Triacylglycerol
TFA	Total fatty acids
TMR	Total mixed ration

ACKNOWLEDGEMENTS

CHAPTER 1

INTRODUCTION

The main goal of the dairy industry in developed nations is to get maximum benefit from milk production. Intense genetic selection of high milk producing cows has led to improvements in milk yield (Royal et al. 2000). However, the reproductive performance of dairy cows has been simultaneously declining for the last 50 years (Butler 2003; de Veth et al. 2009; Sakaguchi 2011). Generally, the cows with higher milk production have lower fertility. Epidemiological surveys indicate that multiple factors contribute to the decreased reproductive performance in dairy cows (Lucy 2001; Thatcher et al. 2006; Sakaguchi 2011). Pregnancy rates to first service have declined from 65% in 1951 to as low as 40% in 1996 (Lucy 2001). Declining fertility in the lactating dairy cows is a major challenge to the Canadian dairy industry. A recent study indicates that an average herd pregnancy rate (Pregnancy rate = Conception rate x heat detection rate) is only 12 – 13 % in Canadian dairies (Ambrose and Colazo 2011). Infertility is a major factor affecting the economics of dairy herds (Lucy 2007b).

In the early lactation of dairy cow, a greater proportion of available nutrient is diverted towards the milk production at the expense of body tissues and reproductive functions (Chagas et al. 2007). Proper herd management and high nutrient profile will improve reproductive function in dairy cows (Britt 1985). In Great Britain, the pregnancy rate to first artificial insemination (AI) declined 1 % per year from 1975 to 1998 (Royal et al. 2000). Previous studies in the USA reported an increase in the number of inseminations per conception from 1.75 to 3.0 and an increase in the calving interval from 13.5 to 14.7 months from 1970 to 1999, respectively (Lucy 2001). Reproductive

problems are the most common reasons, behind production failure, for dairy cattle culling in Canada and most countries around the world (Canadian Dairy Network 2009). Considering all cows across the herds with a valid disposal reason, the number of dairy cows culled for reproductive problems increased significantly from 28.9 % of total culled cows in 2000 to 30.3 % in 2008 (Canadian Dairy Information Centre 2008). It is necessary to optimize the supply of nutrients to dairy cows to enhance reproductive efficiency and also, to meet the demand of the milk market (Nebel and McGilliard 1993).

Fat supplements have been widely used to improve the reproductive performance in dairy cows. Feeding fish meal (2.8 % of dry matter intake) rich in omega-3 polyunsaturated fatty acids (n-3 PUFAs) to dairy cows improved the pregnancy rates (31.9 vs 41.3 %) from Day 24 to 105 postpartum (Burke et al. 1997; Staples et al. 1998). Feeding n-3 PUFAs mainly rich in α-linolenic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may be used as a management tool to enhance the reproductive performance in dairy cattle. However, one challenge with adding n-3 PUFAs to dairy cattle feed is in minimizing the extent of ruminal biohydrogenation (Jenkins 1993). Several studies have used rumen protected fish oil or marine algae to minimize the loss of EPA and DHA to rumen bacterial digestion (Franklin et al. 1999; Mattos et al. 2004).

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is known to play a major role in several reproductive events, including luteolysis, ovulation and pregnancy in dairy cows (Petit et al. 2002; Thatcher et al. 2006). Uterine endometrium secretes $PGF_{2\alpha}$ which induces an irreversible degeneration of the corpus luteum (CL), characterized by a dramatic drop in progesterone (P_4) concentrations in the blood. Progesterone is a major hormone which is essential for

the establishment and maintenance of pregnancy. Omega-3 PUFAs such as EPA may inhibit the uterine synthesis of $PGF_{2\alpha}$ by competing with arachidonic acid (a precursor for the synthesis of $PGF_{2\alpha}$) in dairy cows (Petit et al. 2002). This may delay the regression of CL and potentially increase the chances of embryo survival.

Dairy cows fed diets enriched in PUFAs had increased dominant follicle diameters compared to the cows fed a diet enriched with monounsaturated fatty acids (MUFAs; Bilby et al. 2006b), suggesting that it was PUFAs that were most effective compared to MUFAs. Other studies showed that the size of the dominant follicle was increased in cows which consumed a diet rich in LCFAs from soybean oil (Garnsworthy et al. 2008). However, it appears that animal tallow or protected fish oil have less clear effects on growth dynamics of follicles than Ca-LCFAs derived from palm oil (Lucy et al. 1993; Moussavi et al. 2007b; Santos et al. 2008).

Feeding systems may play a major role in the reproductive performance of lactating dairy cows. Pasture grazing has been employed historically in many countries around the world, though cows are now typically confined and fed a total mixed ration (TMR). Previous studies conducted at two locations, New Zealand and the United States, reported that conception rates were 2 times higher in New Zealand where rotational grazing remains the standard management system for dairy production, compared to the United States where cows were fed a TMR. Grazing cows had lesser plasma P_4 clearance compared to TMR fed cows (Bilby et al. 1998). Boken et al. (2005) reported that cows on pasture fed with soybean oil refined byproduct, composed of sodium salts of LCFAs, had a greater peak of plasma P_4 concentration than cows managed in free-stalls (7.0 vs 5.0

3

ng/mL P≤ 0.05) during the first estrous cycle, postpartum. However, this trend was not observed in subsequent estrous cycles.

Previous experiments of feeding fish meal or oil to lactating dairy cows showed inconsistent effects on follicular and luteal dynamics and P_4 concentrations. Plasma P_4 concentrations did not differ among animals fed control, menhaden fish meal or calcium salts of fish oil fatty acids during the luteal phase (Moussavi et al. 2007b; Childs et al. 2008). However, improved conception rates in dairy cows fed n-3 PUFA have been associated with increased circulating concentrations of P_4 during the luteal phase (Santos et al. 2008). There was no significant effect of n-3 PUFA feeding on the mean numbers or the mean size of developing follicles (Petit et al. 2002). However, Bilby et al (2006a) found that the number and size of developing follicles increased in cows that were fed with calcium salt of transoctadecenoic acids and linseed oil enriched in n-3 PUFA.

Marine algae are the abundant sources of EPA and DHA (Franklin et al. 1999). There are many benefits of using rumen protected fish oil or marine algae as sources of EPA and DHA for reproduction (Hoyt el al. 2007). There is a lack of literature on the effect of protected marine algae supplements on ovarian function in lactating dairy cows. The hypothesis of this study was that ovarian function is affected by dietary lipid and the feeding system. Interaction of these factors may positively affect ovarian dynamics and P_4 profiles in the early post-partum lactating cows. The current study was designed to evaluate the effects of two dietary lipid supplements on ovarian function of lactating dairy cows managed on pasture or in confinement during both conventional estrus and ovulation synchronization.

4

CHAPTER 2

LITERATURE REVIEW

2.1 Estrous Cyclicity in Bovines

2.1.1 Estrous Cycle

Dairy cows are polyestrous animals with the estrous cycle typically ranging between 17 and 24 days, with 21 days being the average length. The estrous cycle in dairy cows begins at estrus and ends at the subsequent estrus (Aerts and Bols 2010b). It can be divided into two distinct phases: follicular and luteal. These are named after the dominant structures present on the ovary (Niswender et al. 1994). The follicular phase begins after luteolysis and ends at ovulation. Gonadotropins, follicle stimulating hormone (FSH) & luteinizing hormone (LH), released from the anterior pituitary, stimulate antral follicles to produce estradiol (E_2). The follicular phase is dominated by E_2 produced by developing follicles (Adams et al. 1999). The luteal phase begins after ovulation and it includes the development of the CL. The CL, the transient intraovarian endocrine gland, produces P_4 during the non-pregnant cycle as well as the pregnancy (Mann 2009). The luteal phase also includes luteolysis, brought about by $PGF_{2\alpha}$, which results in a decline in P_4 secretion (Allrich 1994; Mann 2009).

The estrous cycle can be divided into four stages which are subdivisions within the follicular and luteal phases. The follicular phase includes proestrus and estrus, while the luteal phase includes metestrus and diestrus; these are continuous unless interrupted by pregnancy or diseases associated with reproductive organs (Gordon 1996). Anestrus is characterized by the cessation of the regular estrous cycle which occurs naturally during pregnancy and during the early postpartum period (Roche et al. 1992). Proestrus begins

when P_4 gradually declines as a result of degeneration of the CL and ends at the onset of estrus. This period lasts for 2-5 days and is characterized by a significant rise in E_2 and a gradual decline in P_4 concentrations. Estradiol and FSH reach peak concentrations during the estrus period (Adams 1999). The duration of estrus in dairy cows typically ranges from 0.5-36 hours, with an average length of 7 hours (Dransfield et al. 1998). The cow enters behavioral estrus or standing heat in this period and shows sexual receptivity to the male. The estrus period is also characterized by a surge in LH, which will induce a follicle to ovulate approximately 24 - 32 hours after the onset of estrus (Sakaguchi 2011).

Following ovulation, during metestrus, follicular cells are transformed into luteal cells to form the CL. Metestrus begins at the end of estrus and lasts for 2 to 4 days until a fully functional CL is formed. The metestrus period involves the transition from E_2 dominance to P_4 dominance (Adams et al. 2008; Forde et al. 2011). The developing CL will produce increasing quantities of P_4 during metestrus. Diestrus is characterized by a fully functional CL, producing significant amounts of P_4 (Niswender et al. 1994). The duration of diestrus is usually about 10 to 14 days, depending on the length of time that the CL remains functional (Skarzynski et al. 2008). The sustained high P_4 secretions during diestrus prompt the uterus to prepare for pregnancy and upon conception, this stage persists until parturition. In the absence of pregnancy, the $PGF_{2\alpha}$ secreted from the uterus induces the luteolysis of the CL and subsequent resumption of the estrous cycle (Mann 2009).

2.1.2 Ovarian Follicular Dynamics during Bovine Estrous Cycle

2.1.2.1 Pre-antral Follicular Population

The bovine ovary contains a large pool of pre-antral follicles which are a major source of oocytes. Previous studies have addressed the significance of studying the pre-antral follicles as they account for the vast majority of follicles in the ovary (Aerts and Bols 2010a). However, very few of these follicles grow to maturity and ovulate during the reproductive life time of a cow (Fortune et al. 2000). The pre-antral follicular population can be studied by categorizing follicles into two different pools: the non-growing and the growing pool (Fortune et al. 2000). Primordial follicles are part of the non-growing pool, which is present throughout the reproductive life span of the dairy cow. Primordial follicles transit from the non-growing to the growing pool continuously throughout the reproductive life of the dairy cow (Fortune et al. 2000; Aerts and Bols 2010a). The activation of primordial follicles results in the transition of these follicles into primary follicles.

Before the oocyte in the primordial follicle increases in size, it is surrounded by a single layer of flattened follicular cells (Fortune et al. 2000). As the primordial follicle grows in size, this layer transforms into cuboidal cells surrounding the growing oocyte, forming a primary follicle. Secondary follicles contain two or more granulosa cell layers and are destined to develop into tertiary follicles. Tertiary follicles consist of a fluid filled cavity called the antrum and can be further subcategorized into small and large antral follicles (Figure 2.1; Aerts and Bols 2010a).
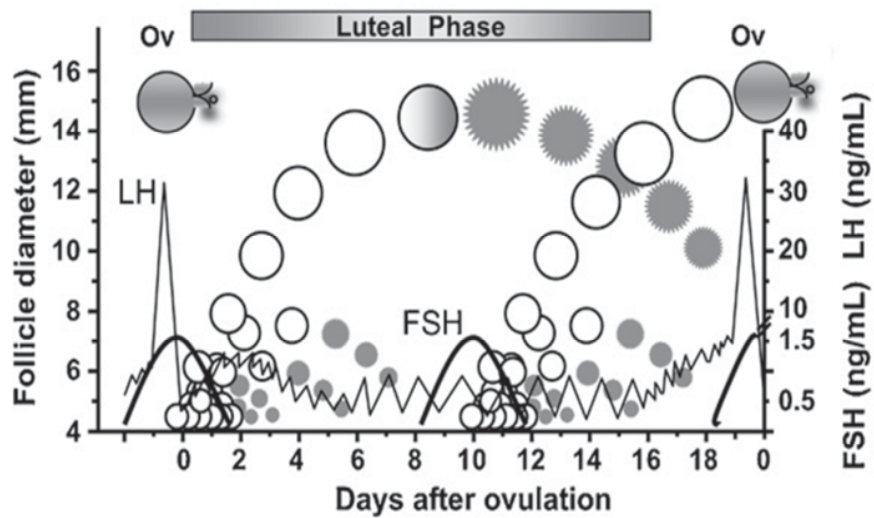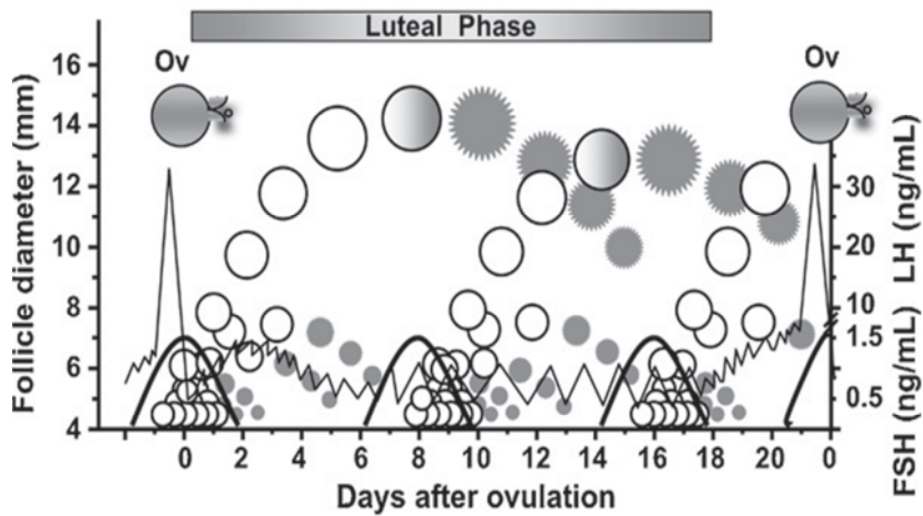
Figure 2.1 Suggested pattern of follicular wave dynamics and pituitary gonadotropin profiles during the two and three wave patterns of estrous cycle in cattle showing FSH levels (dark thick lines) preceding emergence of each wave, LH (faint line) surge before the ovulation, growth pattern of dominant follicle (open circles with smooth border), and subordinate (atretic) follicles (grey shade with corrugated border). As a result of low circulatory P_4 levels, a surge in LH occurs before the ovulation (Source: Adams et al. 2008).

2.1.2.2 Follicular Waves during Estrous Cycle

Previous studies showed that postpartum follicular development in normal cycling cattle occurs in a wave-like fashion, with 2 or 3 waves per estrous cycle (Figure 2.1; Adams et al. 2008). However, estrous cycles with one or four waves have also been reported (De-Rensis and Peters 1999). Predictive factors such as pregnancy rates associated with two or three wave pattern may provide an understanding of mechanisms controlling the pattern and have important implications for the development of effective ovulation synchronization protocols (Townson et al. 2002). The preovulatory follicle in the two-wave pattern grows for a relatively longer period and may contain a relatively aged oocyte. Based on this notion, a higher pregnancy rates can be predicted in the dairy cows (Adams et al. 2008). In contrast, the pregnancy rates did not differ between two-versus three-wave patterns (Ahmad et al. 1997).

The emergence of the follicular wave in normal cycling cattle is characterized by the sudden (within 2–3 days) growth of a pool of small follicles (Figure 2.1). The introduction of transrectal ultrasonography has improved our understanding of bovine follicular and luteal dynamics (Ginther 1993). This technology has enabled the follow-up of growing antral follicles with a diameter of ≥ 2 mm on a daily basis (Aerts and Bols 2010). The first follicular wave typically emerges on the day of ovulation (Figure 2.1; Day 0). With two wave cycles, the second wave of follicles emerges on Days 9 or 10 and with three wave cycles, on Days 8 or 9 (Figure 2.1). A third wave in a three-wave cycle typically emerges on Day 15 or 16 of the estrous cycle (Figure 2.1; Mapletoft et al. 2002). As a result, the growth waves in bovine develop during both the follicular and luteal phases of the estrous cycle.

9

The luteal phase in three wave patterns tends to be significantly longer, as compared to the two wave pattern. As a result of prolonged luteal phase, the cows with three wave patterns typically have longer estrous cycle (Figure 2.1; Ginther et al. 1989). The second dominant follicle in the two wave pattern ovulates whereas, in the three wave pattern, the third dominant follicle ovulates in normal cycling cows (Figure 2.1; Ginther et al. 2000). Townson et al. (2002) showed that the ovulatory follicles from two wave pattern were larger and older than those from three wave pattern, which were more fertile.

2.1.2.3 Recruitment of a Cohort of Follicles

Antral follicular development in dairy cattle proceeds through the following series of events: follicular recruitment, selection, and dominance. Initially, all follicles of a particular wave grow at the same rate for approximately 2 days (Figure 2.1; Adams et al. 2008). A group of small antral follicles escapes from the atresia to grow from the resting ovarian follicular pool, under the influence of increasing circulatory FSH (Figure 2.1).A developing antral follicular group typically consists of 5-10 follicles (Driancourt 2001), but may be as high as 24-41 follicles in a cow (Figure 2.1; Mihm and Austin 2002; Adams et al. 2008). Once the follicles are recruited to grow in size, they are destined to ovulate, under the influence of a surge of LH or undergo an irreversible degeneration (Figure 2.1).

A transient elevation in the blood levels of FSH will occur after the atresia or degeneration of a dominant follicle in a growth wave or after ovulation at the end of the estrous cycle (Figure 2.1). As the follicle grows to become dominant, it exerts a major inhibitory effect on other antral follicles from the recruited cohort (Aerts and Bols 2010).

This inhibitory effect may be due to a combination of the production of inhibin and E_2 by the dominant follicle. Most of the antral follicles undergo atretic degeneration due to suppressed circulatory FSH concentrations and decreased blood supply to some follicles (Driancourt 2001). Therefore, only those growing follicles that receive large blood supplies continue to grow under higher levels of gonadotropins (Figure 2.1).

2.1.2.4 Selection

In mono-ovular species, very few follicles continue to grow from the follicular pool after recruitment while other recruited follicles undergo atresia, leading to irreversible degeneration (Figure 2.1; Ginther et al. 2000). During each follicular growth wave, some of the small antral follicles in a recruited pool start producing moderate amounts of E_2 from the granulosa cells (Lucy 2007a). Usually, one follicle is selected to grow to become dominant, while subordinate follicles undergo atresia in dairy cows (Figure 2.1).

Selection of the dominant follicle may coincide with a decrease in the blood FSH concentrations during the first few days after the wave emergence (Figure 2.1; Ginther et al. 2000). Previous studies found that the growing follicles that are $\geq$ 5mm in diameter have acquired the ability to suppress the FSH release during early stages of selection. In addition to this, the same experiment revealed that follicles which are 3 mm in diameter do not suppress the secretion of FSH (Gibbons et al. 1999).

2.1.2.5 Characteristic Features of the Dominant Follicle

The dominant follicle plays a very important role in bovine reproduction. The selected growing follicle is referred to as dominant when it reaches an average diameter

of 8.5-10 mm (Adams et al. 2008; Aerts and Bols 2010), when the subordinate follicles are inhibited or cease their growth and become atretic (Figure 2.1; Lucy et al. 1992). Ginther and coworkers (1996) used the term deviation to differentiate between the future dominant follicle and the remaining subordinate follicles. All growing follicles have the ability to become dominant before deviation, but after deviation, only the largest follicle will grow to dominance, while others become atretic (Figure 2.1). Previous experiments showed that selection and dominance of the growing follicle are associated with the progressive increase in the capacity of thecal cells to produce androgen from cholesterol and granulosa cells to produce E_2 from androgen (Sartori et al. 2001).

The developing follicle has LH specific membrane receptors located on the theca interna cells. The binding of LH to its specific receptors activates a signaling cascade, leading to the conversion of cholesterol to testosterone (Niswender et al. 1985). Testosterone diffuses out of the theca interna cells into the granulosa cells, where FSH receptors are situated. When FSH binds to the FSH receptors on the granulosa cells, it causes the synthesis of enzymes (aromatase and protein kinase) that are responsible for the conversion of testosterone to E_2 (Sartori et al. 2001). In the dominant ovulatory follicle, the increasing level of E_2 reaches a threshold, inducing the preovulatory surge of LH (Figure 2.1). The subordinate follicles undergo regression due to low circulating FSH and lack of blood supply. The basal level of circulating FSH effectively prevents the emergence of a new cohort of antral follicles (Driancourt 2001). Due to the acquisition of LH receptors under the low FSH blood profile, the dominant follicle shifts its increased gonadotropin dependence to LH (Mihm et al. 2006; Aerts and Bols 2010). In consequence, the dominant follicle survives and matures in spite of the low FSH levels

(Figure 2.1; McGee and Hsueh 2000; Adams et al. 2008). Previous studies revealed that the factors produced by the dominant follicle act either locally or systemically to suppress the development of other follicles (McGee and Hsueh 2000).

2.1.2.6 Final Maturation of Preovulatory Follicle

Due to the acquisition of LH receptors on granulosa cells, the dominant follicle becomes largely dependent on the LH action under the low circulatory FSH levels (Figure 2.1; Ginther et al. 1996). As the pheripheral E_2 level increases, it induces a positive feedback on the GnRH pulse frequency, eventually leading to a surge in the concentration of LH. The LH surge occurs if there is no functional CL on the ovary (Wiltbank et al. 2002). However, the P_4 secreted from the functional CL induces a negative feedback on GnRH pulse frequency and consequently, inhibits the LH surge (Figure 2.1; McCartney et al. 2007). The pre-ovulatory LH surge is crucial for the induction of ovulation and preparation of thecal and granulosa cells for luteinization. Episodic LH pulses are essential for the formation of CL in cows. However, they are not required for the maintenance of luteal activity (Smith et al. 1994).

Maturation of the preovulatory follicle coincides with spontaneous luteolysis of the CL. The CL negatively affects the LH pulse frequency by P_4 secretion during the mid luteal phase (Niswender et al. 1994). A group of hypothalamic nuclei, composed of nerve cell bodies that influence the reproduction are called the surge centre and the tonic centre. Neurons in these regions produce GnRH responsible for controlling the secretion of FSH and LH (Baker and Kragt 1969). The LH dependent dominant follicle developed during the early luteal phase undergoes atresia, due to the lower LH pulse frequency and

13

negative feedback from P_4 on hypothalamic GnRH centre (Rajamahendran et al. 1989). The subsequent development of the dominant follicle in the absence of functional CL results in final maturation under the influence of enhanced LH pulse frequency (Figure 2.1; Aerts and Bols 2010).

2.1.2.7 Ovulation of the Preovulatory Follicle

Ovulation is a complex process characterized by the discharge of a mature ovum from the ovary. A preovulatory surge of LH triggers several signaling cascades that lead to ovulation (Figure 2.1; Algire et al. 1992). After the LH surge, $PGF_{2\alpha}$ and prostaglandins of the E series are produced by the ovary (Aerts and Bols 2010). Prostaglandins of the E series are necessary for the successful rupture of the pre-ovulatory follicle and the release of an oocyte into the periovarian space (Filion et al. 2001). Prostaglandin E_2, along with histamine produced by the blood cells, will cause hyperemia (elevated blood flow at local tissue) in the ovarian tissue (Algire et al. 1992; Arosh et al. 2002). Hyperemia in the dominant follicle causes the theca interna cells to become edematous in response to increased vascular permeability brought about by histamine. This edematous condition eventually leads to increased hydrostatic pressure within the follicle and the release of an oocyte (Mihm et al. 1994).

Following the LH surge, the thecal cells in the follicle begin to produce P_4 enzymatically by protein kinase and mitochondrial enzymes. This P_4 subsequently induces the production of collagenase by the theca interna cells (McCartney et al. 2007). Collagenase breaks down the collagen matrix on the tunica albugenia, the outer covering of the ovary. Concurrently, the follicular fluid volume also increases, leading to follicular

apex (stigma) enlargement (Gordon 1996). Prostaglandin $F_{2\alpha}$ causes the contraction of the smooth muscle cells of the ovary. This recurrent contraction leads the stigma to push itself out of the ovary (Lucy et al. 1992). Prostaglandin $F_{2\alpha}$ causes the rupture of lysosomes to release their enzymes within the granulosa cells. These lysosomal enzymes further degrade the connective tissue around the stigma of the follicle (Sakaguchi 2011). Prostaglandin E_2 is presumed to stimulate a substrate called plasminogen that is converted into plasmin with the help of plasminogen activator. Plasmin is involved in tissue remodeling after ovulation (Dow et al. 2002). Increased plasminogen activator and plasmin activity may be contributing factors in the mechanism of follicular rupture in cows (Dow et al. 2002; Dias et al. 2010). In total, these tightly regulated processes lead to rupture of the ovulatory follicle, evacuation of the oocyte and formation of the CL.

2.1.3 Ovarian Luteal Dynamics during Bovine Estrous Cycle

2.1.3.1 Luteinization, Development and Maintenance of CL

The corpus luteum is a transient endocrine gland established by cells of the ovulated follicle. This transient gland produces P_4 which is necessary for the establishment and maintenance of pregnancy (Skarzynski et al. 2008). Immediately after ovulation, the wall of the follicle collapses into folds. Small blood vessels rupture to cause a hemorrhage and clot. This structure is referred to as a corpus haemorrhagicum (Niswender et al. 1994). The follicular cells in the ovulated follicle transform or differentiate into luteal cells (luteinization) and develop into a fully functional CL in 5-6 days after ovulation (Hawkins et al. 1995). The two key features of the cells after luteinization are their capacity to produce sufficient quantities of P_4 and the capacity to

undergo regression at an appropriate time. Formation of the CL is initiated by reorganization of follicular cells under the influence of pre-ovulatory LH before ovulation (Hayashi et al. 2003). These changes, termed luteinization, are characterized by biochemical changes in the theca interna and granulosa cells of the preovulatory follicle, leading to increased steroid production (Berisha and Schams 2005).

Several biochemical and morphological modifications occur which involve breakdown of the basement membrane between the thecal and granulosa cells. Blood vessels start invading the follicular antral space, leading to development of an extensive vascular network. Thecal cells undergo significant hyperplasia and migrate into the previous antral (follicular) cavity by dispersing themselves between the luteinizing granulosa cells (Niswender et al. 1994). There is a transformation of follicular cells secreting E_2 to the CL, mainly producing P_4. During luteinization, the thecal cells are believed to undergo hyperplasia to form small luteal cells, while the granulosa cells undergo hypertrophy to form large luteal cells (O'Shea et al. 1989). Large luteal cells account for 80% of total P_4 production by the CL (Niswender et al. 1985).

2.1.3.2 Biosynthesis of Progesterone

Progesterone is a steroid hormone synthesized in the CL from pregnanolone, a cholesterol derivative. Proliferating granulosa and thecal cells in the wall of the ovulated follicle gradually produce P_4 after ovulation (Skarzynski et al. 2008). Progesterone concentration in blood is the main indicator of a functional CL. The CL begins to grow during the early luteal stage, reaching a maximum diameter at mid stage, which is maintained until its diameter decreases during late diestrus stage. Both the structural (maintenance of size) and functional (maintenance of P_4 production) integrity of the CL

are important for the prevention of luteolysis and maintenance of pregnancy (Niswender 2002). Progesterone concentration begins to rise by Day 3 of the estrous cycle. The P_4 reaches a high concentration in 5 to 12 days and remains at a plateau until Day 16 of diestrus (Niswender et al. 2002). Then the P_4 concentration rapidly decreases as the CL regresses (Niswender et al. 2002).

2.1.3.3 Regression (Luteolysis) of CL

The required minimal levels of P_4 are crucial throughout pregnancy to maintain the quiescent state of the uterus and functioning of uterine secretions, which are essential for the maintenance of conceptus. In the absence of the conceptus in the uterus, luteolysis of the CL begins on Days 17-19 of the estrous cycle in cattle (Niswender 2000). This process is characterized by structural and functional changes in steroidogenic and non-steroidogenic (accessory) cells of the CL. Regression of the CL in ruminant species, at the end of the estrous cycle, is brought about by the episodic release of $PGF_{2\alpha}$. The life span of the CL in cattle is regulated by the uterus which secretes $PGF_{2\alpha}$ (Berisha and Schams 2005). Uterine $PGF_{2\alpha}$, in most species, including ruminants, is known to be a principal luteolytic factor. Communication between the uterine endometrium and CL is essential for luteolysis (Gordon 1996).

It has been postulated that the P_4 prevents the secretion of $PGF_{2\alpha}$ by downregulating the oxytocin receptors during the first half of the estrous cycle. However, after 10 to 12 days, P_4 loses its ability to block formation of oxytocin receptors (Niswender 2000). The changes in expression patterns of E_2 and P_4 receptors during the estrous cycle are in part due to circulatory steroid concentrations. Progesterone has an

17

effect on function of the bovine early and mid-cycle CL in an autocrine or paracrine fashion. The endometrial steroid receptors, ER and PR, are differentially regulated during the estrous cycle (Niswender et al. 2002). Progesterone up-regulates PR, OT and stimulates $PGF_{2\alpha}$ secretion by early CL, but at late luteal phase, P_4 down-regulates the PR expression. Therefore, P_4 may affect the secretory function of CL in a stage dependent fashion (Skarzynski et al. 2008). Expression of PR is under the control of both P_4, which increases or decreases, and E_2, which increases the PR expression on the endometrium (Skarzynski et al. 2008). Estrogen has been reported to enhance the development of endometrial oxytocin receptors. However, the luteal phase P_4 reduces the concentration of endometrial oxytocin receptors by blocking the action of estrogen (Robinson et al. 2002). In absence of P_4, the luteal oxytocin stimulates the uterine endometrium to secrete $PGF_{2\alpha}$ by binding to its receptors. The small quantity of $PGF_{2\alpha}$ is sufficient to elicit the production of more oxytocin and $PGF_{2\alpha}$ from the CL and uterine endometrium (Skarzynski et al. 2008). Thus, oxytocin and $PGF_{2\alpha}$ stimulate each other in a positive feedback manner. The $PGF_{2\alpha}$ is believed to reach the CL through the counter current system between the uterine vein and ovarian artery (Niswender 2000).

Luteolysis refers to an irreversible degeneration of the CL in a non-pregnant animal and the resumption of a new estrous cycle (Schams and Berisha 2004). The onset of luteal regression is characterized by several events that may include: a rapid loss of synthesis and secretion of P_4 by luteal cells, a morphological change characterized by a decrease in luteal size or weight, a decline in the number of steroidogenic secretary cells, and hyalinization and degeneration of capillaries (Niswender 2000). The sudden decline

in P_4 concentrations in serum does not appear to be due to a loss of steroidogenic cells but by decreased blood flow to the luteal parenchyma (Skarzynski et al. 2008).

2.1.4 Hormonal Interplay Controlling Bovine Estrous Cycle

The neuroendocrine and endocrine factors synthesized and secreted by the hypothalamus, anterior pituitary, and ovaries will reciprocally interact to regulate the ruminant reproductive cycle (Schams and Berisha 2002). Hormones produced at each level of the hypothalamo-pituitary-ovarian-axis are responsible for the tightly regulated events and behavior changes during the estrous cycle (Figure 2.2; Ahmad et al. 1996).
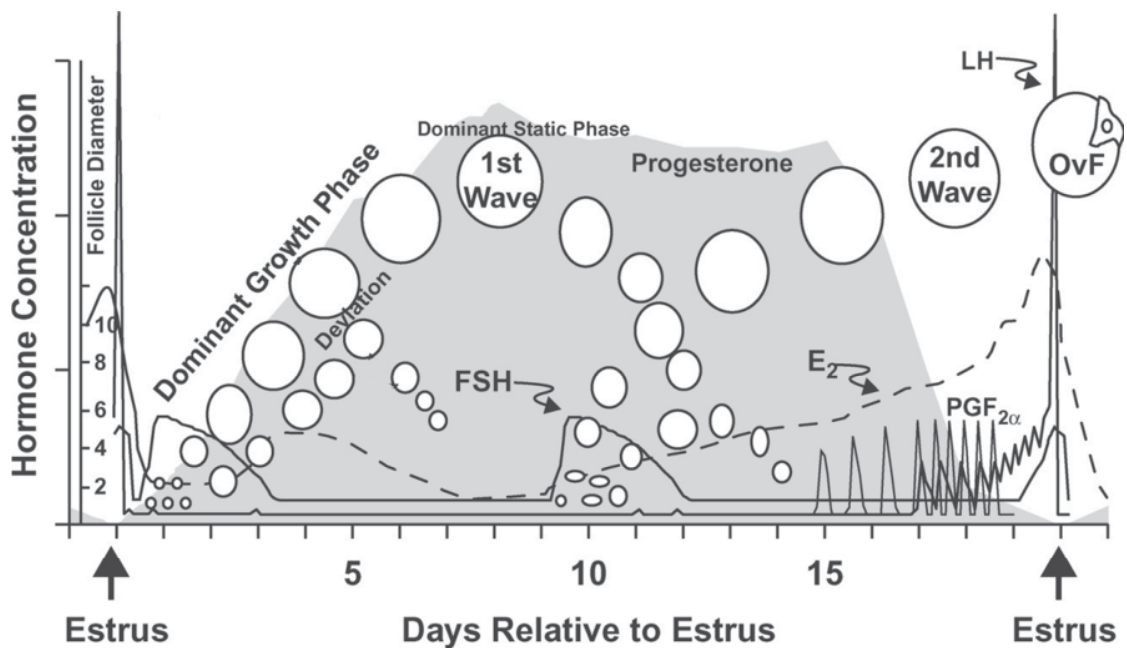
Figure 2.2 Reproductive hormonal profiles correlated with ovarian follicular and luteal development during the estrous cycle of dairy cattle. E_2 = Estradiol, LH = Luteinizing hormone, FSH = Follicle stimulating hormone, $PGF_{2\alpha}$ = Prostaglandin $F_{2\alpha}$, OvF = ovulatory follicle (Source: Moore and Thatcher 2006).

The interaction between the uterus and ovaries is also important, since the $PGF_{2\alpha}$ produced by the uterus causes regression of the CL and cessation of P_4 production (Figure 2.2). The positive and negative feedback mechanisms, elicited from the pituitary and ovary, controls the synthesis and secretion of these hormones (Allrich 1994; Gordon 1996).

2.1.4.1 Gonadotropin-Releasing Hormone

Gonadotropin-releasing hormone (GnRH) is essential for sexual maturation and reproductive functions in mammals. It is a decapeptide synthesized and released by neurons of the hypothalamus (Schallenberger and Prokopp 1985). The GnRH plays a major role by regulating the synthesis and release of gonadotropins, FSH and LH, from the anterior pituitary (Figure 2.2; Ryan et al. 1993). The tonic centre is responsible for basal secretion of GnRH which occurs in a spontaneous rhythmic manner. Small episodes of GnRH secretion occur every 1.5 to 2 hours and every 4 to 8 hours during follicular and luteal phases, respectively (Ryan et al. 1993). They will influence the pulsatile release of FSH and LH from the gonadotropes of the anterior pituitary (Figure 2.2).

The secretion and function of GnRH is regulated by positive and negative feedback mechanisms (Motta et al. 1973). The GnRH in turn controls secretion of FSH and LH from the anterior pituitary. High concentrations of P_4, especially during the luteal phase, cause the negative feedback by inhibiting the GnRH neurons (Figure 2.2; Baker and Kragt 1969). Therefore, GnRH neurons, under the influence of high P_4, release only basal secretions of GnRH. This basal secretion allows for some follicular development. However, it does not allow for sufficient follicular development to elicit secretion of high concentrations of E_2 (Figure 2.2; Ahmad et al. 1996). A high level of E_2, which occurs

20

during the mid to late follicular phase or low P_4 levels, stimulates the preovulatory centre to release large quantities of GnRH (Sartori et al. 2004), which in turn release the LH, that stimulate the ovulation (Figure 2.2; Gordon 1996). The concentration of GnRH begins to rise in proestrus, reaches its maximum level at estrus, decreases at the end of metestrus and reaches its basal level during the diestrus period (Allrich 1994).

2.1.4.2 Profiles of FSH and LH during the Estrous Cycle

Follicle stimulating hormone and LH are the glycoprotein hormones secreted by the gonadotrophs of the anterior pituitary in response to pulsatile GnRH (Schallenberger and Prokopp 1985). They play a major role in controlling follicular wave dynamics by stimulating the steroidogenesis and ovulation in the ovary. Progesterone starts decreasing by approximately Day 16-17 of the estrous cycle (Figure 2.2; Gordon 1996). The drop in P_4 production removes the negative feedback on the hypothalamus. In response, pulses of GnRH, FSH, and LH are released with increased frequency and amplitude (Figure 2.2). A rise or increase in plasma FSH precedes the emergence of the follicular wave in both spontaneous and induced waves (Figure 2.2; Adams et al. 2008). Increasing blood FSH and LH concentrations during proestrus promote the growth of developing follicles into larger diameter follicles (Adams et al. 2008).

The developing antral follicles secrete greater concentrations of E_2 under the influence of FSH and LH (Figure 2.2; Driancourt 2001). Estradiol reaches a threshold concentration, leading to the large preovulatory surge of GnRH, FSH, and LH. The preovulatory FSH surge, in turn, stimulates the more rapid growth of follicles and greater secretion of E_2 (Figure 2.2; Adams et al 2008). The E_2 released by the growing follicle suppresses FSH release of anterior pituitary. The circulating concentrations of FSH

increase after ovulation and also during the mid-static phase of the anovulatory dominant follicle (Figure 2.2; Lucy 2007a). Follicle stimulating hormone concentrations peak (1.5 to 2 fold) 12-24 h before the emergence of a new wave (Figure 2.2). Previous research reported that removal of the existing dominant follicle (follicular ablation) causes a peak in FSH within 12 h and a new wave will emerge within 24 h (Bergfelt et al. 1997).

The secretion of FSH is likely modulated by inhibin, a hormone secreted by the ovaries. Inhibin's effect may also be responsible for atresia of follicles that are in a growing stage due to suppression of FSH secretion (Kengaku et al. 2007). In consequence, the growing follicles may not reach ovulatory size. The selection of the dominant follicle is associated with decreasing concentrations of FSH during the first 3 days of the wave (Figure 2.2; Adams et al. 2008). Therefore, developing follicles grow larger to become dominant in response to LH, while subordinate follicles regress due to lack of response (Figure 2.2; Mihm et al. 2006). Decreasing concentrations of P_4, with increasing concentrations of E_2 after luteolysis, will lead to a further rise in LH pulse frequency, leading to a large preovulatory LH surge (Figure 2.2). This surge occurs during early estrus and stimulates the final maturation of the oocyte and thus, ovulation (Mihm et al. 2002). The preovulatory surge of LH simultaneously stimulates $PGF_{2\alpha}$ to increase follicular pressure. Prostaglandin $F_{2\alpha}$, in-turn, induces contraction of the ovarian smooth muscle and weakens the follicular wall. Finally, it leads to ovulation (Figure 2.2; Mihm et al. 2002).

2.1.4.3 Estradiol-17β (E_2) Profile

Estradiol-17β, produced by the dominant follicle rises significantly during proestrus (Figure 2.2; Bridges et al. 2010). The preovulatory dominant follicle secretes E_2 in sufficient quantities during proestrus to stimulate the hypothalamus to increase its secretion of GnRH. In response to this, increased pulses of high amplitude and frequency of LH and FSH occur, leading to complete follicular development and a surge in E_2 (Figure 2.2; Reames et al. 2011). Thecal cells of the developing antral follicle convert P_4 to androgens. Estradiol is synthesized in granulosa cells where the aromatase enzyme required for the conversion of androgen to E_2 is located (Allrich 1994).

Singh et al. (2003) reported that E_2 content in the follicular fluid of the growing dominant follicle increases at least 20-fold just before the day of selection. This is followed by a threefold decrease in E_2 by the early static phase of the anovulatory dominant follicle (6 days); E_2 returns to base-line (9 days) in the early regressing phase (Figure 2.2). The ovulatory dominant follicle contains twice the peak concentration of the E_2 of the anovulatory dominant follicle (Figure 2.2; Singh et al. 2003). The E_2 concentrations begin to rise (≤ 2 pg/mL) four days before the last day of the estrous cycle, reaching peak at the time of estrus (8.5-10.0 pg/mL; Figure 2.2; Macmillan 2010). This peak concentration of E_2 is necessary for the cow to express behavioural signs of heat (Bridges et al. 2010). Estradiol levels start declining after ovulation and falls to the basal level (≤ 2 pg/mL; Gordon 1996). The moderate increase in E_2 concentrations during metestrus and mid-diestrus may be associated with the selection and growth of the developing follicle (Figure 2.2; Reames et al. 2011).

2.1.4.4 Progesterone (P$_4$) Profile

The CL morphology and plasma P$_4$ concentration are good indicators of P$_4$ synthesis in the CL (Singh et al. 2003). Progesterone concentrations are at basal levels (less than 0.25 ng/mL) on Days 1 and 2 of the estrous cycle. Progesterone levels begin to rise from 1 ng/mL at Day 3 post-ovulation to approximately 3 ng/mL by Day 6 (Figure 2.2). The size of the CL is positively correlated with the amount of P$_4$ produced (Adams et al. 2008). The plasma P$_4$ concentrations ($\geq$ 4 ng/mL) stabilize until Day 16 and decrease after the regression of the CL during the late diestrus period, possibly due to hyalinization of the blood vessels supplying the CL (Figure 2.2). Hypoxic cell death may be induced by the PGF$_{2\alpha}$ released by the endometrium of the non-pregnant cow (Figure 2.2; Adams et al. 2008).

The GnRH secretion during the luteal phase can be inhibited by P$_4$. A high level of P$_4$ decreases the number of GnRH receptors by downregulating the mRNA coding the GnRH receptor (Niswender et al. 2000). It also inhibits the FSH and LH secretion by downregulating the expression of genes, encoding α and β subunits of these hormones. The CL has the capacity to produce a variety of eicosanoids, including PGF$_{2\alpha,}$ prostaglandin E$_2$ (PGE$_2$) and prostaglandin I$_2$ (PGI$_2$), in addition to the products of the lipooxygenase pathway (Wathes et al. 2007). Prostaglandin E$_2$ and PGI$_2$ are luteotrophic, while PGF$_{2\alpha}$ inhibits P$_4$ production. Endometrial production of prostaglandins may cause variation in the concentration of P$_4$, possibly affecting the overall length of the luteal phase (Figure 2.2; Abayasekara and Wathes 1999).

2.1.4.5 Prostaglandin $F_{2\alpha}$ Profile

Prostaglandin $F_{2\alpha}$ is a 20-carbon molecule synthesized from arachidonic acid (AA) or di-homo-γ-linolenic acid. It is primarily produced by the uterine endometrial cells and also by luteal cells of the CL (Danet-Desnoyers et al. 1995). In dairy cows, the uterus plays a major role in luteal regression and cessation of P_4 production by secreting $PGF_{2\alpha}$ (Figure 2.2). In the case of non-pregnant cattle, the regression may start at any time from Day 5 to Day 19 of the estrous cycle (Niswender et al. 1985). Developing large follicles secrete increasing amounts of E_2 during the luteal phase. Estradiol has been found to control endometrial secretion of $PGF_{2\alpha}$ by regulating the number of oxytocin receptors (Silvia et al. 1991). The posterior pituitary secretes the oxytocin that binds to the receptors which are located in the uterine endometrium. This phenomenon elicits the tonic release of $PGF_{2\alpha,}$ which stimulates an episodic release of oxytocin from the CL. This oxytocin further binds to its receptor in the uterine endometrium, leading to an episodic release of $PGF_{2\alpha}$ from the uterus. Thus, endometrial $PGF_{2\alpha}$ elicits a positive feedback loop which leads to secretion of additional luteal and uterine $PGF_{2\alpha}$ and luteal oxytocin (Figure 2.2; Schams and Berisha 2004).

Previous researchers proposed that the uterine $PGF_{2\alpha}$ is released into the uterine vein and transported to the CL by a counter current mechanism which exists between the ovarian artery and utero-ovarian vein. However, high P_4 levels during the luteal phase inhibit the degeneration of CL and this process may lead to pregnancy (Figure 2.2; Silvia et al. 1991). Prostaglandin $F_{2\alpha}$ may reduce the P_4 synthesis by depriving the cellular uptake of cholesterol, depriving the cells of oxygen and reducing the blood flow to the CL (Niswender et al. 2000). The reduced blood supply to the CL may be caused by

PGF$_{2\alpha}$ binding to its receptors on the endothelia cells of the blood vessels supplying the CL (Schams and Berisha 2004). In consequence, luteolysis may set in due to the reduced blood supply to the luteal parenchyma and degeneration of luteal capillary endothelial cells (Berisha and Schams 2005).

2.2 Presynch and Ovsynch Protocol

Estrus synchronization is widely practiced to bring a large percentage of dairy cows into heat at a particular set time by manipulating the estrous cycle (Moore and Thatcher 2006). There are two protocols currently being used in research and commercial dairy herds: 1) use of PGF$_{2\alpha}$ as potent luteolysin to cause premature regression, 2) progestagen as a source of artificial P$_4$ to bring cows into estrus upon withdrawal. Various drugs such as GnRH and its agonists, P$_4$, E$_2$, P$_4$ and E$_2$ combinations are currently being used to synchronize the follicular and luteal growth (Odde 1990; Moore and Thatcher 2006).

Exogenous PGF$_{2\alpha}$ analogue is a potent luteolysin. This hormone is used to synchronize the estrous cycle and induce estrus in dairy cows (Pursley et al. 1995). Prostaglandin F$_{2\alpha}$ is usually applied in one or two injections 10 to 12 days apart. The presynch program begins with PGF$_{2\alpha}$ injection on the first day followed by a 2nd injection 12 days later (Pursley et al. 1995). The first PGF$_{2\alpha}$ injection will lyse any existing and responsive CL and induce ovulation. According to Odde (1990), approximately 70% of the cows will respond by luteolysis, if there is a functional CL. The ovulation due to 1st PGF$_{2\alpha}$ results in the formation of a new CL. The second PGF$_{2\alpha}$ will lyse the new CL or old CL not responding to the 1st PGF$_{2\alpha}$. A new follicular phase begins with the second PGF$_{2\alpha}$ injection (Pursley et al. 1995).

The GnRH is injected 12 d after the 2nd $PGF_{2\alpha}$ injection to induce ovulation in the dominant preovulatory follicle. This results in a new follicular wave as well as a new CL formation. After 7 days, a third $PGF_{2\alpha}$ injection is given to lyse the CL. The cow will enter new follicular phase which result in the development of a new dominant follicle (Moore and Thatcher 2006). A second GnRH injection is given 2 days after $PGF_{2\alpha}$ to cause the dominant follicle to ovulate. Ovulation occurs 24 - 32 hr after the GnRH injection (Pursley et al. 1995; Senger 2003). It is possible to synchronize ovulation when the preovulatory follicles are in similar stages of growth. These preovulatory follicles ovulate because they are responsive to LH at the time of the 2nd GnRH application (Pursley et al. 1995).

2.3 Fatty Acid Classification and Background Information

Dairy cattle diets are supplemented with fat to enhance the energy concentration of the diet and therefore, animal performance (Beam and Butler 1998). Fats are the biological compounds classified as lipids and are soluble in organic solvents. Various biological compounds, including cholesterol and fats such as triacylglycerol and phospholipids, are categorized as lipids (Wathes et al. 2007).

Phospholipids are basic components of cellular membranes and also act as a source of fatty acids in the uterine endometrium and ovary. These fatty acids are required for the synthesis of various effector molecules such as eicosanoids, which include prostaglandins, thromboxanes, and leukotrienes. Another cellular membrane component, cholesterol is the precursor in the synthesis of steroid hormones (Mattos et al. 2000).

The typical dairy cow ration may contain up to 5% fat which provides a dietary energy source. Fatty acids are the basic structural components of fats and oils that are of plant and animal origin. Fatty acid function is determined by length of acyl chain, the number and position of double bonds, and the type of isomer formed by each double bond (Surette 2008). They are classified into saturated fatty acids (no double bond), monounsaturated fatty acids (MUFA) (single double bond) and PUFAs (more than 1 double bond) depending on the presence of a double bond in their chemical structure (Surette 2008).

2.3.1 Classification of Long Chain Polyunsaturated Fatty Acids

Several different systems of nomenclature are used for fatty acids. Commonly used systems are: omega-x (n-x) and delta-x (Δ^x) nomenclature systems. In the omega-x (n-3 or n-6) system, fatty acids are designated based on the position of double bond from the methyl end. For example, linolenic acid (C18:3; cis-9, 12, 15-octadecatrienoic acid) belongs to the n-3 family because it has 18 carbon atoms and three double bonds, with its first double bond at the third position from the methyl end of the fatty acid. However, linoleic acid (C18:2; cis-9, cis-12-octadecadienoic acid) belongs to the n-6 family due to the presence of its first double bond at the sixth carbon position from the methyl end (Mattos et al. 2000). In the delta-x system, fatty acids are designated based on the position of the double bond from the carboxyl end. This system is used in the nomenclature of enzymes involved in the biosynthetic pathways of PUFAs. Each double bond is preceded by a cis or trans prefix, indicating the conformation of hydrogen atom around the double bond. For example, linoleic acid is designated cis Δ^9, cis Δ^{12} octadecadienoic acid (Wathes et al. 2007). The precursors of omega-6 (arachidonic acid)

and omega-3 PUFAs (EPA and DHA) are linoleic acid (LA; 18:2; cis-9, cis-12-octadecadienoic acid) and α-linolenic acid (ALA; C18:3; cis-9, 12, 15-octadecatrienoic acid), respectively (Surette 2008). Nutritionally important n-3 PUFAs are ALA (C18:3; cis-9, 12, 15-octadecatrienoic acid), eicosapentaenoic acid (C20:5; eicosa-5, 8, 11, 14, 17-pentaenoic acid) and docosahexaenoic acid (C22:6; docosa-4, 7, 10, 13, 16, 19-hexaenoic acid). Animal tissues can synthesize the oleic acid (18:1, n-9) family of unsaturated fatty acids. However, linoleic acid (18:2, n-6) and ALA cannot be synthesized endogenously by mammalian cells because of they lack enzymes that desaturate beyond the 9th C in the acyl chain. And hence, they are nutritionally essential fatty acids (Ruxton et al. 2004).

Due to the importance of specific fatty acids and their roles in reproductive processes, the reproductive performance of dairy cows may be improved by including specific long chain fatty acids rather than feeding fat as a whole (Santos et al. 2008). Linoleic acid is required for the synthesis of AA and series 2 prostaglandins. Eicosapentaenoic acid is required for series 3 prostaglandins (Cheng et al. 2001). Series 1 and 3 are less biologically active than series 2 prostaglandins. However, they may be produced in the body at the expense of series 2 prostaglandins depending on the presence of various proportions of precursors in the membrane phospholipids which in turn are supplied through the diet (Cheng et al. 2001). Flaxseed, rapeseed, canola, and soy oils are rich sources of ALA, whereas EPA and DHA are present in high amounts in fish oil and other marine food products such as algae (Moghadasian 2008).

2.3.2 Dietary Omega-3 Fatty Acid Sources of Plant Origin

Forages and oil seeds are excellent sources of PUFAs for dairy cows (Jenkins 1993). Concentrates usually have higher lipid content than do forages. The typical dairy cow ration contains approximately 2 % long chain fatty acids that are predominantly PUFAs of plant origin (Santos et al. 2008). Most of the lipids in concentrates are present in the form of triacylglycerols (TAG; De Vries and Veerkamp 2000). Generally, ALA is present in higher concentrations in fresh forages and leafy vegetables, while LA is mainly present in cereal grains and seeds (Jenkins 1993).

Flaxseed oil, or linseed oil, is a popular source of ALA from a sub-tropical annual crop grown mainly in Canada (Gagnon et al. 2009). Flax seed and its oil have been used widely as a conventional source of n-3 PUFA that contain >500 g of ALA per kilogram of total fatty acid (Moallem 2009). Flax seeds are incorporated into dairy feeds to improve reproduction and milk quality in dairy cows (Petit 2003). Feeding protected flaxseed or its protected oil may enhance reproductive performance and increase the n-3 PUFA content of milk fat (Gonthier et al. 2005). However, the method of protection of fat from ruminal biohydrogenation determines the efficiency of fatty acid transfer into the reproductive tissues and the milk as well (Santos et al. 2008). The circulatory concentrations of myristic acid, palmitic acid, oleic acid and ALA were increased when dairy cows were fed with flaxseed (Petit 2003). However, the increase in ALA was very small (i.e., 13%) suggesting that the greater proportions of PUFA were subjected to extensive ruminal biohydrogenation in supplemented cows (Jenkins and Palmquist1984). The PUFA in oilseeds such as flaxseed can be protected from ruminal biohydrogenation

by heat treatment. The protein matrix around the fat droplets in the seed is denatured by the heat, thus reducing the access of ruminal bacteria to dietary PUFA (Kennelly 1996).

2.3.3 Dietary Omega-3 Fatty Acid Sources of Marine Origin

Currently fish oil is used as the primary source of EPA and DHA to indirectly enrich the milk and milk products. Fish meal fed to dairy cows contains about 8% fat, of which two thirds is present in the form of long chain PUFAs such as EPA and DHA (Burke et al. 1997). Geographical location, season of the year, and species of fish will determine the composition and quality of oil harvested (Van-Vliet and Katan 1990). Cold water oily fish such as salmon, herring mackerel, and shell fish are the most available sources of EPA and DHA. However, these n-3 PUFAs in wild fish originate from marine microbes such as marine microalgae and diatoms (Sayanova and Napier 2011). The increasing demand for n-3 PUFAs from fish and fish oil contributes to depletion of fish stocks. Since wild populations of fish depend on marine microalgae as a primary source of n-3 PUFAs, they are harvested for feeding to fish to enrich their oil with more n-3 PUFAs compared with farmed fish (Van-Vliet and Katan 1990). Long term over fishing is putting great pressure on oceanic ecosystems while commercial fish farming is not sustainable and cannot compensate for the shortage in fish supply (Sayanova and Napier 2011).

Fish oil is also contaminated by environmental pollution and may impart a fishy smell with unpleasant taste (Sijtsma and de Swaaf 2004). Expensive purification may be required for DHA before usage as marine oil is a complex mixture of fatty acids with varying length and degrees of unsaturation (Sijtsma and de Swaaf. 2004). Production of EPA and DHA from fish is insufficient to supply the expanding market. In order to meet

the market demand and to overcome the unpleasant smell from the fish oil, researchers are looking to develop an alternate feed processing technology to harvest EPA and DHA (De Swaaf et al. 2003). The techniques such as refinement of fish oil, purification and synthesis of PUFAs from marine microbial sources might lead to a sustainable production of EPA and DHA (De Swaaf et al. 2003).

Marine microbes such as microalgae and diatoms are the primary producers of EPA and DHA (Sargent 1997). These n-3 PUFAs move up through the aquatic food chain when marine algae are consumed by fish and concentrated in fish lipids. Recent research suggests that fish are dependent on marine microbes for their n-3 PUFAs requirement as animal tissues lack the elongase and desaturase enzyme system required for EPA and DHA biosynthesis (Franklin et al. 1999; Sayanova and Napier 2011). With the growing concern over wild fish stocks, marine microalgae could be exploited to be the potential alternate source of EPA and DHA (Sargent 1997). Two marine microalgae sp, *Pavlova* and *Isochrysis*, were reported to produce substantial amounts of EPA and DHA (Pereira et al. 2004). The expressed sequence analysis of these organisms revealed two novel genes that are involved in DHA synthesis through desaturase and elongase enzymatic pathways (Meireles et al. 2003). Commercial use of marine microalgae sp, *Schizochytrium* has been reported. Franklin and coworkers (1999) reported that feeding *Schizochytrium* (protected microalgae) resulted in higher levels of DHA and decreased the concentrations of saturated fatty acids in milk of dairy cows than cows fed the control diet. The same studies coated the algal supplements with xylose to enable them to escape the ruminal biohydrogenation (Franklin et al. 1999).

2.3.4 Biosynthetic and Metabolic Pathways of Omega-3 Fatty Acids

The essential fatty acids present in mammalian systems come from various sources. These are either absorbed into the system from the diet or generated by *de novo* synthesis (Thatcher et al. 2006). Short chain fatty acids undergo elongation and desaturation in order to generate the long chain fatty acids with various biochemical properties (Jenkins 1993).

The system of elongation and desaturation is catalyzed by two key enzymes: elongase and desaturase. Elongation is a process catalyzed by elongase enzymes which enables the addition of two-carbon units to a primary carbon chain (Figure 2.3). Desaturation involves the insertion of a double bond in the acyl chain through the action of a desaturase. Desaturase enzymes are categorized based on the position of insertion of double bond in the acyl chain (Jenkins 1994). These desaturase enzymes in an animal system will not catalyze the reaction of insertion of a double bond beyond the Δ9 position (Mattos et al. 2000). This will prevent the synthesis of n-3 and n-6 PUFAs in animals and hence, they must be supplied by the diet.

Omega-3 PUFAs

α- Linolenic Acid
(C18:3; cis-9, 12, 15)

↓ Δ6-Desaturase

Stearidonic Acid
(C18:4; cis-6, 9, 12, 15)

↓ Elongase

Eicosatetraenoic Acid
(C20:4; cis-8, 11, 14, 17)

↓ Δ5-Desaturase

Eicosapentaenoic Acid
(C20:5; cis-5, 8, 11, 14, 17)

↓ Elongase

Docosapentaenoic Acid
(C22:5; cis-7, 10, 13, 16, 19)

↓ Δ6-Desaturase

Docosahexaenoic Acid
(C22:6; cis-4, 7, 10, 13, 16, 19)

Omega-6 PUFAs

Linoleic Acid
(C18:2; cis-9, 12)

↓ Δ6-Desaturase

γ -Linolenic Acid (GLA)
(C18:3; cis-9, 12, 15)

↓ Elongase

Dihomogamma linolenic Acid
(DGLA; C20:3; cis-8, 11, 14)

↓ Δ5-Desaturase

Arachidonic Acid
(C20:4; cis-5, 8, 11, 14)

Figure 2.3 Biosynthetic desaturation and elongation pathways of omega-3 and omega-6 polyunsaturated fatty acids. The enzyme system involving Δ6-desaturase, elongase, and Δ5-desaturase are acting sequentially on the precursor fatty acids, linoleic (C18:2, n-6), linolenic (C18:3, n-3) leading to longer chain polyunsaturated fatty acids (Source: Mattos et al. 2000).

Ruminant animals have a unique feature of digesting the lipids supplied with conventional diets. Dietary lipids, after ingestion, enter the ruminal hydrolytic and reductive environment (Nafikov and Beitz 2007). Dietary lipids involving TAG, phospholipids and galactolipids are acted upon by the ruminal flora, bacteria which release the fatty acids of various chain lengths from their glycerol backbone. As a result,

the glycerol is fermented to form volatile fatty acid (VFA) and unsaturated fatty acids released from glycerol backbone will be reduced before absorption (Nafikov and Beitz 2007). This reduction process of the double bonds, associated with unsaturated fatty acids and changes in the orientation of isomers is called fatty acid biohydrogenation (Jenkins 1993).

Previous research reported that EPA and DHA may escape, or undergo negligible biohydrogenation by ruminal bacteria (Ashes et al. 1992). Previous studies on ruminal biohydrogenation resulted in the development of fat supplements that are partially resistant to biohydrogenation (Jenkins and Palmquist 1984). The same researchers indicated that calcium soaps remain relatively inert under the normal pH conditions in the rumen while dissociate completely in the acidic conditions of the abomasum. Fat supplements such as calcium soaps of long-chain PUFAs have been developed which reach the duodenum in increased concentrations after escaping the ruminal biohydrogenation (Jenkins and Palmquist 1984). These protected fatty acids are absorbed at the duodenal level and incorporated into milk and adipose tissue of liver and mammary gland (Mattos et al. 2000).

2.4 Fat Supplementation and Reproductive Performance

Long chain PUFAs play a major role in regulating the reproductive processes in dairy cattle. The potential mechanism by which fat supplements improve reproductive performance may include altered follicular growth and ovulation (Robinson et al. 2002, Moussavi et al. 2007a), increased plasma P_4 levels during the luteal phase (Burke et al. 1998), prolonged life span of the CL and suppressed activity of E_2 and $PGF_{2\alpha}$ around maternal recognition of pregnancy (Figure 2.4; Hightshoe et al. 1991; Mattos et al. 2004).

Dietary fats such as cholesterol and n-6 PUFAs are precursors for steroid and eicosanoid synthesis. These steroids and eicosanoids alter ovarian and uterine function and affect pregnancy rates (Mattos et al. 2000). Altering the concentrations of both n-6 and n-3 PUFAs in feeds can influence $PGF_{2\alpha}$ synthesis and conceptus development. The changing patterns of $PGF_{2\alpha}$ synthesis have a major effect on fertility due to a role in many reproductive functions such as hormone secretion and ovulation (Mattos et al. 2000).
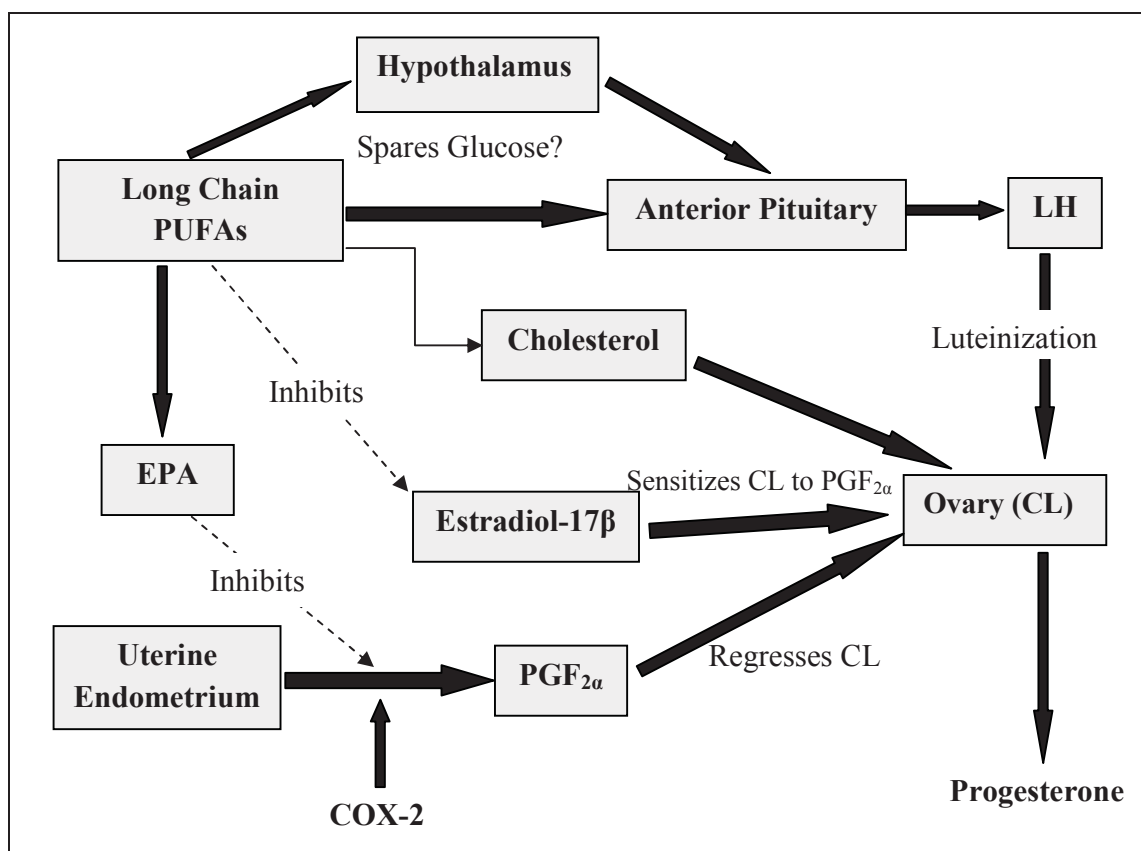
Figure 2.4 Suggested mechanism of action by which LCPUFAs may improve reproductive performance in dairy cattle (Source: Staples et al. 1998).

The conception rate in lactating dairy cows supplemented with PUFAs may be improved by 1) sparing a glucose molecule to stimulate the anterior pituitary to release

LH which may in turn stimulate the differentiation of granulosa cells to luteal cells (luteinization; Figure 2.4), 2) increased production of progesterone associated with improved fertility by increased uptake and circulatory concentrations of cholesterol (Figure 2.4), and 3) inhibiting the estradiol and $PGF_{2\alpha}$ production thereby delaying the luteal regression and prolonged life span of CL associated with survival of the conceptus (Figure 2.4).

Dietary supplementation with n-3 PUFAs might improve fertility by influencing follicular growth and ovulation in dairy cows. Feeding fat supplements such as calcium salts of PUFAs increased the number of large follicles (more than 10 mm) after Day 25 postpartum in a synchronized estrous cycle (Lucy et al. 1993). Similar studies of follicular development found increased diameter and number of follicles in cows fed with n-3 PUFAs (Beam and Butler 1997; Robinson et al. 2002). Cows fed with rolled flaxseed enriched with ALA (56.7% of total FA) had larger mean diameter of the ovulatory follicle (16.9 vs. 14.1 mm) than the cows fed with rolled sunflower seed (0.1% ALA; Ambrose et al. 2006). However, cows fed with flaxseed rich in n-3 PUFAs have a lower number of small follicles than the cows fed soybean rich in n-6 PUFAs (Ponter et al. 2006).

Recent studies indicate that there was a 23% increase in the diameter of large follicles ($\geq$ 10 mm) in cows fed diets rich in n-3 PUFAs (rolled flax seeds and its oil, Ca-LCFA and fish oil) compared with MUFAs (Lucy et al. 1993; Ambrose et al. 2006; Bilby et al. 2006a; Santos et al. 2008). These studies clearly indicate the differential effects of fatty acids on follicular growth. Some researchers reported that fat supplementation may influence early resumption of postpartum cyclicity (Beam and Butler 1997). Therefore,

fat supplementation may reduce the interval from calving to first postpartum ovulation (Lammoglia et al. 1999). However, the estrous cyclicity in early lactation may be due to the improved energy status of the cow.

Previous studies reported that E_2 may stimulate uterine endometrial secretion of $PGF_{2\alpha}$, and may increase CL sensitivity which will cause it to be acted upon by $PGF_{2\alpha}$ resulting in complete luteolysis of the CL (Knickerbocker et al. 1986; Staples et al. 1998). Therefore, the lower concentrations of E_2 may result in the prevention of premature degeneration of the CL and the loss of early conceptus. Cows infused with tallow in their abomasum had lower plasma E_2 levels (2.42 vs. 3.81 pg/mL) on Days 15 to 20 of their synchronized estrous cycle compared with glucose infused control cows (Oldick et al. 1997). Similar results were observed when lipid supplements were fed to beef cows suggesting that fat supplementation may lower the plasma E_2 concentrations (Hightshoe et al. 1991). In contrast, other studies found that there was an increase in E_2 concentrations during the follicular phase in cows fed ALA (Robinson et al. 2002b). Lactating dairy cows fed with 1 kg/d microencapsulated fat diet consisting of 40.8% sunflower oil (which provided 260 g of n-6 PUFAs) had higher E_2 levels in follicular fluid of preovulatory follicles than the cows fed with 1 kg/d microencapsulated fat diet consisted of 40.8% flaxseed oil (which provided 242.2 g of n-3 PUFAs; Zachut et al. 2010). Other studies reported that no change in plasma E_2 levels was observed during the luteal and follicular phase of the estrous cycle of lactating dairy cows fed with Ca-LCFA (Lucy et al. 1993). It may be beneficial to have a prolonged life span of CL as a result of a reduced level of E_2 caused by fat supplementation. However, it might negatively affect estrus behavior and expression (Santos et al. 2008).

Ruminants fed with protected PUFAs were reported to show increased concentrations of P_4 during the luteal phase of estrous cycle (Walsh et al. 2011). The role of P_4 in early maternal pregnancy or the luteal phase of the estrous cycle has attracted much attention. Diskin and Morris (2008) reported that animals with an early rise in the level of P_4 between Days 4 and 7 after artificial insemination had greater chances of maintaining pregnancy in comparison with animals which had slower increase in P_4 concentrations.

When compared to a control group of cows, the cumulative P_4 concentrations were higher during the luteal phase of the estrous cycle before AI in cows fed with calcium salts of LCFA (Sklan et al. 1991). Similar results were reported for increased P_4 concentrations in cows given supplemental fat (Carroll et al. 1990; Son et al. 1996). Garcia-Bojalil et al. (1998) reported an increase in the number and the diameter of CL in cows fed with calcium salts of LCFA than in cows that did not receive the fat supplement. Peak plasma P_4 concentration was 7.9 ng/mL in cows fed 15.7% degradable protein diet (DM) supplemented with 2.2% calcium salts of LCFA against the cows that had 3.9 ng/mL fed with no fat supplement (Garcia-Bojalil et al. 1998).

Steroidogenesis in the ovarian tissues is facilitated by the delivery of cholesterol by both high density and low density lipoproteins (Grummer and Carroll 1991). As compared to the control group of cows, soybean oil (5.4% of diet DM) supplemented beef cows had higher levels of total cholesterol and HDL cholesterol in their serum and follicular fluid, respectively (Ryan et al. 1992). Several studies indicated that the supplementation of fat to dairy cows increased the cholesterol content in plasma, follicular fluid and the CL (Hawkins et al. 1995; Staples et al. 1998). In contrast,

increased P_4 in plasma may not be due to the increased uptake of cholesterol and synthesis by luteal cells but rather due to the reduction in P_4 disappearance from the plasma (Hawkins et al. 1995).

Linoleic acid and ALA can be converted in the liver to their longer chain PUFA relatives by desaturation and elongation enzymes (Jenkins 1993). The long chain PUFAs are transported and stored in the uterine endometrium for the synthesis of $PGF_{2\alpha}$ (Abayasekara and Wathes 1999). Fatty acid desaturase 2 (FADS2) is the rate limiting enzyme in animal tissues (Wathes et al. 2007). Higher tissue concentrations of long chain PUFAs can be achieved by bypassing the rate limiting enzyme step. For example, EPA and DHA may be supplied directly from fish oils and marine microalgae (Robinson et al. 2002b, Ruxton 2004). Eicosapentaenoic acid and DHA are the most potent cyclooxygenase -2 (COX-2) inhibitors (Ringbom et al. 2001). The EPA & DHA alter prostaglandin biosynthesis in a number of cells and tissues in vitro (Weber and Sellmayer. 1991). *In vitro* cell culture studies demonstrated a decreased synthesis of prostaglandins when n-3 or n-6 PUFAs are fed (Achard et al. 1997; Mattos et al. 2004). Mattos et al. (2002) reported a decrease in uterine prostaglandin secretions stimulated by an oxytocin injection in lactating dairy cows fed fish meal. Mattos et al. (2003) showed that EPA and DHA were potent inhibitors of $PGF_{2\alpha}$ production.

Arachidonic acid is present in membrane phospholipids (uterine endometrium) and acts as the primary precursor of $PGF_{2\alpha}$ (Staples et al. 1998). A rate limiting enzyme, cyclooxygenase-2 (COX-2), oxidizes AA to PGH_2. Prostaglandin H_2 is the precursor for PGs, including $PGF_{2\alpha}$ and PGE_2, secreted by the bovine endometrium during the estrous cycle (Danet-Desnoyers et al. 1995). Expression of COX-2 mRNA and protein were

found to be low on days 1 to 12 and high on days 13 to 21 of the estrous cycle (Arosh et al. 2002). Polyunsaturated fatty acids, EPA and DHA, may prevent synthesis of uterine $PGF2_\alpha$ by competing with AA for binding with COX-2 (Mattos et al. 2002). Fish meal as a source of PUFAs in the diet alters the uterine $PGF2_\alpha$ synthesis. Inhibiting uterine secretion of $PGF2_\alpha$ by feeding EPA and DHA may delay regression of the CL and increase fertility by improving embryo survival (Thatcher et al. 2006; Santos et al. 2008; Sakaguchi 2011).

2.5 Feeding System and Reproductive Performance

Housing management and the feeding system may play a role in the reproductive performance of post-parturient lactating cows. Pasture grazing is widely practiced for managing lactating dairy cows around the world. Reproductive behavior is significantly affected by factors such as nutrition, environment, herd size and condition of feet and legs in dairy cows. Cows managed on pasture have the advantage of an environment which is more similar to natural conditions compared to cows confined in tie stalls. Cows managed on pasture may continue to maintain superior foot and leg health, and thus can exhibit uninhibited estrous activity. A previous study monitored the behavioral events associated with estrus by using a radiotelemetric system for continuous 24 h surveillance. They found that 70% of behavioral events such as mounting were noticed between 1900 and 0700 hours when cows were not distracted by the barn activities such as milking and feeding (Dransfield et al. 1998). In contrast, cows managed on pasture are more likely to show mounting activities compared to confined cows. However, if the cow density within the same paddock is too low, the pasture managed cows may not show mounting activity and hence estrus is less likely to be detected.

41

Cow density has a major role in detecting estrus irrespective of the management system. Researchers studied the occurrence of standing events per estrus under the conditions of drylot and free stall housing systems. They reported that fewer standing events per estrus were due in large part to fewer cows rather than feeding system itself (Dransfield et al. 1998). The density per unit area and number of cows housed in an experimental condition may affect estrus behavior and conception. However, the potential reason for altered estrus behavior may be less contact time between farm staff and cows that might lead to poor estrus detection (Dransfield et al. 1998). More research is needed to accurately define the role of feeding system in improving reproducing ability in dairy cattle.

A previous study reported that cows on pasture had a significantly greater peak plasma P_4 concentration than that of cows managed in a free-stall barn (7.0 vs 5.0 ng/mL) during the first estrous cycle after calving. However, this trend was not observed in subsequent estrous cycles. Feeding soybean oil refined byproduct consisting mainly of sodium salts of long-chain fatty acids to pasture cows resulted in increased accumulation of plasma P_4 after 14 weeks postpartum. These cows returned to first estrus earlier after calving and improved pregnancy rates of 62.5 % were achieved (Boken et al. 2005). The conception rate to first service was greater in pasture fed cows compared to those fed total mixed ration (TMR; Bilby et al. 1998). No research has been conducted on the interaction between feeding system and n-3 PUFA supplementation on ovarian function in the ovulation synchronization regime.

Previous studies of feeding fish oil to lactating dairy cows show inconsistent effects on P_4 production, as well as follicular and luteal development (Petit and

Twagiramungu 2006; Moussavi et al. 2007). Plasma P_4 concentrations did not differ among animals fed a control, menhaden fish meal or calcium salts of fish oil fatty acids during the luteal phase. Mean concentrations of P_4 on Days 7 to 24 postpartum did not differ among diets (Mattos et al. 2002, Moussavi et al. 2007). In contrast, Hightshoe et al. (1991) reported an increased blood cholesterol and plasma P_4 during the luteal phase when cows fed with Ca-LCFA from palm oil. Similarly, when Angus and Hereford cows fed with supplement containing 125 g of Ca-LCFA (Espinoza et al. 1995) and in other studies cows fed with Ca-LCFA from palm oil or beef tallow had greater concentrations of blood cholesterol (Marin-Aguilar et al. 2007). There was no significant effect of n-3 PUFAs on the mean numbers and size of developing follicles (Petit et al. 2002). In contrast, Bilby et al. (2006a) found that the number and size of the developing follicles increased in cows fed PUFA enriched diets.

In summary, previous observations indicate that supplemental dietary fatty acids can provide a concentrated energy source, and if protected from the ruminal biohydrogenation, can deliver specific PUFAs to the intestines for absorption. Previous studies report beneficial effects of EPA and DHA when absorbed in increased amounts into the blood that may favor reproductive success. Fish meal or oil has relatively large concentrations of EPA and DHA. Several studies have evaluated the utilization of fish meal or oil as a dairy nutrient to alter the follicular and luteal dynamics in dairy cows during the Ovsynch regime. In addition, supplemental rumen protected fish oil (RPFO) feeding may modulate the endocrine function, prostaglandin cascade and luteal function during the synchronized estrous programs. However, the effects have been inconsistent and more research is needed to accurately characterize the role of EPA and DHA from

43

RPFO. Rumen protected marine algae (RPMA) are the potential alternate source of EPA and DHA which may be utilized to enhance the reproductive performance in dairy cows. No study has been conducted to determine the effect of RPMA on ovarian function in dairy cows during a ovulation synchronization regime. The feeding system also has a major role in enhancing the reproductive performance in dairy cows. There has been a rejuvenated interest in pasture grazing systems worldwide. Cows managed on pasture have the benefit of natural environment compared to cows confined in tie stalls. Previous studies conducted at two locations, New Zealand and United States, reported that conception rates were 2 times higher in New Zealand where rotational stocking is the standard management system for dairy production compared to United States where cows were fed a TMR in confinement. Grazing cows had lower plasma P_4 clearance compared to TMR fed cows (Bilby et al. 1998). However, no research has been conducted on the interaction between feeding system and n-3 PUFA supplementation on ovarian function during the Ovsynch regime. This dissertation aims to elucidate the mechanisms by which n-3 PUFA supplements can improve the ovarian function in dairy cows managed either in pasture or confinement with particular focus on follicular and luteal dynamics and P_4 production during the Ovsynch period.

CHAPTER 3

OBJECTIVES

Feeding n-3 PUFA supplements, high in EPA and DHA have been shown to increase the reproductive performance of dairy cows. Rumen protected fish oil and RPMA rich in EPA and DHA may be used as a management tool to improve the reproductive performance in lactating dairy cows. The overall goal of this research is to evaluate the effects of two rumen-protected n-3 PUFA (RPFO and RPMA) supplements on ovarian function of post-parturient lactating dairy cows managed on a pasture or confinement feeding system.

The ovarian function was evaluated by studying two specific objectives as follows:

1) Ovarian follicular and luteal dynamics in lactating dairy cows managed either in pasture or confinement during conventional estrus and ovulation synchronization treatments.

2) Hormonal profiles of P_4 in lactating dairy cows managed either in pasture or confinement during conventional estrus and ovulation synchronization treatments.

CHAPTER 4

OVARIAN DYNAMICS AND PROGESTERONE PROFILES IN LACTATING DAIRY COWS SUPPLEMENTED WITH OMEGA-3 FATTY ACIDS DURING CONVENTIONAL ESTRUS AND OVSYNCH PERIOD

4.1 ABSTRACT

Dietary lipid supplements, high in content of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), feeding system and ovulation synchronization protocols improve reproductive performance in dairy cows. Pasture grazing has been employed historically in many countries around the world, though cows are now typically confined and fed a total mixed ration (TMR). However, no studies have been conducted to determine the effect of interaction between the lipid supplementation and feeding system on the ovarian function of dairy cows. The objective of this research was to evaluate the interaction between two rumen-protected n-3 PUFA supplements on ovarian function of lactating dairy cows in pasture or confinement during conventional estrus and ovulation synchronization period. Eighteen Holstein cows were assigned to two feeding systems: either grazing pasture (n = 18), or in confinement (n = 18). Cows within each feeding system were blocked by parity and expected date of calving. Cows were assigned randomly within a block to one of three isolipidic dietary treatments, fed from 30 d before to 100 d after calving. Six cows were assigned to the control, 6 to the rumen-protected marine algae (RPMA) and 6 to the rumen-protected fish oil (RPFO) supplements in the pasture or confinement, respectively. Cows were synchronized for estrus and ovulation during Days 35-68 post-partum. The resumption of cyclicity and onset of estrus were not influenced by LS (P > 0.05). Cows fed RPFO had larger follicles (≥ 10 mm) compared to the control during the period between 1st GnRH and 2nd GnRH injections (17.3 ± 1.0 mm and 13.1 ± 1.0 mm, respectively; P < 0.05). However, the RPMA did not differ from the RPFO or the control (P > 0.05). Ovulation was delayed in cows fed RPMA (1.4 ± 0.1 d) and RPFO (1.4 ± 0.1 d) compared to control (1.1 ± 0.1 d) after the 2nd GnRH injection (P < 0.05). The number of CL, in RPMA group was greater than that in the control at the 3rd $PGF_{2\alpha}$ (2.2 ± 0.3 vs. 0.8 ± 0.2) and 2nd GnRH injection (2.0 ± 0.3 vs. 0.7 ± 0.2; P < 0.05). However, RPFO fed cows had a similar number of CL as the cows fed RPMA (P > 0.05). Cows fed RPMA had larger CL compared to control during the Ovsynch period (25.0 ± 1.5 vs. 19.3 ± 1.8 mm; P < 0.05). However, cows fed RPFO did not differ from RPMA or the control (P > 0.05). During the period between 1st GnRH to the 2nd GnRH injections, cows fed RPMA had greater (P < 0.05) concentrations of plasma P_4 compared to cows fed the control diet (2.52 ± 0.28 ng/mL and 1.15 ± 0.29 ng/mL, respectively). However, cows fed RPFO did not significantly differ from cows fed the control or RPMA. These results indicate that RPFO and RPMA supplementation can improve ovarian function in lactating dairy cows managed either in pasture or confinement during conventional estrus and Ovsynch period.

4.2 INTRODUCTION

Dairy cows are fed with 3 - 5 % fat (diet DM) in order to increase the energy concentration and to improve animal performance. However, in recent years, dietary fats rich in n-3 PUFAs have been utilized in attempts to enhance reproductive performance in dairy cows (Thatcher et al. 2006; Juchem et al. 2010; Caldari-Torres et al. 2011). Several potential mechanisms have been proposed by which supplemental fats alter ovarian function (Mattos et al. 2000; Santos et al. 2008). These include: 1) influencing early postpartum ovarian cyclicity (de Veth et al. 2009); 2) influencing follicular dynamics and ovulation (Chagas et al. 2007); 3) increasing the number and diameter of the CL (Garcia-Bojalil et al. 1998); 4) increasing the life span of the CL; and 5) increasing the plasma P_4 concentrations during the luteal phase (Santos et al. 2008). The mechanism and extent of improving reproductive performance likely depend on the fatty acid composition of the supplement and the extent of the dietary PUFAs supply to the blood. Therefore, it is critical to increase the escape of these fatty acids from ruminal biohydrogenation to improve their absorption in the small intestine.

Time to post-partum resumption of ovarian activity was reduced by 7 days in Holstein cows supplemented with 500 g/d of vegetable oil (60 % PUFAs) compared to a control group (Maria-Aguilar et al. 2007). Time to first ovulation post-partum was reduced by 8 days when dairy cows were supplemented with 8 g/d of dietary PUFAs (trans-10, cis-12 CLA; de Veth et al. 2009). These observations demonstrate that PUFA supplementation may influence the resumption of postpartum ovarian cyclicity. Recent studies reported no difference in the proportion of cycling cows at 65 days after calving when 699 dairy cows were fed 400 g of fatty acids from either tallow or palm and fish oil

47

containing Ca-LCPUFA. Subsequently, feeding Ca-LCPUFA of palm oil or a blend of C18:2 n-6 and trans-octadecenoic FA, from 25 days before to 80 days after parturition, resulted in similar mean interval to first postpartum ovulation (31.3 days; Juchem et al. 2010).

Dairy cows fed diets enriched with PUFAs had larger dominant follicles compared with the cows fed a MUFA (Bilby et al. 2006b), suggesting that it was PUFAs that were most effective. The number of 5 to 10 mm size follicles was higher in dairy cows that were fed a diet enriched with 5% n-3 PUFAs derived from fish oil (Moussavi et al. 2007). Robinson et al. (2002) observed similar results when diets containing linolenic (n-3) and linoleic (n-6) acid protected PUFAs were fed to dairy cows. Staples and Thatcher (2005) summarized the studies on the effect of supplemental fat on the diameter of the dominant follicle in dairy cows. These studies concluded that there was a 23% increase (3.2 mm larger) in the size of the dominant follicle when dairy cows were supplemented with lipid containing the protected PUFAs in the ration. Other studies showed that the size of the dominant follicle increased in cows that consumed a diet rich in LCFAs from soybean oil (Garnsworthy et al. 2008). However, it appears that animal tallow or fish oil have been shown to have less clear effects on growth dynamics of follicles than Ca-LCFAs derived from palm oil (Lucy et al. 1993b; Moussavi et al. 2007; Santos et al. 2008).

Dairy cows supplemented with fat generally yielded larger dominant follicles. Subsequently, CL developed out of that follicle after ovulation may also be larger. Previous studies reported that larger dominant follicles in lactating and nonlactating dairy cows and in heifers resulted in larger CL (Sartori et al. 2002). Weekly examination by

rectal palpation of the ovaries revealed that the number of CL (0.85 vs. 1.05) and the diameter of the largest CL (12.2 vs. 17.2 mm) tended to be higher in lactating dairy cows fed either 0 or 2.2% Ca-LCFA beginning from parturition to Day 60 postpartum (Garcia-Bojalil et al. 1998). However, the larger CL may not be due to a larger ovulatory follicle but rather, may be due to a direct steroidogenic and developmental effect on the CL.

Early embryonic loss is one of the major causes for reduced fertility in dairy cows (Thatcher et al. 2006). During early gestation, 25 - 55% of embryos die due to inadequate functioning of various mechanisms which influence the establishment and maintenance of pregnancy. Much of the early embryonic mortality may be due to the insufficient functioning of luteal cells (Niswender et al. 1994). Progesterone secretion is the main function of luteal cells in the CL. Progesterone helps the uterus prepare for the implantation of the conceptus and maintain pregnancy by providing a nourishing environment. Improved conception rates in dairy cows have been associated with increased plasma P_4 concentrations. Likewise, improved fertility has been associated with increased plasma P_4 concentration before and after artificial insemination (Santos et al. 2008). Cholesterol is the main precursor for the synthesis of P_4. Previous studies indicated that the supplementation of fat to dairy cows has consistently increased the cholesterol content in plasma, follicular fluid and CL (Hawkins et al. 1995). Garcia-Bojalil et al. (1998) indicated that cows fed diets of 2.2% calcium salts of palm oil accumulated more P_4 from 0 to 50 DIM, and had improved pregnancy rates with no change in the energy status as compared to the non-fat supplemented cows. Therefore, where fat supplementation increases P_4 secretion, fertility may be enhanced.

Pasture grazing is widely practiced for managing lactating dairy cows around the world. Pasture grazing has been employed historically in many areas; increasingly though, cows are being confined year round and fed a silage and grain mixture. Boken et al. (2005) reported that cows on pasture had a greater peak plasma P_4 concentration than cows managed in a free-stall barn (7.0 vs 5.0 ng/mL $P \leq 0.05$) during the first estrous cycle after calving. However, this trend was not observed in subsequent estrous cycles. Previous studies conducted at two locations, New Zealand and the United States, reported that conception rates were 2 times higher in New Zealand where rotational stocking is the standard management system for dairy production, compared to the United States where cows were fed a TMR in confinement. Grazing cows had lower plasma P_4 clearance compared to TMR fed cows (Bilby et al. 1998).

Fish meal or oil has relatively large concentrations of EPA and DHA, which may prevent synthesis of uterine $PGF_{2\alpha}$ by competing with AA for binding with COX-2 (Mattos et al. 2002). Inhibiting uterine secretion of $PGF_{2\alpha}$ by feeding EPA and DHA may delay regression of the CL and increase fertility by improving embryo survival (Thatcher et al. 2006). Several studies have examined the utilization of fish oil and meal to enhance the reproductive performance of dairy cows (Burke et al. 1997; Moussavi et al. 2007; Juchem et al. 2010).

Previous studies of feeding fish meal or fish oil to lactating dairy cows showed inconsistent effects on ovarian dynamics and P_4 production. There was no significant effect of n-3 PUFA supplementation on the mean number and the size of developing follicles (Petit et al. 2002). Moussavi and coworkers (2007) fed dairy cows with diets that were isolipidic and isoenergetic, containing none (control), 1.25, 2.5, or 5% menhaden

50

fish meal or 2.3% calcium salts of fish oil fatty acids. They found that the size of the non-ovulatory dominant follicle on Day 10 and 14, maximum diameter attained by the non-ovulatory dominant follicle and the number of days until detection of a large follicle ($\geq$10 mm) were the same among treatments. In contrast, Bilby et al. (2006a) found that the number and size of developing follicles increased in cows fed with diets enriched in PUFAs of plant oil origin. Plasma P_4 concentrations did not differ among animals fed control, menhaden fish meal or calcium salts of fish oil fatty acids during the luteal phase (Moussavi et al. 2007; Childs et al. 2008). However, several studies reported increased P_4 concentrations during the luteal phase and improved fertility in dairy cows fed fish meal during early postpartum (Burke et al. 1997; Garcia-Bojalil et al. 1998).

Due to their relatively high concentrations of EPA and DHA, fish meal or oil may alter the uterine $PGF_{2\alpha}$ secretion (Mattos et al. 2000). Inhibiting the uterine endometrial secretion of $PGF_{2\alpha}$ may prolong the life span of CL, leading to prolonged secretion of P_4 (Staples et al. 1998). Marine algae (MA) are the potential alternate source of EPA and DHA which may be utilized to improve the reproductive performance of dairy cows. However, no studies have been conducted to determine the effect of marine algae on the ovarian function in postpartum lactating dairy cows during estrus and ovulation synchronization period.

The objective of the current study was to evaluate the effect of sources of rumen-protected n-3 PUFAs (RPFO and RPMA) on ovarian function in lactating dairy cows managed in pasture or confinement feeding systems during conventional estrus and ovulation synchronization periods.

51

4.3 MATERIALS AND METHODS

4.3.1 Experimental Design, Animals, Feeding and Housing

The current study was a part of an extended nutritional trial. Only reproductive parameters were evaluated in this experiment. Initially, 30 Holstein cows/heifers (*Bos taurus*) from the Nova Scotia Agricultural College farm were used for the study. These 30 cows/heifers were paired by parity, due date and stage of lactation and allocated randomly within pair to one of 2 system treatments and 2 dietary treatments nested within system. Cows/heifers were synchronized and bred during a 6 week period using synthetic $PGF_{2\alpha}$ and GnRH. Animals not in-calf by the end of the period were returned to the herd. At least 26 cows/heifers were in-calf with their due dates between April 1st and May 31st 2008 for the pasture group. This included the 24 animals required for the trial plus two extra in the event of problems at parturition (eg. dystocia, pyometra or retention of fetal membranes) that would prevent the animal from being included in the trial. Extra cattle were returned to the dairy herd. Similarly, at least 26 cows/heifers were in-calf with their due dates between October 26th and December 6th 2008 for the confinement group. Extra cattle were returned to the dairy herd.

The pasture and confinement groups of 24 pre-partum Holstein cows/heifers were blocked by parity and predicted calving date and assigned to either a pasture or a confinement management system. The parity and calving date were recorded for each cow/heifers before the start of the feeding trial. The parity of cows/heifers ranged from 0 to 5 within each feeding systems. Cows/heifers were scored visually for body condition with 1 being emaciated and 5 being obese (Ruegg and Milton 1995). Body condition score (BCS) was assessed by two trained staff. The average body weight (BW), BCS,

milk yield and fat % were recorded for each cow/heifer 31 d prior to the start of the feeding trial. The average BW and BCS of the pasture group were: 747.17 ± 11.04 kg and 3.30 ± 0.06, respectively, while the confined group averaged 711.0 ± 7.22 kg and 3.13 ± 0.10, respectively. Within each feeding system, animals were paired on the basis of parity and calving date and assigned randomly to one of the two isolipidic dietary treatments or control from 30 days before calving to 100 days post calving. The pasture (with concentrate) and TMR experimental diets were formulated to be isoenergetic, isonitrogenous, isolipidic and to meet targeted nutritional requirements of each group. Different diets were formulated for pre-calving as well as early and late lactation. The daily TMR intake of confined animals was determined by recording the amount of feed offered and refused.

The pasture experiment was conducted from May to August 2008. All cows/heifers on pasture calved between April 3rd and May 27th 2008. A rotational management grazing scheme was used for the group. The pasture was a mixed perennial sward pasture. Grazing started 30 ± 5 d in milk (DIM). The sward pasture was composed of approximately 70-80% blue grass (*Poa pratensis*), 20-30% timothy, meadow fescue (*Festuca pratensis*), couch grass (*cynodon dactylon*), white clover (*Trifolium repens*), red clover (*Trifolium pratense*) and alfalfa (*Medicago sativa*). The average BW and BCS of the pasture group during the experimental period were 632.50 ± 5.21 kg and 2.59 ± 0.02, respectively, while the average milk yield and fat % were 34.23 ± 0.69 kg/d and 3.27 ± 0.14 %, respectively. The pasture group (24 Holstein cows/heifers) was synchronized for estrus and ovulation during 33-68 days post-partum. In total, 8 cows were assigned to the control (no supplement), 8 to the RPFO and 8 to the RPMA. Supplements were started 30

d prepartum. The amount of supplement fed was similar throughout the trial. The RPFO and RPMA group was supplemented with 300 g of each lipid supplement (RPFO and RPMA) per day. The RPFO supplement provided approximately 25 g/d each of EPA and DHA, whereas the RPMA supplement provided approximately 25 g/d of DHA. Pasture group cows were brought to tie stalls for milking twice daily (0500h and 1600h) and one of the test n-3 PUFA supplements was fed along with 1.0 kg of TMR just after morning milking (0500h) and just before afternoon milking (1600h). The control group was fed with 1.0 kg of TMR with no supplements. Cows were fed with concentrate at a rate of 0.25 times the milk yield (as fed basis) to a maximum of 8 kg/d.

The confinement experiment was conducted from November 2008 to February 2009. All confined cows/heifers calved between October 9th and December 23rd 2008. The confinement group (n = 24) received the same dietary lipid supplements as the pasture group. The average BW and BCS of the confined group during the experimental period was 667.69 ± 7.15 kg and 2.73 ± 0.03, respectively. The average milk yield and fat % were 38.71 ± 0.96 kg/d and 2.73 ± 0.03 %, respectively. The confinement group was maintained on a TMR-based feeding system, housed in tie stalls and fed twice daily (0800h and 1700h) at *ad libitum* intake. The daily TMR intake of each cow was measured by recording the amount of feed offered and refused. Confined cows were milked twice daily and fed one of the treatment supplements, along with 1.0 kg of TMR just after morning milking (0500h) and just before afternoon milking (1600h). Only 18 paired animals were selected based on the parity, due date and stage of lactation in each feeding system. All experimental animals had free access to water during the experimental period. Health checks of all experimental cows were performed daily by trained staff.

Experiments were conducted in accordance with the recommendations and guidelines of the Canadian Council on Animal Care (CCAC, 1993) and approved by the NSAC Animal Care and Use Committee.

4.3.2 Analysis of Feed

Total mixed ration and pasture were pooled by period and subsampled for compositional analysis. Compositional and ingredient analyses for TMR, pasture and concentrate mix were performed at the forage laboratory, Dairy One, Ithaca, New York, USA. Briefly, the dry matter of feed sample was determined by placing it in a drying oven at 100 °C for 48 hours (AOAC, 1990, Method 930.15). Crude protein was determined using the Dumas Method and a Leco FP-528 (LECO Corporation, St. Joseph's, MI, USA). Acid detergent fiber and NDF concentrations were determined (ANKOM Technology. Macedon, NY, USA). Minerals (% calcium, % phosphorus, % potassium, % magnesium, % sodium, ppm manganese, ppm iron, ppm copper, ppm zinc) were determined using AOAC (1990; method 968.08) after dry ashing.

4.3.3 Synchronization of Estrus and Ovulation

Ovulation of experimental dairy cows was controlled by estrus synchronization. Ovulation synchronization of cows was achieved as explained diagrammatically in Figure 4.1, using the Presynch and Ovsynch protocols recommended by Pursley et al. (1995) and Moore and Thatcher (2006). Briefly, at day 35 postpartum, presynchronization was achieved by two injections of $PGF_{2\alpha}$, dinoprost tromethamine (5 mL i.m., Lutalyse[®], Pfizer Animal Health, Pfizer Canada Inc., Kirkland QC, Canada) 11 days apart to synchronize estrus. Cows were visually observed for signs of estrus for 4 days, to confirm cyclicity, following each $PGF_{2\alpha}$ injection.
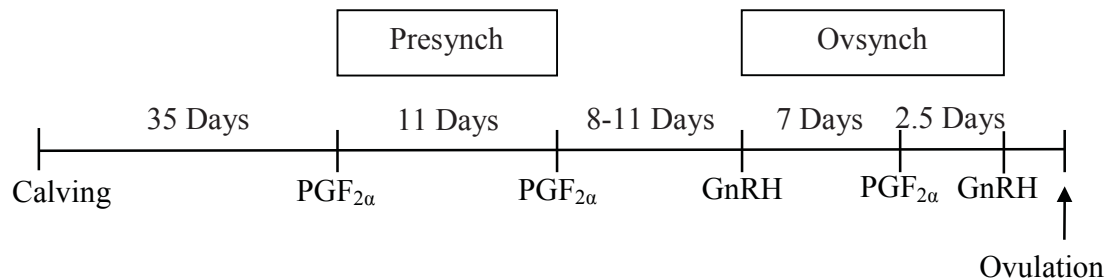
Figure 4.1 Ovulation presynchronization (Presynch) and synchronization (Ovsynch) protocols. Source: Pursley et al. (1995) and Moore and Thatcher (2006).

Synthetic gonadotropin releasing hormone (GnRH, 2 mL i.m., Factrel®, Pfizer Animal Health, Pfizer Canada Inc., Kirkland QC, Canada) was injected 8-11 days after the last presynchronization application of $PGF_{2\alpha}$ to facilitate the Ovsynch protocol. Ovulation synchronization was brought about by a third application of $PGF_{2\alpha}$ (Lutalyze®; 5 mL), injected 7 days after the 1st GnRH injection. The second GnRH (Factrel®; 2 mL) was injected 2.5 days after the third $PGF_{2\alpha}$ injection to ensure ovulation in all lactating cows. Animals were physically observed for signs of heat and behavioral changes during the whole experimental period.

4.3.4 Milk Sampling and Analysis

All experimental cows were milked twice daily at 0500h and 1600h. Milk yield was recorded daily during the trial by calibrated milk meters. Samples of milk from each cow were taken on Days 7, 30, 60 and 90 after calving. Only Day 60 and 90 milk samples were used for compositional analysis. Milk from 4 consecutive milkings from each cow was pooled and sampled as follows: two samples were preserved for compositional analysis and two samples were flushed with nitrogen for fatty acid analysis. All samples

were stored at $-80^{\circ}C$ until analyses. Compositional analysis for fat, protein and lactose content was performed at the Milk Testing Laboratory, Department of Agriculture, Fisheries and Aquaculture, Prince Edward Island, using a Foss electric Milkoscan 4000 (Foss, Brampton, ON, Canada). The Milkoscan 4000 uses infrared technology in order to analyze the components of milk.

4.3.5 Blood Sampling and Processing

Daily afternoon blood sampling started on Day 33 after calving. Blood samples (10 mL) were collected, by coccygeal venipuncture into anticoagulated (Ethylenediamine tetraacetic acid) vacutainer® tubes, prior to the feeding and were kept on ice. Sampling ended 12 d (Day 80) after ovulation was detected after the third $PGF_{2\alpha}$ injection. All blood samples were centrifuged for 20 minutes at 1000 x g at $10^{\circ}C$ and plasma was harvested and transferred into 2 mL micro centrifuge tubes and kept at $-20^{\circ}C$ until assayed for P_4.

4.3.6 Analysis of Fatty Acids

The total lipid content was extracted from 1 mL of milk in 12 mL 2:1 chloroform:methanol solution, using a modified Folch et al. (1957) method (Budge et al. 2006). Methylation of extracted lipid was facilitated by using 0.5 N sulfuric acid and later heated to 110 °C for one hour. Methylated plasma fatty acids were separated using a gas chromatograph with a flame-ionization detector (CP-3800, Varian Inc., Palo Alto, CA, USA) on an Omegawax 250 fused silica capillary column. The oven temperatures were set at: 153 °C for three minutes, increasing by 2.3 °C/minute to 174 °C, which was held for 30 seconds, and continued to increase by 2.5 °C to reach 210 °C, which was later held for 15 minutes. The temperatures of injectors and detectors were 250 °C and 260 °C,

respectively. A 37-component standard (Supelco, Bellefonte, PA, USA) and an omega-3 enriched fish oil sample were used to compare and to identify the fatty acid present in the sample (Budge et al. 2006).

4.3.7 Ultrasound Technique

Daily transrectal ovarian ultrasonography started on Day 33 after calving. Ultrasonographic scanning was conducted with a 7.5-MHz linear array transducer connected to a B-mode, high resolution real-time echo camera (Aloka SSD-500, Aloka Co., Ltd., Tokyo, Japan). This technique has been validated for monitoring ovarian follicular and luteal dynamics in cows (Ginther 1993; Fricke 2002; Adams et al. 2008). To ensure constant ovarian image quality, all images were viewed at magnification 1.5 x with constant gain settings. Ovarian images were recorded on high grade Digital Video Disc (DVD) for retrospective analysis of ovarian data in the laboratory. The number, diameter and relative position of all follicles and corpora lutea were sketched on ovarian charts. To determine the exact timing of ovulation, twice daily ultrasonography was conducted after the GnRH injection (about 2.5 days). Ovarian ultrasonography ended when ovulation was detected after the 3rd PGF$_{2\alpha}$ treatment.

4.3.8 Follicular Data Analysis

The recorded ovarian images on DVD were captured using Sonic Cine Player software (Version 3.2., 2004, Sonic Solutions., Roxio). This software is optimized for ultrasonographic image processing on a computer graphics work station. Follicles appear as hypoechogenic (dark) areas on the ovarian stroma. The shape of the follicles varies from circular to oval or sometimes misshapen. The maximum cross sectional diameter of

follicles was measured by optimized Image-Pro Express software (Image Pro® Version 4.5. Media Cybernetics, Inc). The number and size (maximum cross sectional diameter) of follicles (≥10 mm) were determined for each cow on a daily basis during the experimental period (Ginther 1992; Fricke 2002). To determine the exact time of ovulation, twice daily ovarian ultrasonography was conducted after each injection and follicular number and diameter (≥10 mm) were also determined during this time.

Ovulation was detected with ultrasonography as the sudden disappearance of a large antral follicle (≥ 8 mm) and later confirmed with ultrasonographic detection of corpora hemorrhagicum or CL (Ginther 1993). The number and timing of ovulations in each animal were determined from the follicular data. Duration of estrus was measured as an interval between first and last estrus signs (mounting, chin resting, vaginal mucous discharge, frequent bellowing and swollen vulva) observed. The presence of cystic ovarian follicles was evident in this study. The cystic ovarian follicles may be defined as follicles with a diameter of at least 20 mm that are present on one or both ovaries in the absence of functional luteal tissue (Vanholder et al. 2006). The cystic follicles were included in the follicular data analysis. Ovarian data was combined for both ovaries of each animal (Ginther 1993).

4.3.9 Luteal Data Analysis

Ultrasound images of the CL were analyzed using the same technique as for the follicle analysis. The CL resembles a solid tissue mass within the ovarian stroma and sometimes it contains a fluid-filled cavity (Singh et al. 1997; Fricke 2002). Ultrasonographic attributes of CL, like its cross-sectional diameter, luteal area and echogenicity, have already been correlated with CL function and structure (Singh et al.

2003). The total number and maximum cross-sectional diameter of corpora lutea (CLs) per cow during the trial were determined (Singh et al. 2003; Fricke 2002). The following equation was used to calculate the volume of CL:

$$= \frac{4}{3}$$

where r (radius) was calculated by using the following formula:

$$radius = \frac{\frac{L}{2} + \frac{W}{2}}{2}$$

where L and W were the measured length and width of the CL. In the case of the CL with a fluid-filled cavity, the volume of the cavity was first calculated and then subtracted from the total volume of the CL (Bilby et al. 2006c). Part of the follicular and luteal data from the confinement group were not analyzed and presented due to loss of primary data during the feeding trial.

4.3.10 Progesterone Hormone Assays

Progesterone concentrations were measured by a validated solid phase enzyme immunoassay (EIA) procedure (Munro and Stabenfeldt 1984; Del-Vechhio et al. 1995; Walker et al. 2008). The EIA technique utilized an antibody (anti P_4 polyclonal antibody R4859; supplied by CJ Munro, University of California, Davis, CA), horseradish peroxidase enzyme labeled conjugate (progesterone; prepared according to Munro and Stabenfeldt 1984) and standards (synthetic progesterone; P0130, Sigma-Aldrich, UK). The modified assay procedures were as follows: 1) The antiserum was diluted at 1:6000 for progesterone; 2) The horseradish peroxidase conjugate was used at a dilution of 1:60000; 3) Standards (n = 10) were used in the range of 0.03125 to 16 ng/mL. The P_4 antiserum cross reacted with several P_4 metabolites, including: 5α-Pregnan-3β-ol-20-one

94%; 5β-Pregnan-3β-ol-20-one 12.5%; 4-pregnen-3β-ol-20-one 172%; 20-dione (progesterone) 100%; 5α-Pregnan-3,20-dione 55%; 4-pregnen-3,4-pregnen-3α-ol-20-one 188%; 4-pregnen-11α-ol-3,20-dione 147%; 5α-Pregnan-3α-ol-20-one 64% and ≤ 10% for all other metabolites of P_4 tested previously (Graham et al. 2001).

Progesterone in samples of plasma was extracted with 2.5 mL of petroleum ether in a procedure that involved a freezing step. The tubes were vortexed 3 times for 30 seconds with a vortexer. Then, samples were allowed to sit for a minimum of 60 minutes before the extraction was continued. The process of vortexing the samples for 30 seconds for 3 times was repeated. Near the end of the 60 minute incubation period, a dry ice/ethanol bath was prepared by pouring the ethanol solution into a container and adding 2-3 cubes of dry ice. The tubes were placed in the dry ice/ethanol solution until the plasma was frozen, and then the top layer was poured into the second set of labeled 12 x 75 mm borosilicate disposable tubes. The tubes were placed in the heater block and taken to dryness under nitrogen gas, using the analytical evaporator (Organomation Associates Inc., Berlin, MA). Dryness occurred typically after approximately 8 - 9 minutes. The dried test tubes were reconstituted in 250 μL of EIA buffer and vortexed for 30 seconds. Then, the samples were stored at 4 °C overnight, to allow complete reconstitution of the P_4 into the buffer.

The 96-well flat-bottomed polysterene microtitre plates (NUNC Maxisorb, Kamstrupvej, Denmark) were used to perform the assay. Each plate contained two blank wells (with no anti P_4 polyclonal antibody), used to calculate the background absorbance caused by 2, 2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) reagent. Two non-specific binding wells (NSB) were used to calculate the non-immunological binding

of the progesterone-HRP conjugate to the well. Two total wells (containing progesterone-HRP conjugate) were used to determine the total enzymatic activity of the progesterone-HRP conjugate. Two maximum binding wells were used to determine the maximum amount of progesterone-HRP conjugate that the antibody could bind in the absence of free tissue progesterone. A 10-point standard curve was run in triplicate. The plates were coated with 50 µL per well of the anti P_4 polyclonal antibody (R4859) in coating buffer, at a dilution of 1: 6000, except for the blank and NSB. The microtitre plates were sealed tightly with acetate plate sealers and left overnight at 4°C in a styrofoam chamber, to avoid evaporation. After overnight incubation, the plates were emptied by inversion and washed five times (plate washer) with the wash buffer, and tapped gently on paper towels to ensure that all wash buffer droplets were drained out.

The standards were (50 µL) serially double diluted in the range of 0.03125 ng/mL to 16 ng/mL, using EIA buffer. These were pipetted into the respective microtitre plate wells in triplicates, starting from the highest dilution and continuing until all the serially diluted standards were pipetted into their respective wells. Maximum binding wells and NSB wells were added with 50 µL of EIA buffer. The extracted samples (50 µL) were aliquoted into the wells previously coated with anti P_4 polyclonal antibody, in duplicates. This was immediately followed by 50 µL of diluted progesterone-HRP conjugate (1:60000) pipetted into each well, except the blank and total wells, to initiate the competitive binding reaction. Plates were covered tightly with acetate plate sealer after the addition of HRP conjugate and incubated for 2 hours, at room temperature on an orbital shaker. These plates were covered so that development occured in darkness.

After 2 hours of incubation, the plates were washed five times to remove unbound P_4, and blotted dry. Then, 100 μL of substrate solution made up of 40 μL 0.5 M H_2O_2, 125 μL 40 mM ABTS and 12.5 mL citrate buffer (pH 5.0) was added to each well, except the blank. The plates were again covered and incubated at 37 ^{0}C for 25 to 30 mins. The optical density of each well in the microtitre plates was measured when the maximum binding well read between 0.300 and 0.500 units. The results were generated on a Synergy Mx multi-mode microplate reader (Biotek Instruments Inc., Winooski, VT), set at a wavelength of 405 nm. The amount of P_4 in extracts of plasma or standards was calculated by using equations derived from the regression of logit of percent binding on log of concentration. The sensitivity of the assay for plasma was 0.03125 ng/mL. The P_4 concentrations of low and high plasma P_4 internal controls were ranged from 0.86 - 2.75 ng/mL and 3.60 - 6.15 ng/mL, respectively. The intra-assay coefficients of variation were 8.07% and 7.58%, whereas the interassay coefficients of variation were 8.05% and 7.48% for low and high plasma P_4 internal controls (references), respectively.

4.3.11 Statistical Analyses

The follicular and luteal data from the pasture group repeated in time were analyzed using the PROC MIX of SAS (SAS institute Inc. Cary, NC, USA 2003). Univariate analyses were performed on all variables to verify the normality of data and constant variances and to identify the outliers. The pasture group data was analyzed as a randomized block design. The repeated measure ANOVA was used to determine the effect of lipid supplement on the number of follicles and CL and diameter of follicles and CL, days, or an interaction of these factors. MIXED procedure applies methods based on the mixed model with a special parametric structure on the covariance matrices. The data

63

set was tested for the appropriate covariance structure that provided the best fit for available follicular and luteal data. The covariance structures tested included auto-regressive order 1, compound symmetry, and unstructured. The data consisting of parity and date of calving were used as covariates in the analysis. The follicular and luteal data were analyzed by using the following model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \delta_k + \varepsilon_{ijk}$$

in which Y_{ijk} was the response variable (e.g. size of follicle and CL, number of follicles and CL), μ was the overall mean, α was the effect of lipid supplement (1-3), β the effect of time (day, j = 33rd - 68th PP), δ the effect of block (I = 1-2), $\alpha\beta$ was the interaction effect between lipid supplement and day, and ε_{ijk} was the residual error.

Data for follicular and luteal dynamics of the pasture group that were not repeated in time were analyzed by using the following generalized linear model (SAS PROC GLM):

$$Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij},$$

where Y_{ij} = dependent variable (size of follicle and CL, number of follicles and CL etc.), μ = overall mean, α_i = treatment effect (i = 1, 2, 3), β_j = Block effect (j = 1, 2), and ε_{ij} = residual error.

The plasma P_4 concentrations were analyzed using PROC MIX of SAS. The P_4 data of both the pasture and confinement groups were analyzed as a split-plot design. The repeated measure ANOVA was used to determine the effect of days, lipid supplement, feeding system or an interaction of these factors. The MIXED procedure applies methods which are based on a mixed model with a special parametric structure on the covariance matrices. Covariance structures tested included auto-regressive order 1, compound

symmetry and unstructured. The data consisting of parity and date of calving were used as covariates in the experiment. The progesterone data set was analyzed using the following model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \varepsilon_{ijkl}$$

in which Y_{ijklm} was the response variable (e.g. progesterone concentrations), μ was the overall mean, α was the effect of day (i = 33rd - 80th), β the effect of feeding system (j = 1, 2), γ the effect of lipid supplement (k = 1-3), $\alpha\beta$ was the interaction effect between day and feeding system, $\alpha\gamma$ was the interaction effect between day and lipid supplement, $\beta\gamma$ was the interaction effect between feeding system and lipid supplement, $\alpha\beta\gamma$ was the three way interaction between day, feeding system and lipid supplement, and ε_{ijkl} was the residual error.

Data for plasma P_4 that were not repeated in time (single point measurement) and heat data were analyzed by using the following generalized linear model (PROC GLM, SAS):

$$Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij},$$

where Y_{ij} = dependent variable (plasma P_4 concentrations, onset and duration of heat, number of ovulations), μ = overall mean, α_i = feeding system (i = 1, 2), β_j = lipid supplementation (j = 1, 2, 3), and ε_{ij} = residual error. Interaction of main effects was studied as follows: 1) effect of pasture vs. confinement feeding system, 2) effect of lipid supplementation, 3) interaction of feeding system and lipid supplementation. One cow from the pasture feeding system assigned to RPFO treatment was not cycling during the whole experimental period and was subsequently dropped from all luteal and P_4 data analysis. Part of the follicular and luteal data from the confinement group was not

analyzed due to loss of primary data during the feeding trial. Results are reported as least squared means ± SEM. Significance was declared at P ≤ 0.05 and a trend reported at P < 0.10 unless otherwise stated.

4.4 RESULTS

4.4.1 Feed Composition

Four formulations of TMR were fed from before calving until the end of the trial (namely: dry cow, close-up, low lactation and high lactation). The TMR was mixed twice daily to meet the nutritional requirement of the cow at different stages of lactation. The TMR formulation (high lactation) fed that was most relevant to this study is shown in Table 4.1, along with the composition of the concentrate.

The TMR intake was corrected to ensure the cows were consuming the optimal amount of TMR during the feeding trial. The concentrate formulation fed to the pasture cows is shown in Table 4.1. The pasture, silage, concentrate, and TMR analyses are summarized in Tables 4.2, 4.3, 4.4 and 4.5, respectively.

Pasture had lower dry matter content than that of silage in the TMR. Crude protein content was higher in the TMR samples compared to pasture samples but ADF and NDF content were higher in pasture sample compared to TMR. The pasture and silage samples had higher ADF and NDF content compared to TMR. Ether extract and ash content are almost similar in both the Pasture and TMR samples.

Table 4.1 Ingredient composition of the high lactation total mixed ration (TMR) and concentrate fed to the lactating dairy cows during the feeding trail (Source: Payam Vahmani, Personal Communication).

Composition	TMR	Concentrate
Ingredients, % of DM		
Corn Silage Ag Bag 27149-2	22.01	
1st cut Grass silage 27268-1	28.60	
2nd cut Hay	6.19	
Concentrate	43.19	
Ingredients, % as fed		
Barley Grain, 75%		23.92
corn distillers		9.57
Energy booster		1.91
Vit premix dairy 08		4.78
Soybean meal 48%		23.92
Corn grain (ground medium)		23.92
Calcium Phos Di		0.48
Limestone Ground		1.91
Mag Ox		0.10
Sodium bicarbonate		0.96
Se Premix		0.05
Top Soy		7.18
Diamond V XP		0.48
Copper sulphate 5H$_2$O		0.01
Bioplex copper		0.0003
Sel Plex 1000		0.04
Potassium Iodide		0.0002
Iodised Salt		0.77
Zinpro 180		0.02
Cobalt carbonate		0.0001

Table 4.2 Chemical composition of the pasture fed to the lactating dairy cows during the feeding trial (Source: Payam Vahmani, Personal Communication).

Nutrient	DM
Dry Matter (% as fed)	22.56
Crude protein (% of DM)	16.76
ADF (% of DM)	30.08
NDF (% of DM)	45.57
NE$_L$ (Mcal/kg DM)	1.48
Ether Extract (% of DM)	4.42
Ash (% of DM)	7.58

Table 4.3 Chemical composition of the silage fed to the lactating dairy cows during the feeding trial (Source: Payam Vahmani, Personal Communication).

Nutrient	DM
Dry Matter (% as fed)	45.67
Crude protein (% of DM)	13.90
ADF (% of DM)	32.27
NDF (% of DM)	49.83
TDN (%)	62.95
DE (Mcal/kg DM)	2.77
NE_L (Mcal/kg DM)	1.42

Table 4.4 Chemical composition of the concentrate mix (% DM basis) fed to the lactating dairy cows during the feeding trial (Source: Payam Vahmani, Personal Communication).

Nutrient		Nutrient	
Crude protein	25.68	Calcium (%)	0.98
Soluble protein	5.78	Phosphorus (%)	0.62
NPN	3.26	Magnesium (%)	0.32
ADIP	0.99	Potassium (%)	1.13
NDIP	3.18	Sulfur (%)	0.31
ADF	5.93	Sodium (%)	0.67
NDF	13.93	Chlorine (%)	0.62
peNDF	3.67	Iron (ppm)	210.65
Lignin	0.84	Zinc (ppm)	80.87
Ash	9.67	Copper (ppm)	36.54
Ether Extract	6.05	Manganese (ppm)	22.03
NFC	47.84	Selenium (ppm)	0.86
Sugar	5.87	Cobalt (ppm)	0.68
Starch	35.35	Iodine (ppm)	1.36
Soluble fiber	6.62	Vitamin A (KIU/kg)	33.3
		Vitamin D (KIU/kg)	8.88
		Vitamin E (IU/kg)	74.92

Table 4.5 Chemical composition of the high lactation total mixed ration (TMR) diet and mineral and vitamin supplement fed to the lactating dairy cows managed in confinement during the feeding trial (Source: Payam Vahmani, Personal Communication).

Macro Nutrients		Minerals and Vitamins	
Nutrient		**Nutrient**	
Dry Matter (% as fed)	53.22	Dry Matter (% as fed)	53.22
Crude Protein (% of DM)	17.89		**% of DM**
ADF (% of DM)	18.53	Calcium (%)	0.6
NDF (% of DM)	31.95	Phosphorus (%)	0.43
NE_L (Mcal/kg DM)	1.76	Magnesium (%)	0.22
Ether Extract (% of DM)	4.64	Potassium (%)	1.49
Ash (% of DM)	8.02	Sulfur (%)	0.21
		Sodium (%)	0.31
		Chlorine (%)	0.61
		Iron (ppm)	192.32
		Zinc (ppm)	49.46
		Copper (ppm)	18.73
		Manganese (ppm)	36.65
		Selenium (ppm)	0.37
		Cobalt (ppm)	0.31
		Iodine (ppm)	0.59
		Vitamin A (KIU/kg)	14.38
		Vitamin D (KIU/kg)	3.84
		Vitamin E (IU/kg)	32.36
		DCAD (meq/100g)	21.32

Table 4.6 outlines the fatty acid composition of the two lipid supplements. The RPMA supplement had higher saturated fatty acid (especially myristate and palmitate) content than that of RPFO. However, stearate content was slightly higher in RPFO than in the RPMA supplement. Monounsaturated fatty acids content was slightly higher in RPFO compared to the RPMA supplement. The PUFA content was greater in both the RPFO and RPMA supplements. Eicosapentaenoic acid content was higher in RPFO supplement compared to the RPMA supplement. The DHA content was higher in RPMA supplement compared to the RPFO supplement.

69

Table 4.6 Fatty acid composition of the rumen-protected marine algae (RPMA) and rumen-protected fish oil (RPFO) supplemented to lactating dairy cows managed either on pasture or in confinement during the feeding trial (Source: Payam Vahmani, Personal Communication).

Fatty acid of total fatty acid content (%w/w)	RPMA	RPFO
Saturated fatty acids		
Myristate C14:0	16.3	0
Palmitate C16:0	42.2	2.33
Stearate C18:0	1.32	4.35
Monounsaturated fatty acids		
Palmitoleate C16:1	1.75	0.96
Oleate C18:1	0.11	8.19
cis-11-octadecenoic acid C18:1 n-7	0	3.31
1-eicosaenoic acid C20:1	0	0.74
Erucic acid C22:1	0	3.2
Nervonic acid C24:1	0	0.73
Polyunsaturated fatty acids		
Linoleic Acid C18:2 n-6	0	0
Alpha Linolenic acid C18:3 n-3	0	2.58
Stearidonic acid C18:4 n-3	0	3.71
Eicosatrienoic acid (ETA) C20:3 n-6	0.11	0
Arachidonic Acid C20:4 n-6	0	0
Eicosatetraenoic acid C20:4 n-3	0	1.42
Eicosapentaenoic (EPA) C20:5 n-3	0.54	31.69
Heneicosapentaenoic acid C21:5 n-3	0	1.3
Docosapentaenoic (DPA) C22:5 n-6	10.67	4.01
Docosahexaenoic (DHA) C22:6 n-3	27	24.66

The amount of supplement fed was similar throughout the trial. The RPFO and RPMA group was supplemented with 300 g of each lipid supplement (RPFO and RPMA) per day. The RPFO supplement provided approximately 25 g/d each of EPA and DHA, whereas the RPMA supplement provided approximately 25 g/d of DHA. The TMR intake in the confinement group was similar during the experimental period. No significant difference in TMR intake (28.9 ± 9.7 kg/d) was observed between the treatments (P <

0.05). There was no problem with the palatability of the lipid supplements, as there were no refusals of either the RPFO or the RPMA. The LS had no effect on the BW (631.9 ± 5.3 kg) and BCS (2.5 ± 0.1) in the pasture group during the trial. Similarly, the LS had no effect on the BW (663.9 ± 7.2 kg) and BCS (2.7 ± 0.1) in the confinement group during the trial.

4.4.2 Milk Yield and Composition

Cows on pasture tended to have lower milk yield (35.35 ± 4.34 kg/d) compared to cows in confinement (40.27 ± 4.33 kg/d; $P = 0.08$). The milk yield (37.80 ± 4.44 kg) was not affected by LS ($P > 0.05$). Time did not affect the milk yield during the trial period ($P > 0.05$).

Milk fat percent was significantly decreased in cows fed RPMA (3.0 ± 0.1 %) compared with those fed with the control diet (3.5 ± 0.1 %), but was not altered by feeding RPFO (3.4 ± 0.1 %; $P = 0.005$; Figure 4.2). However, the FS ($P > 0.05$) and time ($P > 0.05$) did not affect the milk fat percent.

Grazing cows (1.2 ± 0.1 kg/d) tended to have lower milk fat yield compared to confined cows (1.4 ± 0.1 kg/d; $P = 0.08$), but, LS ($P > 0.05$) and time ($P > 0.05$) did not affect the milk fat yield in the experimental cows. Grazing cows (31.3 ± 3.5 kg/d) tended to have lower 4 % fat corrected milk (FCM) compared to confined cows (36.8 ± 3.5 kg/d; $P = 0.08$). However, LS ($P > 0.05$) and time ($P > 0.05$) did not affect the FCM in the experimental cows.
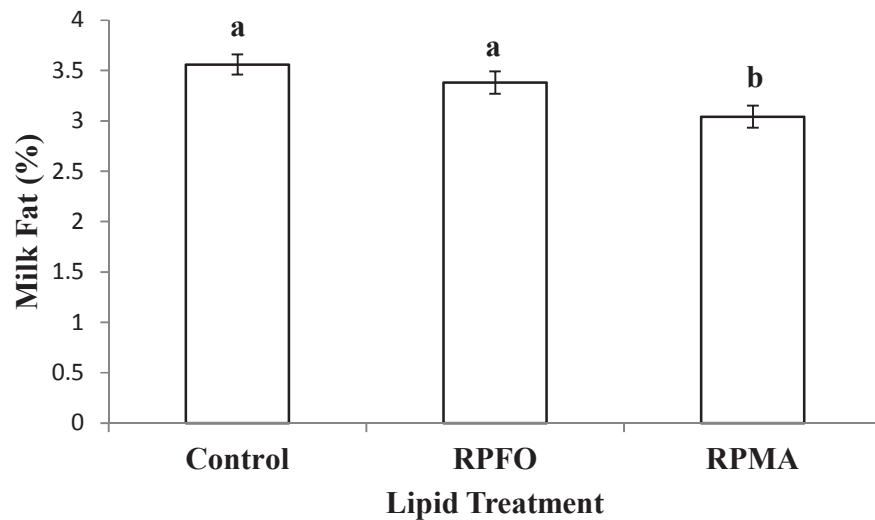
Figure 4.2 Milk fat (%) of lactating dairy cows fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during the Ovsynch period. Number of cycling cows per treatment = 12, 11 and 12 in control, RPFO and RPMA treatment group, respectively. Data from the analysis of variance are expressed as LSM ± SEM for milk fat. Least square means with different letters are significantly different (P < 0.05).

Interaction of FS and time affected the milk protein (%) in this study. Cows managed in confinement (3.1 ± 0.1 %) had greater milk protein (%) at 90 DIM compared to cows managed on pasture (2.8 ± 0.1 %; P = 0.01). However, LS (P > 0.05) did not affect the milk protein (%). Cows managed on pasture (1.0 ± 0.1 kg/d) tended to have lower milk protein yield compared to cows in confinement (1.2 ± 0.1 kg/d; P = 0.06). However, LS (P > 0.05) or time (P > 0.05) did not affect the milk protein yield.

Neither LS (0.003 ± 0.000 %) nor FS (0.003 ± 0.000 %) affected the EPA content of milk on Day 60 (P > 0.05). Feeding system did not impact the DHA content of milk on Day 60 (P > 0.05). The DHA content of milk was significantly increased in both the RPMA (0.3 ± 0.0 %) and RPFO (0.1 ± 0.0 %) groups compared to the control (0.04 ± 0.0 %; P < 0.05).

4.4.3 Postpartum Cyclicity and Estrus Responses

In pastured cows, the LS had no effect on postpartum cyclicity as it did not influence the interval from calving to first ovulation (46.1 ± 4.0 d; $P > 0.05$). The interval from calving to first CL observed was not different among the treatments (39.4 ± 4.5 d; $P > 0.05$). The LS and FS did not influence the onset of estrus after the 1st $PGF_{2\alpha}$ (3.2 ± 0.8 d), 2nd $PGF_{2\alpha}$ (1.8 ± 0.3 d), 1st GnRH (1.9 ± 0.7 d), 3rd $PGF_{2\alpha}$ (1.8 ± 0.2 d), or 2nd GnRH (1.1 ± 0.2 d) injections ($P > 0.05$).

During the Presynch period, the duration of estrus after the 1st $PGF_{2\alpha}$ injection was affected by LS and FS. Rumen protected fish oil (2.6 ± 0.4 d) fed cows tended ($P = 0.07$) to have prolonged estrus compared to RPMA (1.6 ± 0.4 d) and the control diet (1.3 ± 0.4 d) fed cows. Heat was significantly longer in confined cows (2.5 ± 0.3 d) compared to cows managed on pasture (1.3 ± 0.4 d) after the 1st $PGF_{2\alpha}$ injection ($P < 0.05$; Figure 4.3).

Similarly, cows managed in confinement (3.3 ± 0.3 d) had significantly prolonged estrus compared to cows managed on pasture (2.3 ± 0.3 d) after the 2nd $PGF_{2\alpha}$ injection ($P < 0.05$; Figure 4.4). However, the duration of estrus was not influenced by the LS (2.8 ± 0.3 d; $P > 0.05$). Neither LS nor FS affected the duration of estrus after the 1st GnRH (1.5 ± 0.3 d), 3rd $PGF_{2\alpha}$ (1.9 ± 0.4 d), or 2nd GnRH injection (0.9 ± 0.2 d; $P > 0.05$).
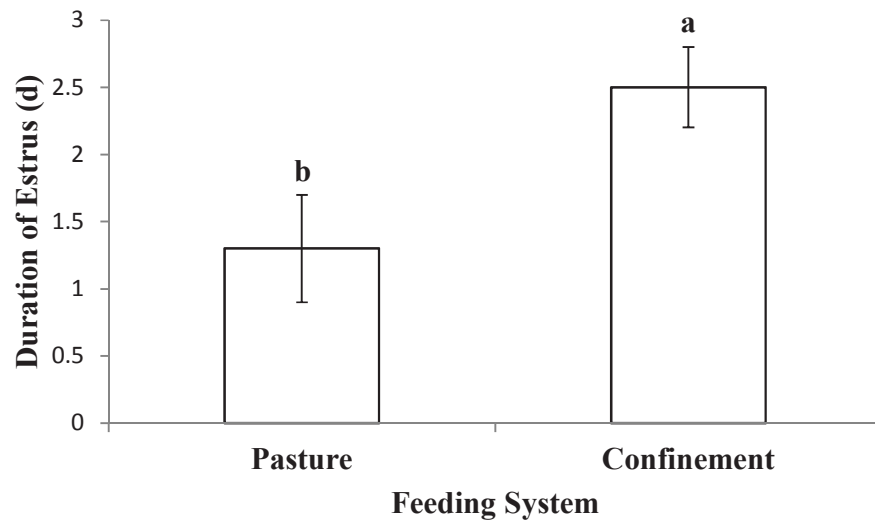
Figure 4.3 Duration of estrus (d) in lactating dairy cows managed either on pasture or in confinement after the 1st $PGF_{2\alpha}$ injection during the Presynch period. Number of cycling cows per treatment = 12 and 17 for pasture and confinement, respectively. Data from the analysis of variance are expressed as LSM ± SEM. Least square means with different letters are significantly different ($P < 0.05$).
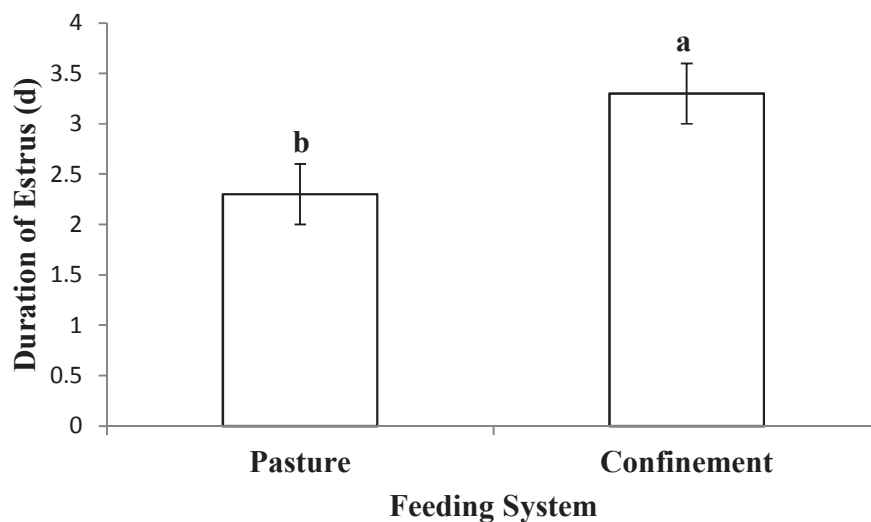
Figure 4.4 Duration of estrus (d) in lactating dairy cows managed either on pasture or in confinement after the 2nd $PGF_{2\alpha}$ injection during the Presynch period. Number of cycling cows per treatment = 16 and 18 for pasture and confinement, respectively. Data from the analysis of variance are expressed as LSM ± SEM. Least square means with different letters are significantly different ($P < 0.05$).

4.4.4 Follicular Dynamics in Pasture Group

During the Presynch period, cows fed RPFO tended to have a greater number of large follicles (≥ 10 mm) compared to cows fed the control diet during the period between 1st $PGF_{2\alpha}$ to 2nd $PGF_{2\alpha}$ injection (2.0 ± 0.2 vs. 1.0 ± 0.2; P = 0.07). However, cows fed RPMA tended to be intermediate (1.5 ± 0.2). During the same period, the number of large follicles was unaffected by time (P > 0.05; Table 4.7). During the period between 2nd $PGF_{2\alpha}$ to 1st GnRH injection, cows fed RPFO had a greater number of large follicles compared to cows fed the control diet (2.0 ± 0.2 vs. 1.0 ± 0.2; P < 0.05). However, cows fed RPMA (1.5 ± 0.2) did not significantly differ from cows fed RPFO or the control diet (P > 0.05). During the same period, the number of large follicles was unaffected by time (P > 0.05; Table 4.7).

Table 4.7 Mean daily number of the large follicles (≥ 10 mm) in lactating dairy cows managed on pasture and fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during and after the Presynch period.

	[1]Large follicle number			
Responses	**Control**	**RPFO**	**RPMA**	**P-Value**
Between the 1st $PGF_{2\alpha}$ to 2nd $PGF_{2\alpha}$ injections	1.0 ± 0.2	2.0 ± 0.2	1.5 ± 0.2	0.07
Between the 2nd $PGF_{2\alpha}$ to 1st GnRH injections	1.0 ± 0.2b	2.0 ± 0.2a	1.5 ± 0.2ab	0.01

[1]Data from the analysis of variance are expressed as LSM $\pm$ SEM of the number of follicles. Least square means followed by different letters within the row are significantly different (Tukey-Kramer adjusted P < 0.05). Number of cycling cows per treatment = 6, 5 and 6 for control, RPFO and RPMA, respectively.

Neither LS nor time affected the mean daily number of the large follicles during the period between 1st GnRH to 3rd $PGF_{2\alpha}$ (1.0 ± 0.2) or 1st GnRH to ovulation in response to the 1st GnRH injection (1.0 ± 0.2; P > 0.05). The mean daily number of the

large follicles was unaltered by the LS (P > 0.05) but was affected by time (P < 0.05) during the period between 1st GnRH to 2nd GnRH injection. There was an interaction of LS and time because the number of large follicles was greater in cows fed RPFO (2.0 ± 0.2) compared to cows fed the control diet (0.5 ± 0.2) on the day of 1st GnRH injection (Figure 4.5).

Figure 4.5 Mean daily number of the large follicles (≥ 10 mm) in lactating dairy cows managed on pasture and fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during the period between the 1st GnRH (Day 0) to 2nd GnRH injection (Day 9.5) of the Ovsynch period. Number of cycling cows per treatment = 6, 5 and 6 for control, RPFO and RPMA, respectively. Data from the analysis of variance are expressed as LSM ± SEM. *Overall LSM for number of large follicles in RPFO group showed an interaction effect of LS over time compared to control group on the day of 1st GnRH injection (P < 0.05).

During the Presynch period, RPFO fed cows had significantly larger follicles (≥10 mm) compared to the control diet fed cows during the period between 1st PGF$_{2\alpha}$ to 2nd PGF$_{2\alpha}$ injection (19.7 ± 1.5 vs. 13.9 ± 1.6 mm; P < 0.05). However, they did not significantly differ from RPMA fed cows (16.9 ± 1.6 mm; P > 0.05). During the same period, there was an interaction (P < 0.05) of LS and time because the diameter of large

follicles in RPFO fed cows was greater on the day (20.9 ± 1.8 mm) and also at 2 ($21.7 \pm$ 1.7 mm), 5 (20.2 ± 1.7 mm) and 6 d (19.6 ± 1.6 mm) compared to the control on the day (14.7 ± 2.1 mm) and also at 2 (13.2 ± 2.0 mm), 5 (13.4 ± 1.9 mm) and 6 d (13.5 ± 1.8 mm) after the 1st $PGF_{2\alpha}$ injection. Cows treated with RPMA had larger follicles at 5 (18.1 ± 1.7 mm), 6 (18.4 ± 1.7 mm), and 9 d (17.8 ± 1.6 mm) compared to control at 5 (13.4 ± 1.9 mm), 6 (13.5 ± 1.8 mm), and 9 d (14.9 ± 1.6 mm) after the 1st $PGF_{2\alpha}$ injection (Figure 4.6).
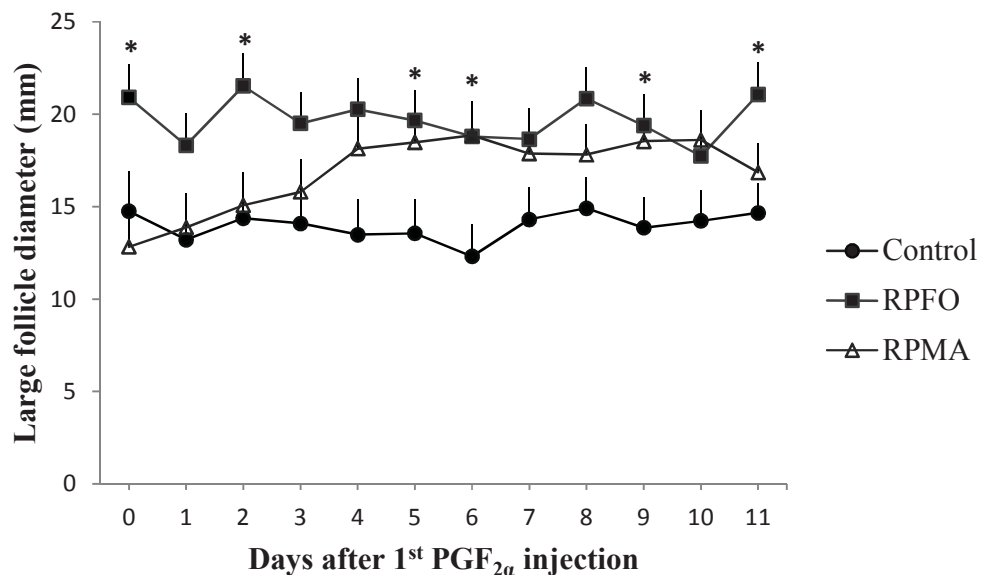
Figure 4.6 Mean daily diameter of the large follicle (≥ 10 mm) in lactating dairy cows managed on pasture and fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during the period between 1st $PGF_{2\alpha}$ (Day 0) to 2nd $PGF_{2\alpha}$ injection (Day 11) of the Presynch period. Number of cycling cows per treatment = 6, 5 and 6 for control, RPFO and RPMA, respectively. Data from the analysis of variance are expressed as LSM ± SEM. *Overall LSM for diameter of the large follicle in RPFO group showed an interaction effect of LS over time compared to control after the 1st $PGF_{2\alpha}$ injection (P < 0.05).

During the period between 2nd PGF$_{2\alpha}$ to 1st GnRH injection, the RPFO fed cows tended to have larger mean large follicles compared to the control diet fed cows (18.8 ± 1.3 vs. 14.9 ± 1.3 mm; P = 0.08). However, the diameter of large follicles in RPMA fed cows remained intermediate (16.3 ± 1.3 mm). A trend in the interaction effect of LS and time (P = 0.053) was observed during the same period. The RPFO fed cows (18.5 ± 1.4 mm) tended to have larger follicles (≥10 mm) on the day of 2nd PGF$_{2\alpha}$ injection compared to the control diet fed cows (15.8 ± 1.2 mm).

The mean daily diameter of the large follicle (≥ 10 mm) was greater in RPMA fed cows compared to the control during the interval between the 1st GnRH injection to ovulation in response to the 1st GnRH injection (17.4 ± 0.7 vs. 13.3 ± 1.0 mm; P < 0.05). However, RPFO group remained intermediate (15.6 ± 0.9 mm) in comparison to the other two groups (P > 0.05). During the same period, the diameter of the large follicle was unaffected by time (P > 0.05; Table 4.8). Mean diameter of the large follicles (≥ 10 mm) in RPFO group tended to increase in comparison with the control group, during the period between 1st GnRH to 3rd PGF$_{2\alpha}$ injection (17.1 ± 1.0 vs. 13.5 ± 1.0 mm; P = 0.06). However, the RPMA group tended to remain intermediate (16.2 ± 0.9 mm) in comparison with the other two groups. During the same period, the diameter of the large follicle was unaffected by time (P > 0.05; Table 4.8).

Later, the RPFO fed group was found to have significantly larger follicles (≥ 10 mm) compared to the control diet fed group during the period between 1st GnRH to 2nd GnRH injection (17.3 ± 1.0 vs. 13.1 ± 1.0 mm; P < 0.05). However, the RPMA group (16.3 ± 1.0 mm) did not significantly differ from RPFO or the control group (P > 0.05; Table 4.8). The mean daily diameter of the large follicles was significantly larger in cows

fed RPMA and RPFO compared to cows fed the control diet between the 3rd $PGF_{2\alpha}$ injection to ovulation, in response to the 2nd GnRH injection (16.6 ± 0.9 mm, 17.3 ± 0.9 mm and 12.2 ± 1.2 mm, respectively; $P < 0.05$; Table 4.8).
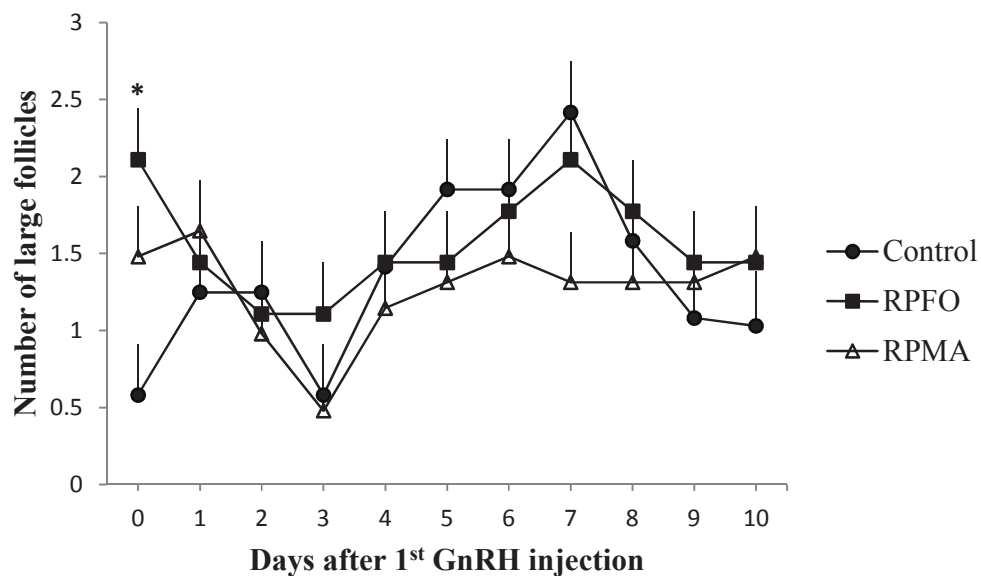
Table 4.8 Mean daily diameter of the large follicle ($\geq$10 mm)[2] in lactating dairy cows managed on pasture and fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during the Ovsynch period.

	[1]**Large follicle diameter (mm)**			
Responses	**Control**	**RPFO**	**RPMA**	**P-Value**
Between the 1st GnRH to ovulation in response to the 1st GnRH injection	13.3±1.0b	15.6±0.9ab	17.4±0.7a	0.01
Between the 1st GnRH to 3rd $PGF_{2\alpha}$ injection	13.5±1.0	17.1±1.0	16.2±0.9	0.06
Between the 1st GnRH to 2nd GnRH injection	13.1±1.0b	17.3±1.0a	16.3±1.0ab	0.01
Between the 3rd $PGF_{2\alpha}$ to ovulation in response to the 2nd GnRH injection	12.2±1.2b	17.3±0.9a	16.6±0.9a	0.01

[1]Data from the analysis of variance are expressed as LSM ± SEM of the diameter of large follicles in mm. Least square means followed by different letters within the row are significantly different (Tukey-Kramer adjusted $P < 0.05$). Number of cycling cows per treatment = 6, 5 and 6 for control, RPFO and RPMA, respectively.

The diameter of the largest follicle present after the 1st (17.2 ± 2.2 mm) or 2nd (16.0 ± 0.9 mm) GnRH injection just before ovulation was not different among the treatments ($P > 0.05$). Ovulation was significantly delayed in cows on pasture (1.4 ± 0.0

d) compared to cows in confinement (1.2 ± 0.0 d) after the 2nd GnRH injection (P < 0.05;

Figure 4.7). Furthermore, ovulation was significantly delayed in cows fed RPMA (1.4 ±

0.0 d) and RPFO (1.4 ± 0.0 d) compared to cows fed the control diet (1.1 ± 0.0 d) after

the 2nd GnRH injection (P < 0.05; Figure 4.8). The number of ovulations after the 1st (1.0

± 0.4) or 2nd (1.0 ± 0.3) GnRH injection was not different among the treatments (P >

0.05).
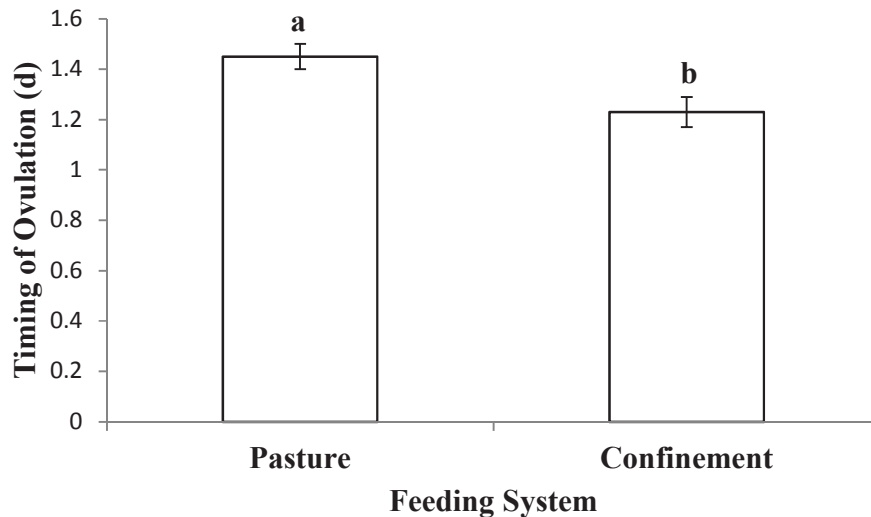
Figure 4.7 Timing of ovulation (d) in lactating dairy cows managed either on pasture or in confinement after the 2nd GnRH injection during the Ovsynch period. Data from the analysis of variance are expressed as LSM ± SEM. Number of cycling cows that ovulated per treatment = 15 and 13 for pasture and confinement, respectively. Least square means with different letters are significantly different (P < 0.05).
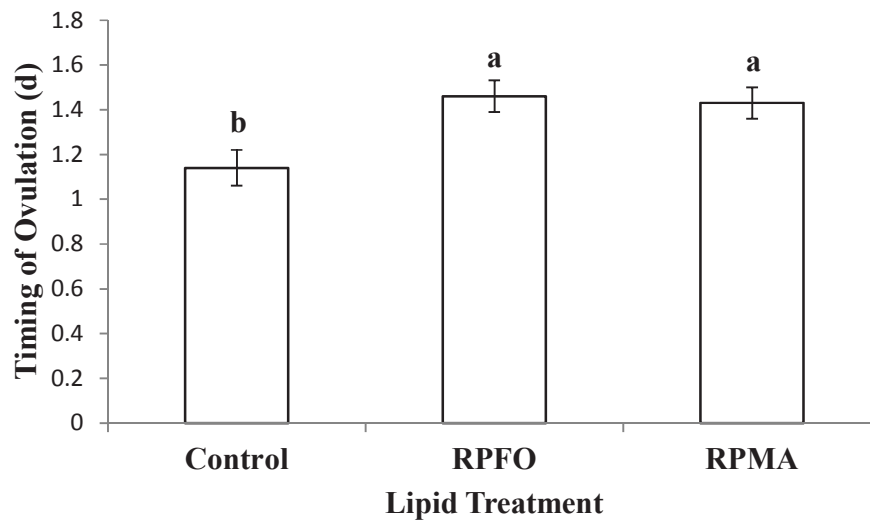
Figure 4.8 Timing of ovulation (d) in lactating dairy cows fed the control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) after the 2nd GnRH injection during the Ovsynch period. Data from the analysis of variance are expressed as LSM ± SEM. Number of cycling cows ovulated per treatment = 7, 10 and 9 for control, RPFO and RPMA, respectively. Least square means with different letters are significantly different ($P < 0.05$).

The presence of cystic ovaries was evident in some experimental cows during the trial. Among the pasture group, 2 cows assigned to RPFO treatment had cystic ovaries around the day of 1st $PGF_{2\alpha}$ injection. In the case of the confinement group, 1 from control, 3 from RPFO and 1 cow from RPMA group had cystic ovaries around the day of 2nd GnRH injection.

4.4.5 Luteal Dynamics in Pasture Group

The number of CLs were not affected by LS at the 1st $PGF_{2\alpha}$ (0.4 ± 0.2), 2nd $PGF_{2\alpha}$ (0.7 ± 0.2) or on the day of 1st GnRH injection (1.0 ± 0.4; $P > 0.05$). However, there were fewer cows in each treatment group (2, 2, and 4 in control, RPFO, and RPMA, respectively) having a functional CL at the 1st $PGF_{2\alpha}$ injection (Table 4.9). The number of CLs were greater in cows fed RPMA compared to cows fed the control diet at the 3rd

PGF$_{2\alpha}$ injection (2.2 ± 0.3 vs. 0.8 ± 0.2; P < 0.05); however, cows fed RPFO (1.7 ± 0.3) had a similar number of CL as the cows fed RPMA or the control diet (P > 0.05; Table 4.9). A similar trend was observed for the number of CLs at the 2nd GnRH injection. Cows fed RPMA had greater number of CLs compared to cows fed the control diet (2.0 ± 0.3 vs. 0.7 ± 0.2; P < 0.05); however, cows fed RPFO (1.7 ± 0.3) had similar number of CLs as the cows fed RPMA or the control diet (P > 0.05; Table 4.9).

Table 4.9 The number of CLs at each injection in lactating dairy cows managed on pasture and fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during conventional estrus and Ovsynch period.

Injection	[1]Number of CLs			
	Control	RPFO	RPMA	P-Value
1st PGF$_{2\alpha}$	0.2±0.3 (n=2)[2]	0.3±0.2 (n=2)	0.8±0.2 (n=4)	0.49
2nd PGF$_{2\alpha}$	0.7±0.3 (n=4)	0.5±0.2 (n=4)	1.0±0.2 (n=6)	0.38
1st GnRH	0.7±0.3 (n=4)	1.3±0.5 (n=5)	1.2±0.3 (n=5)	0.80
3rd PGF$_{2\alpha}$	0.8±0.2b (n=5)	1.7±0.3ab (n=5)	2.2±0.3a (n=6)	0.03
2nd GnRH	0.7±0.2b (n=4)	1.7±0.3ab (n=5)	2.0±0.3a (n=6)	0.04

[1]Data from the analysis of variance are expressed as LSM ± SEM of the number of CLs. Least square means followed by different letters within the row are significantly different (Tukey- Kramer adjusted P < 0.05). [2]n = number of cycling cows with functional CL in each treatment group.

Cows fed RPMA (1.0 ± 0.2) tended to have greater number of CLs in response to the 1st GnRH injection compared to cows fed RPFO (0.5 ± 0.2) and the control diet (0.5 ± 0.2; P = 0.09). Subsequently, cows fed RPMA (1.0 ± 0.2) followed the same trend of

possessing greater number of CLs, in response to 1st GnRH, when detected on the day of 3rd $PGF_{2\alpha}$ injection, while RPFO and control group tended to have lesser number of CLs (0.5 ± 0.2 and 0.5 ± 0.2, respectively; $P = 0.09$). The number of CLs, in response to the 2nd $PGF_{2\alpha}$ detected on the day of 1st GnRH (1.0 ± 0.3), in response to 2nd $PGF_{2\alpha}$ on the day of the 3rd $PGF_{2\alpha}$ injection (1.0 ± 0.5), or during the Ovsynch period (1.7 ± 0.3) was not different among the treatments ($P > 0.05$).

During the Presynch period, neither LS (20.4 ± 1.1 mm) nor time (20.4 ± 1.2 mm) affected the diameter of the CL during the period between the 2nd $PGF_{2\alpha}$ to 1st GnRH injection ($P > 0.05$). The diameter of the CL was greater in RPMA fed cows compared to the control diet fed cows during the period between the 1st GnRH to 3rd $PGF_{2\alpha}$ injection (26.7 ± 1.6 vs. 19.7 ± 2.0 mm; $P = 0.04$). However, the diameter of CL in RPFO fed cows (22.0 ± 1.8 mm) did not significantly differ from RPMA or the control diet fed cows ($P > 0.05$; Table 4.10).

During the Ovsynch period, a similar trend was observed where larger CL were detected in cows fed RPMA compared to cows fed the control diet during the period between 1st GnRH to 2nd GnRH injection (25.0 ± 1.5 vs. 19.2 ± 1.8 mm; $P < 0.05$). However, cows fed RPFO (20.4 ± 1.7 mm) did not significantly differ from cows fed RPMA or the control diet ($P > 0.05$). During the same period, the diameter of the CL was affected by time ($P < 0.05$) because the CL started to increase in diameter (22.2 ± 1.3 mm) 2 d after the 1st GnRH injection. It then reached a plateau of maximum diameter (24.8 ± 1.0 mm) between 5 and 6 d after the 1st GnRH injection and regressed thereafter (16.3 ± 1.6 mm) until the day of 2nd GnRH injection (Table 4.11).

Table 4.10 The diameter of the CL (mm) in lactating dairy cows managed on pasture and fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during the period between the 1st GnRH (Day 0) to 3rd $PGF_{2\alpha}$ injection (Day 7) of the Ovsynch period.

Treatments	[1]Diameter of the CL (mm)		
	Control	**RPFO**	**RPMA**
Lipid Supplement (LS)	$19.7 \pm 2.0b$	$22.0 \pm 1.8ab$	$26.7 \pm 1.6a$
Days after 1st GnRH			
Day 0 (1st GnRH injection)	14.0 ± 3.8	21.0 ± 2.7	27.0 ± 2.2
Day 1	15.1 ± 3.0	22.8 ± 2.7	28.0 ± 2.2
Day 2	17.9 ± 2.7	21.7 ± 2.4	26.9 ± 2.1
Day 3	20.6 ± 2.6	23.0 ± 2.3	26.7 ± 2.1
Day 4	23.9 ± 2.5	23.6 ± 2.2	27.2 ± 2.0
Day 5	23.1 ± 2.5	24.2 ± 2.2	27.1 ± 2.0
Day 6	22.0 ± 2.5	21.1 ± 2.3	25.8 ± 2.0
Day 7 (3rd $PGF_{2\alpha}$ injection)	20.7 ± 2.5	18.3 ± 2.4	25.0 ± 2.1
ANOVA	**P-Value**		
LS	0.04		
Day	0.08		
LS*Day	0.76		
Block	0.85		

[1]Data from the analysis of variance are expressed as LSM ± SEM of diameter of the CL. Least square means followed by different letters within the row are significantly different (Tukey-Kramer adjusted $P < 0.05$). Number of cycling cows with functional CL per treatment = 6, 5 and 6 for control, RPFO and RPMA, respectively.

Table 4.11 The diameter of the CL (mm) in lactating dairy cows managed on pasture and fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during the period between the 1st GnRH (Day 0) to 2nd GnRH injection (Day 9.5) of the Ovsynch protocol.

Treatments	[1]Diameter of the CL (mm)		
	Control	**RPFO**	**RPMA**
Lipid Supplement (LS)	$19.2 \pm 1.8b$	$20.4 \pm 1.7ab$	$25.0 \pm 1.5a$
Days after 1st GnRH			
Day 0 (1st GnRH injection)	14.2 ± 3.7	20.8 ± 2.6	27.0 ± 2.1
Day 1	15.2 ± 2.9	22.6 ± 2.6	28.0 ± 2.1
Day 2	18.1 ± 2.6	21.6 ± 2.3	27.0 ± 2.1
Day 3	20.7 ± 2.5	22.9 ± 2.3	26.7 ± 2.0
Day 4	24.0 ± 2.5	23.5 ± 2.2	27.3 ± 2.0
Day 5	23.2 ± 2.5	24.1 ± 2.2	27.2 ± 2.0
Day 6	22.1 ± 2.4	21.4 ± 2.2	25.8 ± 2.0
Day 7 (3rd $PGF_{2\alpha}$ injection)	20.9 ± 2.4	18.9 ± 2.3	25.1 ± 2.0
Day 8	20.0 ± 2.4	19.2 ± 2.2	20.5 ± 2.1
Day 9	17.7 ± 2.4	14.7 ± 2.2	22.5 ± 2.2
Day9.5(2nd GnRH injection)	15.6 ± 2.8	15.0 ± 3.3	18.4 ± 2.3
ANOVA	**P-Value**		
LS	0.05		
Day	0.002		
LS*Day	0.13		
Block	0.89		

[1]Data from the analysis of variance are expressed as LSM ± SEM of diameter of the CL. Least square means followed by different letters within the row are significantly different (Tukey- Kramer adjusted P < 0.05). Number of cycling cows with functional CL per treatment = 6, 5 and 6 for control, RPFO and RPMA, respectively.

During the Ovsynch period, the diameter of the largest CL, in response to the 2nd $PGF_{2\alpha}$ (31.5 ± 2.1 mm), in response to the 2nd $PGF_{2\alpha}$ on the day of 3rd $PGF_{2\alpha}$ (27.0 ± 1.5 mm), in response to the 1st GnRH (22.1 ± 1.5 mm), in response to the 1st GnRH on the day of 3rd $PGF_{2\alpha}$ (24.5 ± 1.7 mm), on the day of 3rd $PGF_{2\alpha}$ (24.5 ± 1.6 mm) injection, or during the Ovsynch period (26.5 ± 1.6 mm) was not different among the treatments ($P > 0.05$).

The total luteal volume at the 2nd $PGF_{2\alpha}$ (3248.9 ± 1304.1 mm^3) or 3rd $PGF_{2\alpha}$ (4298.9 ± 1344.4 mm^3) injection was not different among the treatments ($P > 0.05$). The volume of the largest CL at the 3rd $PGF_{2\alpha}$ (7302.6 ± 1836.5 mm^3) injection was not different among the treatments ($P > 0.05$).

4.4.6 Progesterone Profiles

4.4.6.1 During & After Presynch Period

During the period between start of the trial (Day 33 PP) to the 2nd $PGF_{2\alpha}$ injection, plasma P_4 concentration tended to be higher ($P = 0.053$) in cows managed on pasture compared to the cows in confinement (1.38 ± 0.24 vs. 0.73 ± 0.23 ng/mL). Neither FS (1.31 ± 0.35 ng/mL) nor LS (0.81 ± 0.42 ng/mL) affected the plasma P_4 concentrations on the day of the 1st $PGF_{2\alpha}$ injection ($P > 0.05$).

During the period between the 1st $PGF_{2\alpha}$ to 2nd $PGF_{2\alpha}$ injections, cows fed RPMA had significantly higher plasma P_4 concentrations compared to cows fed the control diet (1.63 ± 0.24 vs. 0.65 ± 0.27 ng/mL). However, cows fed RPFO (1.17 ± 0.34 ng/mL) did not significantly differ from cows fed the control diet or RPMA (Table 4.12).

Table 4.12 Plasma progesterone (ng/mL) concentrations in lactating dairy cows managed either on pasture or in confinement and fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during the period between the 1st $PGF_{2\alpha}$ (Day 0) to 2nd $PGF_{2\alpha}$ injections (Day 11) of the Presynch period.

Treatments	[1]Progesterone (ng/mL)
Feeding Systems (FS)	
Pasture	1.53 ± 0.26a
Confinement	0.78 ± 0.19b
Lipid Supplements (LS)	
RPMA	1.63 ± 0.24a
RPFO	1.17 ± 0.34ab
Control	0.65 ± 0.27b
Days after 1st $PGF_{2\alpha}$ injection	
Day 0 (1st $PGF_{2\alpha}$ injection)	0.72 ± 0.24bcd
Day 1	0.47 ± 0.25d
Day 2	0.50 ± 0.24d
Day 3	0.59 ± 0.24d
Day 4	0.67 ± 0.24d
Day 5	0.84 ± 0.24d
Day 6	1.02 ± 0.24cd
Day 7	1.42 ± 0.22abc
Day 8	1.64 ± 0.23ab
Day 9	1.96 ± 0.22a
Day 10	1.96 ± 0.22a
Day 11 (2nd $PGF_{2\alpha}$ injection)	2.07 ± 0.22a
ANOVA	**P-Value**
FS	0.03
LS	0.04
Day	0.0004
FS*LS	0.10
FS*Day	0.39
LS*Day	0.61
FS*LS*Day	0.42

[1]Data from the analysis of variance are expressed as LSM ± SEM of progesterone values. Least square means followed by different letters within the individual column are significantly different (Tukey- Kramer adjusted $P < 0.05$). Number of cycling cows with CL per treatment = 8, 7 and 10 for control, RPFO and RPMA, respectively.

Cows managed on pasture (1.53 ± 0.26 ng/mL) had significantly higher plasma P_4 concentrations compared to cows managed in confinement (0.78 ± 0.19 ng/mL) during the period between the 1st $PGF_{2\alpha}$ to 2nd $PGF_{2\alpha}$ injection (P < 0.05). The plasma P_4 concentration was affected by time (P < 0.001) because there were greater concentrations of P_4 found on the day of 2nd $PGF_{2\alpha}$ (2.07 ± 0.22 ng/mL) compared to the day of 1st $PGF_{2\alpha}$ injection (0.72 ± 0.24 ng/mL; Table 4.12).

Cows on pasture had higher (P < 0.05) concentrations of plasma P_4 on the day of the 2nd $PGF_{2\alpha}$ injection compared to the cows in confinement (2.28 ± 0.34 vs. 1.19 ± 0.34 ng/mL; Figure 4.9). However, no difference in the effect of LS was found among the treatments on the day of the 2nd $PGF_{2\alpha}$ injection (P > 0.05).

Figure 4.9 Plasma progesterone concentrations (ng/mL) in lactating dairy cows managed either on pasture or in confinement on the day of the 2nd $PGF_{2\alpha}$ injection during the Presynch period. Number of cycling cows with CL per treatment = 11 and 18 in pasture and confinement feeding systems, respectively. Data from the analysis of variance are expressed as LSM ± SEM. Least square means with different letters are significantly different (P < 0.05).

Table 4.13 Plasma progesterone (ng/mL) concentrations in lactating dairy cows managed either on pasture or in confinement and fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during the period between the 2nd $PGF_{2\alpha}$ (Day 0) to 1st GnRH injections (Day 11) after the Presynch period.

Treatments	[1]Progesterone (ng/mL)
Feeding Systems (FS)	
Pasture	0.96 ± 0.15
Confinement	0.89 ± 0.14
Lipid Supplements (LS)	
RPMA	1.25 ± 0.17
RPFO	0.65 ± 0.18
Control	0.88 ± 0.19
Days after 2nd $PGF_{2\alpha}$ injection	
Day 0 (2nd $PGF_{2\alpha}$ injection)	$1.88 \pm 0.17a$
Day 1	$0.67 \pm 0.17bc$
Day 2	$0.42 \pm 0.17c$
Day 3	$0.44 \pm 0.17c$
Day 4	$0.53 \pm 0.17c$
Day 5	$0.61 \pm 0.17bc$
Day 6	$0.75 \pm 0.17bc$
Day 7	$0.94 \pm 0.18bc$
Day 8	$1.01 \pm 0.20bc$
Day 9	$1.18 \pm 0.24abc$
Day 10	$1.59 \pm 0.26ab$
Day 11 (1st GnRH injection)	$1.11 \pm 0.26abc$
ANOVA	**P-Value**
FS	0.72
LS	0.05
Day	0.0001
FS*LS	0.26
FS*Day	0.11
LS*Day	0.89
FS*LS*Day	0.23

[1]Data from the analysis of variance are expressed as LSM $\pm$ SEM of progesterone values. Least square means followed by different letters within the individual column are significantly different (Tukey- Kramer adjusted $P < 0.05$). Number of cycling cows with functional CL per treatment = 10, 11 and 12 for control, RPFO and RPMA, respectively.

During the period between the 2nd PGF$_{2\alpha}$ to 1st GnRH injections, the plasma P$_4$ concentrations (0.92 ± 0.14 ng/mL) were not affected by FS (P > 0.05). Plasma P$_4$ concentrations in cows fed RPMA tended to be higher compared to cows fed RPFO (1.25 ± 0.17 vs. 0.65 ± 0.18 ng/mL). However, the P$_4$ levels in cows fed the control diet (0.88 ± 0.19 ng/mL) remained intermediate (Table 4.13). During the same period, the plasma P$_4$ concentrations were affected by time (P < 0.001) because there were greater P$_4$ concentrations (1.88 ± 0.17 ng/mL) found on the day of 2nd PGF$_{2\alpha}$ compared to 1 (0.67 ± 0.17 ng/mL), 2 (0.42 ± 0.17 ng/mL) and 3 d (0.44 ± 0.17 ng/mL) after the 2nd PGF$_{2\alpha}$ injection (Table 4.13).

4.4.6.2 During & After Ovsynch Period

Cows fed RPMA had higher (P < 0.05) plasma P$_4$ concentrations compared to the cows fed the control diet during the period between the 1st GnRH to 3rd PGF$_{2\alpha}$ injections (3.20 ± 0.38 vs. 1.44 ± 0.39 ng/mL). However, the P$_4$ concentrations in RPFO fed cows (2.14 ± 0.40 ng/mL) did not significantly differ from the control or RPMA fed cows. During the same period, the plasma P$_4$ concentrations were not affected by FS (2.25 ± 0.31 ng/mL), but affected by time (P < 0.001) because there was a gradual increase in the P$_4$ concentrations, which reached a plateau at Days 5 (2.86 ± 0.34 ng/mL) and 6 (2.84 ± 0.29 ng/mL) after the 1st GnRH injection and declined thereafter (Table 4.14).
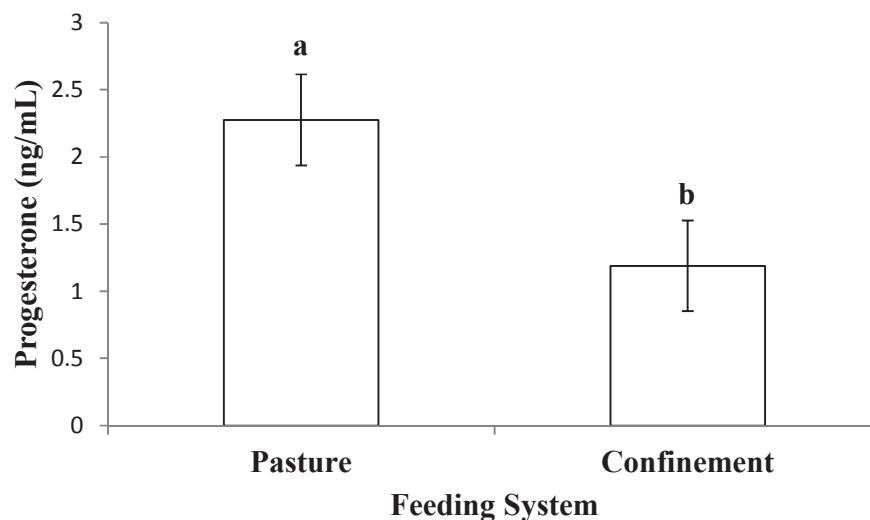
Table 4.14 Plasma progesterone (ng/mL) concentrations in lactating dairy cows managed either on pasture or in confinement and fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during the period between the 1st GnRH (Day 0) to 3rd $PGF_{2\alpha}$ injection (Day 7) of the Ovsynch period.

Treatments	[1]Progesterone (ng/mL)
Feeding Systems (FS)	
Pasture	2.45 ± 0.32
Confinement	2.06 ± 0.31
Lipid Supplements (LS)	
RPMA	$3.20 \pm 0.38a$
RPFO	$2.14 \pm 0.40ab$
Control[4]	$1.44 \pm 0.39b$
Days after 1st GnRH injection	
Day 0 (1st GnRH injection)	$1.42 \pm 0.22d$
Day 1	$1.54 \pm 0.20d$
Day 2	$1.77 \pm 0.20cd$
Day 3	$2.14 \pm 0.24bc$
Day 4	$2.57 \pm 0.28ab$
Day 5	$2.86 \pm 0.34a$
Day 6	$2.84 \pm 0.29a$
Day 7 (3rd $PGF_{2\alpha}$ injection)	$2.94 \pm 0.26ab$
ANOVA	**P-Value**
FS	0.39
LS	0.01
Day	<.0001
FS*LS	0.10
FS*Day	0.18
LS*Day	0.59
FS*LS*Day	0.13

[1]Data from the analysis of variance are expressed as LSM ± SEM of progesterone values. Least square means followed by different letters within the individual column are significantly different (Tukey- Kramer adjusted P < 0.05). Number of cycling cows with functional CL per treatment = 12, 11 and 12 for control, RPFO and RPMA, respectively.

On the day of the 3rd $PGF_{2\alpha}$ injection, cows fed RPMA had greater (P < 0.05) concentrations of P_4 compared to cows fed RPFO and the control diet (3.96 ± 0.44 ng/mL, 2.37 ± 0.46 ng/mL, and 1.86 ± 0.48 ng/mL, respectively; Figure 4.10). During the

period between the 1st GnRH to 2nd GnRH injections, cows fed RPMA had greater ($P < 0.05$) plasma P_4 concentrations compared to cows fed the control diet (2.52 ± 0.28 vs. 1.15 ± 0.29 ng/mL, respectively). However, cows fed RPFO (1.74 ± 0.29 ng/mL) did not significantly differ from cows fed the control diet or RPMA supplements.
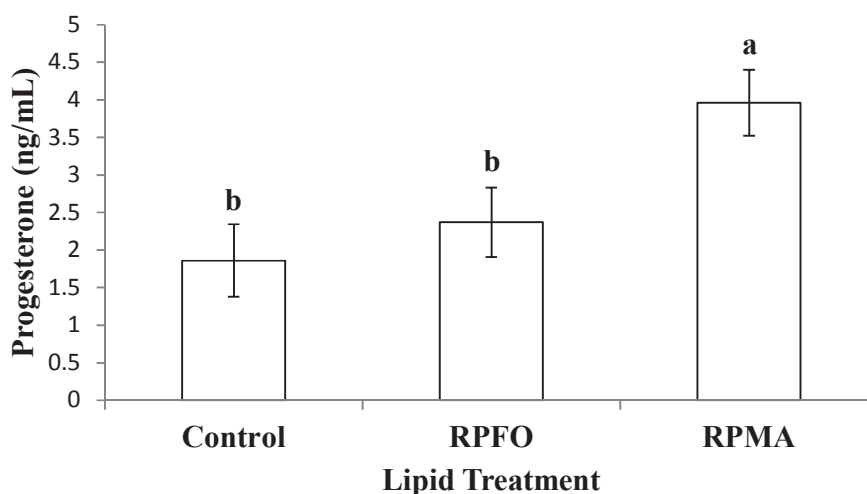
Figure 4.10 Plasma progesterone concentrations in lactating dairy cows fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) at the 3rd $PGF_{2\alpha}$ injection during the Ovsynch period. Number of cycling cows with CL per treatment = 12, 11 and 12 for control, RPFO and RPMA, respectively. Data from the analysis of variance are expressed as LSM ± SEM. Least square means with different letters are significantly different ($P < 0.05$).

The interaction between FS, LS and time was significant ($P < 0.05$) during the period between the 1st to 2nd GnRH injections. The plasma P_4 concentrations began to increase on Day 2 in all treatment groups and reached a plateau on Days 5-7 after the 1st GnRH injection and declined thereafter (Figure 4.11). Cows on pasture fed with RPMA (4.69 ± 0.52 ng/mL) were found to have greater P_4 concentrations across all treatments. The P_4 concentrations started to increase on Day 1 (2.57 ± 0.52 ng/mL) after the 1st

GnRH injection and reached a plateau at a maximum concentration on 4 (4.69 ± 0.52 ng/mL), 5 (4.69 ± 0.52 ng/mL) and 6 d (4.69 ± 0.52 ng/mL) and declined thereafter due to the 3rd $PGF_{2\alpha}$ injection. Cows on pasture fed with RPFO followed a similar trend as RPMA fed cows on pasture. The P_4 concentrations in this group began to rise from 1.04 ± 0.57 ng/mL on the day of 1st GnRH injection and reached a maximum of 3.84 ± 0.58 ng/mL on the Day 6 and declined thereafter (Figure 4.11).
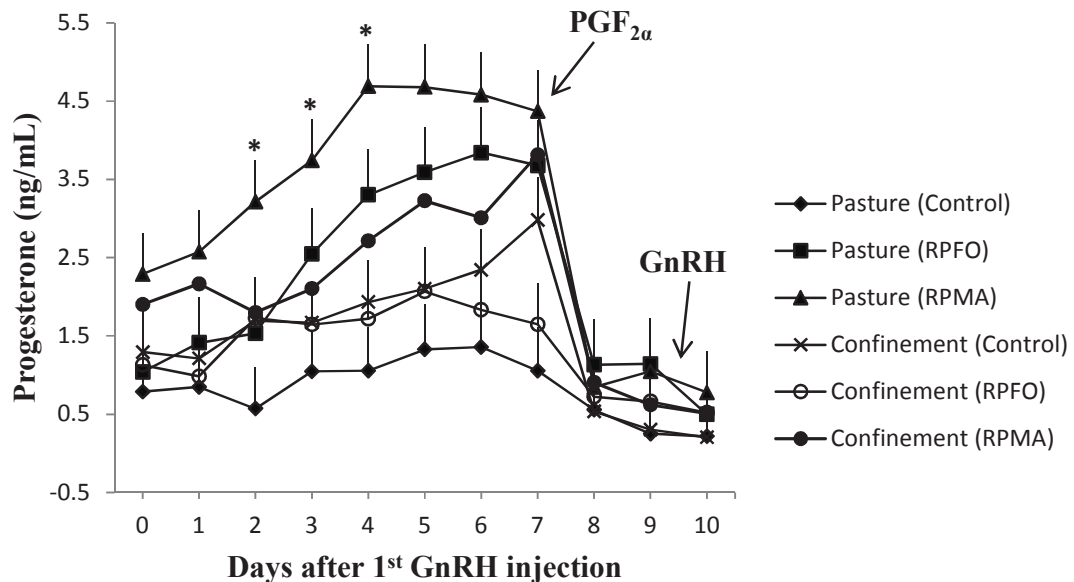
Figure 4.11 Effect of different combinations of treatments on plasma progesterone (ng/mL) concentrations in lactating dairy cows managed on pasture or in confinement and fed control diet (no lipid supplement), rumen-protected fish oil (RPFO) or rumen-protected marine algae (RPMA)during the period between the 1st GnRH (Day 0) to 2nd GnRH injections (Day 9.5) of the Ovsynch period. The different combinations includes: control (n = 6), RPFO (n = 5) and RPMA supplementation (n = 6) in pasture-based feeding system and control (n = 6), RPFO (n = 6) and RPMA supplementation (n = 6) in confinement. Data from the analysis of variance are expressed as LSM ± SEM. *Overall LSM for plasma P_4 showed a significant interaction effect of FS, LS and time for cows on pasture treated with RPMA compared to all other treatments ($P < 0.05$).

The confined cows fed with RPMA started to increase their P_4 concentrations from 1.90 ± 0.53 ng/mL and reached a maximum of 3.82 ± 0.53 ng/mL on the Day 7 and declined due to the 3rd $PGF_{2\alpha}$ injections. However, the P_4 concentrations of this group (3.82 ± 0.53 ng/mL) on the day of 3rd $PGF_{2\alpha}$ injection did not differ from the confined cows fed the control diet (2.74 ± 0.59 ng/mL). The confined cows fed with RPFO (1.54 ± 0.40 ng/mL) did not differ from cows managed on pasture (1.12 ± 0.39 ng/mL) or confinement (2.65 ± 0.78 ng/mL) and fed the control diet on the day of 3rd $PGF_{2\alpha}$ injection (Figure 4.11).

During the period between the 1st GnRH to ovulation, in response to the 2nd GnRH injections, mean plasma concentrations of P_4 showed a significant interaction effect of treatment versus time ($P < 0.05$). The P_4 concentrations of RPMA treated cows achieved a peak during this period at approximately 4.10 ± 0.21 ng/mL on 7 d after the 1st GnRH injection, while RPFO and the control diet fed cows achieved a peak at 2.84 ± 0.23 and 2.02 ± 0.21 ng/mL on 6 and 7 d after the 1st GnRH injection, respectively (Figure 4.12).

Cows fed with RPMA had greater ($P < 0.05$) concentrations of plasma P_4 compared to the cows fed the control diet during this period (2.22 ± 0.25 vs. 1.04 ± 0.26 ng/mL). However, cows fed with RPFO (1.54 ± 0.26 ng/mL) did not significantly differ from cows fed the control or RPMA supplement. Furthermore, the FS did not affect the plasma P_4 values (1.60 ± 0.22 ng/mL; $P > 0.05$; Figure 4.12).
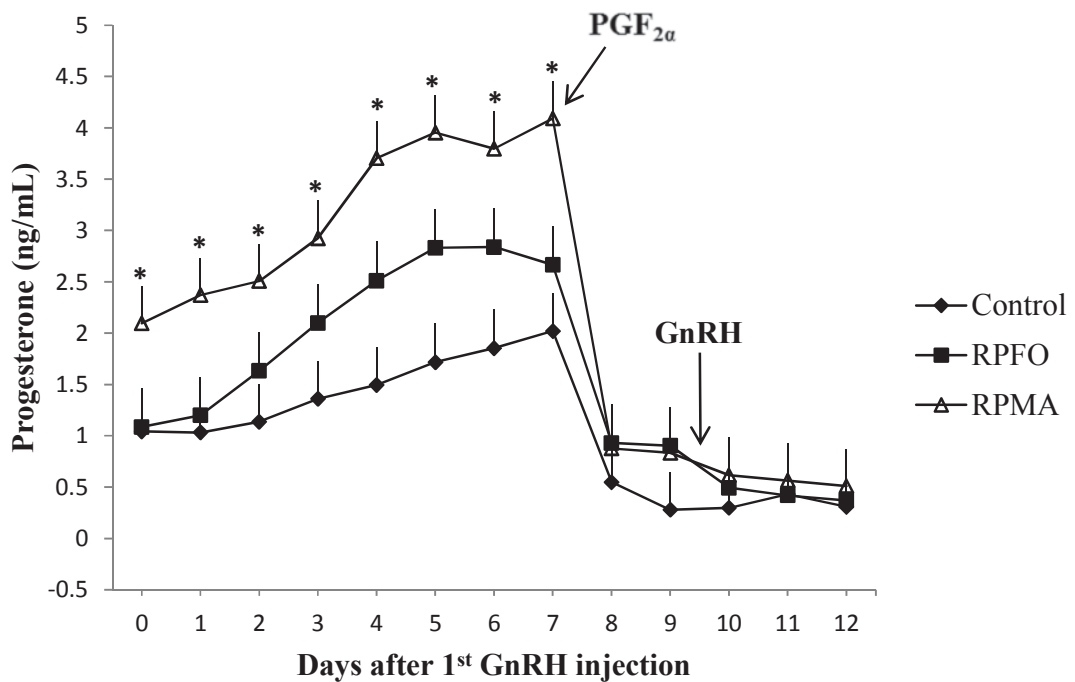
Figure 4.12 Plasma progesterone (ng/mL) concentrations in lactating dairy cows fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during the period between 1st GnRH (Day 0) to ovulation (Day 12) due to 2nd GnRH injections (Day 9.5) of the Ovsynch period. Number of cycling cows with CL per treatment = 12, 11 and 12 for control, RPFO and RPMA, respectively. Data from the analysis of variance are expressed as LSM ± SEM. *Overall LSM for plasma P_4 showed a significant interaction effect of LS and time for cows fed with RPMA compared to all other treatments ($P < 0.05$).

The mean plasma P_4 concentrations were measured for 13 days after the 2nd GnRH injection. Cows fed with RPMA (1.90 ± 0.18 ng/mL) tended to have numerically greater concentrations of P_4 compared to RPFO (1.63 ± 0.18 ng/mL) and the control diet (1.53 ± 0.19 ng/mL) fed cows. However, statistical significance was not achieved for the LS effect ($P > 0.05$) or an interaction effect of LS vs. days ($P > 0.05$). Furthermore, the FS did not affect ($P > 0.05$) the plasma P_4 values (1.69 ± 0.17 ng/mL; Table 4.15).

Table 4.15 Plasma progesterone (ng/mL) concentrations in lactating dairy cows managed either on pasture or in confinement and fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during the period between the 2nd GnRH injection of the Ovsynch (Day 0) to end of the experimental (Day 11) period.

Treatments	[1]Progesterone (ng/mL)
Feeding Systems (FS)	
Pasture	1.71 ± 0.18
Confinement	1.67 ± 0.17
Lipid Supplements (LS)	
RPMA	1.90 ± 0.18
RPFO	1.63 ± 0.18
Control[4]	1.53 ± 0.19
Days after 2nd GnRH injection	
Day 0 (2nd GnRH injection)	$0.51 \pm 0.18f$
Day 1	$0.50 \pm 0.18f$
Day 2	$0.43 \pm 0.18f$
Day 3	$0.68 \pm 0.18f$
Day 4	$0.90 \pm 0.18f$
Day 5	$1.30 \pm 0.18e$
Day 6	$1.59 \pm 0.18de$
Day 7	$1.76 \pm 0.18cde$
Day 8	$2.07 \pm 0.18cd$
Day 9	$2.28 \pm 0.18bc$
Day 10	$2.61 \pm 0.18ab$
Day 11	$2.77 \pm 0.18ab$
Day 12	$3.04 \pm 0.18a$
Day 13	$3.19 \pm 0.18a$
ANOVA	**P-Value**
FS	0.88
LS	0.27
Day	<.0001
FS*LS	0.20
FS*Day	0.43
LS*Day	0.97
FS*LS*Day	0.37

[1]Data from the analysis of variance are expressed as LSM ± SEM of progesterone values. Least square means followed by different letters within the individual column are significantly different (Tukey- Kramer adjusted $P < 0.05$). Number of cycling cows with functional CL per treatment = 12, 11 and 12 for control, RPFO and RPMA, respectively.

Maximum plasma P_4 concentrations were measured throughout the estrus and ovulation synchronization periods. Mean maximum plasma P_4 concentrations of RPMA (5.58 ± 0.34 ng/mL) fed cows were significantly ($P < 0.05$) higher, compared to the RPFO (4.62 ± 0.36 ng/mL) or the control (3.71 ± 0.36 ng/mL; Figure 4.13). Cows managed on pasture had greater ($P < 0.05$) concentrations of plasma P_4 than confined cows (5.08 ± 0.29 vs. 4.19 ± 0.30 ng/mL; Figure 4.14).

Figure 4.13 Maximum plasma progesterone concentrations (ng/mL) in lactating dairy cows fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) during the experimental period. Number of cycling cows with CL per treatment = 12, 11 and 12 for control, RPFO and RPMA, respectively. Data from the analysis of variance are expressed as LSM ± SEM. Least square means with different letters are significantly different ($P < 0.05$).
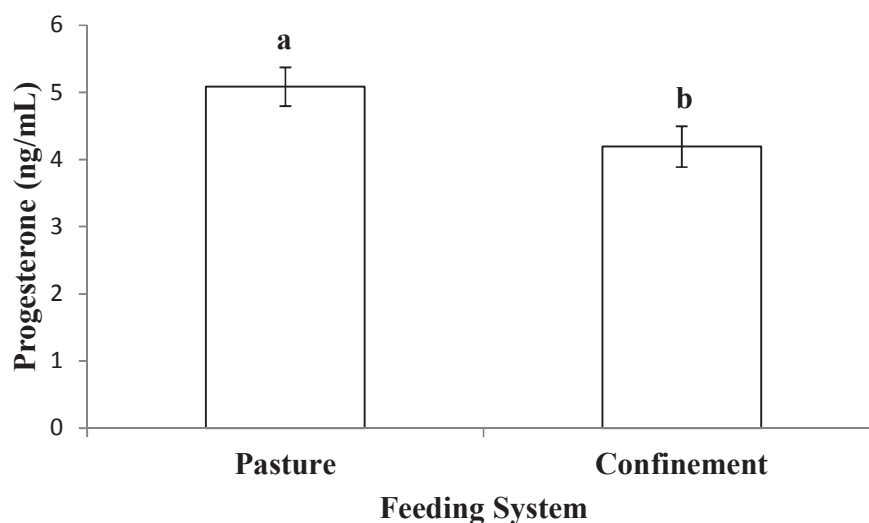
Figure 4.14 Maximum plasma progesterone concentrations (ng/mL) in lactating dairy cows managed either on pasture or in confinement during the experimental period. Number of cows with CL per treatment = 17 and 18 for pasture and confinement feeding systems, respectively. Data from the analysis of variance are expressed as LSM ± SEM. Least square means with different letters are significantly different ($P < 0.05$).

No difference in the interaction effect of FS and LS was evident during the trial (Table 4.16). However, the difference in the interaction effect of FS and LS was evident during the period between the 1st GnRH to 2nd GnRH injections ($P < 0.05$). Cows on pasture fed with RPMA had greater concentrations of plasma P_4 (2.98 ± 0.38 ng/mL) compared to the cows on pasture fed with the control diet (0.83 ± 0.38 ng/mL) and confined cows fed with RPFO (1.33 ± 0.37 ng/mL) or the control diet (1.48 ± 0.38 ng/mL). However, they did not significantly differ from cows on pasture fed with RPFO (2.16 ± 0.41 ng/mL) or confined cows fed with RPMA (2.07 ± 0.38 ng/mL; Table 4.16).
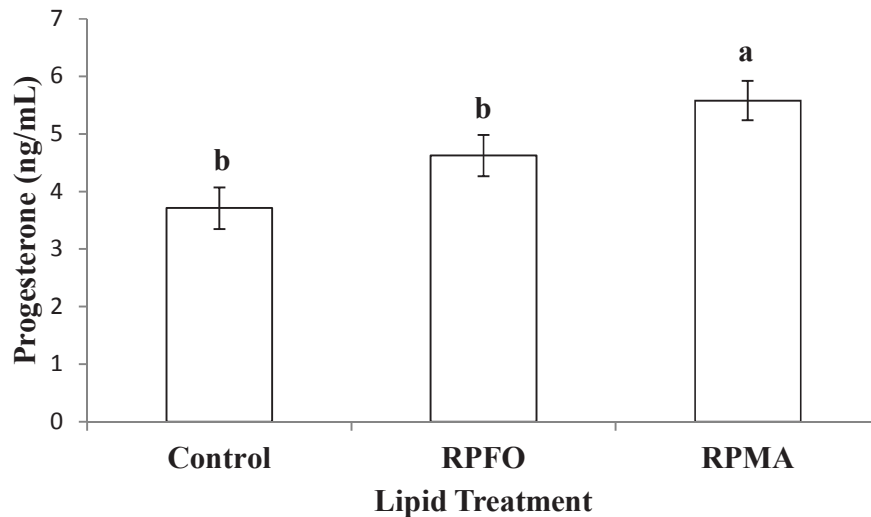
Table 4.16 Plasma progesterone (ng/mL) concentrations in lactating dairy cows managed either on pasture or in confinement feeding system (FS) and fed control diet (no lipid supplement), rumen-protected fish oil (RPFO), or rumen-protected marine algae (RPMA) lipid supplements (LS) during the Presynch and Ovsynch period.

Days	Pasture group			Confinement group			P-Values		
	Control	RPFO	RPMA	Control	RPFO	RPMA	FS	LS	FS*LS
*Day 35-46	[1]0.54±0.42	1.37±0.45	2.35±0.41	0.72±0.41	0.72±0.41	0.87±0.41	0.06	0.05	0.13
*Day 46-57	0.84±0.24	0.61±0.27	1.11±0.23	0.62±0.27	0.67±0.24	1.41±0.26	0.82	0.04	0.60
*Day 57-64	0.91±0.55	2.68±0.60	3.76±0.54	1.96±0.54	1.59±0.54	2.64±0.54	0.39	0.01	0.10
*Day 57-67	0.83±0.38b	2.16±0.41ab	2.98±0.38a	1.48±0.38b	1.33±0.37b	2.07±0.38ab	0.22	0.001	0.05
*Day 67-80	1.82±0.26	1.61±0.28	1.69±0.26	1.44±0.26	1.45±0.25	2.11±0.25	0.88	0.27	0.20

[1]Data from the analysis of variance are expressed as LSM ± SEM for progesterone values in ng/mL. *Day 35-46 = period between the 1st $PGF_{2\alpha}$ to 2nd $PGF_{2\alpha}$ injections, Day 46-57 = period between the 2nd $PGF_{2\alpha}$ to 1st GnRH injection, Day 57-64 = period between the 1st GnRH to 3rd $PGF_{2\alpha}$ injections, Day 57-67 = period between the 1st GnRH to 2nd GnRH injection and Day 67-80 = period between the 2nd GnRH injection to end of the trial. Least square means followed by different letters within a row are significantly different (Tukey- Kramer adjusted $P < 0.05$). Number of cows with functional CL per treatment include: control (n = 6), RPFO (n = 5) and RPMA (n = 6) supplementation in pasture-based feeding system and control (n = 6), RPFO (n = 6) and RPMA (n = 6) supplementation in confinement feeding system.

4.5 DISCUSSION

The composition of diets used in the current study were formulated according to the nutritional requirements of dairy cows with pasture having a lower % DM, higher % ash as well as ADF and NDF compared to TMR. Decreased dry matter intake is associated with reduced palatability and negatively affects ruminal bacterial fermentation. There was no problem with palatability in most animals as there were no refusals of either RPFO or RPMA supplements. However, some animals needed to be drenched with their supplements once in a while during the trial. Reduced dry matter intake was not observed in the current study and is consistent with other studies of supplementing micro-algae (Franklin et al. 1999) and fish meal or oil (Mattos et al. 2002 and 2004). Similarly, when dairy cows were supplemented with fish oil ($0 - 1$ %), dry matter intake was not altered. However, $2 - 3$ % of fish oil supplementation with feed affected the dry matter intake by reducing it from 29 to 20 kg/d (Donovan et al. 2000).

Neither RPFO nor RPMA supplementation affected the milk yield but the effect of FS was evident in this study. Confined cows tended ($P < 0.1$) to produce more milk compared to pastured cows. The differences in milk production between the FS are likely due to differences in feeding habits, exercise and energy expenditure. Confined cows may require less energy compared to pastured cows for walking or grazing activities and may produce more milk. However, confined cows might have diverted a greater amount of energy towards milk production. If the higher energy diversion were to occur, the suboptimal energy status of these cows might impact the GnRH and LH pulsatility (Staples et al. 1998). The suboptimal pulse frequency of LH might negatively impact the

luteinization and P_4 production by the CL (Smith et al. 1994). Therefore, high producing confined cows in this study had lower P_4 concentrations compared to pastured cows.

Feeding n-3 PUFA supplements over an extended time-frame typically altered the large follicle dynamics and luteal responses in the pasture group. Several studies over the past few years reported that feeding fat supplements which are high in n-3 PUFAs to dairy cows may improve the reproductive performance (Bilby et al. 2006b; Caldari-Torres et al. 2011; Garnsworthy et al. 2008; Santos et al. 2008; Silvestre et al. 2011). Due to the fact that early postpartum return to cyclicity is associated with improved fertility, cows should resume the ovarian cyclicity early in the lactation. In the current study, the number of days to first postpartum ovulation or luteal activity was not affected by the RPFO and RPMA. Lammoglia et al. (1996) indicated that 5.2% dietary fat (high concentrations of oleic and linoleic acid) supplementation influences the resumption of postpartum ovulation by enhancing the follicular growth. Consistent with Juchem et al. (2010) and Silvestre et al. (2011), this study indicates that lipid supplements differing in fatty acid profile did not influence the resumption of cyclicity in lactating dairy cows on pasture.

In the current study, there was no significant difference observed in the onset of estrus within treatments in cows managed either on pasture or in confinement. Zachut et al. (2011) reported that the onset of estrus after the $PGF_{2\alpha}$ injection was numerically delayed in E-flax (extruded flaxseed) fed cows compared to control. The onset of estrus after the 3rd $PGF_{2\alpha}$ injection averaged at 1.8 ± 0.2 d in this study compared to 2.5 ± 0.3 d in other studies (Zachut et al. 2011). During the Presynch period, confined cows (2.5 ± 0.3 d) had significantly prolonged estrus compared to pastured cows (1.3 ± 0.4 d). In

contrast, other study reported no difference in the duration of estrus in confined cows (1.8 ± 0.7 d) compared to pastured cows (2.2 ± 0.6 d; Palmer et al. 2010). This difference is possibly explained by confined cows being grouped more tightly and hence being more likely to be contacted by other herd mates. This might have resulted in prolonged observed estrus events in confined cows (Dransfield et al. 1998). The pastured cows were set apart and spent most of the day on pasture field. They might not be in close contact with other herd mates and less likely to show estrus signs. In addition, other possible reason may be that there was more opportunity to observe the estrus events in confined cows. However, the pastured cows were observed for estrus signs only around the milking time. The RPFO fed cows tended to have prolonged estrus (2.6 ± 0.4 d) after the 1st $PGF_{2\alpha}$ injection and is consistent with other studies (1.8 ± 0.5 d; Zachut et al. 2010).

In the current study, RPFO fed cows on pasture had greater number of large follicles during the Presynch period. However, it is not clear whether this effect is due to the RPFO supplementation or the 2nd $PGF_{2\alpha}$ injection. Based on the ovarian data, it raises the possibility that more number of follicles increased in size after the luteolysis due to the 2nd $PGF_{2\alpha}$ injection or the CL might have been in different growth stages. More than one follicle with a diameter of < 10 mm from a follicular pool in RPFO group might have increased in size (≥ 10 mm) and hence the number. In addition, another possible reason is that two cows assigned to RPFO group had cystic follicles during the Presynch period that might have contributed to this increased number. However, the number of ovulations remained similar among the treatments. Furthermore, this trend was not observed in subsequent stages of the Ovsynch period and is consistent with other studies (Petit et al. 2002; Ponter et al. 2006; Carrriquiry et al. 2009). Cows fed fat supplements rich in n-3

PUFAs had similar numbers of large follicles ($\geq$ 10 mm) compared to cows fed the control diet (no fat) or some other source of fat (Ponter et al. 2006; Carriquiry et al. 2009). In contrast, others reported an increase in the numbers of 10 to 15 mm diameter follicles in the lactating cows fed with calcium soaps of fatty acid based supplement (Hightshoe et al. 1991) or a blend of tallow and yellow grease (4.4% of dietary DM; Beam and Butler 1997).

The majority of studies have shown that lipid supplements which are high in LCPUFAs increase the average size of large follicles ($\geq$10 mm) in lactating dairy cows (Ambrose et al. 2006a; Bilby et al. 2006b; Garnsworthy et al. 2008; Mattos et al. 2000). In the current study, the RPFO fed cows had significantly larger follicles ($\geq$ 10 mm) and RPMA fed cows remained intermediate until the 3rd $PGF_{2\alpha}$ injection. Staples et al. (2000) reported that the diameter of the large follicle ($\geq$ 10 mm) was greater in fish oil fed dairy cows compared to the control diet fed cows (17.1 mm vs. 14.3 mm). The differences in follicle diameter in these groups might be explained by differences in EPA and DHA content of lipid supplements. The DHA in the RPMA supplement might have an indirect steroidogenic effect on P_4 production and LH pulsatility. According to Adams et al. (2008), the large follicles grow further to reach ovulatory size under the influence of LH. However, the LH surge will not occur under P_4 dominance. In support of this theory, the RPMA group had a functional CL with greater plasma P_4 levels compared to other groups. The higher levels of P_4 might have restricted follicular growth. Subsequently, the functional CL present at the time of the 3rd $PGF_{2\alpha}$ injection in the RPMA group then regressed and P_4 concentrations decreased to a basal level. The large follicles in the RPMA group reached the diameter similar to the RPFO group which might be due to the

presence of lower P_4 concentrations after the 3rd $PGF_{2\alpha}$ injection. Compared to the control, the diameter of the large follicle in RPMA and RPFO group was increased by an average of 4.3 mm and 3.4 mm, respectively. Santos et al. (2008) reviewed several studies involving the effect of increasing the supplemental fat in the dairy ration on the diameter of large dominant follicle. They concluded that the diameter of large follicles ($\geq$ 10 mm) was increased by 3.2 mm compared to the control. No significant differences were found among treatments for the diameter of largest follicle after the 1st or 2nd GnRH injection just before ovulation and are consistent with other studies (Robinson et al. 2002; Moussavi et al. 2007b). In contrast, some studies reported that the size of the largest follicle after the 1st or 2nd GnRH injection was increased in cows fed with dietary PUFAs (Lucy et al. 1991a; Moallem et al. 1999; Ambrose et al. 2006b; Zachut et al. 2008; Carriquiry et al. 2009).

In the current study, ovulation was delayed in pastured cows compared to confined cows after the 2nd GnRH injection. The difference in the timing of ovulation between the feeding systems may be explained by a delay in preovulatory LH surge. An additional amount of LCPUFAs available from pasture, along with LS, might have directly or indirectly affected the E_2 secretion and preovulatory LH surge. Previous studies have reported that LCPUFA supplementation reduces the concentration of E_2 in the plasma of dairy cows (Hightshoe et al. 1991; Oldick et al. 1997; Santos et al. 2008). In support of this, the E_2 secretions might have either reduced or slower to reach the peak level in pastured cows. Once E_2 concentration reaches a threshold level or peak, it stimulates preovulatory center to release large quantities of GnRH which in turn stimulate anterior pituitary for a preovulatory surge of LH (Allrich 1994). A delay in preovulatory

104

LH surge might have caused a delay in timing of ovulation in pastured cows. However, no studies have reported difference in the timing of ovulation in dairy cows supplemented with either RPFO or RPMA. The greater size of the large follicle appeared to be related to an increased time for follicular growth (Sartori et al. 2002). This was demonstrated in the current study that RPMA and RPFO group had significantly larger follicles (≥ 10 mm) and experienced delayed ovulation after the 2nd GnRH injection. Consistent with Carriquiry et al. (2009), the number of ovulation was not affected by the LS in this study.

Weekly rectal palpation of fish meal (2.2% Ca-LCFA) supplemented cows from 0 - 60 d after calving revealed a greater number of CL (1.05 vs.0.85) compared to the control (Garcia-Bojalil 1993). In the current study, the RPFO has been used which was in pure form compared to fish meal in the previous study. The current study supports the finding of Garcia-Bojalil (1993) in that RPMA fed cows had a greater number of CL compared to control cows on pasture at the time of the 3rd $PGF_{2\alpha}$ or 2nd GnRH injection. However, the RPFO fed cows remained intermediate. Milk fatty acid analysis of RPMA group revealed higher concentrations of DHA compared to EPA in this study. It is possible that there could be greater availability of circulatory DHA to the ovaries. The DHA might have indirectly delayed or prevented the CL turnover. The DHA applies its effect as an alternate substrate that reduces the lipid pool of arachidonic acid in the endometrium. Therefore precursors for series-2 prostaglandins (luteolytic) are reduced and series-3 prostaglandins (luteotropic) are increased (Abayasekara and Wathes 1999). The CL might have been non-responsive to lower concentrations of endogenous $PGF_{2\alpha}$. In addition, a new CL developed from a follicle which ovulated due to the 1st GnRH injection, separate from previously existing functional CL.

During the Ovsynch period, a larger CL detected in the RPMA group on pasture, compared to the control, while the diameter of CL in RPFO group remained intermediate. According to Staples et al. (1998), LCPUFAs stimulate the anterior pituitary to release LH which may, in turn, stimulate the differentiation of granulosa and thecal cells to luteal cells (luteinization). Current data supports this theory in that RPMA and RPFO, differing in fatty acid profiles, might have influenced the differentiation of luteal cells by stimulating LH secretion. Burns et al. (2010) reported that luteal tissue collected from fish meal fed cows had greater content of EPA and DHA compared to tissue collected from corn-gluten meal fed cows. Based on the luteal data, it raises the possibility that DHA might be having an indirect effect on LH secretion, resulting in larger CL producing greater concentrations of P_4 in RPMA fed cows. However, the EPA content of milk was negligible and it is less likely that circulatory EPA levels might affect LH secretion. In response to this, the P_4 levels and the diameter of CL in RPFO group remained intermediate. Supplementation of C18:2 enriched dietary lipid to lactating dairy cows resulted in 5 mm larger CL than the cows that were fed control diet (Garcia-Bojalil et al. 1998; Petit et al. 2002). In the current study, there were 5.8 mm and 1.2 mm larger CL in RPMA and RPFO fed cows than in the control, respectively.

Previous studies indicated that larger preovulatory follicles in dairy cows resulted in the CL with a greater diameter and volume, producing greater concentrations of P_4 after ovulation (Sartori et al. 2002). However, the larger CL may not be due to a larger preovulatory follicle but rather, may be due to the direct steroidogenic and developmental effects on the CL. In the current study, the diameter of the largest follicle present just before ovulation in response to 1st GnRH and 2nd GnRH was similar within the

treatments. However, there were larger CL producing greater concentrations of P_4 during the Ovsynch period. In addition, the current study revealed that there was no effect of n-3 PUFA on the total luteal volume and volume of the largest CL in pastured cows. Others reported that the mean luteal volume was not affected (Sartori et al. 2004) or decreased (Carriquiry et al. 2009) in cows supplemented with dietary fat compared to the control.

Several studies have indicated that feeding dairy cows with tallow (Son et al. 1996), Ca-LCFA (Sklan et al. 1991; Espinoza et al. 1995; Espinoza-Villvicencio et al. 2010), prilled fatty acids (Carroll et al. 1990; Spicer et al. 1993) and beef cows with Ca-LCFA (Hightshoe et al. 1991; Hawkins et al. 1995), whole sunflower seeds (Talavera et al. 1985) or soybean oil (Ryan et al. 1992) increases the plasma P_4 concentrations. Increased P_4 concentrations in these studies suggest that luteal function may be improved by dietary PUFA supplementation.

Feeding fat supplements to dairy cows generally increases blood cholesterol, a precursor for P_4 synthesis by the CL (Carroll et al. 1990; Mattos et al. 2000; Santos et al. 2008). During the Presynch period, cows fed RPMA had greater plasma P_4 concentrations, compared to control and RPFO fed cows. Consistent with Boken et al. (2005), pasture group had significantly higher plasma P_4 compared to the confined cows. Digestible lipids in temperate pasture species consumed by pasture grazed cows are high in ALA (Palmquist et al. 1986). The increased P_4 concentrations in pastured cows may be due to the greater uptake of long chain PUFAs, such as ALA from the pasture. Intra annual variation in the chemical composition of PUFA rich pasture might have affected the peak P_4 levels in pastured cows (Walker et al. 2004).The EPA content of milk was not affected by LS. However, the DHA content was increased in milk. These findings

indicate that there might be greater availability of DHA to the ovaries. Increased uptake of DHA, along with increased availability of cholesterol to the ovaries, might have altered the P_4 levels in RPMA group. Another possibility is that the DHA might have a direct or indirect steroidogenic effect on the ovaries of RPMA fed cows. Consistent with Bilby et al. (2006b), the LS did not affect the plasma P_4 concentrations between the treatments on the day of the 2nd $PGF_{2\alpha}$ injection. Based on the luteal and P_4 data, some lactating cows did not have a functional CL or the CL might have regressed before the 2nd $PGF_{2\alpha}$ injection. In contrast, others reported an increase in plasma cholesterol and P_4 (Son et al. 1996).

The EPA and DHA have been shown to suppress $PGF_{2\alpha}$ synthesis in the uterine endometrium (Bilby et al. 2006a; MacLaren et al. 2006; Hoyt et al. 2007) and may escape microbial biohydrogenation in the rumen of dairy cows (Ashes et al. 1992). If suppression were to occur, the regression of CL might become more dependent on exogenous application of $PGF_{2\alpha}$. In support of this, the RPMA group had higher plasma P_4 compared to the control group. Milk fatty acid profiles revealed higher concentrations of DHA in RPMA group and lower concentrations of EPA in RPFO group. The greater circulatory levels of DHA might have indirectly influenced P_4 production. Other possibilities may include enhanced lipoprotein cholesterol concentrations in the blood due to dietary fat supplementation (Carroll et al. 1992). In turn, the enhanced blood cholesterol may be due to the increased absorption of DHA, along with cholesterol, chylomicrons and VLDL from the duodenum (Staples et al. 1998). However, EPA content of milk in RPFO group was low and it is less likely that EPA was absorbed in greater quantity and indirectly affected the P_4 production. The differences in responses

for P_4 levels in RPMA and RPFO group may be due to differences in EPA and DHA content of these supplements. In addition, a greater number and diameter of CL might have contributed to peak P_4 levels in RPMA group compared to RPFO group during the Ovsynch period. However, this trend was not observed after the ovulation in response to 2nd GnRH injection.

The nature of intact cell coat in marine algae may protect the EPA and DHA, contained in them and thus, may provide protection in the rumen (Barclay et al. 1994). Previous studies report that endogenous antioxidants are also produced in the micro-algae cells (Pereira et al. 2004). Therefore, native marine algae may be more protected from the ruminal biohydrogenation compared to the fish oil. Although fish oil is protected, this study indicates that there is a higher concentration of P_4 in RPMA fed cows managed on pasture. However, it is not clear whether the marine algae are more protected, or whether the cell coat may cause EPA and DHA to escape the biohydrogenation in the rumen.

CHAPTER 5

CONCLUSIONS

5.1 Conclusions

In this study, long term lipid supplementation and FS altered the ovarian function in dairy cows. The dry matter intake in the confined cows was not affected by LS. The resumption of cyclicity and onset of estrus were not influenced by LS. Large follicle dynamics in the pasture group was enhanced by LS, as RPFO feeding increased the number and diameter of large follicles and RPMA feeding increased the diameter of large follicles. Ovulation was delayed due to the LS. However, the number of ovulations was not altered. In the pasture group, possible beneficial effects of LS on the luteal dynamics were evident by the increase in the number and diameter of CL. The interaction effects of FS, LS and time on the plasma P_4 were evident in this study. Feeding RPMA resulted in an increase in plasma P_4 and RPFO was found to be intermediate in influencing the P_4 production. Cows on pasture supplemented with RPMA had greater P_4 levels, among all treatments. Feeding n-3 PUFAs appeared to have beneficial effects on various biological windows that may improve ovarian function. The RPMA supplement provide a superior alternative source of omega-3 PUFAs to the fish oil supplements to improve the ovarian function in lactating dairy cows.

5.2 Future Recommendations

The current study addresses challenges regarding utilization of n-3 PUFA supplements, high in EPA and DHA, of marine origin that has the potential to enhance reproductive performance in dairy cows. However, numerous challenges remain; the

following list of studies explain the approaches that would build on present work and could lead to further advances in improving the reproductive performance in dairy cows and other ruminants.

5.2.1 Hormonal Profiles

In the current study, large follicular dynamics was greatly influenced by lipid supplementation. Therefore, estradiol concentrations in blood and follicular fluid may be studied to accurately define the role of RPFO and RPMA supplements in improving the reproducing ability of dairy cows. Luteinizing hormone plays an important role during and after ovulation. The timing of ovulation was delayed in the current study. Very few studies have been conducted to determine the effect of omega-3 PUFAs on the blood profiles of LH. Therefore, current work could be extended to accurately define the role of LH in dairy cows supplemented with n-3 PUFAs. Although the current study has confirmed the higher plasma P_4 levels during the Ovsynch period, it would be interesting to investigate the effect of n-3 PUFA on P_4 levels in subsequent estrous cycles. In addition to this, the rate of P_4 accumulation and clearance from the body may be determined. This may improve our understanding of whether LS directly affects the P_4 synthesis or delays its clearance from the body.

5.2.2 Cholesterol Estimation and Fatty Acid Profiling of Oocytes

Cholesterol serves as the precursor for the production of P_4 by luteal cells and it is delivered by both high and low density lipoproteins to the ovarian cells. Increased cholesterol availability to luteal cells may improve steroidogenesis. It would be interesting to determine the cholesterol content of luteal tissue and blood as well. Competence of the dominant follicle is related to fatty acid composition of the oocyte.

111

The lipid content of the oocyte plasma membrane plays a major role during and after fertilization and may serve as an energy reserve. More research is warranted into this area to better understand the role of n-3 PUFAs of marine source in improving the oocyte quality and membrane composition. Few *in vitro* studies have been conducted to determine the effect of LCFA (Ca-LCFA of palm oil) on embryo quality and development in lactating dairy cows (Bilby et al. 2006). However, it is not clear which particular LCFA is most beneficial. Therefore, this study may be extended to determine the effect of n-3 PUFAs of marine origin on the embryo quality and uterine lipid content.

5.2.3 Gene and Receptor Expression Studies

A previous study indicated that rumen protected fish oil is associated with increased suppression of the series-2 prostaglandins in response to oxytocin challenge. In addition, there was increased expression of P_4 and E_2 receptors and PGHS-2 in the uterine endometrium. However, rumen protected marine algae had an intermediate response on series-2 prostaglandins and the effects were subtle (Hoyt et al. 2007). This study may be used as advancement to previous studies to accurately determine the role of RPMA in suppressing the series-2 prostaglandins and associated reproductive hormones.

REFERENCES

Abayasekara, D. R. E. and Wathes, D. C. 1999. Effects of altering dietary fatty acid composition on prostaglandin synthesis and fertility. Prostaglandins Leukotrienes Essential Fatty Acids **61:** 275-287.

Achard, F., Gilbert, M., Benistant, C., Ben Slama, S., DeWitt, D. L., Smith, W. L. and Lagarde, M. 1997. Eicosapentaenoic and docosahexaenoic acids reduce PGH synthase 1 expression in bovine aortic endothelial cells. Biochem. Biophys. Res. Commun. **241:** 513-518.

Adams, G. P. 1999. Comparative patterns of follicle development and selection in ruminants. J. Reprod. Fertil. Suppl. **54:** 17-32.

Adams, G. P., Jaiswal, R., Singh, J. and Malhi, P. 2008. Progress in understanding ovarian follicular dynamics in cattle. Theriogenology **69:** 72-80.

Aerts, J. M. and Bols, P. E. 2010a. Ovarian follicular dynamics: a review with emphasis on the bovine species. Part I: Folliculogenesis and pre-antral follicle development. Reprod. Domest. Anim. **45:** 171-179.

Aerts, J. M. and Bols, P. E. 2010b. Ovarian follicular dynamics. A review with emphasis on the bovine species. Part II: Antral development, exogenous influence and future prospects. Reprod. Domest. Anim. **45:** 180-187.

Ahmad, N., Beam, S. W., Butler, W. R., Deaver, D. R., Duby, R. T., Elder, D. R., Fortune, J. E., Griel Jr., L. C., Jones, L. S., Milvae, R. A., Pate, J. L., Revah, I., Schreiber Jr., D. T., Townson, D. H., Tsang, P. C. W. and Inskeep, E. K. 1996. Relationship of fertility to patterns of ovarian follicular development and associated hormonal profiles in dairy cows and heifers. J. Anim. Sci. **74:** 1943-1952.

Ahmad, N., Townsend, E. C., Dailey, R. A. and Inskeep, E. K. 1997. Relationships of hormonal patterns and fertility to occurrence of two or three waves of ovarian follicles, before and after breeding, in beef cows and heifers. Anim. Reprod. Sci. **49:** 13-28.

Algire, J. E., Srikandakumar, A., Guilbault, L. A. and Downey, B. R. 1992. Preovulatory changes in follicular prostaglandins and their role in ovulation in cattle. Can. J. Vet. Res. **56:** 67-69.

Allrich, R. D. 1994. Endocrine and neural control of estrus in dairy cows. J. Dairy Sci. **77:** 2738-2744.

Ambrose, D. J., Kastelic, J. P., Corbett, R., Pitney, P. A., Petit, H. V., Small, J. A. and Zalkovic, P. 2006. Lower pregnancy losses in lactating dairy cows fed a diet enriched in α-linolenic acid. J. Dairy Sci. **89:** 3066-3074.

Ambrose, D. J. and Colazo, M. G. 2011. Strategies to improve the fertility in lactating dairy cow. Pages 496 - 497 In Proceedings of the Canadian Society of Animal Science annual meeting., 60th, Halifax, Nova Scotia, May 2011.

AOAC. 1990. Official methods of analysis. 15th ed. Association of official analytical chemists international, Arlington, VA.

Arosh, J. A., Parent, J., Chapdelaine, P., Sirois, J. and Fortier, M. A. 2002. Expression of cyclooxygenases 1 and 2 and prostaglandin E synthase in bovine endometrial tissue during the estrous cycle. Biol. Reprod. **67:** 161-169.

Ashes, J. R., Siebert, B. D., Gulati, S. K., Cuthbertson, A. Z. and Scott, T. W. 1992. Incorporation of n-3 fatty acids of fish oil into tissue and serum lipids of ruminants. Lipids **27:** 629-631.

Baker, F. D. and Kragt, C. L. 1969. Maturation of the hypothalamic-pituitary-gonadal feedback system. Endocrinology **85:** 522-527.

Barclay, W. R., Meager, K. M. and Abril, J. R., 1994. Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J. Appl. Phycol. **6**: 123-129.

Beam, S. W. and Butler, W. R. 1997. Energy balance and ovarian follicle development prior to the first ovulation postpartum in dairy cows receiving three levels of dietary fat. Biol. Reprod. **56:** 133-142.

Beam, S. W. and Butler, W. R. 1998. Energy balance, metabolic hormones, and early postpartum follicular development in dairy cows fed prilled lipid. J. Dairy Sci. **81:** 121-131.

Bergfelt, D. R., Smith, C. A., Adams, G. P. and Ginther, O. J. 1997. Surges of FSH during the follicular and early luteal phases of the estrous cycle in heifers. Theriogenology **48:** 757-768.

Berisha, B. and Schams, D. 2005. Ovarian function in ruminants. Domest. Anim. Endocrinol. **29:** 305-317.

Bilby, T. R., Block, J., do Amaral, B. C., Sa Filho, O., Silvestre, F. T., Hansen, P. J., Staples, C. R., Thatcher, W. W. 2006a. Effects of dietary unsaturated fatty acids on oocyte quality and follicular development in lactating dairy cows in summer. J. Dairy Sci. **89**: 3891-3903.

114

Bilby, T. R., Guzeloglu, A., MacLaren, L. A., Staples, C. R. and Thatcher, W. W. 2006b. Pregnancy, bovine somatotropin, and dietary n-3 fatty acids in lactating dairy cows: II. endometrial gene expression related to maintenance of pregnancy. J. Dairy Sci. **89**: 3375-3385.

Bilby, T. R., Sozzi, A., Lopez, M. M., Silvestre, F. T., Ealy, A. D., Staples, C. R. and Thatcher, W. W. 2006c. Pregnancy, bovine somatotropin, and dietary n-3 fatty acids in lactating dairy cows: I. ovarian, conceptus, and growth hormone-insulin-like growth factor system responses. J. Dairy Sci. **89**: 3360-3374.

Boken, S. L., Staples, C. R., Sollenberger, L. E., Jenkins, T. C. and Thatcher, W. W. 2005. Effect of grazing and fat supplementation on production and reproduction of Holstein cows. J. Dairy Sci. **88:** 4258-4272.

Bridges, G. A., Mussard, M. L., Burke, C. R. and Day, M. L. 2010. Influence of the length of proestrus on fertility and endocrine function in female cattle. Anim. Reprod. Sci. **117:** 208-215.

Britt, J. H. 1985. Enhanced reproduction and its economic implications. J. Dairy Sci. **68:** 1585-1592.

Budge, S. M., Iverson, S. J. and Koopman, H. N. 2006. Studying trophic ecology in marine ecosystems using fatty acids: a primer on analysis and interpretation. Mar. Mammal Sci. **22:** 759-801.

Burke, J. M., Staples, C. R., Risco, C. A., de la Sota, R. L. and Thatcher, W. W. 1997. Effect of ruminant grade Menhaden fish meal on reproductive and productive performance of lactating dairy cows. J. Dairy Sci. **80:** 3386-3398.

Burns, P. D., White, N. R., Cheatham, R. D., Romero, R., Bruemmer, J. E. and Engle T. E. 2010. Effect of fish meal supplementation on bovine plasma and luteal omega-3 fatty acid content. Pages 46 - 47 In Proceedings of Society for the Study of Reproduction annual meeting., 43rd, Milwaukee, Wisconsin, July-August 2010.

Butler, W. R. 2003. Energy balance relationships with follicular development ovulation and fertility in postpartum dairy cows. Livest. Prod. Sci. **83:** 211-218.

Caldari-Torres, C., Lock, A. L., Staples, C. R. and Badinga, L. 2011. Performance, metabolic, and endocrine responses of periparturient Holstein cows fed 3 sources of fat. J. Dairy Sci. **94**: 1500-1510.

Canadian Council on Animal Care. 1993. A guide to the care and use of experimental animals. Vol. 1, 2nd ed. E.D. Olfert, B.M. Cross and A.A. McWilliam, eds. CCAC, Ottawa, ON.

Canadian Dairy Information Centre, 2008. Statistics of the Canadian Dairy Industry - Complete publication. Culling rate and replacement rate in dairy herds (canada) at 2008. Canadian dairy information centre. (Online). Available: http://www.dairyinfo.gc.ca/pdf/ publication_2008.pdf. (2009 December. 1).

Canadian Dairy Network, 2009. Canadian dairy network information articles. Trends in disposal reasons. Aug 2009. Canadian dairy network. (Online). Available: http://www. cdn. ca/articles.php (2009 August. 30).

Carriquiry, M., Dahlen, C. R., Weber, W. J., Lamb, G. C. and Crooker, B. A. 2009. Postpartum ovarian activity in multiparous Holstein cows treated with bovine somatotropin and fed n-3 fatty acids in early lactation. J. Dairy Sci. **92**: 4876-4888.

Carroll, D. J., Jerred, M. J., Grummer, R. R., Combs, D. K., Pierson, R. A. and Hauser, E. R. 1990. Effects of fat supplementation and immature alfalfa to concentrate ratio on plasma progesterone, energy balance and reproductive traits of dairy cattle. Journal of dairy science. J. Dairy Sci. **73**: 2855-2863.

Carroll, D. J., Grummer, R. R. and Clayton, M. K. 1992. Stimulation of luteal cell progesterone production by lipoproteins from cows fed control or fat-supplemented diets. Journal of dairy science. J. Dairy Sci. **75**: 2205-2214.

Chagas, L. M., Bass, J. J., Blache, D., Burke, C. R., Kay, J. K., Lindsay, D. R., Lucy, M. C., Martin, G. B., Meier, S., Rhodes, F. M., Roche, J. R., Thatcher, W. W. and Webb, R. 2007. Invited review: New perspectives on the roles of nutrition and metabolic priorities in the subfertility of high-producing dairy cows. J. Dairy Sci. **90:** 4022-4032.

Cheng, Z., Robinson, R. S., Pushpakumara, P. G., Mansbridge, R. J. and Wathes, D. C. 2001. Effect of dietary polyunsaturated fatty acids on uterine prostaglandin synthesis in the cow. J. Endocrinol. **171:** 463-473.

Childs, S., Sreenan, J. M., Diskin, M. G., Kenny, D. A., Wathes, D. C., Lynch, C. O., Hennessy, A. A. and Stanton, C. 2008. Effect of dietary enrichment with either n-3 or n-6 fatty acids on systemic metabolite and hormone concentration and ovarian function in heifers. Animal **2**: 883-893.

Danet-Desnoyers, G., Meyer, M. D., Gross, T. S., Johnson, J. W. and Thatcher, W. W. 1995. Regulation of endometrial prostaglandin synthesis during early pregnancy in cattle: effects of phospholipases and calcium in vitro. Prostaglandins **50:** 313-330.

Del Vecchio, R. P., Sutherland, W. D. and Connor, M. L. 1995. A solidphase enzyme immunoassay for the determination of progesterone in bovine, ovine and porcine plasma. Can. J. Anim. Sci. **75:** 525–529.

De Rensis, F. and Peters, A. R. 1999. The control of follicular dynamics by $PGF_{2\alpha}$, GnRH, hCG and oestrus synchronization in cattle. Reprod. Domest. Anim. **34:** 49–59.

De Swaaf, M. E., Sijtsma, L. and Pronk, J. T. 2003. High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga Crypthecodinium cohnii. Biotechnol. Bioeng. **81:** 666-672.

de Veth, M. J., Bauman, D. E., Koch, W., Mann, G. E., Pfeiffer, A. M. and Butler, W. R. 2009. Efficacy of conjugated linoleic acid for improving reproduction: a multi-study analysis in early-lactation dairy cows. J. Dairy Sci. **92:** 2662-2669.

De Vries, M. J. and Veerkamp, R. F. 2000. Energy balance of dairy cattle in relation to milk production variables and fertility. J. Dairy Sci. **83:** 62-69.

Dias, F. C., Colazo, M. G., Kastelic, J. P., Mapletoft, R. J., Adams, G. P. and Singh, J. 2010. Progesterone concentration, estradiol pretreatment, and dose of gonadotropin-releasing hormone affect gonadotropin-releasing hormone-mediated luteinizing hormone release in beef heifers. Domest. Anim. Endocrinol. **39:** 155-162.

Diaz, F. J., Anderson, L. E., Wu, Y. L., Rabot, A., Tsai, S. J. and Wiltbank, M. C. 2002. Regulation of progesterone and prostaglandin F2alpha production in the CL. Mol. Cell. Endocrinol. **191:** 65-80.

Diskin, M. G. and Morris, D. G. 2008. Embryonic and early foetal losses in cattle and other ruminants. Reprod. Domest. Anim. **43 Suppl 2:** 260-267.

Donovan, D. C., Schingoethe, D. J., Baer, R. J., Ryali, J., Hippen, A. R. and Franklin, S. T. 2000. Influence of dietary fish oil on conjugated linoleic acid and other fatty acids in milk fat from lactating dairy cows. J. of Dairy Sci. **83:** 2620-2628.

Dow, M. P., Bakke, L. J., Cassar, C. A., Peters, M. W., Pursley, J. R. and Smith, G. W. 2002. Gonadotropin surge-induced up-regulation of the plasminogen activators (tissue plasminogen activator and urokinase plasminogen activator) and the urokinase plasminogen activator receptor within bovine periovulatory follicular and luteal tissue. Biol. Reprod. **66:** 1413-1421.

Dransfield, M.B.G., Nebel, R.L., Pearson, R.E. and Warnick L.D. 1998. Timing of insemination for dairy cows identified in estrus by a radiotelemetric estrus detection system. J. Dairy Sci. **81:** 1874-1882.

Driancourt, M. A. 2001. Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. Theriogenology **55:** 1211-1239.

Espinoza, J. L., Ramirez-Godinez, J. A., Jimenez, J. A. and Flores, A. 1995. Effects of calcium soaps of fatty acids on postpartum reproductive activity in beef cows and growth of calves. J. Anim. Sci. **73**: 2888-2892.

Espinoza-Villvicencio, J. L., Ortego-Perez, R., Palacios-Espinosa, A. and Guillen-Trujillo, A. 2010. Effect of fat supplementation on production characteristics, pregancy

rates and some lipid metabolites in grazing beef cows. Arch. Vet. Med. **42:** 25-32. (in spanish, English abstract).

Filion, F., Bouchard, N., Goff, A. K., Lussier, J. G. and Sirois, J. 2001. Molecular cloning and induction of bovine prostaglandin E synthase by gonadotropins in ovarian follicles prior to ovulation in vivo. J. Biol. Chem. **276:** 34323-34330.

Folch, J., Lees, M. and Sloane Stanley, G. H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem, **226:** 497-509.

Forde, N., Beltman, M. E., Lonergan, P., Diskin, M., Roche, J. F. and Crowe, M. A. 2011. Oestrous cycles in Bos taurus cattle. Anim. Reprod. Sci. **124:** 163-169.

Fortune, J. E., Cushman, R. A., Wahl, C. M. and Kito, S. 2000. The primordial to primary follicle transition. Mol. Cell. Endocrinol. **163:** 53-60.

Franklin, S. T., Martin, K. R., Baer, R. J., Schingoethe, D. J. and Hippen, A. R. 1999. Dietary marine algae (Schizochytrium sp.) increases concentrations of conjugated linoleic, docosahexaenoic and transvaccenic acids in milk of dairy cows. J. Nutr. **129:** 2048-2054.

Fricke, P. M. 2002. Scanning the future--ultrasonography as a reproductive management tool for dairy cattle. J. Dairy Sci. **85:** 1918-1926.

Gagnon, N., Cortes, C., da Silva, D., Kazama, R., Benchaar, C., dos Santos, G., Zeoula, L. and Petit, H. V. 2009. Ruminal metabolism of flaxseed (*Linum sitatissimum*) lignans to the mammalian lignan enterolactone and its concentration in ruminal fluid, plasma, urine and milk of dairy cows. Br. J. Nutr. **102:** 1015-1023.

Garcia-Bojalil, C. M., Staples, C. R., Risco, C. A., Savio, J. D. and Thatcher, W. W. 1998. Protein degradability and calcium salts of long-chain fatty acids in the diets of lactating dairy cows: reproductive responses. J. Dairy Sci. **81:** 1385-1395.

Garcia-Bojalil, C. M. 1993. Reproductive, productive, and immunological responses by Holstein dairy cows fed diets varying in concentration and ruminal degradability of protein and supplemented with ruminally-inert fat. Ph.D. Diss., Univ.Florida, Gainesville.

Garnsworthy, P. C., Lock, A., Mann, G. E., Sinclair, K. D. and Webb, R. 2008. Nutrition, metabolism, and fertility in dairy cows: 1. dietary energy source and ovarian function. J. Dairy Sci. **91**: 3814-3823.

Gibbons, J. R., Wiltbank, M. C. and Ginther, O. J. 1999. Relationship between follicular development and the decline in the follicle-stimulating hormone surge in heifers. Biol. Reprod. **60:** 72-77.

Ginther, O. J., Knopf, L. and Kastelic, J. P. 1989. Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. J. Reprod. Fertil. **87:** 223-230.

Ginther, O. J. 1993. A method for characterizing ultrasonically-derived follicular data in heifers. Theriogenology **39:** 363-371.

Ginther, O. J. 1995. Ultrasonic imaging and animal reproduction: fundamentals. Equiservices Publishing, Cross Plains, Wisconsin.

Ginther, O. J., Wiltbank, M. C., Fricke, P. M., Gibbons, J. R. and Kot, K. 1996. Selection of the dominant follicle in cattle. Biol. Reprod. **55:** 1187-1194.

Ginther, O. J., Bergfelt, D. R., Kulick, L. J. and Kot, K. 2000. Selection of the dominant follicle in cattle: role of estradiol. Biol. Reprod. **63:** 383-389.

Gonthier, C., Mustafa, A. F., Ouellet, D. R., Chouinard, P. Y., Berthiaume, R. and Petit, H. V. 2005. Feeding micronized and extruded flaxseed to dairy cows: effects on blood parameters and milk fatty acid composition. J. Dairy Sci. **88:** 748-756.

Gordon, I. 1996. Controlled reproduction in cattle and buffaloes. Vol 1. CAB International. Wellingford, United Kingdom.

Graham, L., Schwarzenberger, F., Möstl, E., Galama, W. and Savage, A. 2001. A versatile enzyme immunoassay for the determination of progestogens in feces and serum. Zoo Biology **20**: 227-236.

Griffin, P. G. and Ginther, O. J. 1992. Research applications of ultrasonic imaging in reproductive biology. J. Anim. Sci. **70:** 953-972.

Grummer, R. R. and Carroll, D. J. 1991. Effects of dietary fat on metabolic disorders and reproductive performance of dairy cattle. J. Anim. Sci. **69:** 3838-3852.

Hawkins, D. E., Niswender, K. D., Oss, G. M., Moeller, C. L., Odde, K. G., Sawyer, H. R. and Niswender, G. D. 1995. An increase in serum lipids increases luteal lipid content and alters the disappearance rate of progesterone in cows. J. Anim. Sci. **73:** 541-545.

Hayashi, K., Acosta, T. J., Berisha, B., Kobayashi, S., Ohtani, M., Schams, D. and Miyamoto, A. 2003. Changes in prostaglandin secretion by the regressing bovine corpus luteum. Prostaglandins Other Lipid Mediators. **70:** 339-349.

Hightshoe, R. B., Cochran, R. C., Corah, L. R., Kiracofe, G. H., Harmon, D. L. and Perry, R. C. 1991. Effects of calcium soaps of fatty acids on postpartum reproductive function in beef cows. J. Anim. Sci. **69:** 4097-4103.

Horan, B., Mee, J. F., O'Connor, P., Rath, M. and Dillon, P. 2005. The effect of strain of holstein-friesian cow and feeding system on postpartum ovarian function, animal production and conception rate to first service. Theriogenology **63**: 950-971.

Hoyt, J. M., Fredeen, A. F., Glover, K., Enright, C. and MacLaren, L. A. 2007. Marine microalgae effects on endometrial expression of key molecules in pregnant dairy cows. Biol. Reprod. **77**: 103.

Jenkins, T. C. 1993. Lipid metabolism in the rumen. J. Dairy Sci. **76**: 3851-3863.

Jenkins, T. C. 1994. Regulation of lipid metabolism in the rumen. J. Nutr. **124:** 1372S-1376G.

Jenkins, T. C. and Palmquist, D. L. 1984. Effect of fatty acids or calcium soaps on rumen and total nutrient digestibility of dairy rations. J. Dairy Sci. **67:** 978-986.

Juchem, S. O., Cerri, R. L., Villasenor, M., Galvao, K. N., Bruno, R. G., Rutigliano, H. M., DePeters, E. J., Silvestre, F. T., Thatcher, W. W. and Santos, J. E. 2010. Supplementation with calcium salts of linoleic and trans-octadecenoic acids improves fertility of lactating dairy cows. Reprod. Domest. Anim. **45**: 55-62.

Kengaku, K., Tanaka, T. and Kamomae, H. 2007. Changes in the peripheral concentrations of inhibin, follicle-stimulating hormone, luteinizing hormone, progesterone and estradiol-17 β during turnover of cystic follicles in dairy cows with spontaneous follicular cysts. J. Reprod. Dev. **53:** 987-993.

Kennelly, J. J. 1996. The fatty acid composition of milk fat as influenced by feeding oilseeds. Anim. Feed Sci. Technol. **60**:137–152.

Knickerbocker, J. J., Thatcher, W. W., Foster, D. B., Wolfenson, D., Bartol, F. F. and Caton, D. 1986. Uterine prostaglandin and blood flow responses to estradiol-17 beta in cyclic cattle. Prostaglandins **31:** 757-776.

Lammoglia, M. A., Willard, S. T., Oldham, J. R. and Randel, R. D. 1996. Effects of dietary fat and season on steroid hormonal profiles before parturition and on hormonal, cholesterol, triglycerides, follicular patterns, and postpartum reproduction in Brahman cows. J. Anim. Sci. 74:2253–2262.

Lammoglia, M. A., Bellows, R. A., Grings, E. E., Bergman, J. W., Short, R. E. and MacNeil, M. D. 1999. Effects of feeding beef females supplemental fat during gestation on cold tolerance in newborn calves. J. Anim. Sci. **77:** 824-834.

Lucy, M. C., Staples, C. R., Michel, F. M. and Thatcher, W. W. 1991a. Energy balance and size and number of ovarian follicles detected by ultrasonography in early postpartum dairy cows. J. Dairy Sci. **74**: 473-482.

Lucy, M. C., Staples, C. R., Michel, F. M., Thatcher, W. W. and Bolt, D. J. 1991b. Effect of feeding calcium soaps to early postpartum dairy cows on plasma prostaglandin F2 alpha, luteinizing hormone, and follicular growth. J. Dairy Sci. **74**: 483-489.

Lucy, M. C., De La Sota, R. L., Staples, C. R. and Thatcher, W. W. 1993. Ovarian follicular populations in lactating dairy cows treated with recombinant bovine somatotropin (sometribove) or saline and fed diets differing in fat content and energy. J. Dairy Sci. **76:** 1014-1027.

Lucy, M. C. 2001. Reproductive loss in high-producing dairy cattle: where will it end? J. Dairy Sci. **84:** 1277-1293.

Lucy, M. C. 2007a. The bovine dominant ovarian follicle. J. Anim. Sci. **85:** 89-99.

Lucy, M. C. 2007b. Fertility in high-producing dairy cows: reasons for decline and corrective strategies for sustainable improvement. Soc. Reprod. Fertil. Suppl. **64:** 237-254.

Lucy, M. C., Savio, J. D., Badinga, L., De La Sota, R. L. and Thatcher, W. W. 1992. Factors that affect ovarian follicular dynamics in cattle. J. Anim. Sci. **70:** 3615-3626.

MacLaren, L. A., Guzeloglu, A., Michel, F. and Thatcher, W. W. 2006. Peroxisome proliferator-activated receptor (PPAR) expression in cultured bovine endometrial cells and response to omega-3 fatty acid, growth hormone and agonist stimulation in relation to series 2 prostaglandin production. Domest. Anim. Endocrinol. **30**: 155-169.

Macmillan, K. L. 2010. Recent advances in the synchronization of estrus and ovulation in dairy cows. J. Reprod. Dev. **56:** S42-S47.

Mann, G. E. 2009. Corpus luteum size and plasma progesterone concentration in cows. Anim. Reprod. Sci. **115:** 296-299.

Mapletoft, R. J., Steward, K. B. and Adams, G. P. 2002. Recent advances in the superovulation in cattle. Reprod. Nutr. Dev. **42:** 601-611.

Marín-Aguilar, M.A., Tinoco-Magaña, J.C., Herrera-Camacho, J., Sánchez-Gil, L.G., Sánchez-Parra, V.M., Solorio-Rivera, J.L. and García-Valladares, A. 2007. Resumption of ovarian activity and level of lipid metabolites in dairy cows supplemented with vegetable oil during the early postpartum. Interciencia. **32:** 180-184. (in spanish, English abstract).

Mattos, R., Staples, C. R. and Thatcher, W. W. 2000. Effects of dietary fatty acids on reproduction in ruminants. Rev. Reprod. **5:** 38-45.

Mattos, R., Staples, C. R., Williams, J., Amorocho, A., McGuire, M. A. and Thatcher, W. W. 2002. Uterine, ovarian, and production responses of lactating dairy

cows to increasing dietary concentrations of menhaden fish meal. J. Dairy Sci. **85:** 755-764.

Mattos, R., Guzeloglu, A., Badinga, L., Staples, C. R. and Thatcher, W. W. 2003. Polyunsaturated fatty acids and bovine interferon-tau modify phorbol ester-induced secretion of prostaglandin F2 alpha and expression of prostaglandin endoperoxide synthase-2 and phospholipase-A2 in bovine endometrial cells. Biol. Reprod. **69:** 780-787.

Mattos, R., Staples, C. R., Arteche, A., Wiltbank, M. C., Diaz, F. J., Jenkins, T. C. and Thatcher, W. W. 2004. The effects of feeding fish oil on uterine secretion of PGF2alpha, milk composition, and metabolic status of periparturient Holstein cows. J. Dairy Sci. **87:** 921-932.

McCartney, C. R., Blank, S. K. and Marshall, J. C. 2007. Progesterone acutely increases LH pulse amplitude but does not acutely influence nocturnal LH pulse frequency slowing during the late follicular phase in women. Am. J. Physiol. Endocrinol. Metab. **292:** 900-906.

McGee, E. A. and Hsueh, A. J. 2000. Initial and cyclic recruitment of ovarian follicles. Endocr. Rev. **21:** 200-214.

Meireles, L. A., Guedes, A. C. and Malcata, F. X. 2003. Lipid class composition of the microalga *Pavlova lutheri*: eicosapentaenoic and docosahexaenoic acids. J. Agric. Food Chem. **51:** 2237-2241.

Mihm, M. and Austin, E. J. 2002. The final stages of dominant follicle selection in cattle. Domest. Anim. Endocrinol. **23:** 155-166.

Mihm, M., Baguisi, A., Boland, M. P. and Roche, J. F. 1994. Association between the duration of dominance of the ovulatory follicle and pregnancy rate in beef heifers. J. Reprod. Fertil. **102:** 123-130.

Mihm, M., Baker, P. J., Ireland, J. L., Smith, G. W., Coussens, P. M., Evans, A. C. and Ireland, J. J. 2006. Molecular evidence that growth of dominant follicles involves a reduction in follicle-stimulating hormone dependence and an increase in luteinizing hormone dependence in cattle. Biol. Reprod. **74:** 1051-1059.

Mihm, M., Crowe, M. A., Knight, P. G. and Austin, E. J. 2002. Follicle wave growth in cattle. Reprod. Domest. Anim. **37:** 191-200.

Moallem, U., Folman, Y., Bor, A., Arav, A., Sklan, D. 1999. Effect of calcium soaps of fatty acids and administration of somatotropin on milk production, preovulatory follicular development, and plasma and follicular fluid lipid composition in high yielding dairy cows. J. Dairy Sci. **82**: 2358-2368.

Moallem, U. 2009. The effects of extruded flaxseed supplementation to high-yielding dairy cows on milk production and milk fatty acid composition. Anim. Feed Sci. Technol. **152:** 232-242.

Moghadasian, M. H. 2008. Advances in dietary enrichment with n-3 fatty acids. Crit. Rev. Food Sci. Nutr. **48:** 402-410.

Moore, K. and Thatcher, W. W. 2006. Major advances associated with reproduction in dairy cattle. J. Dairy Sci. **89**: 1254-1266.

Motta, M., Piva, F. and Martini, L. 1973. New findings on the central control of gonadotropin secretion. J. Reprod. Fertil. Suppl. **20:** 27-52.

Moussavi, A. R., Gilbert, R. O., Overton, T. R., Bauman, D. E. and Butler, W. R. 2007a. Effects of feeding fish meal and n-3 fatty acids on milk yield and metabolic responses in early lactating dairy cows. J. Dairy Sci. **90:** 136-144.

Moussavi, A. R. H., Gilbert, R. O., Overton, T. R., Bauman, D. E. and Butler, W. R. 2007b. Effects of feeding fish meal and n-3 fatty acids on ovarian and uterine responses in early lactating dairy cows. J. Dairy Sci. **90:** 145-154.

Munro C. J. and Stabenfeldt G. H. 1984. Development of a microtitre plate enzyme immunoassay for the determination of progesterone. J Endocr **101**:41–49.

Nafikov, R. A. and Beitz, D. C. 2007. Carbohydrate and lipid metabolism in farm animals. J. Nutr. **137:** 702-705.

Nebel, R. L. and McGilliard, M. L. 1993. Interactions of high milk yield and reproductive performance in dairy cows. J. Dairy Sci. **76:** 3257-3268.

Niswender, G. D., Schwall, R. H., Fitz, T. A., Farin, C. E. and Sawyer, H. R. 1985. Regulation of luteal function in domestic ruminants: new concepts. Recent Prog. Horm. Res. **41:** 101-151.

Niswender, G. D., Juengel, J. L., McGuire, W. J., Belfiore, C. J. and Wiltbank, M. C. 1994. Luteal function: the estrous cycle and early pregnancy. Biol. Reprod. **50:** 239-247.

Niswender, G. D., Juengel, J. L., Silva, P. J., Rollyson, M. K. and McIntush, E. W. 2000. Mechanisms controlling the function and life span of the corpus luteum. Physiol. Rev. **80:** 1-29.

Niswender, G. D. 2002. Molecular control of luteal secretion of progesterone. Reproduction **123:** 333-339.

Odde, K. G. 1990. A review of synchronization of estrus in postpartum cattle. J. Anim. Sci. **68:** 817-830.

Oldick, B. S., Staples, C. R., Thatcher, W. W. and Gyawu, P. 1997. Abomasal Infusion of Glucose and Fat - Effect on Digestion, Production, and Ovarian and Uterine Functions of Cows. J. Dairy Sci. **80:** 1315-1328.

O'Shea, J. D., Rodgers, R. J. and D'Occhio, M. J. 1989. Cellular composition of the cyclic corpus luteum of the cow. J. Reprod. Fertil. **85:** 483-487.

Palmer, M. A., Olmos, G., Boyle, L. A. and Mee, J. F. 2010. Estrus detection and estrus characteristics in housed and pastured Holstein-Friesian cows. Theriogenology **74:** 255-264.

Palmquist, D. L., Jenkins, T. C. and Joyner, A. E., Jr. 1986. Effect of dietary fat and calcium source on insoluble soap formation in the rumen. J. Dairy Sci. **69**: 1020-1025.

Pereira, S. L., Leonard, A. E., Huang, Y. S., Chuang, L. T. and Mukerji, P. 2004. Identification of two novel microalgal enzymes involved in the conversion of the omega3-fatty acid, eicosapentaenoic acid, into docosahexaenoic acid. Biochem. J. **384:** 357-366.

Petit, H. V., Dewhurst, R. J., Scollan, N. D., Proulx, J. G., Khalid, M., Haresign, W., Twagiramungu, H. and Mann, G. E. 2002. Milk production and composition, ovarian function, and prostaglandin secretion of dairy cows fed omega-3 fats. J. Dairy Sci. **85:** 889-899.

Petit, H. V. 2003. Digestion, milk production, milk composition, and blood composition of dairy cows fed formaldehyde treated flaxseed or sunflower seed. J. Dairy Sci. **86:** 2637-2646.

Petit, H. V. and Twagiramungu, H. 2006. Conception rate and reproductive function of dairy cows fed different fat sources. Theriogenology **66:** 1316-1324.

Pierson, R. A. and Adams, G. P. 1995. Computer-assisted image analysis, diagnostic ultrasonography and ovulation induction: Strange bedfellows. Theriogenology **43:** 105-112.

Pierson, R. A. and Ginther, O. J. 1988. Ultrasonic imaging of the ovaries and uterus in cattle. Theriogenology **29:** 21-37.

Ponter, A. A., Parsy, A., Saadé, M., Mialot, J., Ficheux, C., Duvaux-Ponter, C. and Grimard, B. 2006. Effect of a supplement rich in linolenic acid added to the diet of post partum dairy cows on ovarian follicle growth, and milk and plasma fatty acid compositions. Reproduction Nutrition Development **46:** 19-29.

Pursley, J. R., Mee, M. O. and Wiltbank, M. C. 1995. Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. Theriogenology **44:** 915-923.

Rajamahendran, R., Burton, B. and Shelford, J. 1993. A field study on the usefulness of milk progesterone determination to confirm estrus and pregnancy of dairy cows in the Fraser Valley area of British Columbia. Can. Vet. J. **34:** 349-352.

Rajamahendran, R., Robinson, J., Desbottes, S. and Walton, J. S. 1989. Temporal relationships among estrus, body temperature, milk yield, progesterone and luteinizing hormone levels, and ovulation in dairy cows. Theriogenology **31:** 1173-1182.

Reames, P. S., Hatler, T. B., Hayes, S. H., Ray, D. L. and Silvia, W. J. 2011. Differential regulation of estrous behavior and luteinizing hormone secretion by estradiol-17β in ovariectomized dairy cows. Theriogenology **75:** 233-240.

Ringbom, T., Huss, U., Stenholm, A., Flock, S., Skattebol, L., Perera, P. and Bohlin, L. 2001. Cox-2 inhibitory effects of naturally occurring and modified fatty acids. J. Nat. Prod. **64:** 745-749.

Robinson, R. S., Pushpakumara, P. G., Cheng, Z., Peters, A. R., Abayasekara, D. R. and Wathes, D. C. 2002. Effects of dietary polyunsaturated fatty acids on ovarian and uterine function in lactating dairy cows. Reproduction **124:** 119-131.

Roche, J. F., Crowe, M. A. and Boland, M. P. 1992. Postpartum anoestrus in dairy and beef cows. Anim. Reprod. Sci. **28:** 371-378.

Royal, M. D., Darwash, A. O., Flint, A. P. F., Webb, R., Woolliams, J. A. and Lamming, G. E. 2000. Declining fertility in dairy cattle: Changes in traditional and endocrine parameters of fertility. Anim. Sci. **70:** 487-502.

Ruegg, P. L. and Milton, R. L. 1995. Body condition scores of Hostein cows on Prince Edward Island, Canada: relationships with yield, reproductive performace, and disease. J. Dairy Sci. **78:** 552-564.

Ruxton, C. 2004. Health benefits of omega-3 fatty acids. Nurs. Stand. **18:** 38-42.

Ruxton, C. H., Reed, S. C., Simpson, M. J. and Millington, K. J. 2004. The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. J. Hum. Nutr. Diet. **17:** 449-459.

Ryan, D. P., Snijders, S., Condon, T., Grealy, M., Sreenan, J. and O'Farrell, K. J. 1993. Endocrine and ovarian responses and pregnancy rates in dairy cows following the administration of a gonadotrophin releasing hormone analog at the time of artificial insemination or at mid-cycle post insemination. Anim. Reprod. Sci. **34:** 179-191.

Ryan, D. P., Spoon, R. A. and Williams, G. L. 1992. Ovarian follicular characteristics, embryo recovery, and embryo viability in heifers fed high-fat diets and treated with follicle-stimulating hormone. J. Anim. Sci. **70:** 3505-3513.

Sakaguchi, M. 2011. Practical aspects of the fertility of dairy cattle. J. Reprod. Dev. **57:** 17-33.

Sakaguchi, M., Sasamoto, Y., Suzuki, T., Takahashi, Y. and Yamada, Y. 2004. Postpartum ovarian follicular dynamics and estrous activity in lactating dairy cows. J. Dairy Sci. **87:** 2114-2121.

Santos, J. E., Bilby, T. R., Thatcher, W. W., Staples, C. R. and Silvestre, F. T. 2008. Long chain fatty acids of diet as factors influencing reproduction in cattle. Pages 23-30 Reproduction in Domestic Animals. Proceedings of the 16th International Congress on Animal Reproduction; in Budapest; Hungary; July 2008.

Sargent, J. R. 1997. Fish oils and human diet. Br. J. Nutr. **78 Suppl 1:** S5-13.

Sartori, R., Fricke, P. M., Ferreira, J. C., Ginther, O. J. and Wiltbank, M. C. 2001. Follicular deviation and acquisition of ovulatory capacity in bovine follicles. Biol. Reprod. **65:** 1403-1409.

Sartori, R., Rosa, G. J. M., Wiltbank, M. C. 2002. Ovarian structures and circulating steroids in heifers and lactating cows in summer and lactating and dry cows in winter. Journal of Dairy Science. J. Dairy Sci. **85**: 2813-2822.

Sartori, R., Haughian, J. M., Shaver, R. D., Rosa, G. J. M. and Wiltbank, M. C. 2004. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. J. Dairy Sci. **87:** 905-920.

SAS Institute. 2003. SAS Statistics Users Guide, Statistical Analysis System, 9.1 version, Carry, NC, SAS Institute Inc.

Sayanova, O. and Napier, J. A. 2011. Transgenic oilseed crops as an alternative to fish oils. Prostaglandins, Leukotrienes and Essential Fatty Acids **85:** 253-260.

Schallenberger, E. and Prokopp, S. 1985. Gonadotrophins and ovarian steroids in cattle. IV. Re-establishment of the stimulatory feedback action of oestradiol-17β on LH and FSH. Acta Endocrinol. **109:** 44-49.

Schams, D. and Berisha, B. 2002. Steroids as local regulators of ovarian activity in domestic animals. Domest. Anim. Endocrinol. **23:** 53-65.

Schams, D. and Berisha, B. 2004. Regulation of corpus luteum function in cattle-an overview. Reprod. Domest. Anim. **39:** 241-251.

Scott, T. A., Shaver, R. D., Zepeda, L., Yandell, B. and Smith, T. R. 1995. Effects of rumen-inert fat on lactation, reproduction, and health of high producing Holstein herds. J. Dairy Sci. **78**: 2435-2451.

Senger, P. L. 2003. Pathways to pregnancy and parturition. 2nd ed. Current Conceptions, Inc. Pullman, Washington.

Sijtsma, L. and de Swaaf, M. E. 2004. Biotechnological production and applications of the omega-3 polyunsaturated fatty acid docosahexaenoic acid. Appl. Microbiol. Biotechnol. **64:** 146-153.

Silvestre, F. T., Carvalho, T. S. M., Francisco, N., Santos, J. E. P., Staples, C. R., Jenkins, T. C. and Thatcher, W. 2011. Effects of differential supplementation of fatty acids during the peripartum and breeding periods of Holstein cows: I. uterine and metabolic responses, reproduction, and lactation. J. Dairy Sci. **94**: 189-204.

Silvia, W. J., Lewis, G. S., McCracken, J. A., Thatcher, W. W. and Wilson, L. J. 1991. Hormonal regulation of uterine secretion of prostaglandin F$_{2\alpha}$ during luteolysis in ruminants. Biol. Reprod. **45:** 655-663.

Singh, J., Adams, G. P. and Pierson, R. A. 2003. Promise of new imaging technologies for assessing ovarian function. Anim. Reprod. Sci. **78:** 371-399.

Skarzynski, D. J., Ferreira-Dias, G. and Okuda, K. 2008. Regulation of luteal function and corpus luteum regression in cows: hormonal control, immune mechanisms and intercellular communication. Pages 57-65 Reproduction in Domestic Animals. Proceedings of the 16th International Congress on Animal Reproduction; in Budapest; Hungary; July 2008. International Congress on Animal Reproduction.

Sklan, D., Moallem, U. and Folman, Y. 1991. Effect of feeding calcium soaps of fatty acids on production and reproductive responses in high producing lactating cows. J. Dairy Sci. **74:** 510-517.

Smith, M. F., McIntush, E. W. and Smith, G. W. 1994. Mechanisms associated with corpus luteum development. J. Anim. Sci. **72:** 1857-1872.

Son, J., Grant, R. J. and Larson, L. L. 1996. Effects of tallow and escape protein on lactational and reproductive performance of dairy cows. J. Dairy Sci. **79:** 822-830.

Spicer, L. J., Vernon, R. K., Tucker, W. B., Wettemann, R. P., Hogue, J. F. and Adams, G. D. 1993. Effects of inert fat on energy balance, plasma concentrations of hormones, and reproduction in dairy cows. J. Dairy Sci. **76**: 2664-2673.

Staples, C. R., Burke, J. M. and Thatcher, W. W. 1998. Influence of supplemental fats on reproductive tissues and performance of lactating cows. J. Dairy Sci. **81:** 856-871.

Staples, C. R., Wiltbank, M. C., Grummer, R. R., Guenther, J. Sartori, R. Diaz, F. J., Bertics, S., Mattos, R. and Thatcher, W. W. 2000: Effect of long chain fatty acids on lactation performance and reproductive tissues of Holstein cows. J. Dairy Sci. **83**(Suppl. 1), 278 (Abstr.)

Staples, C.R. and Thatcher, W.W. 2005. Effects of fatty acids on reproduction of dairy cows. In: Garnsworthy PC, Wiseman, J. (Eds.), Recent Advances in Animal Nutrition. Nottingham University Press, Nottingham, UK, 229-256 p.

Surette, M. E. 2008. The science behind dietary omega-3 fatty acids. Canadian Medical Association Journal **178:** 177-180.

Talavera, F., Park, C. S. and Williams, G. L. 1985. Relationships among dietary lipid intake, serum cholesterol and ovarian function in holstein heifers. J. Anim. Sci. **60**: 1045-1051.

Tanaka, T., Arai, M., Ohtani, S., Uemura, S., Kuroiwa, T., Kim, S. and Kamomae, H. 2008. Influence of parity on follicular dynamics and resumption of ovarian cycle in postpartum dairy cows. Anim. Reprod. Sci. **108**: 134-143.

Thatcher, W. W., Bilby, T. R., Bartolome, J. A., Silvestre, F., Staples, C. R. and Santos, J. E. 2006. Strategies for improving fertility in the modern dairy cow. Theriogenology **65**: 30-44.

Thompson, L. U., Robb, P., Serraino, M. and Cheung, F. 1991. Mammalian lignan production from various foods. Nutr. Cancer **16**: 43-52.

Townson, D. H., Tsang, P. C. W., Butler, W. R., Frajblat, M., Griel Jr., L. C., Johnson, C. J., Milvae, R. A., Niksic, G. M. and Pate, J. L. 2002. Relationship of fertility to ovarian follicular waves before breeding in dairy cows. J. Anim. Sci. **80:** 1053-1058.

Vanholder, T., Opsomer, G. and De Kruif, A. 2006. Aetiology and pathogenesis of cystic ovarian follicles in dairy cattle: A review. Reprod. Nutr. Dev. **46:** 105-119.

van Vliet, T. and Katan, M. B. 1990. Lower ratio of n-3 to n-6 fatty acids in cultured than in wild fish. Am. J. Clin. Nutr. **51:** 1-2.

Walker, G. P., Dunshea, F. R. and Doyle, P. T. 2004. Effects of nutrition and management on the production and composition of milk fat and protein: A review. Australian Journal of Agricultural Research. Aust. J. Agric. Res. **55**: 1009-1028.

Walker, S.L., Smith, R.F., Jones, D.N., Routly, J.E. and Dobson, H. 2008. Chronic stress, hormone profiles and estrus intensity in dairy cattle. Hormones and Behavior. **53:** 493-501.

Walsh, S. W., Williams, E. J. and Evans, A. C. 2011. A review of the causes of poor fertility in high milk producing dairy cows. Anim. Reprod. Sci. **123:** 127-138.

Wang, C., Harris, W. S., Chung, M., Lichtenstein, A. H., Balk, E. M., Kupelnick, B., Jordan, H. S. and Lau, J. 2006. n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. Am. J. Clin. Nutr. **84:** 5-17.

Wathes, D. C., Abayasekara, D. R. and Aitken, R. J. 2007. Polyunsaturated fatty acids in male and female reproduction. Biol. Reprod. **77:** 190-201.

Weber, P. C. and Sellmayer, A. 1991. Modification of the eicosanoid system and cell signalling by precursor fatty acids. Adv. Prostaglandin Thromboxane Leukot. Res. **21:** 217-224.

White, N. R., Burns, P. D., Charumilinda, J., Bryant, A. D., Prosser, Z. T., Bruemmer, J. E. and Engle, T. E. 2010. Effect of omega-3 fatty acids on prostaglandin F2alpha-induced cyclooxygenase-2 (Cox-2) gene expression in bovine luteal cells in vitro. Page 46 In Proceedings of the Society for Study of Reproduction annual meeting., 43rd, Milwaukee, Wisconsin, July-August 2010.

Wiltbank, M. C., Gumen, A. and Sartori, R. 2002. Physiological classification of anovulatory conditions in cattle. Theriogenology **57:** 21-52.

Zachut, M., Arieli, A., Lehrer, H., Argov, N. and Moallem, U. 2008. Dietary unsaturated fatty acids influence preovulatory follicle characteristics in dairy cows. Reproduction **135**: 683-692.

Zachut, M., Dekel, I., Lehrer, H., Arieli, A., Arav, A., Livshitz, L., Yakoby, S. and Moallem, U. 2010. Effects of dietary fats differing in n-6:n-3 ratio fed to high-yielding dairy cows on fatty acid composition of ovarian compartments, follicular status, and oocyte quality. J. Dairy Sci. **93:** 529-545.

Zachut, M., Arieli, A. and Moallem, U. 2011. Incorporation of dietary n-3 fatty acids into ovarian compartments in dairy cows and the effects on hormonal and behavioral patterns around estrus. Reproduction **141:** 833-840.