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Effects of dietary starch on ovarian physiology, intra-follicular milieu of the preovulatory follicle, and plasma metabolites in postpartum dairy cows

By

Elango Subramaniam

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EXAMINING COMMITTEE

Dr. Divakar Ambrose, Alberta Agriculture and Rural Development

Dr. Masahito Oba, Department of Agricultural, Food and Nutritional Science

Dr. Michael Dyck, Department of Agricultural, Food and Nutritional Science

Dr. John Chang, Department of Biological Sciences

DEDICATION

This thesis is dedicated to my parents for their support and encouragement

ABSTRACT

Effects of dietary starch content on ovarian dynamics in lactating dairy cows and intrafollicular milieu of preovulatory follicles were studied. Diets containing two levels of starch (29.2% and 19.1%) were fed until 84 d post-calving. Diets had no effect on the interval from calving-to-ovulation, but a greater proportion of cows on high starch diet ovulated two or more follicles at first ovulation. Cows consuming a high starch diet had higher concentrations of insulin in plasma, IGF-1 in follicular fluid, and lower concentrations of non-esterified fatty acids (NEFA) in both plasma and follicular fluid. Primiparous cows had higher concentrations of IGF-1 and NEFA, and lower concentrations of urea in plasma than multiparous cows. Reproductive hormones and gene expression in granulosa cells were affected neither by diet nor parity. Although a high starch diet increased insulin and IGF-1, and reduced NEFA, it did not hasten resumption of cyclicity in postpartum dairy cows.

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LIST OF ABBREVIATIONS

AI	artificial insemination
BCS	body condition score
BHBA	β-hydroxybutryate
BW	body weight
C	carbon
cDNA	complementary deoxy-ribonucleic acid
CH_4	methane
CG	corn grain
CGM	corn gluteal meal
CL	corpus luteum
CO_2	carbon-di-oxide
COC	cumulus oocyte complex
CP	crude protein
Ct	cycle threshold
CYP19	cytochrome p450 aromatase
d	day
DDGS	dry distiller's grain with soluble
dl	decilitre
DM	dry matter
DMI	dry matter intake
DIM	days in milk
FFC	follicular fluid collection
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
GC	granulosa cells
GH	growth hormone
GnRH	gonadotropin releasing hormone
GOI	gene of interest
GPX3	glutathione peroxidase 3
h	hour
HSD	high starch diet
IGF-I	insulin-like growth factor I
IU	international units
L	litre
LH	luteinizing hormone
LSD	low starch diet
min	minute
mEQ	milli-equivalents
ml	millilitre

mg	milligram
mRNA	messenger ribonucleic acid
MSD	medium starch diet
NEB	negative energy balance
NEFA	non-esterified fatty acids
NE_L	net energy for lactation
ng	nanogram
N_2O	nitrous oxide
PGF2α	prostaglandin F2α
PUN	plasma urea nitrogen
RT-PCR	reverse transcription-polymerase chain reaction
RUP	rumen undegradable protein
SAS	statistical analysis system
SBM	soybean meal
SEM	standard error of the mean
TAI	timed artificial insemination

Chapter 1:

General Introduction

For dairy cows to maintain continuous milk production cycles, it is necessary that parturition occurs each year. However it is often an arduous experience for the cow during the transition from pregnant, non-lactating state to non-pregnant, lactating state. During the period of pregnancy and lactation, the alterations in homeostasis pose a huge challenge to dairy cows (Goff and Horst., 1997).

In the past few decades, milk production has increased dramatically due to factors like genetic selection, improved nutrition and management (van Knegsel et al., 2005). However, there are some negative consequences such as increased incidence of metabolic diseases and poor fertility associated with this increase in milk production. The energy requirement for milk production and maintenance exceeds the feed intake during early lactation period leading to energy loss from the body (Jorritsma et al., 2003).

During negative energy balance (NEB), dairy cows adapt by obtaining the fuel for their body from lipid derived sources (Herdt, 2000; Vernon, 2002). NEB has been reported to impair immune function of dairy cows after calving (Goff and Horst, 1997). Beam and Butler (1999), Butler (2000) studied that NEB affects LH pulsatility and resumption of cyclicity after calving. Roche (2006) found that the dominant ovarian follicles of cows that were in NEB took longer time to increase the circulating estradiol concentrations needed for an ovulation to occur. Stevenson (2000) reported that the average interval from calving to first ovulation was 10 d longer in modern dairy cows and the percentage of anestrous cows was

considerably higher than seen in the past. The main reason for increased interval from calving to first ovulation was greater NEB found in modern dairy cows (Lucy, 2001).

In vitro studies from Spicer and Echternkamp (1995) showed that insulin and IGF-1 play important roles in follicular cell proliferation and steroidogenesis. The concentrations of insulin and IGF-1 are reported to decrease after calving (Beam and Butler, 1999) and the decline was linked to severe NEB and delayed calving-to-ovulation interval (Gong et al., 2002). In the same study, Gong et al. (2002) demonstrated that feeding a diet to induce propionate production in the rumen (26% starch) of post-partum dairy cows increased the proportion of cows ovulating before 50 d from 55 to 90% compared to the diet that induce rumen acetate production (10% starch). Armstrong et al. (2001) reported that the elevated concentration of insulin and IGF-1 in plasma was due to feeding cows with high energy (816 KJ/kg BW $^{0.75}$) diet than low energy diet (408 KJ/kg BW $^{0.75}$). A recent study showed that cows fed diets containing high starch (26.7%) had decreased number of days from calving to first ovulation (30.6 d vs. 38.1 d and 43.2 d) than those fed medium (25.2%) or low (23.3%) starch diets but the concentration of plasma insulin was not different between the diets (Dyck et al., 2011).

In the same study, 46% of cows on high-starch diet ovulated more than one follicle during first postpartum ovulation compared to 31% and 0% in medium and low starch diets, respectively. IGF-1 concentration in serum and follicular fluid was greater in cows selected for twins compared to that of control

cows (Echternkamp et al., 1990). In the recent study (Dyck et al., 2011), plasma IGF-1 concentrations were not different between the diets, even though HSD cows had more double first ovulations.

Even though the re-establishment of positive energy balance and correct endocrine signaling would trigger ovulation, reproduction success is not assured (Leroy et al., 2005). Negative energy balance would affect the ovarian follicles and oocyte, leading to ovulation of inferior oocyte (Leroy et al., 2005). This is because growing and maturing oocytes were affected by the biochemical changes occurring in the early post-partum period (Leroy et al., 2005). Serum concentrations of glucose, NEFA and urea were reflected in follicular fluid. They also suggested that higher glucose and lower NEFA concentrations in follicular fluid than serum may protect the oocyte and granulosa cells.

Therefore we hypothesized that feeding high starch diet would increase the concentrations of insulin and IGF-1 in follicular fluid and plasma, decrease the interval from calving to first ovulation and increase the number of cows ovulating two or more follicles.

The main objective of the first study (Chapter 3) was to determine the effects of feeding high and low starch diets on ovarian dynamics and the onset of cyclicity in postpartum dairy cows.

In the second study (Chapter 4), the main objective was to determine the effects of feeding high and low starch diet on follicular fluid metabolite concentration like insulin, IGF-1, glucose, NEFA, urea and gene expression

profiles in granulosa cells of preovulatory follicles (approximately 58 days post-partum).

CHAPTER 2.

REVIEW OF THE LITERATURE

2.1 Introduction

Dried distiller's grain with solubles (DDGS) is a by-product obtained from distillery industry. In North America, distiller's grain with solubles (DGS) available in dried and wet form comes from the ethanol plant. This by-product is an increasingly available and cost-effective feed ingredient to dairy cattle. According to the reports from Wilbur-Ellis Co. Canada, the price of wheat DDGS ranged from $120 to $150 from January to September 2010. During the same time period the price of barley ranged from $129 to $150 as reported by Alberta canola producers commission (http://www.albertabarley.com/ marketing / pricing/ price_signal.html). Eun et al. (2009) reported that DDGS are rich in rumen undegradable protein (RUP) while primary energy source is from highly digestible fiber and fat. Wheat DDGS contains 38-40% crude protein (CP) and up to 60% of CP is available as RUP.

Külling et al. (2001) reported that the emission of greenhouse gases like methane (CH_4) from dairy slurry is considerably reduced in cows fed with low protein diet compared to high protein diet. According to Hao and Larney (2011), the greenhouse gases associated with livestock management are CH_4 and N_2O and their global warming potentials are found to be 25 and 298 times greater than carbon-dioxide (CO_2). Studies showed that DDGS contains two to three times more nitrogen content than unprocessed grain (Spiehs et al., 2002 and Widyaratne and Zijlstra, 2007). Therefore, inclusion of DDGS in animal diets at levels of

>40% of dry matter would increase the emission of nitrogen (N) and nitrous oxide (N_2O) (Hao et al., 2009). The emission of nitrous oxide is affected by pH, moisture, carbon (C), C/N ratio and temperature of the manure.

The concentration of starch in DDGS is low and could benefit the dairy cows by maintaining normal ruminal pH as energy in DDGS is mainly from digestible fiber and fat, unlike barley, where starch is a predominant energy source which decreases rumen pH due to its increased degradation leading to acidosis (Eun et al., 2009). Therefore, DDGS can be introduced as a primary feedstuff to dairy cattle rations. However, from a reproductive perspective, dietary starch is an essential nutrient for early lactating dairy cows. Studies conducted by Gong et al. (2002) showed that a high starch diet alters the concentrations of metabolic hormones like insulin and decreases the interval from calving to first ovulation.

2.2 Postpartum dairy cow

2.2.1. Lactation cycle and energy requirements after calving

At the beginning of the early lactation period, dairy cows experience a high energy demand due to increase in milk production, resulting in a lag in peak dry matter intake after calving which leads to mobilization of body stored lipids and weight loss. According to van Knegsel et al. (2005), energy balance is the difference between net energy intake and net energy expenditure for maintenance and milk yield. According to the 2001 Nutrient Requirements of Dairy Cattle Guidelines (7^{th} edition revised, published by the National Research Council) dairy cows attain peak milk yield during 4-8 weeks after calving while peak dry matter

intake lags behind until 10-14 weeks after calving. Bewley et al. (2008) reported that dry matter intake decreases for those cows with high body condition at calving, as body fat was found to reduce dry matter intake. However increased rate of body condition loss was associated with increased milk production as Bewley et al. (2008) reported that cows calving with body condition scoring (BCS) of 3.25 to 3.5 have increased milk production.

High producing dairy cows in first, second and third lactation requires maximum energy intake at weeks 7, 5 and 6 postpartum respectively as stated by De Vries et al. (1999). However the maximum energy intake occurred for these groups at weeks 12, 14 and 16 respectively. The energy requirement and energy intake on day 10 postpartum for first lactation cows was reported to be 31.1 and 25.1 Mcal NE_L/day. While for second and third lactation cows the energy requirement and energy intake was found to be 44.2 and 32.3 Mcal NEL/day respectively, indicating a greater energy deficit for this group. This is in agreement with reports of Coffey et al. (2002) as cows losing body weight in first lactation would be in severe energy deficit in subsequent lactation as cows may not regain the energy in the same lactation. The resulting energy deficit in early lactation leads to mobilization of body reserves, mainly fat and to a small extent of protein. Non-esterified fatty acid (NEFA) levels in blood increase after the fat mobilization from adipose tissue, while glucose levels decrease as the mammary gland uses it for lactose production (Vernon, 2002). According to Leroy et al. (2008) the drop in plasma insulin levels is associated with the down-regulation of

growth hormone (GH) 1 A receptors in the liver which uncouples the GH:insulin like growth factor 1 axis thereby suppressing IGF-1 concentration.

During early lactation, apart from mobilizing body fat, dairy cows also mobilize body proteins during extreme negative energy status, which results in increased levels of urea concentrations (Jorritsma et al., 2003).

2.2.2. Association between metabolites and fertility

Increased milk production in dairy cows is associated with a decline in fertility. Butler (2003) reported that an increase in energy demand that is related to a rise in milk yield leads to negative energy balance and results in concentration fluctuations of metabolites and metabolic hormones in early lactation that in turn lead to infertility.

2.2.2.1. Insulin and IGF-1

Beam and Butler (1999) reported that negative energy balance influences fertility by decreasing plasma insulin and IGF-1 concentrations. In vitro studies conducted by Spicer and Echternkamp (1995) showed that insulin stimulates granulosa cell proliferation and steroid production in the ovary. In the same study they found that insulin concentrations of 0.1 and 1.0 ng/ml stimulated estradiol production in bovine granulosa cells by up to three-fold. Insulin also plays an important role in recruitment of small follicles and increases follicular growth (Webb et al., 1999). These authors also reported that estradiol production from dominant follicle increased as serum insulin concentration increased. There is also a strong correlation between plasma IGF-1 and estrogen levels (Beam and Butler, 1999). IGF-1 is involved in the proliferation and mitogenesis of granulosa cells

and also stimulates follicular stimulating hormone (FSH)-induced steroid production in the ovary (Spicer and Echternkamp, 1995). Both IGF-1 and gonadotropins (FSH and LH) are reported to have synergistic action on follicular growth and differentiation (Lucy 2000); whereas, IGF-II is produced from thecal cells and its concentration was found to be greater in smaller follicles. The ovary also synthesizes IGF binding protein (IGFBP) apart from producing IGF-1 and IGF-II. Health of the follicle depends on the amount of IGFBP in follicular fluid, as according to Lucy (2000), IGFBP prevents both IGF-I and IGF-II from binding to their receptors. In addition, atretic follicles had higher concentrations of IGFBP.

Leroy et al. (2008) reported that negative energy balance would influence insulin and IGF-1 concentrations after calving and have an impact on follicular growth, either directly by affecting the ovarian sensitivity to gonadotropins, or indirectly by suppressing luteinizing hormone (LH) concentrations and pulsatility. The reduced LH pulsatility is associated with decreased estrogen production in dominant follicles of lactating dairy cows. This delays the ovulation of first dominant follicle postpartum as it requires a prolonged period of growth to stimulate the LH pulsatility and LH surge as described by Webb et al. (2004). There is an increase in the levels of plasma IGF-1of 40-50% in cows that have ovulatory dominant follicles compared to non-ovulatory follicles after 2 weeks postpartum.

2.2.2.2. Glucose

During negative energy balance there is a decrease in glucose with increased β-hydroxy butyric acid (BHBA) concentrations in plasma, and lower glucose and cholesterol concentrations in plasma reportedly increase the calving to conception interval (Westwood et al. 2002).Based on an in vivo study using ewes and cows, Rabiee et al. (2000) found that glucose plays a major role in promoting cholesterol uptake by the ovary. Factors that influence glucose metabolism in CL *in vitro* include physiological condition of the animal, estrous cycle, postpartum period (Chase et al., 1992), and metabolic hormones like insulin and IGF-1 (Gluckman et al., 1987 and Lucy et al., 1992). The administration of LH to rats also increased glucose uptake by the ovary in a study conducted *in vitro* (Gafvels et al., 1987). Murahashi et al. (1996) reported that the area postrema situated in the lower brain stem acts as a gluco-sensor and was found to play a role in modulation of LH secretion. Glucose is also involved in the regulation of gonadotropin- releasing hormone (GnRH) release (Foster and Nagatani, 1999).

Other metabolic hormones like ghrelin (gut-derived hormone) and leptin (adipose tissue-derived satiety hormone) also regulate the reproductive function. Plasma concentrations of leptin were reported to be positively correlated with concentrations of glucose and insulin in cattle (Diskin et al., 2003).In the same study, Diskin et al. (2003) reported that LH pulsatility was reduced due to decreased circulating leptin concentrations when post-pubertal heifers were

subjected to fasting for 48 h. *In vitro* studies showed that administration of ghrelin decreased the responsiveness of LH to GnRH (Tena-Sempere, 2005).

"Kisspeptins are natural ligands encoded by kiSS-1 gene for G-protein coupled receptor" and are pivotal regulators of GnRH neurons; the administration of kisspeptin-10 stimulates LH and GH secretion in prepubertal heifers (Kadokawa et al., 2008).

2.2.2.3. NEFA

During negative energy balance, a low level of insulin in plasma stimulates lipolysis and the subsequent increase in NEFA will serve as an alternative energy source for the body (Leroy et al., 2008). Abnormal levels of NEFA in liver tissues will cause fatty degeneration and suppress pancreatic function. Studies conducted by Rukkwamsuk et al. (1999) showed that plasma NEFA levels and tri-acyl-glycerides (TAG) were positively correlated. Increases in TAG levels in liver would delay the interval from calving to first ovulation (Rukkwamsuk et al., 1999), as higher NEFA levels reduces follicular growth and development (Leroy et al., 2008) and decreases estrogen production in dominant follicle (Beam and Butler, 1997). *In vitro* studies conducted by Leroy et al. (2005) showed that NEFA, especially saturated fatty acids like palmitic acid, oleic acid and stearic acid, induces apoptosis and cumulus cell necrosis during oocyte maturation indicating possible detrimental effects of NEFA on oocyte quality.

2.2.2.4. Urea

In early lactation, protein catabolism and lipid mobilization takes place in dairy cows. Protein de-amination and detoxification increases the urea levels in

circulation (Leroy et al., 2008). When dairy cows were fed with high protein in early lactation, negative energy balance associated with postpartum period became severe as the animal required extra energy to excrete urea (Roche et al. 2006). The decreased energy levels may then have an impact on fertility. Elevated plasma urea and ammonia levels were reported to influence the LH release during early lactation (Jordan and Swanson, 1979, Sinclair et al., 1995) and modify the composition of uterine fluids, decrease uterine pH and reduce the conception rates (Jordan et al., 1983; Elrod and Butler, 1993; Elrod et al., 1993). Concentrations of plasma urea nitrogen (PUN) exceeding 20 mg/dl are reported to decrease the conception rates (Ferguson et al., 1988; 1993). *In vitro* studies have determined that the cows fed with high urea diets have decreased CL weight and subsequent decrease in progesterone production (Garverick et al., 1971).

2.3. Reproductive function

2.3.1. Postpartum uterine health

Uterine involution plays a significant role in postpartum reproductive health of dairy cows. It is the process in which the uterus reaches its normal non-pregnant size and function after parturition (Senger, 2003). Dairy cows require approximately 45-50 days for complete involution of uterus after calving. A shorter period of uterine involution is more desirable in dairy cows as this would facilitate maximum milk yield in the cows' lifetime and prepare the uterus rapidly for subsequent pregnancies. Wathes et al. (2007b) explained that negative energy balance during early postpartum period may delay the uterine recovery by depressing the immune system leading to inflammation of the uterus. The factors

that predispose the uterus to infections include endometritis, retained placenta and metritis (Wathes et al., 2007b). As well, metabolic disturbances like hypocalcemia, ketosis and acidosis also hamper uterine recovery after calving (Leroy et al. 2008).Therefore delayed uterine involution after calving may reduce the reproductive efficiency.

2.3.2. Resumption of ovarian follicle growth

2.3.2.1. LH pulsatility

LH is a glycoprotein hormone produced from the anterior lobe of the pituitary that causes ovulation and then stimulates CL to produce progesterone (Senger, 2003). The hypothalamic centers that control GnRH release are the tonic and surge (or preovulatory) centers. Hypothalamus has different sensitivities to positive and negative feedback of gonadal steroids. The tonic center is reported to respond mainly to negative feedback while the surge center is believed to respond mostly to positive feedback. The latter plays a significant role in preovulatory release of GnRH thereby stimulating LH surge while tonic release of GnRH stimulates the production of FSH and LH from the anterior pituitary which is necessary for ovarian follicular development. The preovulatory GnRH release from neurons present in surge center occurs once during the entire estrous cycle while tonic secretion of GnRH occurs throughout estrous cycle (Senger, 2003).

For ovulation to occur, LH pulse frequency should occur at a rate of 1 pulse per hour and this result in the production of estradiol which is necessary for the positive feedback on GnRH which in turn induces LH surge and ovulation (Roche, 2006). Therefore the restoration of LH pulsatility during early lactation is

necessary for the growth of preovulatory follicle and production of estradiol for ovulation of dominant follicle (Butler, 2000).

2.3.2.2. Ultrasonography

Ultrasound is a valuable and sensitive diagnostic tool for use in bovine reproductive management and research (Rajamahendran et al., 1994; Colazo et al., 2010). The term ultrasound describes the sound waves that have frequencies in range of 15-20 kHz. High frequencies of 7.5-10 MHz are usually used for examination of ovaries and pregnancy diagnosis in early stages (DesCoteaux et al., 2009).

This technology can be used to examine ovarian follicles and CL. During ultrasound examination, follicles appear as black circumscribed image because the follicular fluid is non-echogenic (Pearson and Ginther, 1984). When ovarian scans are performed repeatedly at fixed intervals of about twice a week, ovulation can be confirmed when a large follicle from the previous scanning disappears and is replaced by a CL (Pearson and Ginther, 1984 and Dyck et al., 2011).The new CL following the ovulation is usually visible in about 3 d. When CL grows, the ultrasound examination shows hypo-echogenic image of the structure as they are richly supplied with blood vessels (DesCoteaux et al., 2009).

Diagnosing pregnancy using ultrasound was reported to be safe and inexpensive procedure in dairy cows (DesCoteaux et al., 2009). The use of transrectal ultrasonography for early pregnancy diagnosis in dairy cows is well-adapted (Ambrose et al. 2006; Colazo et al., 2009; Dyck et al., 2011).

Postpartum examination of uterus using ultrasound is efficient in detection of uterine infections like clinical metritis, endometritis and pyometra that impairs the reproductive performance of dairy cows (DesCoteaux et al., 2009).

2.3.3. Follicular growth overview

According to Senger (2003), follicular dynamics is defined as "the sum of intra-ovarian processes involved in follicular development and degeneration". During recruitment stage, cohorts of small antral follicles of 3 mm in diameter are recruited following an increase in FSH which is necessary for the growth of antral follicles. In postpartum dairy cows, the negative feedback exerted by high progesterone concentrations on GnRH production is removed) between 7-14 days post-calving and the subsequent increase in FSH concentrations lasts for 3-5 days resulting in first post-partum follicular wave (Roche, 2006). FSH levels in blood increases at the beginning of each follicular wave. During the first follicular wave, FSH surge occurs a day before ovulation while second wave occurs during the 10^{th} day of estrous cycle; in case of cows with three-wave cycles the follicular wave also occurs at 16^{th} day of estrous cycle (Ginther et al. 1996).Sartori et al. (2004) reported that the cows may also have more than 3 follicular waves per cycle. The concentration of FSH declines at the time of deviation and this decline was found to be the major part leading to the selection of follicles (Gastal et al., 1997 and Ginther et al., 1998).

The time of deviation is defined as "the difference in growth rates between two largest follicles when the second-largest follicle reaches the maximum diameter" (Ginther et al., 1996). They showed that the mean time of deviation of

two largest follicles is 2.8 days after the follicular wave emergence, when the largest follicle is 8.5 mm in diameter while the second largest follicle reaches the diameter of 7.2 mm. Wiltbank et al. (2000) examined the growth profiles of dominant and largest subordinate follicles for the first follicular wave. Their study showed that the future dominant follicle was larger (76%) compared to next subordinate follicle before the time of deviation. Ginther et al. (1996) found that the future dominant follicle emerges as 3 mm follicle, 6 hours earlier compared to largest subordinate follicle or 10 hours earlier than second largest subordinate follicle. These findings revealed that dominant follicle has a size advantage such that the deviation mechanism is completed before the largest subordinate follicle reaches a similar diameter as the dominant follicle, as a result only one follicle reaches dominance.

In cattle, there is an accumulation of LH in the future dominant follicle at the time of deviation (Kulick et al., 1999) due to increased expression of LH receptors in granulosa cells of dominant follicle (Xu et al., 1995; Goudet et al., 1999; Beg et al., 2001). Increases in estradiol levels associated with increase in LH will depress the FSH levels such that subordinate follicles undergo atresia resulting in dominance of one follicle (Ginther et al., 2000). Another hormone that suppresses FSH concentration is reported to be inhibin (Ginther et al., 2001 and Gibbons et al., 1999). Alteration in the follicle selection mechanism leads to selection of two or more follicles attaining co-dominance, in such instances multiple ovulations will occur (Kulick et al., 1999; Beg et al., 2003; Rivera et al., 2001; Rivera et al., 2003; Wiltbank et al., 2000). Low levels of inhibin found in

co-dominant follicles leads to increased concentrations of FSH in the case of cows with multiple ovulations (Lopez et al., 2005).

Milk production and FSH play important roles in regulating the double ovulation. Wiltbank et al. (2000) reviewed the mechanisms associated with double ovulation and reported, based on a preliminary study, that heifers with co-dominant follicles had higher FSH concentrations than heifers with a single dominant follicle, 8 h before follicular deviation; however, FSH concentrations were similar between the two groups 8 h after follicular deviation. In the same study, Wiltbank et al. (2000) proposed that increased milk production leads to double ovulation in dairy cows. High milk production associated with high dry matter intake can lead to increased metabolism of steroids like estradiol in the liver, resulting in the elevation of FSH at the time of deviation, which can contribute to the physiological selection of more than one dominant follicle.

2.3.4. Effect of energy balance on (a) follicular growth and size (b) calving to ovulation interval (c) conception rates

As discussed, in lactating dairy cows an increase in energy demand during the onset of lactation due to increased milk production results in negative energy balance (Butler, 2000). Reduced energy availability during the period of negative energy balance decreases the pulsatile LH secretion, reduces the response of ovary to LH (Butler, 2000) and influences follicular growth (Lucy, 2000). These factors may have an impact on dominant follicle growth during negative energy balance, as they require a longer time and size to produce estradiol to initiate ovulation (Roche, 2006). For an ovulation to occur, estradiol produced from dominant

follicles should exert a positive feedback on the hypothalamus to stimulate GnRH surge followed by LH surge (Senger, 2003).

Preovulatory follicles were reportedly larger in lactating dairy cows compared to heifers, but cows had lower estradiol concentrations in circulation (Roche, 2006). The reason for reduced circulating estradiol concentrations in lactating dairy cows is not clear. Stagg et al. (1998) reported that formation of a large dominant follicle in the early postpartum is not a limiting factor for calving to ovulation interval. However the first dominant follicle postpartum may either ovulate (30-80%), become atretic (15-60%) or cystic (1-5%) as reported by several studies (Savio et al., 1990; Beam and Butler, 1997; Sartori et al., 2004; Sakaguchi et al. 2004). Those follicles that are emerging after low (nadir) NEB reported to have increased diameter and increased quantity of estradiol and have more chances to ovulate (Beam and Butler, 1997), the reason may be due to increases in IGF-1 levels associated with reversal of energy balance (Beam and Butler, 1999).

Anovulatory anestrous associated with NEB may influence fertility in 30% of cows (Staples et al., 1990; Rhodes et al., 1998). Prolonged negative energy balance observed early postpartum may reduce the number of ovulatory estrous cycles before AI as an adequate number (2-3 cycles) of estrous cycles is required to prime the uterus (Butler, 2003). Villa-Godoy et al. (1998) reported that the cows in negative energy balance have lower progesterone concentrations during their first three ovulatory estrous cycles in the postpartum period. This is in agreement with Sartori et al. (2004); however in this study lactating dairy cows

had a larger volume of luteal tissue but produced less progesterone compared to non-lactating heifers. The lower progesterone concentration negatively affects fertility (Lucy, 2001). Postpartum uterine diseases and metabolic disorders during early lactation may affect the oocytes within the follicles, indicating that oocyte developmental competence was determined in the immediate postpartum period before ovulation (Leroy et al., 2005). Therefore the follicles that are expected to ovulate during the service period that undergo their early stage of development during energy balance nadir (Wathes et al. 2007b), tend to have inferior quality oocyte (Leroy et al., 2008). The reason is that growing oocytes in early postpartum period are exposed to metabolite changes associated with negative energy balance (Leroy et al. 2004) thereby affecting fertility.

2.4. Intrafollicular milieu

2.4.1. Structure of the ovarian follicle

Follicles are classified as primordial, primary, secondary or tertiary, and antral based on the size of the oocyte, granulosa cell morphology and number of layers of granulosa cells surrounding the oocyte, presence of antrum, etc. (Lussier et al., 1987). Senger (2003) described that the smallest and immature follicle found in the ovarian cortex is the primordial follicle. The primordial follicle grows and reaches the next stage called primary follicle. The oocyte in primary follicle is surrounded by a single layer of cuboidal epithelial cells, while in secondary follicle it is surrounded by thick translucent layer called zona pellucida. Unlike the secondary follicle, the antral follicle has a fluid filled cavity called the antrum, which contains the follicular fluid. Three distinct layers in the antral

follicles include theca externa, theca interna and granulosa cell layer (Figure 2.1). The theca externa forms the outer layer surrounding the follicle and consists of connective tissue. The theca interna forms the inner layer and produces androgen under LH stimulation. The preovulatory dominant follicle is also referred to as the Graafian follicle.

2.4.2. Granulosa cells and oocyte

Senger (2003) reported that the granulosa cell layer or membrane granulosa is present beneath the theca interna, a thin basement membrane separates both theca interna and granulosa cells. Granulosa cells contain receptors for FSH, while theca cells contain receptors for LH. Binding of FSH to its receptors result in conversion of testosterone to estradiol. When the level of estradiol reaches the threshold, preovulatory LH surge occurs. During this period, granulosa cells start synthesizing LH receptors and as a result the preovulatory LH surge causes ovulation by exerting its effect on the follicle. After ovulation, a new structure referred to as the CL is formed from granulosa cells and theca interna of the ovarian follicle which produce progesterone.

The oocyte is surrounded by several layers of cumulus cells to form cumulus-oocyte complex (COC). COC requires different compounds like fatty acids, metabolites, electrolytes, purines and pyrimidines for the oocyte maturation, mature COC's consume two fold more glucose than immature COC's in bovine (Sutton et al., 2003). In the same study, Sutton et al. (2003) reported that oocyte has a poor capacity to metabolize glucose, as a result cumulus cells play a significant role in glucose metabolism and transfers metabolic

intermediates like pyruvates to oocyte. The transfer of nutrients is accomplished by gap junctions and paracrine signaling between cumulus cells and oocyte (Gilchrist et al. 2004). Oocytes secrete paracrine growth factors that are necessary for normal function of cumulus cells (Sutton et al. 2003), and also for differentiation of cumulus cells from mural granulosa cells.

2.4.3. LH surge and gene expression in granulosa cells

The shift in dependency from FSH to LH during follicular deviation results in the granulosa cells acquiring LH receptors, thereby increasing LH binding to the dominant follicle. LH mRNA expression was reported to be abundant in dominant follicle, 1 day after (Xu et al., 1995) and also 8 hours before the selection process (Beg et al., 2001 and Bao et al., 1997). Thus within 15 minutes of peak in LH pulse there is a peak in estradiol (Rhodes et al., 1995), followed by an increase in estradiol levels in follicular fluid which results in follicular deviation, when the mean diameter of largest follicle reaches 8.5 mm, the mean day is 2.8 post-ovulation (Ginther et al., 1996). After follicular deviation, the selected dominant follicle depends on LH for its continued growth as studies have shown that an increase in LH pulse frequency increases the life span of dominant follicle (Fortune et al., 1991 and Savio et al., 1993). Estradiol and inhibin produced by the granulosa cells of dominant follicle suppress FSH release from the anterior pituitary resulting in regression of subordinate follicles. Activin is also produced from granulosa cells of dominant follicle, have structure similar to that of inhibin but vary in their function (Findlay, 1993).This

glycoprotein hormone was found to stimulate the release of FSH from pituitary cells in culture (Senger, 2003).

The increased expression of enzyme cytochrome P450 aromatase in dominant follicle is required for synthesis of estradiol from androstenedione (Badinga et al., 1992 and Bao et al., 1997); estradiol is also associated with mitosis of granulosa cells (Richard et al., 1976 and Ireland et al., 1978). Mihm et al. (2006; 2008) reported that the genes such as cyclin D2 (CCND2), GADD45B, OSAP, SFRS9 and aromatase variant DQ004742 are elevated in granulosa cells of dominant follicle. They are considered to play a significant role in estrogen synthesis, cell proliferation and survival, organ development, tissue remodeling and prevention of apoptosis as these genes are necessary for enhancing the tissue growth and to synthesize steroids in dominant follicles. High intra-follicular concentrations of estradiol are positively correlated with mRNA expression of estrogen receptor-β, LH receptor, aromatase, DICE 1, MCL 1. The tumor associated genes like DICE 1 is involved in DNA repair while MCL-1 prevents apoptosis (Evans et al., 2004) of granulosa cells of dominant follicle.

2.5. Knowledge gaps

The major factor that influences fertility in dairy cows is nutrition. Nutrition can induce changes in concentrations of metabolic hormones like insulin and IGF-1, influencing follicular growth (Gutierrez et al., 1997). *In vivo* studies conducted by Armstrong et al. (2001) showed that the insulin and IGF-1 concentrations in circulation are positively correlated with ovulation rates and follicular development. Even though there is a good understanding of the

relationship between effects of starch and circulating insulin, IGF-1 on reproductive performance in postpartum dairy cows, further research is needed to better understand the role of insulin and IGF-1with respect to interval from calving to first ovulation and double ovulations. Leroy et al. (2008) reported that the NEB in the early postpartum period will influence primary follicle growth and would have a carry-over effect on pre-ovulatory follicles 2-3 months later and result in an inferior quality oocyte because the primary follicle would take approximately 2-3 months to become a preovulatory follicle and this follicle ovulating at the time of breeding may contain oocyte that decreases fertility. Therefore research should concentrate on analyzing the follicular fluid metabolic profiles from the pre-ovulatory follicles 2-3 months after calving to have a clearer understanding on follicular fluid composition and oocyte quality.

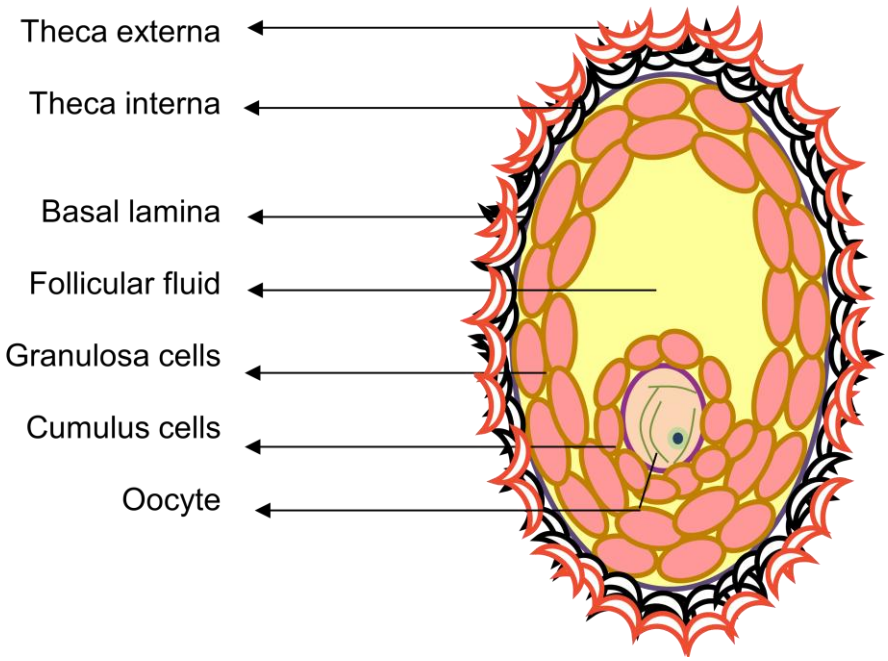


Figure 2.1. Schematic diagram showing the structure of a bovine ovarian follicle. The basement membrane of the follicle separates both thecal cells and granulosa cells. It also forms the blood-follicle barrier. Follicular fluid forms the biochemical environment for the oocyte. Granulosa cells provide nutrients to the oocyte while oocyte secretes factors necessary for the differentiation of the granulosa cells (Sutton et al. 2003; Leroy et al. 2004).

CHAPTER 3:

DIETARY STARCH AND REPRODUCTIVE FUNCTION IN POSTPARTUM DAIRY COWS

3.1. Introduction

Alterations in dairy herd management practices and the physiological adaptation of cows in response to genetic selection for high milk production have negatively influenced the reproductive performance of dairy cows (Lucy, 2001). This could be improved by adopting management practices that advance postpartum ovarian function by alleviating the constraints on metabolic and hormonal factors (Butler, 2000). The nutritional status of cattle was reported to influence the follicular growth, maturation and ovulatory capacity of dominant follicle (Diskin et al., 2003), as negative energy balance (NEB) after calving hampers uterine involution and influences the fertility (Sheldon et al., 2006).

The common approach followed to decrease the negative energy balance is to increase the dietary energy concentration in the early lactation period. This would alter the levels of metabolic hormones like insulin, positively influencing the ovarian follicular function (Webb et al. 2004). Similarly, insulin-like growth factor (IGF-1) plays an important role in follicular differentiation by stimulating the action of gonadotropins like follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Webb, 2004). This is in agreement with the studies of Taylor et al. (2004) as they reported that IGF-1 enhances the effect of FSH and LH by acting as a co-gonadotroph, by stimulating the growth and differentiation of follicles.

Previous studies reported that inclusion of dietary starch in postpartum dairy cow rations will increase the circulating concentrations of insulin and IGF-1. Gong et al. (2002) found that feeding dairy cows a diet (isoenergetic and isonitrogenous) containing 260g of starch per kg dry matter (DM) compared to 100g per kg DM increased the circulating levels of insulin and increased the proportion of cows ovulating before 50 d, from 55 to 90%. Houseknecht et al. (1988) reported that cows fed a low energy diet had decreased concentrations of IGF-1 in serum. Increased circulating concentrations of insulin and IGF-1 were observed in heifers fed a high energy diet which contributed to increase in growth of dominant follicle (Armstrong et al., 2001). Dyck et al. (2011) fed the dairy cows with 26.7%, 25.2% and 23.3% starch in the diet and found that the interval from calving to first ovulation was decreased in cows fed a 26.7% starch diet and significantly higher proportion of cows in 26.7% and 25.2% starch diet ovulated two follicles in the first ovulation postpartum. However no differences were observed in circulating insulin and IGF-1 concentrations.

Based on the findings from above studies, a high starch diet (HSD) decreases the interval from calving to first ovulation, but inconsistent results were reported for circulating insulin and IGF-1 concentrations. Studies conducted by Echternkamp et al. (1990; 2004) showed a positive correlation between IGF-1 concentration and twinning rate in cattle.

Previous studies have shown the positive association between dietary starch and early onset of cyclicity in postpartum dairy cows. Even-though there are several studies reporting the relationship between metabolites and metabolic

hormones, and parity; studies showing the effects of parity on ovarian dynamics are limited.

Zhang et al. (2010) found that dry distiller's grain with soluble (DDGS) can be used as an energy source by partially replacing barley silage or barley grain in lactating dairy cow diets. They reported that the partial replacement of barley silage (20% DM) by DDGS (70% corn DDGS and 30% wheat DDGS) in diets of lactating dairy cows increased milk production. However the effect of DDGS on reproduction was not reported in their study.

The overall objective of this study was to determine the effect of feeding high and low starch diets on reproductive function in postpartum dairy cows. Specific objectives were to assess the reproductive parameters like ovarian dynamics in early postpartum period and onset of cyclicity, as well as to determine plasma concentrations of insulin, IGF-1, glucose, urea and non-esterified fatty acids (NEFA) for up to 12wkpostpartum in lactating dairy cows fed rations containing barley grain (high starch diet; HSD) or wheat DDGS (low starch diet; LSD). The second objective was to investigate the effects of parity on plasma metabolites and reproductive parameters.

3.2. Materials and methods

3.2.1. Animals and diets

Sixty Holstein cows (22 primiparous, 38 multiparous) were used in this study. The experiment was conducted between January 2010 and September 2010 at the University of Alberta, Dairy Research and Technology Centre (DRTC), Edmonton, Canada. All experiments conducted on animals were

approved by the University of Alberta's Livestock Animal Care and Use Committee and cows were cared for according to the guidelines of Canadian Council on Animal Care (1993).

Cows were individually housed in a tie-stall barn and let out for exercise most mornings for about 2 h. Cows were assigned to one of the two experimental diets immediately after calving and remained on the diets until 84 d in milk (DIM) [Figure 3.1]. Diets were formulated according to NRC (2001) to meet the requirements of a 650 kg lactating cow. The experimental diet contained 29.2 % starch, 17.3% protein and 3.7% fat in high starch diet (HSD) group while the low starch diet (LSD) group contained 19.1% starch, 19.4% protein and 4.4% fat. Ingredient composition of the diets is presented in the Table 3.1. Cows were fed the assigned diets once daily as total mixed rations (TMR) at 0730 h and had access to *ad libitum* water.

3.2.2. Ultrasonography and reproductive management:

Transrectal ultrasonography (Aloka-500V scanner equipped with a 7.5 MHz linear transducer, Aloka Co., Tokyo, Japan) was performed twice weekly (Mondays and Thursdays, approximately 6 h after milking or 3 h after feeding) from 7 ± 2 d after calving until 56 DIM to monitor the ovarian function. Cows that did not ovulate by 56 DIM were considered not ovulated. While the larger follicles in each ovary were measured by built-in calipers after immobilizing the ultrasound image, smaller follicle sizes were estimated using the 1cm scale-guide on the display screen. If the follicles were not spherical then the longest and widest points were measured and the average of two measurements was the

follicular diameter. Similarly the longest and widest point measurements were used to calculate the size of corpus luteum (CL). All these procedures were performed as previously reported by Pearson and Ginther (1984).

At each scanning, diameter of the follicles ≥ 3mm was recorded and ovulation was presumed if the large follicle (≥ 10mm) detected during previous examination had disappeared and was replaced by a CL (Dyck et al., 2011). Although the new CL was not always visible early, later ultrasound examination showed a hypo-echogenic image of CL as it was richly supplied with blood vessels (DesCoteaux et al., 2009).

Timed artificial insemination (TAI) was performed approximately 73 d postpartum after placing the cows on a protocol for synchronization of ovulation (Ovsynch; Pursley et al., 1997; Figure 3.2). Out of 60 cows, 40 (HSD=19, LSD=21) that responded to Ovsynch protocol were subjected to TAI. The Ovsynch protocol (Pursley et al., 1997) was described in the figure 3.2. Treatments used in the protocol include GnRH (100µg, i/m, Gonadorelin acetate; Fertiline®, Vetoquinol N.A. Inc., Laval-trie, QC, Canada) and prostaglandin F2α (PGF) (500µg, i/m, Cloprostenol, Estrumate ®, Schering-Plough, Animal Health, Pointe-Claire, QC, Canada). Ultrasonography was performed at TAI and the next day to confirm the ovulatory response to second GnRH treatment as described by Colazo et al. (2003). Pregnancy was diagnosed 32 d after TAI and repeated on the 60^{th} d for confirmation by ultrasonography.

3.2.3. Blood sampling schedule:

Blood samples were collected from all 60 cows four times a wk within a 72 h period, with 18 h intervals between samplings. Blood sampling began one wk before calving for a baseline value and was repeated on wks 1, 2, 3, 4, 6, 8, 10 and 12 after calving (Figure 3.1 and Table 3.2). Samples were collected from a coccygeal blood vessel using evacuated Vacutainer® tubes containing sodium heparin (Becton Dickinson and Company, New Jersey, USA) as an anti-coagulant. After collection, samples were immediately placed on ice and centrifuged at 3000 *x* g for 20 min at 4°C, plasma harvested and kept in a freezer at -20°C. At the end of the experimental period all frozen samples were thawed and equal volumes of the plasma samples were pooled by week and refrozen until further analysis.

At the 10^{th}wk postpartum, blood samples were collected from 40 cows (HSD=19, LSD=21) that responded to Ovsynch protocol before TAI to determine the plasma estrogen concentrations at different time periods of Ovsynch protocol. The first blood sample was collected 15-18 h after the administration of PGF2α, which was given to lyse the CL and the second blood sample was collected approximately 1 hour before TAI, whereas a single blood sample collected 12 d after first TAI from those 40 cows were used to determine progesterone concentration.

3.2.4. Laboratory analyses:

Plasma insulin and IGF-I concentrations were determined from all 60 cows, whereas, glucose, urea and NEFA concentrations were determined only from a subset of 24 cows.

3.2.4.1. Insulin:

A solid phase radioimmunoassay kit was used to determine the concentrations of insulin in plasma (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA).The kit was previously designed for human insulin analyses and later validated by Reimers et al. (1982) in cattle. Insulin was assayed in triplicates using 200 µl of plasma. The average intra- and inter-assay coefficients of variation were 5.82% and 17.93% and the assay sensitivity was 1.3 IU/ml.

3.2.4.2. IGF-1:

Plasma samples were sent to the Laboratory Analytical Facilities (Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, Saskatoon, SK, Canada) for analyzing IGF-1. The assay was carried out using a solid phase, enzyme-labeled chemi-luminescent immunometric assay (IMMULITE, Diagnostic Products Corporation, Los Angeles, CA, USA) in singlicate as previously described by Elmlinger et al. (2005). The samples were diluted 1:10 with the pretreatment solution provided with the kit. Intra-assay coefficients of variation were 3.7% and 5.8% for reference sera. The assay sensitivity for IGF-1 was < 25 ng/ml.

3.2.4.3. Glucose, urea and NEFA:

Plasma glucose was analyzed using glucose oxidase/peroxidase enzyme and O-dianisine-dihydrochloride (Sigma No. P7119 and No. F5803). The concentration of plasma urea nitrogen was measured enzymatically (Fawcett and Scott, 1960), while NEFA was determined in plasma using a commercial kit (NEFA-HR (2) Procedure, Wako Chemicals, USA, Richmond, VA).

3.2.4.4. Estrogen and progesterone:

A solid phase radio-immunoassay kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) was used to determine the concentrations of estrogen in plasma samples by following the manufacturer instructions with the following modification. The assay was carried out in triplicates using 200 µl instead of 100 µl of plasma. Samples were analyzed in one assay with intra-assay coefficient of variation of 18.64% and the assay sensitivity of 0.75pg/ml.

A solid phase radio-immunoassay kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) was used to determine the concentrations of progesterone in plasma samples as described by Colazo and Ambrose (2011). The assay was carried out in triplicates using 100 µl of plasma. Samples were analyzed in one assay with intra-assay coefficient of variation of 3.75% and the assay sensitivity of 0.08ng/ml.

3.2.5. Statistical analyses:

Baseline measurements recorded for body condition scores (BCS) and plasma samples were used as covariates. Covariates that were not significant were removed from the model. All repeated measurements data (plasma insulin,

IGF-1, glucose, urea, NEFA and estrogen) were analyzed using PROC MIXED in SAS (version 9.1; SAS Institute Inc., Cary, NC). The statistical model included cow, treatment, wk/d, parity, treatment x wk/d, treatment x parity, treatment x parity x wk/d interactions. Cow was used as a subject term, while the repeated measures included wk/d of sample collection. The fixed effect includes treatment, wk/d, and parity while random effect was cow. Single measurement data (plasma progesterone) was analyzed using MIXED procedure in SAS with treatment, parity and treatment x parity interactions in the model.

Interval from calving to first ovulation was analyzed using LIFETEST procedure in SAS. Events of multiple first ovulation and multiple ovulation at TAI, and conception rate at first service were analyzed using GENMOD procedure in SAS. Other reproductive parameters like mean diameter of largest follicle at first ultrasound, days to reach a dominant follicle of $\geq$ 10mm diameter and diameter of pre-ovulatory follicle at TAI were analyzed using MIXED procedure in SAS. The model included treatment, parity and treatment x parity interactions with treatment; parity was considered as fixed effect while cow was a random effect. Correlations were calculated between diameter of dominant follicle at first ultrasound, days needed for a dominant follicle to reach $\geq$ 10mm in diameter and interval from calving to first ovulation using Pearson correlation in SAS. Significant differences were reported if the *P* value was ≤ 0.05; *P*-values > 0.05 and ≤ 0.10 were considered trends.

3.3. Results

3.3.1. Reproductive parameters

3.3.1.1. Ovarian dynamics and onset of cyclicity in early postpartum period

The mean diameter of the largest follicle observed in either ovary at the first ultrasound examination was 11.21 ± 0.42 mm, while the mean interval to reach a follicle of ≥ 10mm diameter was 11.75 d; neither of the parameters was affected by diet(Table 3.3) or parity (Table 3.4). There was no significant effect of diet on interval from calving to first ovulation (Table 3.3), however primiparous cows tended to have a longer interval from calving to first ovulation compared to multiparous cows (Table 3.4). A greater (P=0.04) proportion of cows in the HSD group ovulated more than one follicle at first ovulation compared to cows in LSD group (Table 3.3); neither parity nor treatment by parity interactions were observed. No treatment differences were observed for ovarian dynamics in the early postpartum (Table 3.3), however days required for a dominant follicle to attain ≥ 10mm diameter tended to be shorter in multiparous than primiparous cows (Table 3.4). The interval from calving to first ovulation and the days required for a dominant follicle to attain ≥ 10mm diameter were positively correlated (R=0.26, P=0.04). However, no correlation existed between diameter of dominant follicle at first ultrasound and days to reach ≥10mm in diameter, or diameter of dominant follicle at first ultrasound and interval from calving to first ovulation.

3.3.1.2. Reproductive parameters at TAI

A greater proportion of cows in the HSD group tended (*P*=0.10) to ovulate two or more follicles even at TAI (Table 3.3). The mean diameter of pre-ovulatory follicle at TAI was 16.14 ± 0.68 mm (Table 3.3) and was not affected by treatment or parity. Neither diet (Table 3.3) nor parity (Table 3.4) effect was observed for conception rate at first service.

3.3.1.3. Reproductive hormones

No dietary effects were observed for plasma estrogen concentrations from 2 individual samples (the first blood sample was collected 15-18 h after the administration of $PGF_{2\alpha}$, which was given to lyse the CL and the second blood sample was collected approximately 1 hour before TAI) collected during Ovsynch protocol before TAI (Table 3.5). No differences were observed for parity or interaction between the treatment and parity (Table 3.6).

Plasma progesterone concentrations on day 12 post-TAI were affected by neither diet nor parity; however, there was an interaction between diet and parity (*P*=0.04; Table 3.6). Multiparous cows in the LSD group had higher plasma progesterone concentrations than primiparous cows of the same dietary group (7.9 ± 0.8 vs. 5.4 ± 1.0; *P*<0.05) and multiparous LSD cows tended to have higher plasma progesterone concentrations than multiparous HSD cows (7.91 ± 0.80 vs 5.7 ± 0.9; *P*=0.07).

3.3.2. Metabolic profiles- plasma

3.3.2.1. Insulin and IGF-1 (n=30/diet)

Insulin concentrations were higher in cows fed HSD compared to those fed LSD (Table 3.7). Insulin concentrations in plasma increased with time from week 1 to week 12 postpartum (Figure 3.3). Plasma insulin concentration tended to be higher for HSD cows from week1 to 12 postpartum (P=0.08; Figure 3.4). Primiparous cows tended to have higher insulin levels postpartum compared to multiparous cows (Table 3.8), however the interaction between treatment and parity was not significant.

No dietary effects were observed for plasma IGF-1 concentrations (Table 3.7). However the IGF-1 concentrations increased from wk 1 to wk 6 postpartum (Figure 3.5) while the interaction between treatment and IGF-1 concentrations from wk 1 to wk 6 after calving was not significant (Figure 3.6). Whereas primiparous cows had significantly higher IGF-1 concentrations than multiparous cows (Table 3.8), there was no interaction between treatment and parity. Plasma insulin and IGF-1 concentrations did not differ between cows with single and multiple ovulations.

3.3.2.2. Plasma glucose, urea and NEFA (n=12/diet)

No treatment effects were observed for glucose and urea concentrations in plasma (Table 3.9); however the plasma urea concentrations increased from wk 1 to 12 postpartum (Figure 3.7). HSD cows had lower NEFA concentrations compared to LSD cows (Table 3.9) and the concentrations decreased from wk 1 to 12 postpartum (Figure 3.8).

Primiparous cows tended (P=0.08) to have higher glucose concentrations compared to multiparous cows while plasma urea concentration was found to be significantly higher in multiparous cows (Table 3.10). Primiparous cows had higher NEFA concentrations than multiparous cows (Table 3.10), but no interactions between treatment and parity were observed.

3.4. Discussion

The results partially supported the hypothesis, as plasma insulin concentrations and the proportion of cows ovulating two or more follicles were higher in the HSD group, but neither IGF-I nor the interval from calving to first ovulation was affected by diets.

3.4.1. Reproductive parameters

3.4.1.1. Ovarian follicular dynamics after calving

In the present study, we found that the days to reach a dominant follicle of ≥ 10 mm in diameter was positively correlated with the interval from calving to first ovulation, which means that the fewer days it took for a dominant follicle to reach ≥10 mm, the sooner the first postpartum ovulation occurred, which is in agreement with the studies of Dyck et al. (2011).

3.4.1.2. Interval from calving to first ovulation

Contrary to our hypothesis, no treatment differences were observed for calving to first ovulation between cows fed diets containing 29.2% starch (HSD) and 19.1% starch (LSD) groups. Our findings contrast that of Gong et al. (2002), Burke et al. (2006) and Dyck et al. (2011).In the study by Dyck et al. (2011), dairy cows fed diets containing 26.7% starch (HSD) had shorter interval from

calving to first ovulation compared to cows fed 25.2% (MSD) and 23.3 % (LSD) starch diets (30.6 d vs. 43.2 & 38.1 d). In Gong et al. (2002) study, when dairy cows were fed diets containing high starch (26%) increased the proportion of cows ovulating before 50 DIM from 55 to 90% compared to low starch diet (10%). In another study Burke et al. (2006) described that cows fed with additional non-structural carbohydrates ovulated sooner postpartum.

While we expected that cows in the HSD group would have a shorter interval from calving-to-first-ovulation, the reason for the lack of such difference is not clear. Unlike in the study by Dyck et al. (2011), we did not record the pulsatility of LH in the early postpartum period. They found that the cows with greater LH pulses at 15 d postpartum had a shorter interval from calving to first ovulation; however, they did not find any dietary effects on mean LH concentration. LH is essential for the development of dominant follicle (Savio et al., 1993), and inadequate LH secretion would affect the growth of follicle beyond 9 mm (Gong et al., 1995; Gong et al., 1996; Ambrose et al., 1998).

The mean interval from calving to first ovulation for primiparous cows tended to be higher (36 d) compared to that of multiparous cows (30 d), which is in agreement with many studies. Dyck et al. (2011) reported that primiparous cows took 45 d compared to multiparous cows (32 d) before first ovulation occurred postpartum. Similar findings were reported by Rosenberg et al. (1977) and Lucy et al. (1992). The slight delay in first postpartum ovulation in primiparous cows could be due to higher energy demands for both growth and lactation in primiparous cows than in multiparous cows (Tanaka et al., 2008).

3.4.1.3. Role of insulin and IGF-1 on the onset of cyclicity

In vitro studies conducted by Stewart et al. (1995) found that insulin and IGF-1 are responsible for regulating the LH-stimulated production of androgens from the thecal cells. In the current study, HSD cows had higher circulating insulin concentrations, yet no differences were observed in the interval from calving to first ovulation. This is in contrast to the findings of Dyck et al. (2011) who reported that cows in HSD group had shorter calving-to-ovulation interval, although circulating insulin levels did not differ among their cows.

Increased level of dietary starch in the present study did not affect plasma IGF-1 concentrations in agreement with the findings of Dyck et al. (2011). Rutter et al. (1989), Nugent et al. (1993) and Roberts et al. (1997) found that the low concentrations of IGF-1 after calving delayed the calving-to-first-ovulation interval in beef cows. *In vitro* studies performed by Spicer and Stewart (1996) show that IGF-1 plays a significant role in increasing the responsiveness of follicles to LH thereby increasing the estradiol production essential for an ovulation to occur. Measurement of LH pulsatility, which was not performed in the present study, might have given us a better understanding about the relationship of LH with IGF-1 concentration and interval from calving to first ovulation.

3.3.1.4. Multiple first ovulations

Cows are monovular and generally produce only one offspring per pregnancy. However the twinning rate in dairy cattle has reportedly increased in recent years (Wiltbank et al., 2000). The hormones that play an important role in regulating double ovulation are FSH, inhibin and estradiol. In case of multiple

ovulations in high producing dairy cows, it has been reported that the increased DMI results in increased metabolism of estrogen, progesterone and inhibin causing elevation of FSH during follicular deviation, potentially leading to the selection of two or more dominant follicles (Wiltbank et al., 2000). High concentrations of IGF-1 can also contribute to double ovulation; it has been reported that IGF-1 concentrations in serum and follicular fluid are higher in cows selected for twins than in control cows (Echternkamp et al., 1990).

Supporting our hypothesis, a greater proportion of cows in HSD treatment group ovulated two or more follicles compared to cows in the LSD group during first postpartum ovulation. This is in agreement with the findings of Dyck et al. (2011) as cows fed 26.7% and 25.3% starch had a greater proportion of double first ovulation, compared to those fed 23.3% of starch. We also observed that cows in the HSD group tended to ovulate two or more follicles at TAI than cows in the LSD group. However an increased number of multiple first ovulations in this study could not be attributed to increase in circulating IGF-1concentrations as there was no significant dietary effect on IGF-1 levels observed. In the case of cows ovulating multiple follicles, Lopez et al. (2005) reported that circulating FSH concentrations was increased 24 h before deviation resulting in continued growth of largest subordinate follicle even after deviation. As circulating FSH concentrations were not measured, the contribution of FSH to multiple ovulation in the present study is not known.

3.4.1.5. Conception rate at first service

Determination of conception rate at first TAI was not our main objective. Yet, there was an opportunity to evaluate conception rate within the study

population, even though only a small number of animals (HSD=19, LSD=21) were subjected to TAI. In an earlier study from our laboratory, Dyck et al. (2011) reported no treatment differences in the pregnancy at first, second or third AI when 3 levels of starch were fed. In the present study also no differences existed between the two groups of cows in conception rate.

3.4.2. Reproductive hormone

Plasma progesterone concentrations were higher approximately 13 d post AI in cows that conceived (Bulman et al., 1978) compared to those that did not. As it has been proposed that luteolysis is initiated in non-conceived cows at about day 13 (Bulman et al., 1978), determining plasma progesterone concentration at this stage (12 d post AI) was considered essential in this study, and to investigate the effect of diets on progesterone concentrations. In this study, multiparous cows on LSD had higher plasma progesterone concentrations than primiparous LSD cows however this difference was not observed for conception rate at first service.

3.4.3. Metabolic profiles

3.4.3.1. Concentration of metabolites after calving until 84 DIM

In our study, NEFA decreased with time while urea, insulin and IGF-1 increased from d 7 after calving to 84 DIM.

Similar to our study, Gong et al. (2002) observed an increase in plasma insulin levels with time from d 7 to d 50 postpartum when cows were fed diets containing 260g of starch compared to 100g of starch per kg DMI. Doepel et al. (2002) reported that IGF-1 reached the peak concentration during first wk postpartum then decreased subsequently to reach lower (nadir) levels at wk 4

post-calving when cows were fed for 21 d before calving with high or low concentrations of energy and high or low concentrations of protein. This was in contrast with the patterns observed in this study as low IGF-1 concentrations were observed during first wk postpartum and reached the peak during sixth wk postpartum indicating that the energy availability increased from week after calving to six weeks postpartum.

There were no differences in plasma glucose concentrations from wk 1 to wk 12 postpartum, which is also in contrast with the studies of Dyck et al. (2011) as they found that plasma glucose levels decreased from calving to first wk postpartum and then started increasing until 70 d postpartum.

Plasma NEFA concentrations reached its peak during wk 1 postpartum and then decreased subsequently thereafter to reach nadir. In early lactation the imbalance between energy intake and energy requirements for maintenance and milk production leads to NEB. As a result, body fat is mobilized and increases the NEFA levels in blood to produce alternative energy source to the body (de Vries and Veerkamp, 2000). This is in agreement with the results observed in the present study for increased circulating NEFA levels during early postpartum.

In this study, circulating urea concentrations increased from first wk postpartum and the increased concentrations in subsequent weeks could be due to either the increase in dietary protein availability compared to dietary energy in early lactation or due to increased amino acid mobilization from the skeletal muscles as stated by Wathes et al. (2007a).

3.4.3.2. Effects of dietary starch on plasma metabolites concentration

In our study, cows in HSD group had significantly higher insulin concentration compared to cows in LSD group, which is in agreement with the findings of Gong et al. (2002). In their study, Gong et al. (2002) fed diets containing either 10% or 26% starch and found that cows placed on the 26% starch diet had higher insulin concentration compared to those placed in the 10% starch diet. In a recent study, Dyck et al. (2011) found no differences in insulin concentrations in dairy cows that were given diets containing 26.7% (HSD), 25.2% (MSD) and 23.3% starch (LSD) respectively. They explained that the relatively small differences in starch percentages among the diets might have contributed to not finding a difference in circulating insulin concentrations.

Minor et al (1997) reported that cows and heifers fed high non-fiber carbohydrates (NFC) (46.5% of DM) diet had significantly lower NEFA concentration compared to those fed standard NFC (41.7% of DM).This is in agreement with the findings observed in our study as cows in HSD group had significantly less NEFA concentration compared to those in LSD group. Lower NEFA and higher insulin concentrations were observed when starch was infused directly into either rumen or abomasum (Knowlton et al. 1998). No dietary differences in glucose concentrations were observed in Knowlton et al. (1998) study, which is similar to the findings, observed in the present study. In another study, no dietary differences were observed for plasma glucose concentration post-calving and the possible reason could be the reduction in propionate absorption as a result of decrease in dry matter intake postpartum and the

increased demand of glucose for milk production (Doepel et al., 2002), as the glucose is mostly used by mammary gland to form lactose (Vernon, 2002).

No dietary differences were observed for plasma IGF-1 concentrations in this study, which is similar to the findings observed by Dyck et al. (2011) as they did not observe any differences in IGF-1 concentrations in dairy cows fed diets containing three levels of starch. In contrast, Houseknecht et al. (1988) described that the dietary energy rather than dietary source positively influence serum IGF-1 levels in beef heifers. Elsasser et al. (1989) conducted a study in steers and found that circulating concentrations of IGF-1 were influenced by plane of nutrition. This is in agreement with the study conducted by Diskin et al. (2003) as they reported that the plasma IGF-1 levels were positively correlated with nutrient intake and body condition. Some caution should be exercised in interpreting the results in the current study, as the plasma metabolites results for glucose, urea and NEFA were reported from a subset of only 24 cows while the results for circulating insulin and IGF-1 concentrations were discussed for all 60 cows.

3.4.3.3. Effect of parity on plasma metabolites concentration

IGF-1 concentration was found to be higher in primiparous cows after calving (Taylor et al. 2004) which is in agreement with the current study and also with studies of Wathes et al. (2007a). In the same study, Wathes et al. (2007a) found that age is the main factor rather than parity that affects IGF-1 concentrations as the levels considerably reduced between 2 and 3 years of age. Therefore in our study, younger cows (2 to 3 years of age) had higher IGF-1 concentrations than older cows aged between4 and 9 years. In Wathes et al.

(2007a) study, insulin followed the same pattern as IGF-1, as the growth promoting factor declined with age and is similar to the observations from the present study.

According to Santos et al. (2001) primiparous cows have higher glucose concentrations in the peri-partum period which is attributed to the lesser amount of glucose being spared for milk yield and they produce less milk compared to second or third parity cows and this could be the case in the current study as primiparous cows tended to have higher glucose levels compared to multiparous cows even-though milk yield was not reported in this study.

Similar to the findings of the present study, Cisse et al (1991) and Meikle et al. (2004) found that primiparous cows had greater NEFA levels than that of multiparous cows. Unlike the findings of this current study, Wathes et al. (2007a) reported that primiparous cows had greater urea concentration compared to multiparous cows. However in the same study, Wathes et al. (2007a) stated that the reason for increased urea levels in mature cows is due to poor conditioning at calving as a result they mobilize more protein to produce energy.

3.5. Conclusion

In this study, increasing dietary starch did not decrease the interval from calving to first ovulation, but it increased insulin and decreased NEFA concentrations in plasma. Increasing dietary starch could benefit primiparous cows by decreasing NEFA and increasing IGF-1 in plasma, although this did not translate into improved reproductive performance in the present study. While starch inclusion in dairy cows has the potential to improve reproductive function

further research is needed with a larger population of cows to assess its effects on fertility of dairy cows post-partum.

Table 3.1. Ingredient composition of dietary treatments

Ingredient composition (%DM)	HSD[1]	LSD[2]
Barley silage	43.1	43.1
Corn grain rolled	21.6	21.6
Barley rolled	17.3	0.0
Wheat DDGS[3]	0.0	17.2
Beet pulp	3.2	12.3
Corn gluten meal	8.3	0.0
Vegetable oil	2.4	1.9
Urea	0.3	0.0
Minerals & Vitamins	3.9	3.9

[1] High Starch Diet

[2] Low Starch Diet

[3] Dry distiller's grain with soluble

Table 3.2.Weekly blood sampling schedule for plasma metabolites and reproductive hormones

Metabolites/hormones	No. of cows	Sampling week Pre and Post-calving	Samples Individual/pooled
Insulin	60	-1, 1, 2, 3, 4, 6, 8, 10 & 12	Pooled (4 X/week)
IGF-1	60	1, 2, 3, 4 and 6	Pooled (4 X/week)
Glucose, urea and NEFA	24	-1, 1, 2, 3, 4, 6, 8, 10 & 12	Pooled (4 X/week)
Estradiol	40	10	Individual (2 samples)
Progesterone	40	12	Individual (1 sample)

Pooled samples – 4 plasma samples per week were pooled before analyzing the plasma metabolite concentrations.

Table 3.3. Effect of dietary starch on reproductive parameters

	HSD	LSD	*P*
Mean diameter of largest follicle @ first U/S (mm)[1]	11.25 ± 0.42	11.17 ± 0.42	0.85
Days to reach a follicle of ≥ 10mm in diameter[1]	12.38 ± 1.06	11.13 ± 1.06	0.24
Interval from calving to first ovulation[1]	33.9 ± 2.3	30.7 ± 2.6	0.75
Multiple first ovulations, n (%)[1]	12/30 (40)	6/30 (20)	0.04
Multiple ovulations at first TAI (70d), n (%)[2]	4/19 (21)	2/21 (9)	0.10
Diameter of pre-ovulatory follicle (mm)[2]	16.32 ± 0.68	15.96 ± 0.68	0.59
Conception rate at first service, n (%)[2]	7/19 (36)	7/21 (33)	0.97

[1] Number of cows used were 60

[2] Number of cows used for TAI were 40. Out of 60, only 40 cows responded to Ovsynch protocol

Table 3.4.Effect of parity on reproductive parameters

	Primiparous	Multiparous	*P*
Mean diameter of largest follicle @ first U/S (mm)[1]	11.25 ± 0.42	11.18± 0.42	0.87
Days to reach a follicle of ≥ 10mm in diameter[1]	12.6 ± 1.06	10.8 ± 1.06	0.09
Interval from calving to first ovulation[1]	36.27 ± 2.66	29.97 ± 2.18	0.08
Multiple first ovulations, n (%)[1]	6/22 (27)	12/38 (32)	0.49
Multiple ovulations at first TAI (73d), n (%)[2]	2/17 (11)	4/23 (17)	0.24
Diameter of pre-ovulatory follicle (mm)[2]	15.81± 0.68	16.47± 0.68	0.34
Conception rate at first service, n (%)[2]	6/17 (35)	8/23 (34)	0.97

[1] Number of cows used were 60

[2] Number of cows used for TAI were 40. Out of 60, only 40 cows were responded to Ovsynch protocol

Table 3.5.Effect of dietary starch on plasma estradiol and progesterone concentration

	Treatment			
	HSD	LSD	SEM	*P*
N	19	21		
Estradiol, (pg/ml)[1]	1.68	1.73	0.23	0.84
Progesterone, (ng/ml)[2]	6.35	6.66	0.91	0.73

[1] Plasma estradiol analyzed from 2 individual samples per cow (The first blood sample was collected 15-18 h after the administration of PGF2α, which was given to lyse the CL and the second blood sample was collected approximately 1 hour before TAI) during Ovsynch protocol before TAI

[2] Plasma progesterone analyzed from the sample collected 12 d after TAI

Table 3.6. Effect of parity on plasma estradiol and progesterone concentrations

	Parity		SEM	*p*	
	Primiparous	Multiparous		Parity	Treatment*parity
N	19	21			
Estradiol, pg/ml*	1.48	1.92	0.23	0.17	0.20
Progesterone, ng/ml[1]	6.19	6.81	0.91	0.50	0.04

* Plasma estradiol analyzed from 2 individual samples per cow (The first blood sample was collected 15-18 h after the administration of PGF2α, which was given to lyse the CL and the second blood sample was collected approximately 1 hour before TAI) collected during Ovsynch protocol before TAI

[1] Plasma progesterone analyzed from the sample collected 12 d after TAI

Table 3.7.Effect of dietary starch on concentrations of insulin and IGF-1 in all 60 cows

	Treatment		SEM	*p*		
	HSD	LSD		Treatment	Week	Treatment * week
N	30	30				
Insulin (IU/ml)[1]	2.52	1.64	0.29	0.003	0.004	0.08
IGF-1 (ng/ml)[2]	75.92	70.67	5.75	0.36	<0.01	0.90

[1]Plasma samples collected on week -1 (P=0.002) before calving was used as a covariate adjustment and found to affect insulin concentrations post calving. However BCS as a co-variate didn't affect circulating insulin concentrations and was removed from the statistical model.

[2] Insulin-like-growth factor

IGF-1 results were reported for plasma samples collected from week 1 to 6 postpartum. Base line BCS values (P< 0.01) were used as a covariate and found to influence IGF-1 levels postpartum.

Table 3.8. Effect of parity on concentrations of insulin and IGF-1 in all 60 cows

	Parity			*p*	
	Primiparous	Multiparous	SEM	Parity	Treatment*Parity
N	30	30			
Insulin (IU/ml)	2.33	1.83	0.29	0.09	0.17
IGF-1 (ng/ml)*	84.35	62.23	5.85	<0.01	0.18

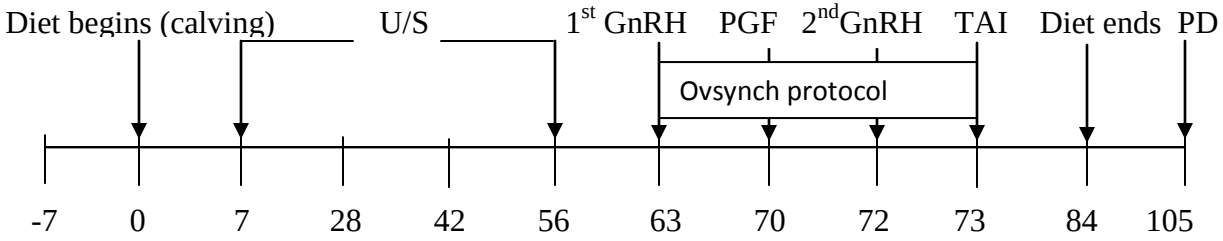
* IGF-1 results were reported for plasma samples collected from week 1 to 6 postpartum. Base line BCS values ($P< 0.00$) were used as a covariate and found to influence IGF-1 levels postpartum

Table 3.9. Effect of dietary starch on plasma glucose, Urea and NEFA concentrations in a subset of 24 cows from week 1 to week 12 postpartum

	Treatment		SEM	*p*		
	HSD	LSD		Treatment	Week	Treatment* Week
N	12	12				
Glucose, mg/dl	56.17	55.99	1.87	0.92	0.25	0.58
Urea, mg/dl	15.02	14.04	1.27	0.45	0.01	0.63
NEFA, m EQ/l	131.96	181.51	22.91	0.04	<0.01	0.58

Table 3.10. Effect of parity on plasma glucose, urea and NEFA concentrations in a subset of 24 cows from week 1 to week 12 postpartum

	Parity			*p*	
	Primiparous	Multiparous	SEM	Parity	Treatment*Parity
N	12	12			
Glucose, mg/dl	57.78	54.38	1.87	0.08	0.42
Urea, mg/dl	13.16	15.91	1.27	0.04	0.55
NEFA, mEQ/l	180.13	133.33	22.91	0.05	0.63



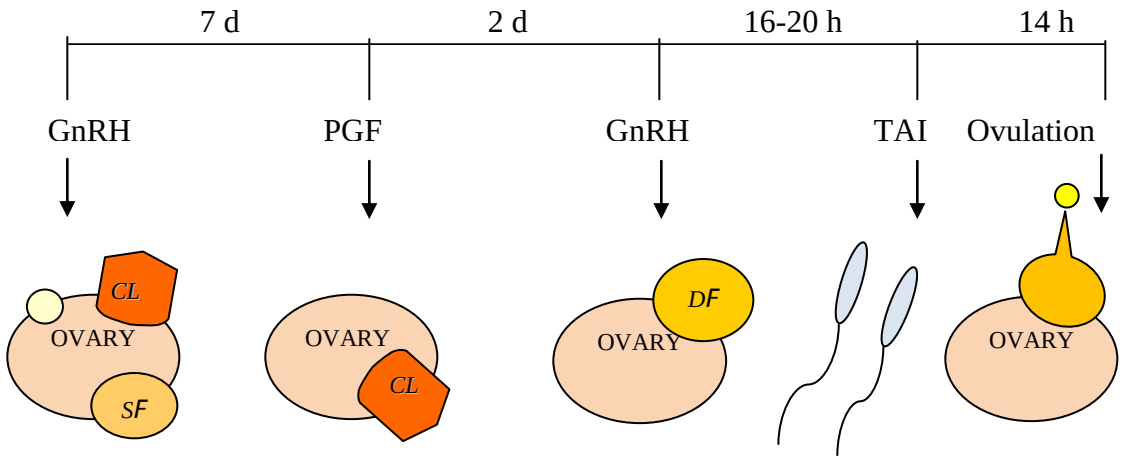
Blood sampling – 4X/week U/S- Ultrasonography 2X/wk

GnRH – Gonadotropin releasing hormone PGF- Prostaglandin $F_{2\alpha}$

PD- Pregnancy diagnosis TAI – Timed artificial insemination

Ovsynch – Ovulation synchronization protocol

Figure 3.1. Ultrasonography and blood sampling schedule. Ultrasonography was performed twice a week from day 7 to day 56 postpartum to determine resumption of cyclicity. Ovsynch protocol is explained in Figure 3.2. Blood sample was collected 4 times a week from day 7 to day 84 postpartum to analyze the concentrations of plasma insulin, IGF-1, glucose, urea and NEFA.



Initiates new follicular growth

Lyses CL

Synchronizes ovulation

PG – Prostaglandin $F_{2\alpha}$ GnRH – Gonadotropin releasing hormone

SF – Subordinate follicle

DF – Dominant follicle

CL – Corpus Luteum

TAI – Timed artificial insemination

Figure 3.2. Ovulation synchronization (Ovsynch) protocol. In Ovsynch protocol, two treatments of GnRH was administered 9 d apart with single treatment of prostaglandin F2α (PGF) given 7 d after first GnRH treatment and TAI was performed 16-20 h after second GnRH treatment. The ovulation was reported to occur approximately 30 h after the second GnRH treatment (14 h after TAI).

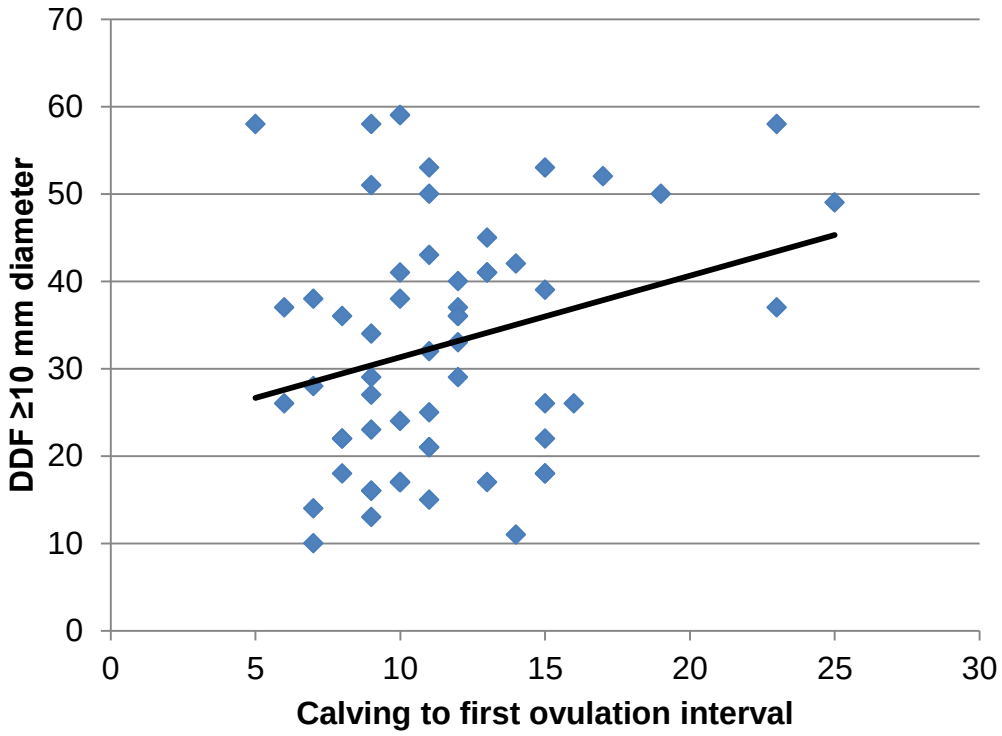


Figure 3.3. Correlation between days to reach a dominant follicle of ≥10 mm diameter and calving to first ovulation interval. A positive correlation was observed for the above two parameters (r=0.26; *P*=0.04)

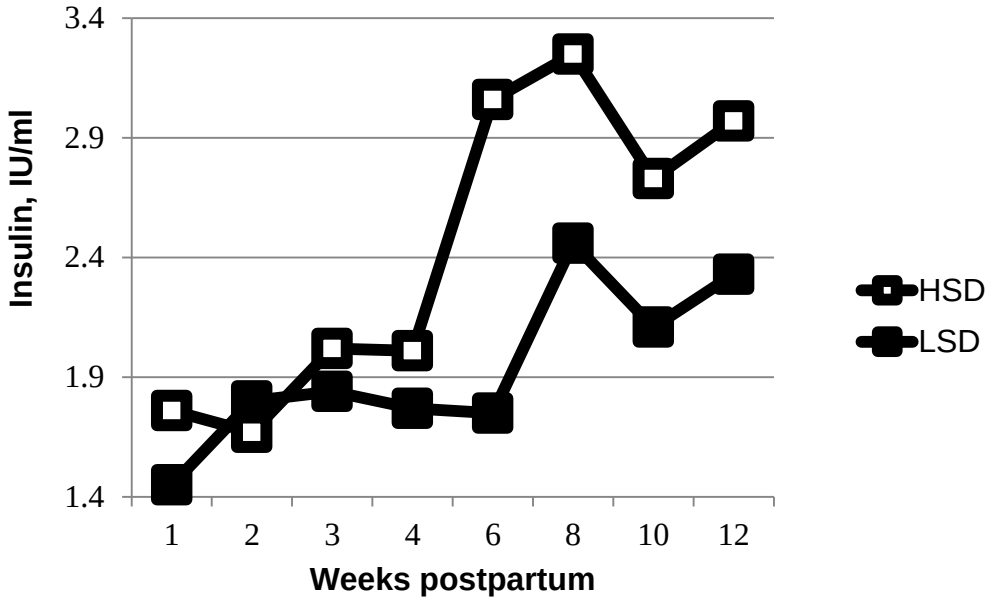


Figure 3.4. Effect of diet on plasma insulin concentrations in all 60 cows from week 1 to 12 postpartum (n=30/diet). High starch diet (HSD) significantly affected plasma insulin concentrations (P<0.003, SEM=0.29). Cows were fed two levels of starch (HSD and LSD) from the day of calving to 84 days in milk (DIM).

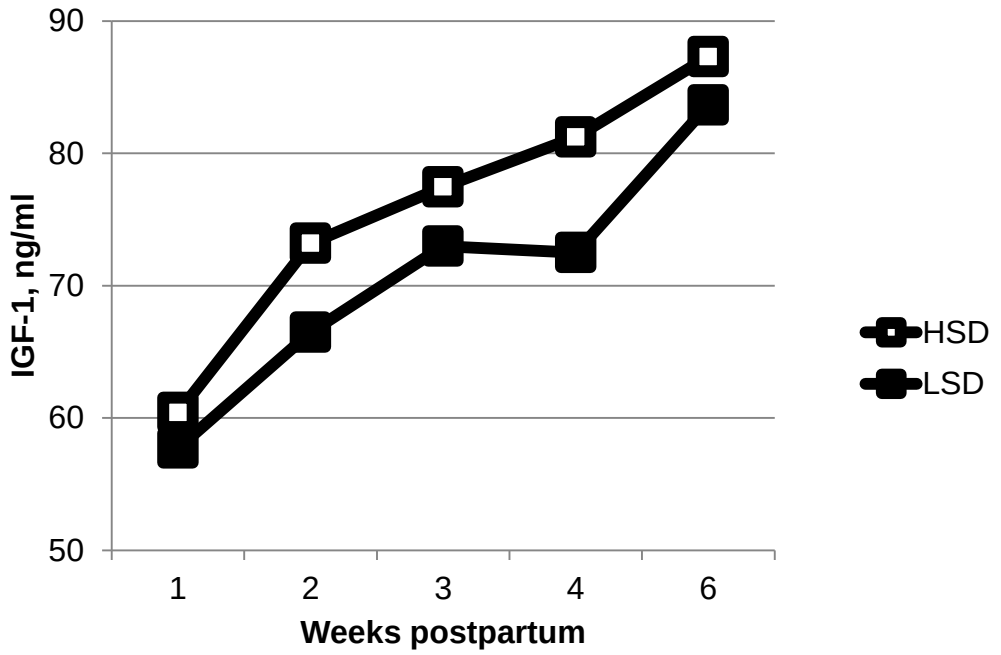


Figure 3.5. Effect of diet on plasma IGF-1 concentrations in all 60 cows from week 1 to 6 postpartum (n=30/diet). Diet had no effect on plasma IGF-1 concentrations (*P*=0.36, SEM=5.75). Cows were fed two levels of starch (HSD and LSD) from the day of calving to 84 days in milk (DIM).

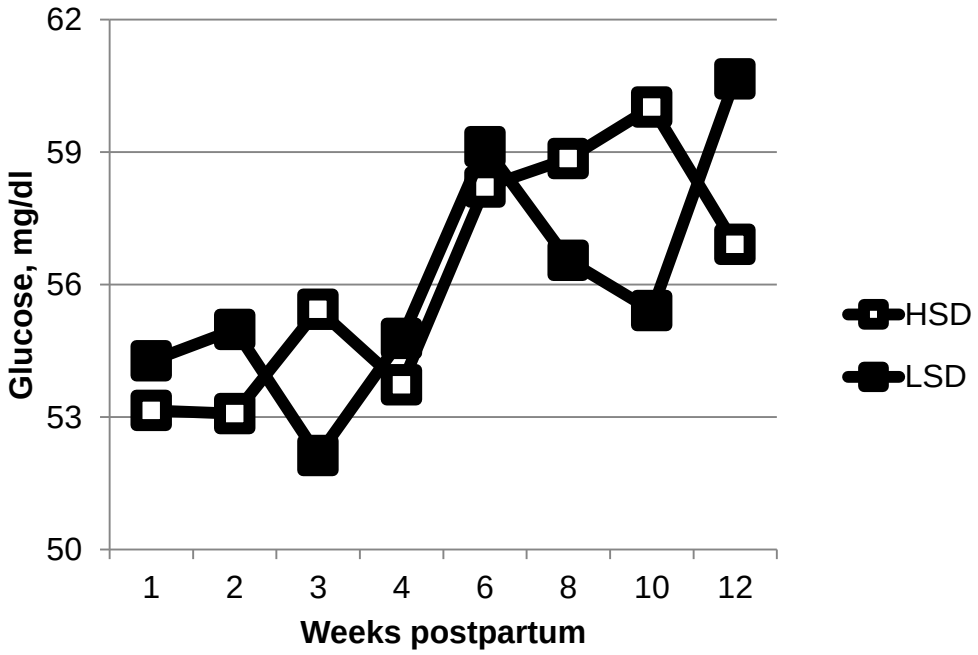


Figure 3.6. Effect of diet on plasma glucose concentrations in a subset of 24 cows from week 1 to 12 postpartum (n=12/diet). Diet had no effect on plasma glucose concentrations (*P*=0.92, SEM=1.87). Cows were fed two levels of starch (HSD and LSD) from the day of calving to 84 days in milk (DIM).

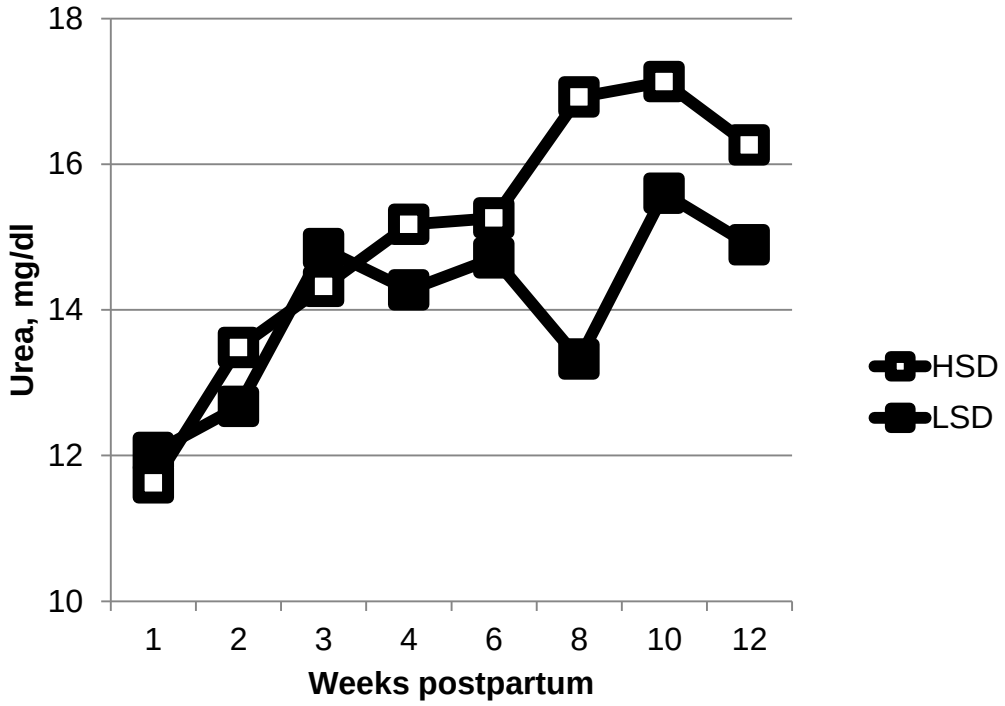


Figure 3.7. Effect of diet on plasma urea concentrations in a subset of 24 cows from week 1 to week 12 postpartum (n=12/diet). Diet had no effect on plasma urea concentrations (*P*=0.45, SEM=1.27). Cows were fed two levels of starch (HSD and LSD) from the day of calving to 84 days in milk (DIM).

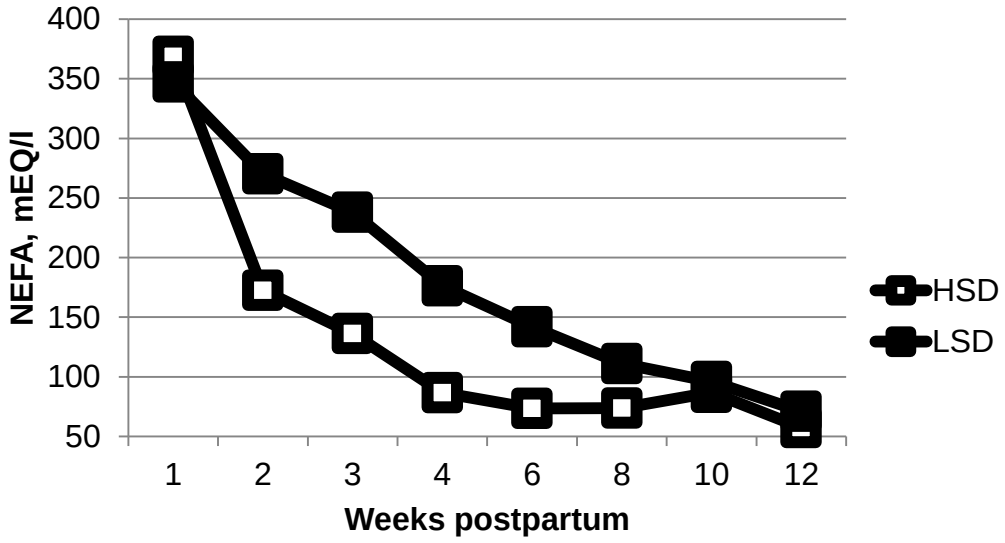


Figure 3.8. Effect of diet on plasma NEFA concentrations in a subset of 24 cows from week 1 to 12 postpartum (n=12/diet). High starch diet (HSD) significantly affected plasma NEFA concentrations (*P*=0.01, SEM=22.91). Cows were fed two levels of starch (HSD and LSD) from the day of calving to 84 days in milk (DIM).

CHAPTER 4

EFFECT OF FEEDING HIGH OR LOW STARCH DIETS ON FOLLICULAR FLUID METABOLITE CONCENTRATIONS AND GENE EXPRESSION PROFILES IN GRANULOSA CELLS OF THE PREOVULATORY FOLLICLE

4.1. Introduction

Follicular fluid is an exudate of serum and it contains substances that are produced locally, and associated with metabolic activity of follicular cells (Gerard et al. 2002). The biochemical environment for the oocyte is formed by the follicular fluid before ovulation. Previous studies have shown that the composition of follicular fluid can be changed by feeding high energy and protein diets to high producing dairy cows, and this could affect oocyte and embryo quality (Leroy et al., 2004).

Inferior oocyte and embryo quality in dairy cows contributes to decline in fertility (O'Callaghan and Bolland, 1999). The oocyte quality is affected by changes in concentrations of gonadotropins, growth factors and steroids (Wehrman et al., 1991, Izadyar et al., 1997 and Driancourt et al., 1998). Adverse metabolic conditions in early lactation will affect the growth of primary follicles and would have a carryover effect on pre-ovulatory follicles 2-3 months later, and such follicles have been reported to contain inferior oocytes (Leroy et al., 2005). These poor quality oocytes that are ovulated at the time of first insemination would detrimentally influence fertility (Leroy et al., 2005).

Leroy et al. (2008) showed that diet composition may affect the oocyte quality by altering the endocrine and metabolic profiles. Feeding a diet that lowers plasma insulin concentrations in dairy cows during the mating period favours oocyte developmental competence, however a diet that increases circulating concentration of insulin is essential during early postpartum period for follicular growth (Garnsworthy et al., 2009). *In vitro* studies showed that non-esterified fatty acids (NEFA) affect granulosa cell proliferation and survival, which may be an explanation for negative energy balance influencing folliculogenesis in immediate postpartum period (Vanholder et al., 2005). Schoppee et al. (1996) reported that dietary restriction in early lactation decreases IGF-1 levels in follicles before selection and also affects follicular growth, as reduced IGF-1 levels also negatively influences FSH concentration. Landau et al. (2000) reported that feeding dairy cows with isoenergetic diet containing corn gluten meal (CGM), soybean meal (SBM) or corn grain (CG) for short period of time positively influences plasma insulin concentrations and also increases insulin and glucose levels in follicular fluid. In addition, *in vitro* studies conducted by Garverick et al. (1971) showed that high urea diet reduces the ability of CL to produce progesterone and also reduces the weight of CL. Elevated urea levels in follicular fluid were reported to affect the oocyte quality (Sinclair et al., 2000). Therefore, alterations in metabolite concentrations caused by diet may affect the key genes which regulate early embryo development (Wrenzycki et al., 2000).

Even-though there is a substantial amount of data in this area, the information on the effects of dietary starch on follicular fluid metabolites like

insulin, IGF-1, glucose, NEFA and urea is limited. By investigating these factors, we can have a better idea of how feeding high starch diet (HSD) to dairy cows in immediate postpartum period affects the follicular fluid metabolite concentrations and how this influences subsequent fertility.

The main objective of this study was to determine the effects of feeding a high or low starch diet on follicular fluid metabolites like insulin, IGF-1, glucose, NEFA and urea. A specific goal was to assess the effects of parity on follicular fluid metabolites concentrations, as well as to understand the correlation between follicular fluid and plasma metabolite concentrations. Another goal was to determine dietary effects on gene expression in the granulosa cells of preovulatory follicles.

4.2. Materials and methods:

4.2.1 Animals and diets:

Twenty-four Holstein cows (9 primiparous, 15 multiparous cows) were used in this study. The experiment was conducted between March 2010 and June 2010 at the University of Alberta, Dairy Research and Technology Centre (DRTC), Edmonton, Canada. All experiments conducted on animals were approved by University of Alberta's Livestock Animal Care and Use Committee and were cared for according to the guidelines of Canadian Council on Animal Care (1993).

Cows were individually housed in a tie-stall barn and let out for exercise daily in the morning period for 2 h. Cows were assigned to one of the two experimental diets immediately after calving and fed until 84 d in milk (DIM).

The experimental diets were formulated to contain 29.2 % starch, 17.3% protein and 3.7% fat in high starch diet (HSD) group while 19.1% starch, 19.4% protein and 4.4% fat in low starch diet (LSD) group. Ingredient composition of the diets is presented in the Table 4.1. Cows were fed once daily the experimental diets as total mixed rations (TMR) at 0730 h and had access to *ad libitum* water.

4.2.2. Ultrasound examination and follicular fluid collection:

Before follicular fluid collection, ovarian status of cows were pre-synchronized with 2 treatments of prostaglandin $F_{2\alpha}$(PGF) (500µg, i/m, Cloprostenol, Estrumate ®, Schening-Plough, Animal Health, Pointe-Claire, QC, Canada) given 14 d apart as described as reported by Senger (2003), and Moore and Thatcher (2006). Two days after second PGF treatment, cows were subjected to ultrasound guided transvaginal follicular aspiration (Figure 4.1) at approximately 58 d after calving. As a preparation for follicular aspiration, rectum was emptied, the perineum, external genitalia and portion of tail was cleaned to be free of fecal contamination. Epidural anesthesia was induced by administration of 2-4ml of 2% Lidocaine hydrochloride (Bimeda-MTC, Animal Health Inc., Cambridge, ON) to prevent cows from straining. Ovaries were examined by trans-rectal ultrasonography (Aloka-500V scanner equipped with a 5 MHz convex transducer, Aloka Co., Tokyo, Japan) to check for the dominant follicle and then trans-vaginal probe was inserted in preparation for aspiration.

The setup for the aspiration includes the long aspiration needle attached to one end of the long tube while the other end is attached to the Falcon tube® (Becton Dickinson and Company, NJ, USA) through clear plastic tubing. Vacuum

was created in the Falcon tube using aspiration pump that creates negative pressure in the tube and the follicular fluid (FF) was drawn into the tube when the needle penetrates the preovulatory follicle. The tubing was monitored for blood and aspiration was stopped and tube disconnected if blood was observed. After each collection, the tube containing dominant follicular fluid was properly labeled and immediately placed on ice, it was then centrifuged at 4500 RPM for 3 min at 4°C to separate the follicular fluid from the cumulus-oocyte-complexes (COCs) holding the oocyte and granulosa cells. The follicular fluid supernatant was transferred to cryo-vials (Nalgene®, New York, USA) for storage at -80°C freezer until further analysis for estradiol, progesterone, insulin, IGF-1, glucose, NEFA and urea.

The pellet resulting from the 4500 RPM centrifugation for 3 min at 4°C described above, was re-suspended in IVF-TALP (Tyrode's albumin lactate pyruvate) medium before searching the COC under the stereoscope. IVF-TALP medium holding both mural and cumulus granulosa cells was centrifuged and the supernatant was discarded and the pellet was suspended in as little fluid as possible and stored in the -80º C freezer until further analysis to determine the differential expression of genes [Luteinizing hormone receptor (LH r), estrogen receptor β, aromatase receptor (CYP 19) and glutathione peroxidase receptor (GPX 3)] in granulosa cells of preovulatory follicles.

4.2.3. Blood sampling:

Four blood samples were collected from each animal (18 h, 30, 15 and 0 minutes) before aspiration, the sample which was collected 18 h before aspiration

was used as a baseline value for measuring LH concentrations (Figure 4.2). Plasma LH concentration was measured to confirm that the follicle is not exposed to LH surge prior to follicular aspiration. Samples were collected from jugular vein using evacuated Vacutainer® tubes containing sodium heparin (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) as an anti-coagulant. After collection, samples were immediately placed on ice and centrifuged at 3000 g for 20 min at 4°C and the plasma obtained was kept in a freezer at -20°C until further analysis. Insulin, Insulin-like growth factor-1 (IGF-1), glucose, NEFA and urea were analyzed by pooling the samples collected close to aspiration (30, 15 and 0 min before) to determine the correlation for above metabolites in follicular fluid and plasma. While plasma analyzed from all the four individual samples was used to determine luteinizing hormone concentrations.

4.2.4. Laboratory analyses:

4.2.4.1. Metabolites and metabolic hormones in follicular fluid and plasma:

Each follicular fluid and plasma sample was analyzed for insulin, IGF-1, glucose, NEFA and urea. A pre-sample run was performed with the follicular fluid samples to determine the amount of fluid required for insulin, glucose, NEFA and urea assay.

A solid phase radioimmunoassay kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) was used to determine the concentrations of insulin in follicular fluid and plasma as previously described by Landau et al. (2000). Follicular fluid was diluted three times with the zero standards provided with the kit. The samples were analyzed in triplicates, in one assay with intra

assay variance of 8.37% and 7.98% for follicular fluid and plasma respectively and the detection limit was 1.3 IU/ml.

Follicular fluid and plasma samples were sent to the Laboratory Analytical Facilities (Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, Saskatoon, SK, Canada) for IGF-analysis 1. Follicular fluid and plasma IGF-1 assays were carried out using a solid phase, enzyme-labeled chemi-luminescent-immunometric-assay (IMMULITE, Diagnostic Products Corporation, Los Angeles, CA, USA). The procedure for both follicular fluid and plasma analysis was carried out as previously described by Elmlinger et al. (2005) for plasma IGF-1 assay. The samples were diluted 1:10 with the pretreatment solution provided with the kit. Intra-assay coefficients of variation was 3.7% and 5.8% for a reference sera in plasma while 5.2% and 4.1% for a reference sera in follicular fluid. The assay sensitivity for IGF-1 was < 25 ng/ml.

Follicular fluid and plasma glucose were analyzed using glucose oxidase/peroxidase enzyme and O-dianisine-dihydrochloride (Sigma Cal. No. P7119 and No.F5803) in triplicates. Volume of plasma and follicular fluid used was 10 µl.

NEFA was determined in plasma and follicular fluid samples using a commercial kit (NEFA-HR (2) Procedure, Wako Chemicals, USA, Richmond, VA) in triplicates. The volume used for determining both follicular fluid and plasma concentrations in NEFA was 5 µl.

The concentration of urea in follicular fluid and plasma was measured enzymatically (Fawcett and Scott, 1960) in triplicates. The volumes used for measuring urea in follicular fluid and plasma were 2 µl and 20 µl respectively.

4.2.4.2. Reproductive hormones in follicular fluid and plasma:

Follicular fluid estrogen and progesterone concentration was analyzed in all samples to differentiate dominant follicles from subordinate follicles. A follicle was considered to be active or dominant when the estrogen: progesterone ratio was greater than 1, while for inactive or subordinate follicles the estrogen: progesterone was lesser than 1, as reported by Landau et al. (2000), and Leroy et al. (2004). Before analysis, the follicular fluid samples were thawed at 37ºC for 10 min and then centrifuged at 3000 g for 20 min at 4ºC as described by Thangavelu et al. (2008).

Follicular fluid estrogen was determined using a solid phase radio-immunoassay kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) in triplicates. The samples were diluted 1:500 in phosphate buffer saline (0.01M PBS, pH=7.5) with 0.1% gelatin as reported by Guzeloglu et al. (2001). A total volume of 100µl aliquot per sample was used for the assay.

Follicular fluid progesterone was determined using a solid phase radio-immunoassay kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) in triplicates. The samples were diluted 1:100 with zero standards provided with the kit as reported by Thangavelu et al. (2008). A total volume of 100 µl aliquot per sample was used for the assay. Samples were analyzed in single assay,

the intra assay variance was 3.47% and 5.81%, and the detection limit was 6pg/ml and 0.04ng/ml for estrogen and progesterone respectively.

Plasma samples were sent to the Laboratory Analytical Facilities (Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, Saskatoon, SK, Canada) for measuring LH concentrations. The assay was performed using previously validated double antibody radio-immunoassay (Rawlings and Evans, 1995). The samples were analyzed in one assay with intra-assay co-efficient of variation of 4.2% and 9.6% for reference sera. The sensitivity of the assay was 0.1 ng/ml.

4.2.4.3. RNA isolation

Total RNA was isolated from granulosa cells of preovulatory follicles, collected from lactating dairy cows, using TRIzol® reagent (Invitrogen, CA, USA) following manufacturer's instructions with some modifications to the protocol. Briefly, 1 ml of TRIzol reagent was added to frozen granulosa cell samples kept on ice and the samples were homogenized using PowerGen 35 homogenizer (Fisher Scientific, USA). The homogenized samples were incubated at 25-30 ºC for 20 min; then 0.2 ml of chloroform was added to each samples, vortexed, and incubated at 25-30 ºC for 2-3 min. After this step, samples were centrifuged at 12,000 g at 4 ºC for 15 min. After centrifugation the micro-centrifuge tube (Fisher Scientific, USA) containing TRIzol lysates and chloroform (Fisher Scientific, USA) separated into 3 phases, the colorless aqueous phase containing RNA forms the upper layer while middle layer was the white phase containing DNA and the bottom layer was red or organic phase containing protein or lipids. The

clear supernatant or upper layer was transferred to a clean, fresh 2 ml micro-centrifuge tube. Then 0.25 ml of isopropyl alcohol and high salt solution was added, and the contents were vortexed, incubated at 25-30˚C for 15 min followed by centrifugation at 12,000 g for 10 min to precipitate the RNA. The supernatant was then discarded, the tube was drained, a white RNA pellet was observed at the bottom of the tube. 1ml of 75% ethanol was added to wash the pellet and centrifuged at 7700 g for 3 min. The pellets were dried at room temperature for 5 min and dissolved in 20µl of RNase-free water (Ambion).

4.2.4.4. RNA quantitation and reverse transcription

The concentration of RNA was determined by measuring the absorbance at 260 and 280 nm using Nanodrop (ND-1000, Nanodrop technologies, USA). The absorbance ratio (260:280) for all samples was between 1.71 and 2.04, indicating high RNA purity. All the samples were diluted using nuclease-free water (Ambion) to contain 92.78 ng/µl of RNA because the lowest concentration of RNA found in one of the experimental sample was 92.78 ng/µl, thus initial concentration of RNA for all the samples were equal. All samples were stored at -80º C until c-DNA synthesis.

The RNA samples extracted from granulosa cells of 9 HSD and 11 LSD cows were treated with DNase I (Invitrogen) to remove the DNA contamination. Then Superscript II (Invitrogen) kit was used to synthesize c-DNA by reverse transcription according to manufacturer's instructions. Then the c-DNA samples were stored at -20 ºC until further analysis.

4.2.4.5. Real time PCR

Primers and Taqman-MGB probes for each gene (Table 4.8) were designed using Primer Express® software v3.0 (Applied Biosystems, Foster City, CA, USA) based on species specific sequences reported in GENBANK. Real-Time PCR was performed in triplicates in 96-well plates using the Taqman® Universal PCR Master Mix (Applied Biosystems, New Jersey, USA) and the ABI 7900HT thermocycler (Applied Biosystems, Foster City, CA, USA). The amplification efficiency for all the genes was determined by serial dilution of c-DNA and was found to be ≥ 90%.

The cycle threshold (Ct) is defined as the point at which the fluorescence of PCR product reaches a threshold. Using the ΔCt method, real-time PCR data for the target gene of interest (GOI) was normalized against the respective means of housekeeping gene i.e. glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Therefore ΔCt value was obtained by subtracting Ct value of GAPDH from the respective cow's Ct value of GOI. To determine the gene expression between treatments, the sample that had the most stable expression for all GOI was taken as a calibrator. The ΔΔ Ct value was calculated by using ΔCt value of the calibrator as a reference value. Therefore the ΔΔ Ct value for the sample used as a calibrator was 0. The relative changes in gene expression were analyzed using $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen, 2001.

4.2.5 Statistical analyses:

All single measurement data (follicular fluid and plasma metabolites like insulin, IGF-1, glucose, NEFA and urea; follicular fluid reproductive hormones

like estradiol and progesterone) were analyzed using MIXED procedure in SAS (version 9.1; SAS Institute Inc., Cary, NC). Baseline measurements recorded for body condition scores (BCS) was used as a covariate adjustment. The model includes treatment, parity and treatment x parity interactions with treatment, parity was considered as fixed effect while cow was a random effect. Correlations were calculated between plasma and follicular fluid metabolites like insulin, IGF-1, glucose, NEFA and urea using Pearson correlation in SAS. Correlations also were determined between follicular fluid estradiol concentration and mRNA expression of luteinizing hormone receptor (LH receptor), estrogen receptor-β and aromatase receptor in GC. The individual fold change for each GOI was analyzed using MIXED procedure of SAS. Significant differences were reported if the *P* value was ≤ 0.05; *P*-values > 0.05 and ≤ 0.10 were considered trends.

4.3. Results

4.3.1. Follicular fluid and plasma metabolites

The effect of treatment and parity on follicular fluid profiles for insulin, IGF-1, glucose, NEFA and urea are presented in the Table4.2 and 4.3. A treatment effect was observed for follicular fluid IGF-1 concentrations as cows in HSD group had significantly higher concentrations of IGF-1 than those in LSD group. Primiparous cows had significantly higher follicular fluid IGF-1 concentrations than multiparous cows. Follicular fluid NEFA levels were found to be significantly lower in cows fed HSD than LSD, however no parity effect was observed in intrafollicular NEFA concentrations. No treatment or parity effect was observed for insulin, glucose and urea concentrations in follicular fluid,

however treatment and parity interaction was observed for insulin concentrations in follicular fluid (P=0.04; Table 4.3). There was no effect of treatment, parity or interaction observed for plasma insulin, IGF-1, glucose, NEFA and urea (Table 4.4 and 4.5). A positive correlation existed between estradiol and insulin concentration in follicular fluid (R=0.47, P=0.02) however no correlation was found between follicular fluid estradiol and IGF-1 concentrations. No correlations were observed between follicular fluid and plasma concentrations for insulin, IGF-1, glucose, NEFA and urea.

4.3.2. Reproductive hormones in follicular fluid and plasma

No treatment, parity or interaction effects were observed for estradiol and progesterone concentrations in follicular fluid of preovulatory follicle (Table 4.6 and 4.7). A positive correlation was observed between the pre-ovulatory follicular diameter measured before follicular aspiration and follicular fluid estradiol concentrations (R=0.44, P=0.04) However there was no effect of diet or parity was observed on the diameter of the pre-ovulatory follicles.

4.3.3. Gene expression

No diet or parity differences were observed for changes in expression of the targeted GOI in the granulosa cells of preovulatory follicles (Table 4.9).

No correlation was observed between follicular fluid estrogen concentration and gene expression of LH receptor, estrogen receptor-β, aromatase receptor in granulosa cells of preovulatory follicle. However a strong positive correlation was observed between mRNA expression of aromatase receptor and estrogen receptor-β in granulosa cells (r=0.98, P<0.0001).

4.4. Discussion

A positive correlation was observed between follicular fluid estradiol content and preovulatory follicular diameter in this study. This was in agreement with Ginther et al. (1996), as they reported that estradiol content in follicular fluid was greater in dominant follicles that were selected after deviation reflecting the estrogenic potential of those follicles.

4.4.1. Metabolites profiles in follicular fluid

No dietary differences were observed for follicular fluid insulin concentrations in this study and no correlations were found between plasma and follicular fluid insulin concentrations. This was in contrast to the studies of Landau et al. (2000) as they reported that cows fed corn grain (CG) had 26% and 32 % higher insulin concentrations than those in the corn gluten meal (CGM) and soybean meal (SBM) group (0.44 ng vs. 0.21 & 0.31 ng, respectively). But dry matter intake was not different between the diets in their study. However in their study, smaller numbers of cows were used (5 in SBM and CG, 6 in CGM) which was in contrast to 7 cows in HSD and 12 cows in LSD in the present study. In their study, experimental period was initiated when cows were on average 76.7 d postpartum compared to 58 d postpartum in the current study.

A positive correlation was observed between follicular fluid insulin and estradiol concentrations in the present study, which was in agreement with the studies of Landau et al. (2000). Among insulin and IGF-1, the most potent stimulator of estradiol production by granulosa cells was reported to be insulin (Spicer and Echternkamp, 1995). This is in agreement with the current study as

we found a positive relationship between follicular fluid concentrations of insulin and estradiol, but not for IGF-1 and estradiol concentrations.

Feeding HSD increased the concentrations of IGF-1 in follicular fluid than LSD in the present study. This was in agreement with the studies of Kendrick et al. (1999) as they found that cows fed high energy diet had higher mean follicular fluid IGF-1 concentrations from d 30-100 post-partum than those in low energy group. Schoppee et al. (1996) found that chronic feed restriction in cattle decreased the intra-follicular IGF-1 concentrations indicating that dietary restriction negatively affects IGF-1 concentrations in follicular fluid. Despite the fact that Leeuwenberg et al. (1996) reported that follicular fluid IGF-1 is mostly derived from circulation, in the current study we did not find a correlation between plasma and follicular fluid IGF-1 concentrations. This is also in contrast with the studies of Echternkamp et al. (1990) as they reported that IGF-1 concentrations from large bovine follicles and blood were positively correlated.

A positive correlation was reported to exist between serum IGF-1 and circulating estradiol concentrations during the dominant follicle growth (Beam and Butler, 1998). Various studies analyzed the relationship between follicular fluid IGF-1 and estradiol, however inconsistent results were reported(Spicer and Echternkamp, 1995). Therefore, we were interested to determine the relationship between IGF-1 and estradiol in follicular fluid. The results showed no correlation between follicular fluid IGF-1 and estradiol in the present study, which suggests that the production of estradiol was not regulated consistently by IGF-1 in bovine follicles (Spicer and Echternkamp, 1995).

Concentrations of IGF-1 in follicular fluid were higher in primiparous cows than in multiparous cows in this study. While several studies have shown the relationship between plasma IGF-1 concentrations and parity, there is little information on the association between follicular fluid IGF-1 and parity. Wathes et al. (2007a) found that primiparous cows had higher plasma IGF-1 concentrations, but age was reported to be the major factor rather than parity that positively influence the concentration of IGF-1. As well, the levels decreased considerably between second and third years of age, which may be the case in current study for elevated levels of IGF-1 in the primiparous cows.

In this study, no differences in diet were observed for glucose concentrations in follicular fluid, which was in contrast to the studies of Landau et al. (2000). In their study, they reported that intra-follicular glucose content was 46% and 32% higher for cows in CG than those in CGM and SBM group (0.99 mg vs. 0.53 & 0.54 mg, respectively). As discussed earlier in this chapter, the experimental period in Landau et al. (2000) study was different from the current study, as cows in their study were fed the rations containing CG, CGM and SBM from d 10 to 16 of synchronized estrous cycles and the follicle was aspirated during luteal phase i.e. on day 18 of estrous cycle.

Landau et al. (2000) and Leroy et al. (2004) reported a positive correlation between plasma and follicular fluid glucose concentrations which was not the case in the present study. Leroy et al. (2004) found that follicular fluid glucose content was higher than in serum reflecting that the follicular fluid of dominant follicles was well exposed to post-partum changes in glycemia. However, their

interpretations were based on repeated measurements for serum (d 0 to 46) and follicular fluid samples (d 14 to 46) collected during early post-partum, compared to a single measurement in our study on approximately 58th day post calving.

For the current study, cows in HSD group had lower follicular fluid NEFA concentrations compared to those in LSD group. This was in agreement with the studies of Comin et al. (2002) as they found that follicular fluid NEFA concentrations was lower in cows fed according to the requirements for maintenance and production (fed diet) at 60 d postpartum than those fed only wheat straw and water (restricted diet) at 90 d postpartum. No correlation was observed between plasma and follicular fluid NEFA concentrations in this study which was in contrast to the findings of Comin et al. (2002) and Leroy et al. (2004). Leroy et al. (2004) found that the concentration of follicular fluid NEFA was considerably lower than in serum and they suggested that an unknown mechanism was protecting the oocyte and granulosa cells as *in vitro* studies showed that higher content of NEFA was detrimental to oocyte quality.

No dietary differences were observed for follicular fluid urea concentrations and also no correlations were observed between plasma and follicular fluid urea concentrations in the current study. Unlike our study, Leroy et al. (2004) reported that the concentration of urea in plasma was reflected in follicular fluid in the early postpartum period and suggested that the increased concentrations of urea may be toxic to oocytes.

4.4.2. Gene expression profiles in granulosa cells of preovulatory follicles

No differences in diet or parity were observed for expression of the genes for the LH receptor, estrogen receptor-β, aromatase receptor and glutathione peroxidase receptor 3 (GPX 3) in GC of preovulatory follicles.

In the current study, no correlation was observed between LH mRNA expression in GC and preovulatory follicle diameter (mean diameter was 15.94 ± 1.41 mm). However Beg et al. (2001) reported that the expression of LH mRNA was positively associated with the diameter of the follicle as the mRNA expression was higher in the dominant follicle with the mean diameter of 10.8 mm, however their study was independent of dietary effects. They also reported that an increase in LH mRNA expression would increase the LH receptor in granulosa cells, as the later plays an important role in the events associated with follicular deviation or selection.

During follicular deviation, estradiol production in dominant follicle depends on aromatase enzyme activity in the granulosa cells (Ginther et al., 1996). No correlation was observed between aromatase receptor gene expression and follicular fluid estradiol concentration in this study, which was in agreement with the studies of Tian et al. (1995). In their study, aromatase mRNA was not expressed in dominant follicles even-though there was an increase in serum estradiol and LH concentrations. Therefore the expression of aromatase mRNA was not a limiting factor in follicular fluid estradiol synthesis (Calder et al., 2001). However we found a positive association between aromatase mRNA expression levels and estrogen receptor-β. In the present study, we measured the expression

of estrogen receptor-β in granulosa cells because it is the pre-dominant estrogen receptor found in the ovary compared to estrogen receptor-α (Byers et al., 1997).

In our study, no correlation was observed between follicular fluid estradiol concentrations and mRNA expression levels of LH receptor, estrogen receptor-β and aromatase receptor. Conversely, Evans et al. (2004) found a positive association between estradiol concentrations in follicular fluid and m-RNA expression for the above 3 GOI in dominant but not in subordinate follicles, implying that estradiol plays an important role in continued growth of dominant follicles after follicular selection or deviation.

4.5. Conclusion

This study demonstrated that increasing dietary starch can alter the follicular fluid metabolites especially by increasing IGF-1 and decreasing NEFA concentrations. However this alteration in follicular fluid composition did not affect the expression of selected genes in granulosa cells. Further studies are required to improve the understanding of dietary effects on intra-follicular milieu.

Table 4.1.Ingredient composition of dietary treatments

Ingredient composition (%DM)	HSD[1]	LSD[2]
Barley silage	43.1	43.1
Corn grain rolled	21.6	21.6
Barley rolled	17.3	0.0
Wheat DDGS	0.0	17.2
Beet pulp	3.2	12.3
Corn gluteal meal	8.3	0.0
Vegetable oil	2.4	1.9
Urea	0.3	0.0
Minerals & Vitamins	3.9	3.9

[1] High Starch Diet

[2] Low Starch Diet

[3] Dry distiller's grain with soluble

Table 4.2. Effect of dietary starch on follicular fluid metabolites and metabolic hormone concentrations in the samples collected during follicular fluid collection (approximately 58 d postpartum)

	Treatment		SEM	*p*
	HSD	LSD		
N	7	12		
Insulin, IU/ml	10.56	10.71	0.33	0.65
IGF-1, ng/ml	108.00	69.25	15.93	0.02
Glucose, mg/dl	56.70	56.53	1.96	0.93
NEFA, mEQ/l	148.58	222.23	30.36	0.02
Urea, mg/dl	7.01	7.00	0.30	0.97

Table 4.3. Effect of parity on follicular fluid metabolites and metabolic hormone concentrations in the samples collected during follicular fluid collection (approximately 58 d postpartum)

	Parity			*p*	
	Primiparous	Multiparous	SEM	Parity	Treatment*parity
N	7	12			
Insulin (IU/ml)	10.39	10.89	0.33	0.15	0.04
IGF-1 (ng/ml)	105.13	72.12	15.93	0.05	0.85
Glucose (mg/dl)	57.68	55.55	1.96	0.29	0.97
NEFA (mEQ/l)	199.01	171.80	30.36	0.38	0.50
Urea (mg/dl)	7.00	7.01	0.30	0.96	0.53

Table 4.4. Effect of dietary starch on plasma hormones and metabolites concentration in the samples collected before follicular fluid collection (approximately 58 d postpartum)

	Treatment			
	HSD	LSD	SEM	*p*
N	7	12		
Insulin, IU/ml	3.14	3.05	0.63	0.89
IGF-1, ng/ml	115.14	107.39	17.13	0.65
Glucose, mg/dl	70.14	71.15	3.48	0.77
NEFA, mEQ/l	372.91	537.11	146.85	0.27
Urea, mg/dl	7.24	5.84	0.91	0.14

Table 4.5. Effect of parity on plasma hormones and metabolites concentration from the samples collected before follicular fluid collection (approximately 58 d postpartum)

	Parity		SEM	*p*	
	Primiparous	Multiparous		Parity	Treatment*parity
N	7	12			
Insulin (IU/ml)	3.41	2.78	0.63	0.33	0.96
IGF-1 (ng/ml)	107.96	114.57	17.13	0.70	0.98
Glucose (mg/dl)	71.5	69.7	3.48	0.62	0.66
NEFA (mEQ/l)	438.27	471.75	146.8	0.82	0.52
Urea (mg/dl)	6.25	6.83	0.91	0.53	0.68

Table 4.6. Effect of dietary starch on follicular fluid reproductive hormone concentrations from the samples collected during follicular fluid collection (approximately 58 d postpartum)

	Treatment		SEM	*p*
	HSD	LSD		
N	9	12		
Estrogen, ng/ml	1150.18	1198.88	319.3	0.88
Progesterone, ng/ml	78.07	61.56	15.48	0.30

Table 4.7. Effect of parity on follicular fluid hormone concentrations from the samples collected during follicular fluid collection (approximately 58 d postpartum)

	Parity			*p*	
	Primiparous	Multiparous	SEM	Parity	Treatment*parity
N	9	12			
Estrogen (ng/ml)	1079.31	1269.74	319.3	0.55	0.43
Progesterone (ng/ml)	58	81.63	15.48	0.14	0.28

Table 4.8. Details of primers and probes used for RT-PCR analysis

Gene name	Gene accession number	Primers and probes	Primers and probes sequences	Position
Estrogen receptor-β	NM_174051.3	Forward	CCATTGCCAGCCGTCAGT	267-284
		Reverse	GGTTTCACGCCAAGGACTCTT	324-304
		Probe	CTGTATGCAGAACCTC	286-301
LH receptor	U20504.1	Forward	TCCCTGGAGCTGAAGGAAAA	615-634
		Reverse	CGGAAGGCGTCGTTGTG	670-654
		Probe	CACGCCTGGAGAAGA	637-651
Aromatase	U18447.1	Forward	TCGTCCTGGTCACCCTTCTG	1367-1386
		Reverse	CGCACCGACCTTGCAAA	1423-1407
		Probe	ACGCTTCCACGTGCAG	1389-1404
GPX3	NM_174077.3	Forward	AACCCATGAAGGTCCATGACA	533-553
		Reverse	GGCCCCACCAGGAACTTCT	587-569
		Probe	CCGGTGGAACTTT	555-567

Gene number	Gene accession number	Primers and probes	Primers and probes sequences	Position
GAPDH	U85042.1	Forward	TGCCGCCTGGAGAAACC	715-731
		Reverse	CGCCTGCTTCACCACCTT	771-754
		Probe	CCAAGTATGATGAGATCAA	734-752

Table 4.9. Summary of statistical analysis showing the fold change in the expression of target genes in granulosa cells of preovulatory follicle.

Gene name (Gene abbreviation)	Fold change			*P*	
	HSD	LSD	SEM	Diet	Parity
Luteinizing hormone receptor (LHr)	0.06	0.16	0.15	0.52	0.58
Estrogen receptor-β (ER-β)	0.21	0.84	0.48	0.23	0.37
Aromatase receptor (CYP 19)	0.22	0.28	0.18	0.72	0.37
Glutathione peroxidase3 (GPX 3)	0.38	0.39	0.18	0.94	0.44

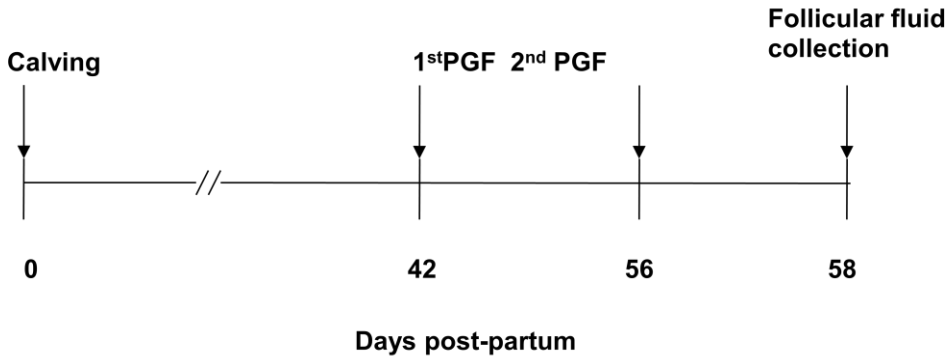


Figure 4.1. Protocol for synchronization of ovarian status prior to collection of follicular fluid. Ovarian statuses of dairy cows were synchronized approximately 42 d postpartum by administering 2 treatments of prostaglandin $F_{2\alpha}$ (PGF), 14 d apart. Follicular fluid was collected from preovulatory follicle 2 d after 2nd PGF administration to analyze insulin, IGF-1, glucose, NEFA and urea. On the day of follicular fluid collection, blood samples were also collected to assess the correlation between follicular and plasma metabolites. Blood sampling schedule is shown in Figure 4.2.

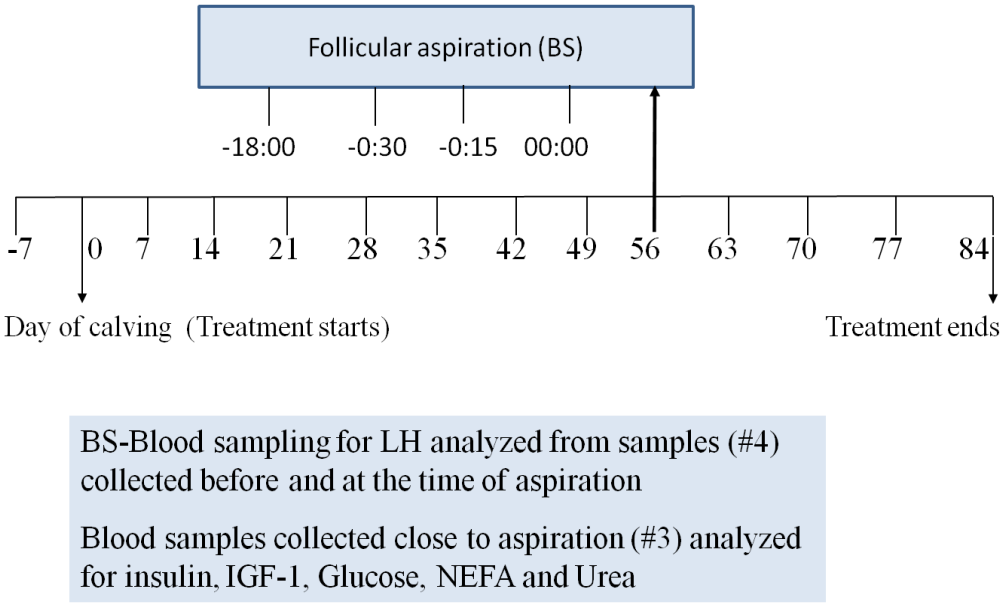


Figure 4.2. Blood sampling schedule during follicular fluid collection (FFC) or follicular aspiration. Four blood samples were collected before follicular fluid collection for plasma separation and all 4 samples were analyzed for LH concentrations to confirm that the preovulatory follicle was not exposed to LH surge. Whereas, 3 blood samples collected close to FFC were pooled before analysis to quantify insulin, IGF-1, glucose, NEFA and urea.

CHAPTER 5

SUMMARY OF FINDINGS, FUTURE RESEARCH, INDUSTRY PERSPECTIVE AND FINAL CONCLUSION

5.1. Summary of findings

The objective of the first study was to determine the effects of feeding high and low starch diet on reproductive function (ovarian dynamics, and plasma concentrations of insulin, IGF-I, glucose, urea and NEFA) in postpartum dairy cows. The main findings are summarized below:

- Increased dietary starch did not affect the interval from calving to first ovulation.
- A greater proportion of cows in high starch diet (HSD) ovulated two or more follicles.
- HSD cows had higher concentration of insulin and lower concentration of non-esterified fatty acids (NEFA) in plasma compared to those in LSD group.
- Multiparous cows tended to have shorter calving to ovulation interval compared to primiparous cows.
- Primiparous cows had higher concentration of plasma IGF-1, NEFA and lower concentration of urea and tended to have higher plasma insulin and glucose concentrations.

The objective of the second study was to determine the effects of feeding high and low starch diet on intra-follicular milieu (follicular fluid composition of insulin, IGF-1, glucose, urea and NEFA, and gene expression in granulosa cells) in postpartum dairy cows. A summary of the main findings are presented below:

- HSD cows had higher concentrations of insulin-like growth factor (IGF-1) and lower concentration of NEFA in follicular fluid compared to those in LSD group.
- No dietary differences were observed for follicular fluid insulin, glucose and urea concentrations.
- No dietary differences were observed for gene expression profiles in GC of preovulatory follicles.
- Primiparous cows had higher IGF-1 concentrations in follicular fluid.
- No correlations were observed between follicular fluid and plasma concentrations of insulin, IGF-1, glucose, NEFA and urea.
- A positive correlation was observed between insulin and estradiol concentration in follicular fluid.
- A strong positive correlation was observed between m-RNA expression of aromatase receptor and estrogen receptor-β in granulosa cells (GC) of preovulatory follicles.

5.2. Future research

Although the findings from this study provided us with valuable information about the effects of dietary starch on reproductive function in dairy cows, further research is certainly needed in the following areas:

It could be beneficial to measure the circulating concentrations of FSH, LH and inhibin around the time of follicular deviation in cows ovulating two or more follicles as these hormones play an important role around follicular selection. Wiltbank et al. (2000) reported that follicular factors like estrogen, progesterone were metabolized rapidly in high producing dairy cows resulting in elevation of FSH in cows leading to selection of two or more dominant follicles around follicular deviation. Therefore determination of FSH, LH and inhibin may give a clear understanding about the follicular dynamics around selection of two or more dominant follicles, as we did not find a difference in IGF-1 concentration in plasma to support the findings of Echternkamp et al. (1990). In their study, cows selected for twins had more serum concentrations of IGF-1 compared to control cows.

In vitro studies conducted by Vanholder et al. (2005) demonstrated that high concentration of NEFA was toxic to bovine granulosa cells. In the current study, we found that LSD cows had higher concentrations of NEFA in follicular fluid compared to HSD cows, however we did not determine whether diet-induced changes in follicular fluid NEFA concentrations have an impact on oocyte developmental capacity. By knowing the oocyte environment and quality, we could better understand their association with conception rate.

It could also be recommended to repeat the study on intrafollicular milieu in larger number of cows to better understand the dietary effects on follicular fluid composition.

5.3. Industry perspectives

In this study, the proportion of multiple first ovulations was increased in cows fed a HSD compared to those fed a LSD (40 vs. 20%). Although multiple ovulations can contribute to increased progesterone concentrations in the early lactation period and possibly improve postpartum reproductive function, the implications of multiple ovulations in the early postpartum period and their effects on reproductive outcome require further study. We also found that cows fed HSD tended to have greater multiple ovulations than LSD cows (21 vs. 9%) at the time of breeding. Even though multiple ovulations were considerably decreased at time of breeding compared to the early postpartum period (i.e., first ovulation), this phenomenon could increase the risk of twinning in dairy cows, which is not a desirable trait. The use of transrectal ultrasonography to diagnose pregnancy in dairy cows would be helpful in identifying cows bearing twins as these cows must be monitored and provided special care at calving time to reduce complications associated with calving and to improve calf survival rate.

Wheat DDGS used at levels of < 20% of DM, as in the present study, could be beneficial to dairy cows as a potential feed ingredient, and at these levels of intake, potentially reduce the emission of greenhouse gases.

5.4. Final conclusion

Increasing the levels of dietary starch increased the plasma insulin and follicular fluid IGF-1 concentrations and also decreased NEFA concentrations in both plasma and follicular fluid. These alterations in plasma and follicular fluid

metabolites did not benefit the HSD cows in advancing the interval from calving to first ovulation but increased the number of multiple first ovulations.

More research needs to be conducted in this area involving large numbers of cows to determine whether high starch diet in early lactation dairy cows would affect plasma and follicular fluid metabolic profiles, oocyte quality and subsequent fertility. As multiple ovulations at breeding could increase the incidence of twinning, further investigations are also essential on the association between increased intake of starch and the risk of twinning in dairy cattle.

CHAPTER 6.

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