THE ROLE OF ADIPONECTIN IN REGULATION OF METABOLISM IN DAIRY COWS

By

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The real constitution of things is accustomed to hide itself.....

Everything flows.....

Heraclitus 540 BCE–480 BCE
Greek philosopher

It is dedicated to my father, Demetrios, and my mother, Agori.

DECLARATION

I hereby declare that this thesis is my own work and that it has not been submitted anywhere for any other degree or award. The work presented herein is my own work and where other sources of information have been used, they have been acknowledged. I also declare that this thesis is less than 100,000 words in length, inclusive of tables, figures, appendices, footnotes, and references.

Konstantinos D. Kalamaras

ABSTRACT

Body condition score has been considered an indirect measure of nutritional status and nutrition has been demonstrated to interact with reproduction. Adiponectin and leptin, because of its insulin sensitising actions and their association with body condition could be potential regulators of metabolism during the transition from pregnancy to lactation. The work described in this P*h*D project was designed to investigate the role of metabolic, body condition, and dietary factors in regulation of productive and reproductive performance in dairy cows, with particular regard to glucose homeostasis and adipokines. A special focus was directed to circulating adiponectin and its association with metabolic and hormonal status, and reproduction. Interestingly, adiponectin was found to be present in bovine milk at concentrations similar those of plasma. Body condition score, GH, insulin, leptin, and adiponectin showed a potential to modulate glucose homeostasis and reproductive output. Results from this study demonstrated a negative effect of long-term moderately elevated insulin concentration on reproductive performance. Additionally, BCS at calving and ΔBCS are determinant factors influencing postpartum reproductive recovery and they should be basic components of farming monitoring schemes. Although further investigation is needed to address the proposed negative relationship between adiponectin and GH, and to identify other dietary factors that may modulate circulating adiponectin, data from this study support regulatory roles of adiponectin in metabolism and reproduction. Moreover, adiponectin holds great promise to serve as a putative target molecule that integrates metabolism, reproduction and feeding behaviour. More importantly, hypoadiponectinemia could be another alternative mechanism that contributes to poor fertility in dairy cows. The incorporation of this new knowledge into the development of better nutritional strategies is a key point that is being considered to improve the welfare, and reproductive and productive performance in dairy cows.

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ABBREVIATIONS

AA	Amino acids
AdipoR1	Adiponectin receptor type I
AdipoR2	Adiponectin receptor type II
ADP	Adiponectin
AIC	Akaike's information criterion
AMP	Adenosine monophosphate
AMPK	5' AMP-activated protein kinase
AMY	Aggregated (cumulative) milk yield
ATP	Adenosine triphosphate
AUC_{ROC}	Area under the ROC curve
BCS	Body condition score
BMI	Body mass index
BOHB	β-Hydroxybutyrate
bST	Bovine somatotropin
cAMP	Cyclic adenosine monophosphate
CFI	Comparative fitness index
CI	Confidence interval 95%
CL	Corpus luteum
CLA	Commencement of luteal activity
C*o*A	Coenzyme A
CP	Crude protein
CV	Coefficient of variation
DIC	Deviance information criterion
DM	Dry matter
DMI	Dry matter intake
DOV1	Delayed ovulation type I
DOV2	Delayed ovulation type II
EB	Energy balance
ED_{80}	Effective dose of 80%
EGF	Epidermal growth factors
ELISA	Enzyme-linked immunosorbant assay
ERK	Extracellular signal-regulated kinases
Exp (*b*)	Exponentiated beta coefficient
FAO	Fatty acid oxidation
FFA	Free fatty acids
FSH	Follicle-stimulating hormone
GC	Glucocorticoids

GDM	Gestational diabetes mellitus
GEE	Generalized estimating equations
GH	Growth hormone
GHR	Growth hormone receptors
GHRH	Growth hormone releasing hormone
GLM	Generalized linear models
GLUGON	Glucagon
GLUT	Glucose transporter
GnRH	Gonadotropin releasing hormone
HF	High fat diet
HPO	Hypothalamic pituitary ovary axis
HS	High starch diet
HSL	Hormone sensitive lipase
ICV	Intra-cerebro-ventricular
IGF-I	Insulin-Like Growth Factor I
INS	Insulin
IRS	Insulin receptor substrates
LH	Luteinizing hormone
LPL	Lipoprotein lipase
LPN	Leptin
LSD	Least square differences
LWT	Live weight
MANOVA	Multivariate analysis of variance
MAPK	Mitogen-activated protein kinase
MAR	Missing at random
ME	Metabolizable energy
MEBAL	Metabolizable energy balance
MI	Multiple imputation
ML	Maximum likelihood
***m*RNA**	Messenger ribonucleic acid
MY	Milk yield
***n*-3 PUFA**	Omega-3 polyunsaturated fatty acids
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NFI	Normed fit index
NPY	Neuropeptide Y

NS	Non-significant
Ob-R	Short form receptors of leptin
Ob-Rb	Long form receptor of leptin
OM	Organic matter
OR	Odds ratio
P4	Progesterone
PCL1	Persistent corpus luteum type I
PCL2	Persistent corpus luteum type II
PCOS	Polycystic ovary syndrome
PDA	Path diagrams analysis
PMY	Previous milk yield
POMC	Pro-opiomelanocortin
PPA	Postpartum anoestrus
PPAR-α	Peroxisome proliferator-activated receptor alpha
QIC	Quasi-likelihood under the independence model criterion
REML	Restricted maximum likelihood
RIA	Radio immuno assay
RMSEA	Root-mean-square error of approximation
ROC	Receiver operating characteristic
SE	Standard error of means
SED	Standard error of the difference between treatment means
SEM	Structural equation modeling
TAG	Triacylglycerols
TG	Triglycerides
TGF	Transforming growth factors
TNF-α	Tumor necrosis factor-alpha
TZD	Thiazolidinediones
VFA	Volatile fatty acids
WAT	White adipose tissue
χ^2	Chi-square

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1. Introduction and purpose of the study

Milk yield is influenced by genetic, nutritional and environmental factors (Rook & Thomas, 1983; Garnsworthy & Webb, 1999). On a short term basis, the efficiency of nutrient use for milk production is primarily dependent on milk volume, but as milk yield increases a lower proportion of total feed intake is used for maintenance (Chilliard, 1992). Consequently, the modern high yielding cow loses weight and mobilizes adipose and muscle tissue depots to support milk production. Thus, high producing dairy cows experience a metabolic stress involving negative energy balance (NEB) in early lactation (Bauman & Currie, 1980). The main characteristics of this condition are loss of body weight and mobilization of body fat stores because feed intake cannot support the energy required for milk yield and maintenance (Beam & Butler, 1999; Butler, 2000; De Vries & Veerkamp, 2000).

In general milk production and reproductive performance are highly negatively correlated in dairy cows (Macmillan *et al.*, 1996; Royal *et al.,* 2000; Stevenson, 2001; Lucy, 2002; Opsomer *et al.,* 2006). In recent decades, genetic improvement in dairy cows has led to a dramatic increase in milk yield, which has been accompanied by an inherent decline in reproductive performance (Butler, 2000; Royal *et al.*, 2000; Evans *et al.*, 2006; Garnsworthy *et al.,* 2008a). Probably, many other reasons (such as nutrition, management, and poor expression or detection of oestrus) are at least in the same degree accountable for the decreased reproduction performance in dairy cows (Garnsworthy & Webb, 1999; Lucy, 2001; Dobson *et al.*, 2007).

Nutrition interacts with reproductive performance and this interaction has prominent influences on livestock productivity and viability (Butler & Smith, 1989; Garnsworthy & Webb, 1999). This interaction is still being investigated, but it seems that the relationship between nutrition and reproduction is dynamic, complex, and not well understood (Gordon 1996; Boland & Lonergan, 2003). The reproductive capacity of the postpartum cow is a critical element that determines overall biological and productive efficiency. Thus, failure

of cows to resume cyclicity after calving is a critical point that influences the economical profitability of the cattle industry (Roberts *et al.*, 1997; Stott *et al.*, 1999; Evans *et al.,* 2006).

Body condition score (BCS) is an indirect measure of nutritional status (Macmillan *et al.*, 1996; Garnsworthy, 2006). It is well known that BCS of a cow, especially at certain points of its lactation cycle, directly influences fertility (Butler, 2003; Garnsworthy, 2006). This is true for a variety of important fertility indices, especially calving interval (Lucy, 2001; Oltenacu & Algers, 2005; Dobson *et al.*, 2007). BCS at calving is believed to influence reproductive performance due to its influence on tissue mobilization and on uterine involution. Moreover, BCS at calving is strongly related with condition loss and postpartum NEB, and influences circulating metabolic hormones and metabolites (Roche *et al.,* 2009).

Adipose tissue releases a variety of adipokines, which are implicated in energy metabolism and reproduction. Leptin and adiponectin are two of the better-studied adipokines, and they are considered to have local and systemic effects (Antuna-Puente *et al.*, 2008). Leptin is positively correlated with BCS, and NEB causes a notable reduction in plasma leptin concentrations (Chilliard *et al.* 2005). Moreover, leptin has been found to modulate nutrient transfer and partitioning by interaction with other hormones including insulin, glucagon, glucocorticoids, growth hormone (GH), insulin-like growth factor I (IGF-I), cytokines and thyroid hormones (Hill, 2004; Garnsworthy *et al.,* 2008a). Adiponectin expresses insulin-sensitizing actions and may be involved in insulin resistance and glucose homeostasis (Antuna-Puente *et al.*, 2008). Fat mass may exert direct negative feedback on adiponectin secretion (Gordon *et al.*, 2007) and a negative correlation between circulating adiponectin and fat mass has been reported in many animal and human models (Kadowaki & Yamauchi, 2005). The link between leptin and reproduction is strong and well documented (Chilliard *et al.* 2005), whereas there is only limited evidence that adiponectin can directly regulate reproductive functions (Mitchell *et al.,* 2005; Dupont *et al.*, 2008).

Insulin is a key hormone in endocrine control which facilitates movement of glucose across cell membranes, and thereby regulates the concentration of blood glucose (Guyton & Hall, 2006). Glucose is the principal source of energy for life processes in the mammalian cell (Kaneko, 2008a). In postpartum cows, decreased insulin reduces glucose uptake by insulin-dependent organs such as adipose and muscle tissue, and increases glucose availability for the insulin-independent mammary gland (Bossaert *et al.,* 2009). Decreased insulin (McGuire *et al.*, 1995) and increased GH (Rhoads *et al.,* 2004) concentrations also promote adipose tissue mobilization, resulting in elevated levels of circulating NEFA and BOHB, which favours milk yield. During early lactation, glucagon concentration increases relative to the dry period to stimulate lipolysis and gluconeogenesis, in order to provide the body with the required energy to support the high milk production (De Boer *et al.*, 1985). Feeding dairy cows on diets to increase circulating insulin concentrations for the first 50 postpartum increased the proportion of cows ovulating during this period (Gong *et al.*, 2002). Control of glucose homeostasis through dietary modulation of circulating insulin has been a key nutritional strategy to alleviate the postpartum dairy cow from the detrimental effects of NEB, and improve reproductive performance (Garnsworthy *et al.*, 2009).

The poor fertility of high yielding dairy cows has been the main scientific focus for many research groups around the world. Because the genetic makeup of the modern high yielding cow is established and it is difficult to change in the short term, much attention has been paid to the interaction between nutrition, condition and adipose with reproductive performance. Work at the University of Nottingham, in collaboration with other researchers, addressed the problem of reduced fertility in dairy cows. The main output of this programme was the development of a nutritional strategy which possibly helps cows to improve fertility without depressing milk production (Gong *et al.*, 2002; Adamiak *et al.,* 2005, 2006; Fouladi-Nashta *et al.,* 2005, 2007; Garnsworthy *et al.,* 2008a, 2008b, 2008c, 2009). This P*h*D project builds on that programme by utilising existing data and blood samples in further laboratory and data analyses.

The overall hypothesis was that the reproductive performance of lactating dairy cows is affected by nutrition and body condition, and that the related hormonal and metabolic profile plays a role in cow fertility. Moreover, the present P*h*D project was to study the effects of different nutritional and condition challenges on metabolic and hormonal profile, circulating adipokines, and reproductive performance in dairy cows. A special emphasis was directed to circulating adiponectin and its association with metabolic and hormonal status, and reproduction. Finally, the major scope of this project was to find evidence for the mechanism which possibly associates changes in body condition, diet, and physiological stage with changes in hormonal and metabolic profile. This mechanism is suggested to be implicated not only in nutrient partitioning but also in reproductive success or failure.

2. Review of literature

2.1. Postpartum nutritional strategies

The interaction of nutrition with reproduction can be characterized as complicated, unstable, and variable (Boland *et al.,* 2001). Such interaction involves both past and present nutritional status, but other factors, such as genetic makeup, animal condition, environmental influence, and physiological stage are determinant regulators of nutritional effects on reproductive performance (Short *et al.,* 1990; Garnsworthy & Webb, 1999; Lucy, 2001; Stevenson, 2001; Robinson *et al.,* 2006). The detrimental impact of negative energy balance (NEB) on reproduction evidences how closely nutrition is related with reproduction (Nebel & McGilliard, 1993; Butler & Smith, 1997; Santos, 2007, 2009). Many nutritional approaches have been applied in periparturient cows in order to overcome the adverse consequences of NEB in reproduction, and to maximize cow profitability and welfare (Grummer, 1995; Friggens *et al.,* 2004; Beever, 2006). Generally, such nutritional strategies are aimed at maintenance of body condition score (BCS) above the figure of 2.0 or better 2.5 throughout the year; BCS should not fall below 2.0 during the year, and BCS should be 2.5 to 3.0 at parturition (Garnsworthy, 2006). All approaches are based on the finding that fatter cows at calving eat less food and lose more body fat than thinner cows (Garnsworthy, 2006; Chagas *et al.,* 2007). Given that a periparturient cow suffers from severe NEB due to decreased appetite and increased milk production (Grummer, 1995), most nutritional regimes aim at stimulating cow appetite or increasing the energy concentration of feeds, along with preserving high milk production. Consequently, these strategies often fail in terms of reproduction because they totally ignore the time taken for reproduction recovery after the calving (Beever, 2006).

More recently, researchers have attempted to find if high milk yield could be maintained along with high fertility rates in high yielding cows (Gong *et al.,* 2002a, 2002b; Fouladi-Nashta *et al.,* 2005; Garnsworthy *et al.,* 2008a, 2008b, 2008c, 2009). The most common strategy for reducing the degree of NEB in early lactation is to increase dietary energy

concentration by increasing the starch or fat components of the ration at the expense of forage components (Garnsworthy *et al.,* 2009). High fat diets with total fat concentration over 50 *g/kg* of dry matter depressed plasma insulin concentration in cows at the onset of the breeding period (Garnsworthy *et al.,* 2008b). Conversely, high starch diets induced high plasma insulin concentration and increased the proportion of cows ovulating within 50 days of calving, reduced the intervals from calving to first ovulation, and tended to reduce the intervals from calving to first service and to conception (Gong *et al.,* 2002b). Moreover, Adamiak *et al.* (2005, 2006) and Fouladi-Nashta *et al.* (2005) demonstrated that diets designed to increase plasma insulin concentration had negative effects on blastocyst rate in heifers and in lactating cows respectively. In addition, Fouladi-Nashta *et al.* (2007) reported that diets with a high fat content had beneficial effects on blastocyst rate in lactating dairy cows, although they decreased circulating insulin. The main conclusion of all these studies is that dietary induced increases in insulin can be beneficial for resumption of oestrous cycles postpartum. However, high insulin concentration might have detrimental effects on oocyte developmental processes, basically at the stage of blastocyst. Garnsworthy *et al.* (2009) tested the hypothesis that pregnancy rate will be enhanced by feeding a diet that increased plasma insulin until cows resume ovarian cycles, and then switching to a diet that decreased plasma insulin during the mating period. This hypothesis was confirmed and it is a paradigm how nutritional strategies can impact on reproductive defects in high yielding cows without compromising animal welfare and productivity.

2.2. Body condition score and body condition score management in dairy cows

Although live weight (LWT) change is a poor indicator of tissue mobilisation during early lactation, LWT is widely used as an index of tissue mobilisation. However, simultaneous increases in feed intake, gut contents, and water repletion of body tissues may disguise the real magnitude of tissue mobilisation measured by LWT (Sutter & Beever, 2000; Garnsworthy, 2006; Bewley & Schutz, 2008). Body condition score is a rapid, noninvasive, inexpensive, but subjective way of assessing body reserves of cow. It is easy applicable at the farm level and might give a more realistic view of the lipid and muscle reserves than LWT (Garnsworthy, 2006). Condition score assessment of cow is based on palpation and visual examination of the animal at certain anatomical and morphological body regions (*i.e.* assessing the level of fat cover over the transverse processes of the lumbar vertebrae and around the tailhead) (Roche *et al.,* 2009). A variety of body condition scoring systems were introduced from 1970 to 1980 (Garnsworthy, 2006). All are based on the same basic principle of physical and visual examination of the animal, but different scales, anatomical regions, and emphasis weights on body reserves assessment are given. Garnsworthy (2006), Bewley & Schutz (2008), and Roche *et al.* (2009) give a list of equations to convert BCS from other BCS systems to 1 to 5 UK/US system which is the most commonly used.

In recent decades, BCS has been proved a useful management tool for assessing nutritional status and energy balance (EB) during lactation in dairy cows. Estimates of BCS are significantly correlated with subcutaneous fat (Garnsworthy & Topps, 1982; Domecq *et al.,* 1995; Heuer *et al.,* 1999; Garnsworthy, 2006). Ruegg & Milton (1995) reported that excessive loss of BCS during early lactation was related to metabolic diseases and Gearhart *et al.* (1990) described BCS at calving as a risk factor for poor reproductive performance in dairy cows. However, the major drawback of BCS application and usefulness are; the scale of observations may be subjective; and BCS determined by different persons and in different studies are not directly comparable. Moreover, although BCS is positively correlated with LWT, in early lactation it is not unusual for cows to maintain or gain LWT whilst losing condition, due to the variability of gut contents

(Garnsworthy, 1988; Bewley & Schutz, 2008). It is known that subcutaneous fat correlates strongly with total body fat (Faulkner *et al.,* 1990; Bewley & Schutz, 2008). Thus, the disadvantages of BCS application can be overridden by using ultrasound technology to assess subcutaneous fat depth.

Absolute BCS at critical points of the lactation cycle (*e.g.* BCS at calving, nadir BCS, and BCS at drying-off) is as important as BCS change or loss, which is the difference between body condition scores assessed at two critical points (Bewley & Schutz, 2008). It is well known that body condition loss and especially BCS at calving affect directly dry matter intake (DMI), NEB, fertility, milk yield and composition, and health of high yielding cows (Butler, 2003; Garnsworthy, 2006). Garnsworthy & Topps (1982) studied the effect of BCS at calving on DMI and milk yield. Their study clearly indicated that fat cows at calving ate less and lost more condition than thin or moderate cows over the first 12 weeks of lactation. Many other studies (Broster & Broster, 1998; Roche *et al.,* 2009) have shown the negative correlation of BCS at calving and BCS loss with DMI in early lactation. It is now clear that over-conditioned cows at calving are prone to experience deeper and longer NEB and mobilize their body reserves due to reduced feed intake in early lactation. Consequently higher BCS at calving is not a recommended approach to alleviate cows from severe NEB postpartum because the unfavourable depression in DMI triggers longer and deeper NEB along with greater BCS loss (Garnsworthy, 2006).

Body condition score has been considered as an indirect measure of nutritional status (Short *et al.,* 1990; Garnsworthy, 2006; Bewley & Schutz, 2008). This implies that BCS is affected by diet composition and DMI (Short *et al.,* 1990; Garnsworthy, 2006). According to Short *et al.* (1990) the effect of nutrition on reproduction depends on whether nutritional differences exist before or after calving. In addition, they stated that BCS at calving is more important than postpartum BCS loss, and consequently nutritional management of animals in the dry period is a crucial factor to ensure timely reproductive functionality after calving. This study also suggested that the relationship between postpartum anoestrus (PPA) and BCS at calving is nonlinear, and that BCS at calving influences postpartum DMI. The effect of different diet compositions on thin and fat cows was reviewed by

Garnsworthy (2006). The main conclusion was that positive EB in early lactation is not unrealistic and it can be achieved under certain combination of BCS and dietary regimes (*e.g.* thin cows fed a high energy diet).

Milk production is the major objective of intensive dairy systems, but genetic improvement of cows for higher milk yield results in greater BCS loss in early lactation (Veerkamp *et al.,* 2001, 2003; Dechow *et al.,* 2002; Berry *et al.,* 2003; Pryce & Harris, 2006). Dechow *et al.* (2002) suggested that modern genetic selection should be directed to increase milk yield, but to minimize the BCS loss postpartum. Buckley *et al.* (2003) reported that for every 100 kg increase in genetic merit for milk yield, there is a loss of 0.02 BCS units between calving and first service. Holstein-Friesian is the main breed used in dairy systems. Increasing the proportion of Holstein genes from 50 to 100% within this breed, results in a mean decrease of BCS by 1 unit (Veerkamp *et al.,* 2001). Body condition score is related to milk composition. Pryce *et al.* (2001), Veerkamp *et al.* (2001) and Berry *et al.* (2003) proposed that there is a negative genetic correlation between BCS and fat and protein contents of milk. Roche *et al.* (2007a) found that butter fat was positively correlated with increasing calving and nadir BCS for a period from 60 to 270 days in milk, but milk protein was influenced positively by nadir BCS and negatively by BCS loss.

Poor or excessive BCS have been associated with health problems and decreased fertility in many studies. According to Mulligan *et al.* (2006a, 2006b), over-fat cows at calving had a greater probability of expressing fatty liver, ketosis, retained placenta, calving difficulties, and milk fever. Butler & Smith (1989) and Villa-Godoy *et al.* (1990) demonstrated that when NEB coincides with excess BCS then the result is declined fertility. Over the last three decades numerous studies showed the negative impact of excess or inadequate BCS at calving, BCS loss, and nadir BCS on postpartum reproductive performance (Garnsworthy & Topps, 1982; Heuer *et al.,* 1999; Moreira *et al.,* 2000; Yamada *et al.,* 2003; Lopez-Gatius *et al.,* 2003; Agenas *et al.,* 2003; Roche *et al.,* 2007b). The main conclusions from these studies were that reproductive indices determining reproductive efficiency of the herd, such as calving interval, days to first oestrus, days to first service, first service conception rate, and number of services, are related to BCS.

Garnsworthy (2006) proposed that cows have a target BCS (about 2.5 units) that they try to attain in early lactation. Moreover, he stated that the biological drive for a cow to attain the target BCS appears to be as strong as the drive to attain a genetically-determined peak milk yield. Furthermore, the genetic correlation between BCS and milk yield is negative; the genetic correlations between health and fertility indices and milk yield are negative; and BCS loss is positively related to milk yield and negatively to health and fertility indices (Veerkamp *et al.,* 2001; Berry *et al.,* 2003; Pryce & Harris, 2006). It is easily conceivable that there are marginal BCS targets to be achieved during the lactation cycle of the cow for satisfying health, milk yield, and reproductive efficiency.

According to Garnsworthy (2006) BCS management (Figure 2.1) must rely on the following basic principles:

1. Body condition score at calving should be allowed to vary no more than 0.5 BCS units above cow target BCS.
2. Cows of low genetic merit for milk yield (target BCS 2.5-3.0) should calve with BCS of 3.0 or less.
3. Cows of high genetic merit for milk yield (target BCS 2.0-2.25) should calve with BCS of 2.75 or less.
4. Body condition score should not fall below 2.0 during the whole lactation.
5. Body condition score at service should not fall below 2 to 2.5.

According to Chagas *et al.* (2007) the perfect BCS fluctuation to minimize the effects of NEB on health and reproduction is BCS at calving 3.0-3.5 with a nadir BCS of 2.5-3.0. Mulligan *et al.* (2006b) suggested BCS targets of 2.75 at drying-off, 3.0 at calving, ≥ 2.75 at service, and a nadir of 2.75. Coffey *et al.* (2004) suggested that if there is an ideal BCS or BCS loss, then they should be dependent on stage of lactation or/and the productive system under which cows are maintained.

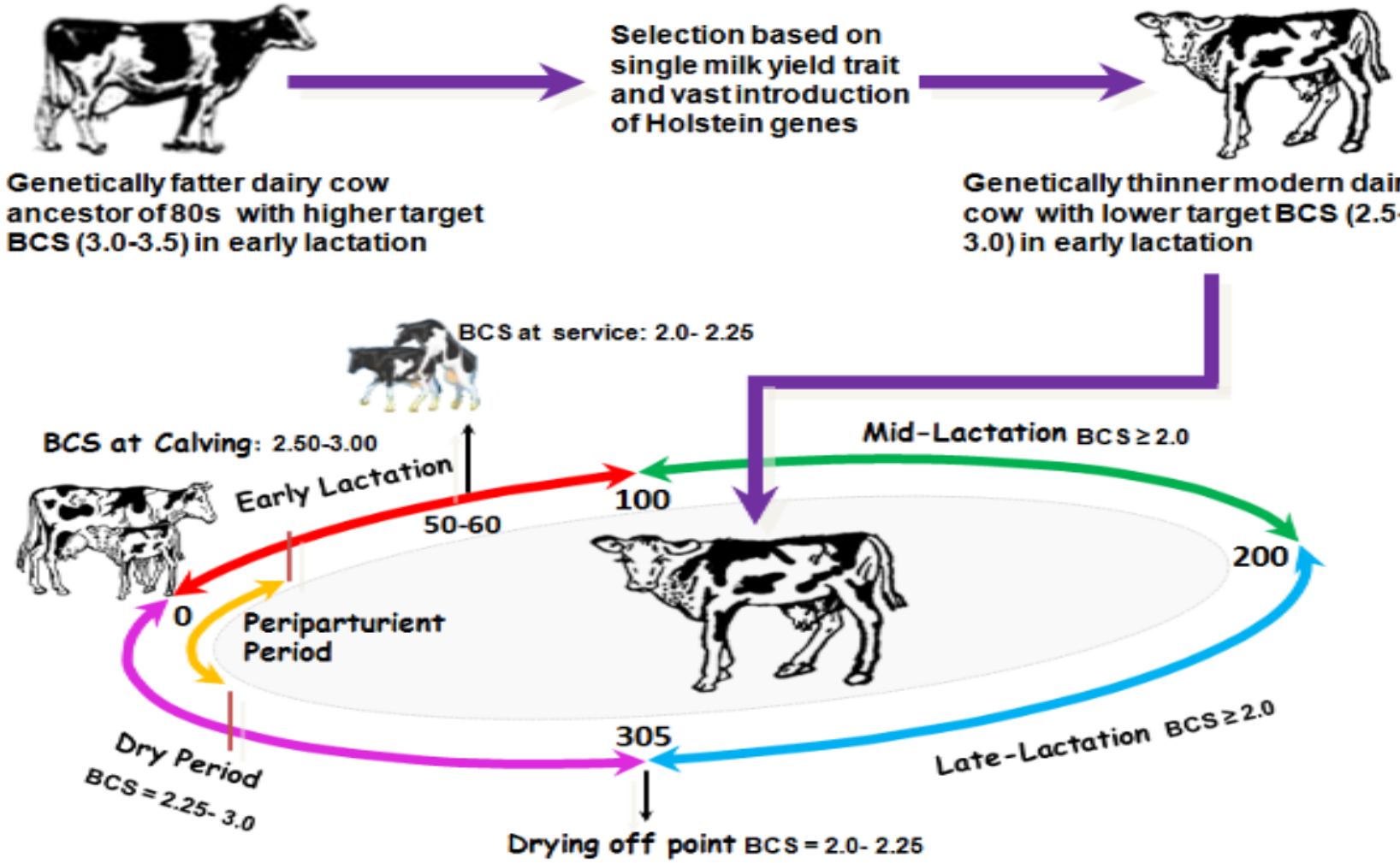


Figure 2.1: BCS management in the modern dairy cow (Based on the suggestions of Garnsworthy (2006), see text for details).

2.3. Glucose homeostasis and homeorhesis

The energy contained in foods is made usable by the animal by intricate biochemical events that are generally defined as metabolism (Kaneko, 2008a). Traditionally, these biochemical events have been separated into the metabolism of three important ingredients of food; carbohydrates, proteins, and lipids (Guyton & Hall, 2006). These also are the main constituents of animal body tissue and animal products (*e.g.* milk) (Bauman & Currie 1980). The dominant circulating carbohydrate is glucose, which is the principal source of energy for life processes in the mammalian cell (Guyton & Hall, 2006; Nafikov & Beitz, 2007). Thus, the major function of glucose is to serve as an energy source for the animal cell, and its storage capacity (in the form of glycogen which is a large polymer of glucose) is nearly zero (lactating cows have the ability to store only 1% glucose in their livers in the form of glycogen) (Kaneko, 2008a). Cells require a constant supply of this essential nutrient, and only relatively small changes are tolerable without adverse effects on the health and productivity of the animal (Kaneko, 2008a; Nafikov & Beitz, 2007). Fundamentally, glucose, protein and lipid metabolism are coupled to support the mammalian cell with a continuous constant flux of glucose which is tightly homeostatically and homeorhetically controlled (Bauman & Currie, 1980). The liver plays a central role in buffering blood glucose concentration because it supplies and removes glucose from circulation. Hepatic glucose metabolism is mostly directed towards supplying rather than using glucose (Kaneko, 2008a; Ghanassia *et al.,* 2007). Thus, the ruminant liver takes up only small amounts of glucose under normal physiological conditions and it is not an insulin dependent organ (Hayirli, 2006). On the other hand, nervous tissue, muscle tissue and the mammary gland during lactation are the primarily glucose-utilizing tissues of the animal (Brockman, 1978, 1979; Bauman & Currie, 1980; Bauman, 2000; Reynolds, 2005). Muscle can deposit small quantities of glycogen, but is not able to liberate free glucose into the blood stream (without liver intervention) because of deficiency of the enzyme glucose-6-phosphatase (Kaneko, 2008a). Foetal tissue in pregnancy and the mammary gland during lactation are heavily dependent on maternal circulating glucose (Bauman & Currie, 1980; Bell & Bauman, 1997). The nutrient demands for pregnant animals at the end of pregnancy are 75% greater than those of non-pregnant animals of a

similar LWT. The developing foetus, foetal membranes, gravid uterus, and mammary gland contribute to the increased glucose and nutrient demand (Bauman & Currie, 1980). Homeothermy and antioxidant balance are also closely related to glucose homeostasis and homeorhesis (Ghanassia *et al.,* 2007; Collier *et al.,* 1982; Kadzere *et al.,* 2002).

Centrally, there is a developed anatomical and functional network that monitors glucose availability that is involved in glucose homeostasis and food intake regulation (Bruning *et al.,* 2000; Krasnow & Steiner, 2006). Basic components of this centrally operating system are glucose-sensing neurons (Moran, 2010). These centrally placed glucose-sensing neurons are located at different spots of the brain (from the hindbrain to the hypothalamus) and together with peripheral glucose sensors, represent an integrated glucose monitoring system (Langhans & Geary, 2010). Glucose phosphorylation by glucokinase is the rate-limiting step in ATP production and it is essential for the effects of glucose on membrane potential and ion channel function of glucose-sensing neurons (Moran, 2010). It is hypothesized that sensitive glucose-sensing neurons in the brain change their signalling in response to the presence (or the amount) of other metabolites and hormones such as insulin, leptin, non-esterified fatty acids (NEFA), and Amino Acids (AA). Glucose availability influences the expression and turnover of several catabolic and anabolic neuropeptides which presumably mediate the effects of glucose-sensing neurons on eating (Krasnow & Steiner, 2006; Cone & Elmquist, 2008; Langhans & Geary, 2010).

Fatty acid oxidation (FAO) is closely related to glucose homeostasis and eating behaviour (Wade & Schneider, 1992; Drackley, 1999; Ingvartsen & Andersen, 2000; Allen & Bradford, 2007). Inhibition of FAO is usually accompanied by a stimulation of eating in animals and man and FAO is tightly related to glucose availability by body tissues (Langhans & Geary, 2010). Fatty acids and/or fatty acid metabolism can also be sensed centrally, in the mediobasal hypothalamus, and this also affects food consumption (Moran, 2010).

Cows are herbivorous and consume diets that are higher in structural carbohydrates and lower in fat (Lewis & Hill, 1983; Allen *et al.,* 2005). Nutrients absorbed by the cow differ

markedly from those that are consumed because of the intervention of rumen microflora and the subsequent fermentation of organic matter (OM) of feed (Van Houtert *et al.,* 1993). OM is partially fermented to volatile fatty acids (VFA: acetic, propionic, and butyric). Feed protein is partially degraded to AA and ammonia, which are incorporated into microbial protein of high biological value (Allen *et al.,* 2005). Moreover, unsaturated fatty acids of feed are biohydrogenated and isomerized to varying degrees (Lewis & Hill, 1983). The major fuels for cows are; VFA from ruminal and intestinal fermentation of OM; glucose from starch digestion in the small intestine (metabolized primarily to lactate by intestinal tissue); NEFA and AA absorbed from the gastrointestinal tract and mobilized from body reserves (Lewis & Hill, 1983; Nafikov & Beitz, 2007). Many ruminant tissues preferentially utilize acetate rather than glucose, but the mammary gland requires glucose, and large quantities are required for milk lactose synthesis (Reynolds, 2005). Insulin induced hypoglycaemia in lactating goats and cows resulted in a depression of milk yield (Smith, 1971).

The liver is the predominant site of glucose synthesis in the ruminant. Hexokinase activity is low in bovine liver and gluconeogenic capacity is high (Brockman, 1978). Consequently, for meeting the high demands of mammary epithelial cells for glucose precursors, net hepatic glucose production is over 4 *kg/d* for cows producing about 60 *kg/d* of milk (Reynolds, 2005; Nafikov & Beitz, 2007). An interesting difference between ruminants and pigs is that when in positive EB, gluconeogenesis and lipogenesis increase in ruminants but only lipogenesis increases in pigs (Nafikov & Beitz, 2007). Ruminants normally absorb little glucose, and propionate is the primary gluconeogenic substrate. Almost all propionate is taken up by the liver and used for glucose synthesis (Allen *et al.,* 2005). Propionate is responsible for as much as 80% of glucose produced in lactating cows. Underfeeding decreases utilization of propionate and other precursors derived from the diet, while the relative usage of lactate, glycerol and AA as gluconeogenic substrates increases. Most AAs are potential gluconeogenic substrates and they are considered to be the second most important glucose precursor (Reynolds, 2005; Nafikov & Beitz, 2007). NEFA is the major source of energy in bovine liver, but the liver has limited capacity for fatty acid synthesis or triglyceride export (Allen *et al.,* 2005; Nafikov & Beitz, 2007).

Milk yield exerts a pronounced drain on glucose and energy resources (Nafikov & Beitz, 2007). In some studies, dairy cows selected for milk yield had lower circulating glucose (Harrison *et al.,* 1990; Gutierrez *et al.,* 1999), but this finding was not in line with other studies (Barnes *et al.,* 1985; Westwood *et al.,* 2000). Lukes *et al.* (1989) reported that glucose concentrations in high yielding cows are typically low at parturition. Moreover, the lower circulating glucose in higher genetic merit cows was observed later in lactation and lasted longer than in lower genetic merit cows (Westwood *et al.,* 2000). In terms of circulating glucose, genetic merit for milk yield and underfeeding seem to interact (Veerkamp *et al.,* 2003).

In dairy cows, two types of physiological regulation are instrumental to metabolic coordination during the transition period: homeostasis, which is the maintenance of physiological equilibrium, and homeorhesis, which is the orchestrated change in metabolism necessary to support a successful transition from one physiological state to another (Bauman & Currie, 1980; Bauman, 2000). Given the species of the animal and the genetic make-up, environmental factors (such as management, nutrition, disease) can modify the interaction between homeostatic and homeorhetic circuits. In many cases of improper animal management, the homeostatic control for survival dominates the homeorhetic mechanism which is operated to support a physiological function (*e.g.* lactation or reproduction), resulting in a problematic or incomplete adaptation of the organism from one state to another (Bauman & Currie, 1980; Friggens, 2003). Such maladaptations are common in transition cows when animals, in a short time, need to adapt to three completely different conditions (parturition, lactation, and reproductive recovery) (Grummer, 1993; Bell, 1995; Bell & Bauman, 1997; Beever, 2006).

Regulation of glucose metabolism and nutrient partitioning involve interaction between homeorhetic and homeostatic circuits to ensure maintenance, production, and reproduction of the animal. The metabolic adaptations support the successful transition to lactation, leading to increased glucose synthesis by the liver and to decreased glucose oxidation by peripheral tissues at the beginning of lactation. However, sometimes the great demand of glucose is not achieved by the cow, resulting in reproductive failure, decreased

productivity, and increased health problems (Bauman, 2000; Bauman *et al.,* 2004). Nutrient partitioning and glucose economy are tightly endocrinologically regulated and this involves coordination of many organs, tissues, metabolites, and hormones (Bell & Bauman, 1997; Leroy *et al.,* 2008, 2010). Pancreatic hormones, adipokines, growth hormone (GH), and their relationships appear to play a key role in regulating glucose homeostasis and homeorhesis in periparturient cows. These hormones will be discussed in the next section.

2.4. Pancreatic hormones and adipokines

2.4.1. Insulin

Pancreatic hormones play a key role in endocrine regulation and facilitate the movement of glucose across cell membranes, thereby adjusting the concentration of blood glucose (Brockman 1978, 1979; Sasaki, 2002). When food is consumed by an animal, especially carbohydrates, insulin is secreted (Marieb & Hoehn, 2007) and there is no circadian pattern of insulin secretion in ruminants (Trenkle, 1978). In general, circulating insulin is increased by feeding diets rich in starches but it is decreased by feeding diets rich in fat. However, the factors that influence insulin release are poorly documented in cows (Bossaert *et al.,* 2008). Insulin plays an important role in storing excess energy and regulates glucose homeostasis along with its counterpart glucagon, (Figure 2.2) (Marieb & Hoehn, 2007; Cryer*,* 2008, Guyton & Hall, 2006). Nevertheless, the role of insulin is not constrained only to regulate glucose homeostasis and homeorhesis. Insulin interrelationships with GH, insulin like growth factor- I (IGF-I), ovarian function and steroidogenesis are partially understood (Webb *et al.,* 2004; Webb *et al.,* 2007). Milk yield and milk composition are indirectly associated with the dynamics of nutrient availability in mammary gland (Veerkamp *et al.,* 2003), whereas nutrient partitioning is mostly governed by insulin (Hayirli, 2006). Also, milk composition may or may not be controlled by insulin, and it is uncertain if selection for milk yield has any influence on insulin secretion, clearance and responsiveness in the modern dairy cow (Veerkamp *et al.,* 2003).

2.4.1.1. Insulin receptor and glucose transporters

The insulin receptor is ubiquitous but liver, muscle tissue, and adipose tissue are the main areas exhibiting insulin bioactivity (Etherton, 1982). The insulin receptor is a trans-membrane glycoprotein which belongs to the large class of tyrosine kinase receptors and consists of two α subunits and two β subunits (Sasaki, 2002). Insulin receptors are embedded in cell plasma membranes of insulin dependent tissues, with the β subunits

passing through the cellular membrane, and α and β subunits are linked by disulphide bonds (Kido *et al.,* 2001; Sesti *et al.,* 2001; Youngren, 2007). The binding of insulin to its receptor is the first step of a signalling pathway, triggering the consumption and the metabolism of glucose (Sasaki, 2002). Bound by insulin, the insulin receptor is phosphorylated from ATP to several proteins in the cytoplasm, including insulin receptor substrates (IRS-1 and IRS-2). This latter step activates phosphatidylinositol 3-kinase (PI-3-K) and leads to an increase in glucose transporter 4 (GLUT4) molecules in the outer membrane of *myocytes* and adipocytes. By this mechanism, there is finally an increase in uptake of glucose from blood into muscle and adipose tissue (Kido *et al.,* 2001; Sesti *et al.,* 2001; Ben-Shlomo, 2002; Youngren, 2007). According to Sasaki (2002) possession of insulin receptor by tissue cells does not necessarily mean the tissue cells are responsive to insulin. For instance, bovine mammary epithelial cells have insulin receptors during lactation, but the tissue appears to be inert to insulin challenges (Laarveld *et al*., 1985). Furthermore, overexposure of adipose cells to high concentration of insulin, down-regulates expression of insulin receptors, and leads to a loss of receptors from the membrane surfaces (Sasaki, 2002).

The uptake of glucose by tissue cells occurs by facilitating diffusion and it requires a carrier protein known as glucose transport (GLUT) (Klip *et al.,* 1994; Thorens, 1996; Goodyear & Kahn, 1998). GLUT in the plasma membrane exists in two conformational states. Extracellular glucose binds to the GLUT, which then alters its shape, discharging glucose in to the cell. Two types of GLUT are recognized (Thorens, 1996):

(i) Insulin Independent Glucose Transporters

GLUT1 is ubiquitously distributed in various tissues (erythrocyte, brain cells, mammary epithelial cells, adipose and muscle cells, and endothelial cells)

GLUT2 (liver, intestine, pancreatic β cells and kidney)

GLUT3 (brain and/or foetal muscle cells)

GLUT5 (intestine)

(ii) Insulin dependent glucose transporters

GLUT4 is primarily located in insulin-sensitive tissues, such as skeletal muscle, cardiac muscle, and adipose tissue.

Different tissues contain different types of GLUT (Herman & Kahn, 2006). The tissue specific allocation of GLUT determines tissue responsiveness to insulin for uptake of glucose (Klip *et al.,* 1994). Among these, only GLUT4 is found in insulin-sensitive tissues (muscle and adipose tissue) and requires insulin for uptake of glucose (Sasaki, 2002). In the bovine follicle and corpus luteum GLUT1 and GLUT3 act as the major transporters of glucose, although GLUT4 may play a supporting role (Nishimoto *et al.,* 2006). GLUT1 is the major glucose transporter of lactating mammary epithelial cells (Bell & Bauman, 1997). GLUT1 expression level was decreased from early to late lactation and become barely detectable in the non-lactating udder (Zhao & Keating, 2007). According to Hayirli (2006) exogenous insulin may decrease availability of glucose for mammary tissue because insulin does not regulate uptake of glucose through GLUT1.

2.4.1.2. Effects of Insulin on glucose metabolism and body reserves

In general, insulin plays numerous roles in metabolism of carbohydrates, lipids, and proteins in adipose tissue, muscle, and liver in mammals (Aronoff *et al.,* 2004). It is the main anabolic hormone in ruminants and acts to preserve nutrients in favour of the animal by directing feed nutrients to body stores rather than to milk production (Brockman, 1978, 1979; Brockman & Laarveld, 1986). The hypoglycaemic effect of insulin action is antagonized by glucagon, adrenalin, glucocorticoids, and GH in cattle (Veerkamp *et al.,* 2003). Plasma insulin concentration is negatively correlated with NEB in postpartum cows (Jorritsma *et al.,* 2003) and insulin treatment increases feed intake, LWT, and body fat (Wade & Schneider, 1992). The most important effect of insulin in mammals is its interplay with glycogen (Aronoff *et al.,* 2004). This short term regulatory mechanism is critical for survival of the animal. After a meal, glucose is absorbed and insulin causes its storage in the liver in the form of glycogen. Between meals, when blood glucose concentration falls, insulin secretion decreases rapidly and liver glycogen splits back into

glucose. Glucose is released back into the blood stream and prevents glucose concentration from falling too low (Cryer, 2008; Guyton & Hall, 2006). This mechanism of controlling glucose homeostasis is stepwise and depends on the simultaneous activation and inactivation of several enzymatic circuits in the liver, including inactivation of liver phosphorilase, activation of glucokinase, and increased activity of the glycogen synthase (Allen & Bradford, 2007; Vernon, 2005; Kokta *et al.,* 2004; Guyton & Hall, 2006). Another mechanism which is critical in glucose homeostasis is gluconeogenesis. Insulin is a potent inhibitor of gluconeogenesis by decreasing the quantities and activities of the liver enzymes which are involved in gluconeogenesis (Hayirli, 2006). Therefore, the main impact of insulin on glucose homeostasis is the control of two separate pathways, glyconeogenesis and gluconeogenesis (Aronoff *et al.,* 2004; Nafikov & Beitz, 2007).

Insulin was the first hormone discovered to have an impact on adipose tissue depots (Kokta *et al.,* 2004; Cryer, 2008). Generally, insulin acts as fat sparer, and this effect is serious and just as important as the control of glucose homeostasis (Guyton & Hall, 2006). In fact, glucose homeostasis and adipose tissue are linked under the spectrum of insulin activity (Ghanassia *et al.,* 2007). That is because insulin increases uptake of glucose by most of the body tissues and automatically damps down lipid usage. In adipose tissue, insulin facilitates entry of glucose into adipose cells through GLUT4 (Hayirli, 2006). In turn, glucose is oxidized to form α-glycerophosphate, which is used for esterification of free fatty acids during lipogenesis. Furthermore, insulin promotes fatty acid synthesis and storage in fat tissue depots, especially when dietary carbohydrate intake is high (Kokta *et al.,* 2004; Guyton & Hall, 2006). The mechanism by which insulin exerts its effect on adipose tissue is not clearly elucidated. However, this mechanism is believed to be based on the capacity of insulin to influence the activity of major enzymatic cascades such as hormone sensitive lipase (HSL) (insulin blocked), lipoprotein lipase (LPL) (insulin activated), and acetyl-CoA carboxylase (insulin activated) (Carmen & Victor, 2006; Guyton & Hall, 2006). Moreover, in adipose and muscle tissue, insulin stimulates triacylglyceride (TAG) synthesis by supplying fatty acids through stimulation of LPL (Ghanassia *et al.,* 2007). Additionally, insulin suppresses lipolysis by lowering the level of cAMP and inhibits the activity of protein kinase A and hormone sensitive-lipase

(Ghanassia *et al.,* 2007; Brockman, 1978, 1979). Unlike non-ruminants, in which glucose is the main precursor for lipogenesis in adipose tissue, ruminants utilize acetate for the same purpose (Vernon & Pond, 1997; Nafikov & Beitz, 2007). Although precursors for lipogenesis are different, the stimulatory role of insulin on lipogenesis in adipose tissue of ruminants and non-ruminants is the same as in hepatic lipogenesis (Brockman & Laarveld, 1986).

In the liver, insulin promotes lipogenesis and inhibits ketogenesis in both ruminants and non-ruminants (Brockman, 1978, 1979). Free fatty acids are taken up by the liver and are re-esterified with glycerophosphate, which is derived either from insulin-stimulated glycolysis or from glycerol formed by glycerophosphate kinase. Mitochondrial acetyl-CoA, which is generated by pyruvate dehydrogenase during glycolysis is transferred to the cytoplasm and converted to malonyl-CoA by acetyl-CoA carboxylase (Vernon & Pond, 1997; Vernon, 2005). This is the rate limiting-step for hepatic lipogenesis and it is stimulated by insulin. Insulin exerts its antiketogenic effect on the liver by decreasing NEFA uptake (Grummer, 1993; Jorritsma *et al.,* 2003). Insulin accomplishes this by altering lipogenesis to lipolysis ratio in adipose tissue in favour of lipogenesis, by increasing peripheral tissue ketone usage, by altering enzyme activities (carnitine palmitoyltransferase-I and acetyl-CoA carboxylase), and by changing the availability of substrates (malonyl-CoA) involved in ketogenesis in the liver (Brockman, 1979; Vernon & Pond, 1997; Hayirli, 2006; Vazquez-Vela *et al.,* 2008).

The ability of insulin to lower blood glucose concentrations is impaired during late pregnancy and early lactation in small ruminants (Prior & Christenson, 1978; Debras *et al.,* 1989). This phenomenon is defined as insulin resistance and it is commonly observed in cattle (Sano *et al.,* 1991, 1993). Insulin resistance is the state in which physiological levels of insulin produce less potent biological responses (Pasquali, 2006; Zavalza-Gomez *et al.,* 2008) due to decreased insulin responsiveness to glucose and/ or increased metabolic clearance (Sano *et al.,* 1993). The molecular mechanism of insulin resistance may be placed at; (1) pre-receptor level, which includes decreased insulin production, increased insulin degradation, or both; (2) receptor level, which includes decreased number of

receptors and decreased binding affinity; and (3) post-receptor level, which includes impaired intracellular signalling and translocation of GLUT (Hayirli, 2006). The current evidence suggests that the number and affinity of insulin receptors in adipose and muscle cells do not change during lactation and that the defect may be downstream of the insulin receptors (Ben-Shlomo, 2002; Barbour *et al.,* 2007; Zavalza-Gomez *et al.,* 2008). Glucose utilization by insulin dependent tissues is lower in pregnant animals than in non-pregnant and lactating animals (Nieuwenhuizen *et al.,* 1998), and ketosis and ketoacidosis decrease tissue responsiveness to insulin in cows (Sakai *et al.,* 1993; Steen *et al.,* 1997). During late pregnancy and early lactation, the cow directs nutrients towards embryo and mammary tissue at a high-level metabolic priority (Bauman & Currie, 1980; Vernon & Pond, 1997). This priority coincides with lowered sensitivity of extra-hepatic tissues to insulin (Sano *et al.,* 1991, 1993; Nieuwenhuizen *et al.,* 1997, 1998), which presumably plays a key role in the development of hepatic lipidosis and ketosis (Steen *et al.,* 1997). Gestational and lactational insulin resistance may be a homeorhetic adaptation that serves successful development of the foetus and survival of the neonate (Bauman & Currie, 1980; Schoenberg & Overton, 2010). Thus, the term insulin resistance may be misleading for characterizing this unique and physiological short-term condition in periparturient cows, because it may falsely refer to complications or diseases in other species that are largely irrelevant to the periparturient bovine model (Schoenberg & Overton, 2010).

Circulating insulin and cerebrospinal levels of insulin are correlated with body adiposity and insulin receptors are present in the brain and hypothalamus of many species (Havrankova *et al.,* 1979; Masters *et al.,* 1987; Bruning *et al.,* 2000), and cattle (Miller *et al.,* 1994; Liu *et al.,* 2009). Male and female mice with genetic deletions of central insulin receptors are obese (Moran, 2010), indicating that insulin receptor signalling in the brain is important for the control of LWT. Insulin is a potent regulator of feed intake and nutrient partitioning in ruminants (Ingvartsen & Andersen, 2000). Moreover, insulin increases VFA absorption indirectly by stimulating growth of gut papillae. This latter effect may prevent accumulation of VFA, which stabilizes ruminal pH and allows greater feed intake (Hayirli *et al.,* 2002). According to Hayirli (2006) insulin in ruminants interacts directly with other

metabolites to regulate feed intake, but ruminants do not overcome hypoglycaemia simply by increasing feed intake.

2.4.1.3. Effects of Insulin on milk yield and milk composition

Insulin is postulated to play an indirect but very important role in milk yield volume and composition due to its regulatory control in nutrient partitioning (Herbein *et al.,* 1985; Veerkamp *et al.,* 2003). Low insulin decreases the uptake of glucose by insulin-sensitive tissues and enhances glucose availability for the mammary gland, which is insulin-insensitive (Rose *et al.,* 1997; Bossaert *et al.,* 2008). Low circulating insulin is genotypically (Veerakamp *et al.,* 2003; Gutierrez *et al.,* 2006) and phenotypically associated (Veerakamp *et al.,* 2003) with high milk yield, and insulin has been shown to be essential for ruminant mammary tissue development and cell maintenance *in vitro* (Collier *et al.,* 1977; Mackle *et al.,* 2002; Neville, 2006). Correlations between insulin levels and breeding values for milk production, butterfat and milk protein have been reported to be positive (Veerkamp *et al.,* 2003). However, circulating insulin is unchanged between high yielding and low yielding cows (Lukes *et al.,* 1989; Westwood *et al.,* 2000) or even low in high genetic merit cows (Gutierrez *et al.,* 1999; Gong *et al.,* 2002b). Circulating insulin is lower in high yielding dairy cows than in beef cows, and this difference was probably due to decreased insulin secretion in the dairy cows (Veerkamp *et al.,* 2003).

The glucogenic-insulin theory is based on the effects of insulin on nutrient partitioning. This theory explains, at least partially, milk fat depression and proposes regulatory influences of insulin on butterfat variation in milk (Griinari *et al.,* 1996; Bauman & Griinari, 2001). Insulin stimulates fat deposition and inhibits fat mobilization, but the ruminant mammary gland is insensitive to changes in circulating insulin. Milk fat depression occurs when cows consume diets high in digestible carbohydrates and low in fibre, which generate large quantities of propionate and glucose (Bauman & Griinari, 2001; Rudolph *et al.,* 2007). High circulating glucose induces release of insulin from the pancreas. Increased circulating insulin (two- to five-fold normal) inhibits fat mobilization

in adipose stores (Vernon & Pond, 1997; Bauman & Griinari, 2001). Lipogenic precursors such as acetate, beta hydroxy butyrate, and diet-derived long chain fatty acids are preferably directed to adipose depots rather than to the mammary gland for butterfat synthesis. Finally, these changes result in a lack of lipogenic precursors for milk fat synthesis and depression of butterfat in milk (Bauman & Griinari, 2001).

In general, insulin increases AA transport and protein synthesis (Veerkamp *et al.,* 2003). Circulating AA has been shown to be the major precursors for milk protein synthesis (Petitclerc et *al.,* 2000; Baldi *et al.,* 2008). It has previously been demonstrated that lactating and non-lactating ruminants respond in the same way to exogenous insulin administration by decreasing circulating AA (Brockman, 1978; McGuire *et al.,* 1995; Griinari *et al.,* 1997; Mackle *et al.,* 1999). Therefore, there is no evidence to suggest that insulin acts directly on the mammary gland to stimulate AA uptake. The decrease in circulating AA may be caused by the effect of insulin on other tissues, such as liver and muscle. In some studies using the hyper-insulinaemic euglycaemic clamp technique in lactating cows (McGuire *et al.,* 1995, Griinari *et al.,* 1997; Mackle *et al.,* 2000), decreases in circulating AA was associated with increases in milk protein content. However, this needs to be further elucidated.

It has been shown that short term changes in circulating insulin have no direct effect on glucose uptake or milk synthesis by mammary epithelial cells in lactating ruminants (Laarveld *et al.,* 1985; Nielsen *et al.,* 2001). The mammary gland cannot synthesize lactose from any precursor except from glucose (Ohtani *et al.,* 2011). Moreover, there was no effect of insulin administration on mammary gland glucose uptake or lactose synthesis and glucose turnover in the mammary gland (Brockman, 1979). According to Kronfeld (1963), variations in circulating insulin might occur without observable changes in mammary consumption of glucose, but low circulating glucose is a more direct cause of milk depression than insulin.

2.4.1.4. Insulin and the GH/IGF-I system

Insulin may regulate the efficiency of GH signalling in liver and extrahepatic tissues of dairy cows (Rhoads *et al.,* 2004). Secretion of GH is elevated in early lactation, and the correlation of insulin with GH is negative in dairy cows (Block *et al.,* 2001; Veerkamp *et al.,* 2003). Moreover, GH exerts two types of effects on adipose tissue and skeletal muscle; insulin-like and anti-insulin-like (Davidson, 1987). The insulin-like effects occur shortly after GH exposure and involve increased glucose usage and decreased lipolysis. When tissues undergo long-term exposure to GH, cells responsive to insulin-like actions become unresponsive and this phenomenon is termed the refractory effect of GH (Mauras & Haymond, 2005). The secretory pattern of GH throughout the day (biorhythm) is considered to cause a constant refractory state. The anti-insulin-like effects of GH are considered to better reflect the physiological action of the hormone. These effects include impaired glucose utilization, hyperglycaemia, stimulation of lipolysis, and induction and maintenance of the refractory state to insulin-like effects (Davidson, 1987; Mauras & Haymond, 2005). Thus, insulin and GH have antagonistic roles in terms of glucose homeostasis, with minor insulin-like GH effects to serve possibly as parts of a homeorhetic and homeostatic mechanism (Bauman, 1999).

Growth hormone exerts its actions in almost every organ of the body, directly after binding to specific GH receptors (GHR) and/or indirectly after binding to GHR, stimulating the production of insulin-like growth factor-I (IGF-I) (Hull & Harvey, 2000, 2001; Webb *et al.,* 2004). The liver is the major source of circulating IGF-I (Rosen & Pollak, 1999), but many tissues of the body, including ovary, uterus and mammary gland, can produce IGF-I (Hull & Harvey, 2000, 2001, 2002; Webb *et al.,* 2004). IGF-I serves as the main negative feedback signal on GH secretion under normal conditions in which GHR expression increases proportionally to circulating levels of GH (Hull & Harvey, 2000, 2001, 2002). IGF-I controls cell growth, differentiation, and maintenance of differentiated cell function in many organs. IGF-I biological activity involves both insulin-like metabolic action and growth promoting action via endocrine, autocrine, and paracrine mechanisms (McGuire *et al.,* 1992; Webb *et al.,* 2004). IGF-I binds to specific cell surface receptors

designated type I and type II IGF receptors with different affinity (Rajaram *et al.,* 1997) and IGF-I can also interact with the insulin receptor (Rechler & Nissley, 1985). Generally, pituitary derived GH or bovine somatotrophin (bST) administration stimulates the synthesis and secretion of hepatic IGF-I, but the combination of high GH and low IGF-I is common during the postpartum period and may be associated with NEB, underfeeding and low circulating insulin in dairy cows (Lucy, 2000; Veerkamp *et al.,* 2003). Moreover, selection for milk production in dairy cows increases circulating GH whilst circulating IGF-I is lower for selected cows (Veerkamp *et al.,* 2003).

In early lactating dairy cows, GH action to stimulate IGF-I synthesis is compromised, resulting in low circulating IGF-I (Vicini *et al.,* 1991; Veerkamp *et al.,* 2003; Rhoads *et al.,* 2004). Insulin may be involved in hepatic expression of GHR. It has been shown that chronically elevated circulating insulin increases plasma IGF-I in mid and late lactating dairy cows, and stimulates hepatic GHR gene expression (Mashek *et al.,* 2001). Also, Rhoads *et al.* (2004) showed that there was a positive effect of insulin on GHR levels in liver and adipose tissue and that this positive effect was accompanied by an enhanced hepatic IGF-I synthesis in periparturient dairy cows. According to Veerkamp *et al.* (2003) the stimulatory effect of GH on IGF-I synthesis may be partly abolished during NEB when insulin levels are low. This might be because GH receptor numbers are decreased in the liver or because the function of GHR second messenger system has failed. According to Lucy (2008), as lactation progresses the somatotrophic axis recovers due to improved EB, increased circulating insulin, and enhanced expression of GHR in the liver.

2.4.1.5. Insulin and reproduction

Insulin also has the ability to relay metabolic information to the neuroendocrine pathways that influence reproduction and ovarian function (Krasnow & Steiner, 2006; Garnsworthy *et al.,* 2008a). Many observations from humans and animals with insulin-dependent diabetes mellitus or similar defects suggested that insulin plays a key role in reproduction (Veerkamp *et al.,* 2003; Kalro, 2003; Krasnow & Steiner, 2006). Rats bearing mutations to

central receptors of insulin experienced reduced reproductive performance due to reduction in gonadotrophin secretion and impaired folliculogenesis (Bruning *et al.,* 2000). Before the advent of insulin in 1921, pregnancies in women who had diabetes mellitus were rare (Kalro, 2003). Low circulating insulin, which may occur in diabetic patients, may also characterize high genetic merit dairy cows (Veerkamp *et al.,* 2003). Low conception rate and low follicular phase progesterone levels have been associated with low circulating glucose and insulin in high genetic merit cows (Pryce *et al.,* 1999; Snijders *et al.,* 2001; Veerkamp *et al.,* 2003; Webb *et al.,* 2004).

Insulin plays roles in reproduction much more complicated than its glucose regulatory action, and it is implicated in follicular development and functionality (Veerkamp *et al.,* 2003; Garnsworthy *et al.,* 2008a). The ovary has been demonstrated to be the site of action for insulin and IGF-I in several species, including dairy cows (Spicer *et al.,* 1990; Spicer & Echternkamp, 1995; Webb *et al.,* 2004; Webb *et al.,* 2007; Velazquez *et al.,* 2008). Growth factors such as insulin like growth factors (IGF), transforming growth factors (TGF), and epidermal growth factors (EGF) directly influence follicular growth by enhancing granulosa cell proliferation (Webb *et al.,* 2004). The IGF family is likely to be important in the process of selection of dominant follicles by potentiating the action of follicle-stimulating hormone (FSH) on granulosa cell differentiation, and by increasing the responsiveness of granulosa cells to FSH during final follicular growth (Garnsworthy & Webb, 1999; Webb *et al.,* 2004; Velazquez *et al.,* 2008; Garnsworthy *et al.,* 2008a). In the ovary, insulin is present in follicular fluid, insulin receptors are expressed by granulosa cells, and insulin stimulates ovarian steroidogenesis (Webb *et al.,* 2004; Krasnow & Steiner, 2006). *In vitro* insulin and/or IGF-I increase progesterone secretion in human and bovine granulosa cells (Spicer *et al.,* 1993; Taketani *et al.,* 2008), and IGF-I, along with gonadotrophins, stimulates ovarian steroidogenesis (Webb *et al.,* 2004). This implies that the stimulatory effects of insulin on steroidogenesis, at least in part, may be exerted by the IGF-I receptor since IGF-I acts together with gonadotrophins to stimulate ovarian steroidogenesis (Webb *et al.,* 2004).

Several studies related changes in circulating insulin to alterations in pulsatile luteinizing hormone (LH) secretion (Downing & Scarramuzi, 1997, Downing *et al.,* 1999; Tanaka *et al.,* 2000; Bucholtz *et al.,* 2000). *In vitro* and *in vivo*, insulin stimulates GnRH release but this occurs only when glucose is available (Arias *et al.,* 1992; Tanaka *et al.,* 2000). On the one hand, short term food deprivation is associated with decreased circulating insulin and LH pulsatility. On the other hand, refeeding after food deprivation is accompanied by increases in both insulin and LH secretion (Krasnow & Steiner, 2006). Thus, it appears that reduced insulin concentrations may signal the hypothalamus and pituitary inhibiting LH secretion during periods of limited feed intake (Wade & Schneider, 1992). Furthermore, dietary induced increases in circulating insulin in early lactation cows may prevent the short luteal phase which is characteristic of the first postpartum oestrous cycle (Miyoshi *et al.,* 2001). Moreover, insulin seems to influence embryo survival and ovulation in dairy cows. Adamiak *et al.* (2005, 2006) and Fouladi-Nashta *et al.* (2007) reported that diets designed to increase plasma insulin concentration had negative effects on blastocyst rate in heifers and in lactating cows, whereas Gong *et al.* (2002b) observed that the percentage of cows ovulating within 50 days postpartum increased from 55 to 90% in cows fed a high starch diet that induced higher insulin release in response to feeding. Garnsworthy *et al.* (2009), by adopting a feeding system that combined a glucogenic diet for the first 50 days postpartum and a lipogenic diet thereafter, demonstrated that insulin stimulus may play different roles in early lactation and in the breeding period in dairy cows.

Experiments in which animals were treated with insulin gave varying results with respect to reproduction (Krasnow & Steiner, 2006). The interpretation of experiments in which insulin is injected peripherally is complicated due to the hypoglycaemia induced by administration of large doses of insulin (Hayirli, 2006). In many instances, adverse effects of systemic insulin treatment on reproductive function did not occur if normal circulating glucose was maintained (Downing *et al.,* 1999). There are clear indications that postpartum glucose concentrations are lower in high genetic merit cows than in low genetic merit cows (Snijders *et al.,* 2001; Veerkamp *et al.,* 2003). McClure *et al.* (1967), Downie & Gelman (1976) and Easdon *et al.* (1985) stated that low circulating glucose may be responsible for infertility in lactating cows and mice. In addition, pregnancy rate was higher in cows with

high circulating glucose than in cows with low circulating glucose, and there was a trend for pregnancy rate to decline in cows with very high circulating glucose (Pehrson *et al.,* 1992). Krasnow & Steiner (2006) reviewed many experiments conducted with a variety of animal models in order to assess the implications of hypoglycaemia for reproduction. They reported that hypoglycaemia may reduce GnRH pulse generator activity, suppress pulsatile LH secretion, disrupt oestrous cyclicity and ovulation, decrease the magnitude of the steroid-induced LH surge, and impair mating behaviour. Selvaraju *et al.* (2002) reported that insulin and glucose concentrations were higher in cows which subsequently became pregnant than in non-pregnant animals. Downing *et al.* (1999) stated that there is a synergism between insulin and glucose at the ovarian level and it is likely the effects of short-term nutrition on ovulation rate may be mediated by direct ovarian actions of insulin and glucose. Mammals primarily rely on intracellular oxidation of glucose and fatty acids to get the energy necessary to support most physiological processes (Wade & Schneider, 1992). Although peripheral tissues may utilize both glucose and fatty acids, the ovary uses glucose as its principal source of energy (Rabiee *et al.,* 1999; Crooker *et al.,* 2007; Scaramuzzi *et al.,* 2010). Glucose is available for cellular oxidation and energy production not only when its quantity is sufficient in the body, but also when it is capable of entering cells, and that latter implies a role for insulin (Wade & Schneider, 1992). It seems that the effect of glucose on fertility is primarily related to its properties as a metabolic fuel (Veerkamp *et al.,* 2003). Insulin and functional insulin signalling are necessary for full reproductive competence. However, insulin by itself is usually insufficient to restore reproductive function in the face of extreme metabolic challenges, especially hypoglycaemia (Krasnow & Steiner, 2006). This suggests that insulin might influence fertility mostly through metabolic fuel partitioning, mainly of glucose, rather than through direct effects (Veerkamp *et al.,* 2003). Nowadays, it is believed that the follicle has a well developed system to sense glucose and nutritional status (Webb *et al.,* 2004; Gransworthy *et al.,* 2008a; Scaramuzzi *et al.,* 2010). This system allows follicle to determine its growth and development, mainly by altering FSH induced effects on local oestradiol synthesis, in accordance with glucose availability (Webb *et al.,* 2004; Scaramuzzi *et al.,* 2010).

2.4.2. Glucagon

Glucagon is released into the circulation when glucose is low (Brockman, 1978; Aronoff *et al.,* 2004). The main physiological role of glucagon is to stimulate hepatic glucose output (glycogenolysis) leading to increased circulating glucose. In this way, Glucagon provides the major countregulatory mechanism for glucose homeostasis (Figure 2.2) (Aronoff *et al.,* 2004). Another main effect of glucagon on glucose metabolism is stimulation of gluconeogenesis in the liver (Brockman, 1978; Jiang & Zhang, 2003; Guyton & Hall, 2006). Enhanced ability of the liver for gluconeogenesis in the presence of glucagon is related to increased activity of key gluconeogenic enzymes such as hepatic pyruvate carboxylase, and to high exportation rates of gluconeogenic substrates such as alanine, pyruvate, lactate, and glutamine (Roden & Bernroider, 2003; Jiang & Zhang, 2003; Aronoff *et al.,* 2004). In adipose tissue there are two major effects of glucagon: (1) activation of adipose cell lipase that makes increased quantities of fatty acids available to other organs of the body; (2) inhibition of triglyceride storage in the liver that prevents the liver from removing fats from the blood stream. The latter makes additional amounts of fatty acids available for other energetic needs, for example milk production (Roden & Bernroider, 2003; Jiang & Zhang, 2003). In ruminants, the effect of glucagon on adipose tissue metabolism seems to be the same as with non-ruminants. Glucagon infusions resulted in increased mobilization of fatty acids and glycerol provided that insulin secretion was abolished (Brockman, 1978). However, a lipolytic role for glucagon in ruminants has not been confirmed *in vitro*. This suggests that if glucagon has an effect on adipose tissue, this is not as potent as the effect of insulin (Brockman, 1978, 1979). Glucagon has no direct effect on peripheral AA metabolism, but it promotes uptake of glucogenic aminoacids by the liver of sheep *in vivo* and enhances the conversion of alanine to glucose (Brockman, 1978). *In vivo* studies in sheep suggest that high concentrations of glucagon may be ketogenic. Glucagon might exert this action in the liver by activating carnitine acyltransferase reaction which is the first step of oxidation of fatty acids (Brockman, 1979).

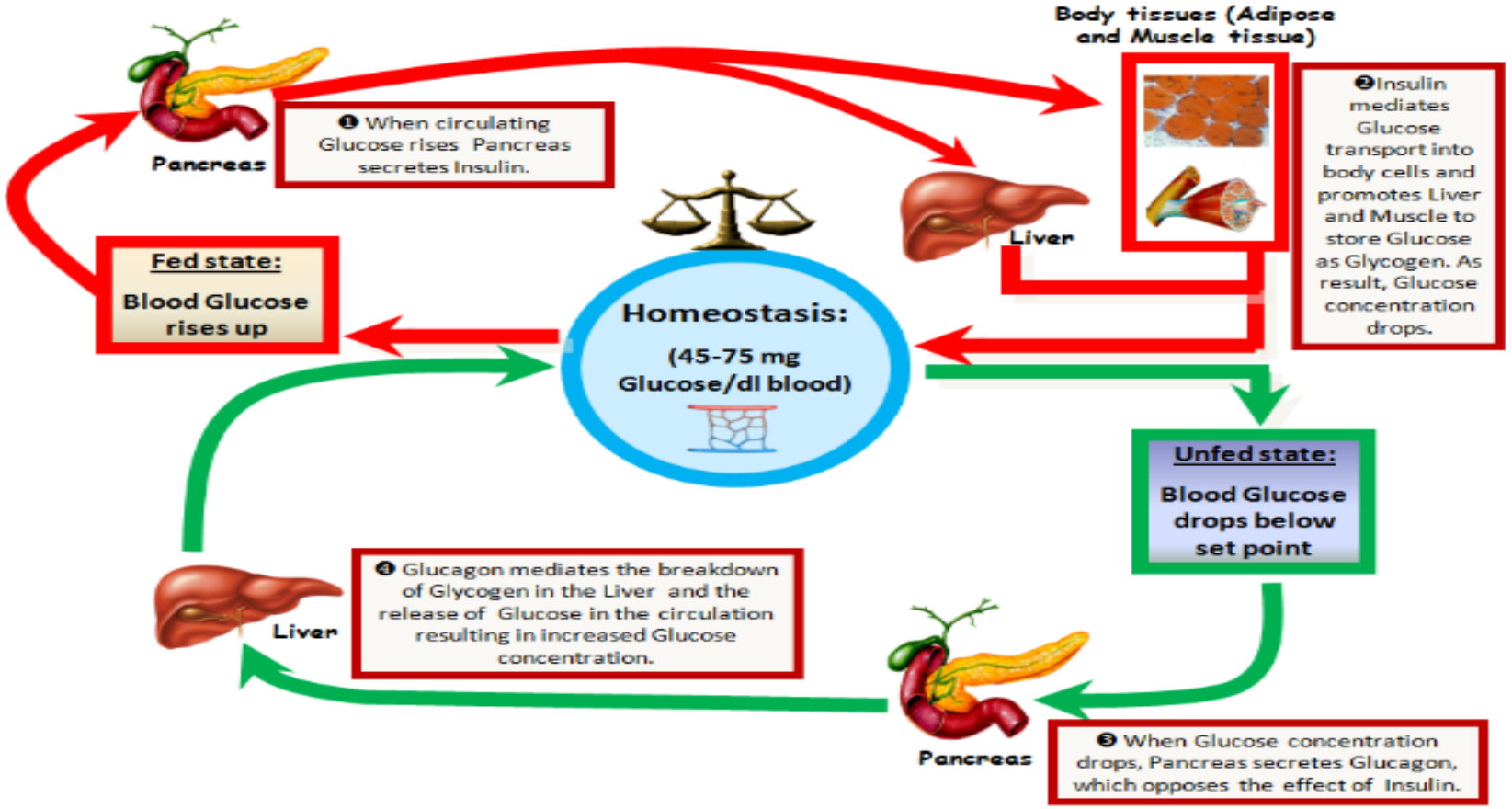


Figure 2.2: Short term homeostatic regulation of circulating glucose by the coordinated action of pancreatic hormones. Increased circulating glucose in the fed state stimulates secretion of the pancreatic hormone insulin by β cells and subsequent release of insulin into the blood stream. Postprandially, a high concentration of insulin stimulates liver cells to increase glucose uptake and storage in the form of glycogen. Moreover, cells from insulin dependent tissues, mainly adipose tissue and muscle, increase glucose utilization and as a result circulating glucose declines to the set point and the insulin effect is diminished. Preprandially, when circulating glucose is low, α cells in the pancreas are stimulated to secrete the pancreatic hormone glucagon and gradually its circulating levels are increased. Glucagon exerts its effects mainly on the liver, where it increases glycogen breakdown resulting in increased circulating glucose. Shortly, after glucagon action circulating glucose increases to the set point and the glucagon effect is diminished (Adapted from Marieb & Hoehn, 2007).

Generally, insulin is the primary hormonal signal that controls lipid and ketone body metabolism in ruminants while glucagon has only secondary effects when circulating insulin is low (Brockman, 1979). Glucose availability has been implicated as a limiting factor for milk production and the ratio of insulin to glucagon during the lactation is believed to be strongly related to the metabolic processes facilitating milk yield (Herbein *et al.,* 1985). According to De Boer *et al.* (1985, 1986), circulating glucagon increases from the dry period to early lactation in order to stimulate lipolysis and gluconeogenesis providing the body with the required energy to support high milk production. However, other studies (Herbein *et al.,* 1985; Kokta *et al.,* 2004) report that circulating glucagon does not change during lactation in dairy cows, suggesting almost minimal dependence of cows on gluconeogenesis. The role of glucagon stimulus has recently been revaluated in periparturient cows. Many studies were conducted to examine any positive effect of exogenous glucagon administration on postpartum NEB, glucose homeostasis, milk yield, and fatty liver syndrome (Steen *et al.,* 1997; Bobe *et al.,* 2003a, 2003b, 2004, 2007; Nafikov *et al.,* 2006; Osman *et al.,* 2008, 2010). The results of these studies indicated a positive influence of glucagon treatment on prevention of fatty liver without any effect on milk yield.

2.4.3. Leptin

Adipose tissue is no longer considered to be just a store of dormant TAGs which are mobilized during NEB (Fruhbeck *et al.,* 2001; Vernon, 2005; Ghanassia *et al.,* 2007). TAGs are partly interchangeable with glucose, and in this way fat and glucose metabolism are coupled (Vernon, 2005; Marieb & Hoehn, 2007). After the discovery of leptin, the scientific views about adipose tissue have changed considerably. Adipose tissue is now considered to be not only an active regulator of body weight but also an endocrine organ (Houseknecht *et al.,* 1998; Williams *et al.,* 2002; Macajova *et al.,* 2004). It has recently become clear that lipid stores play a critical role in regulation of energy homeostasis, insulin action, and glucose metabolism (Yildiz & Haznedaroglu, 2006; Kokta *et al.,* 2004; Block *et al.,* 2003). Moreover, the amount of body fat is the principal determinant of circulating levels of leptin. This means that there is a strong positive correlation between body fat and plasma leptin (Schneider, 2004; Zieba *et al.,* 2005). Leptin is positively correlated to LWT and adiposity in ruminants. Also, leptin in growing ruminants is positively correlated with BCS when other confounding factors are absent. However, the correlation between leptin and BCS appears to weaken postpartum when many other physiological influences are in action (Hill, 2004).

Leptin effects can be divided to two main categories: (1) central effects in the brain, affecting the amount of food consumption, energy homeostasis, and reproduction; (2) peripheral effects in the majority of body tissues, affecting nutrient partitioning (Schneider, 2004; Macajova *et al.,* 2004). Centrally, leptin exerts its function after binding to its long form receptor Ob-Rb. In the periphery, leptin action is inextricably associated with its binding to short form receptors (Ob-R) and there are at least six isoforms of Ob-R (Houseknecht *et al.,* 1998; Fruhbeck *et al.,* 2001; Macajova *et al.,* 2004; Liefers *et al.,* 2005). Ob-Rb is the primary mediator of leptin actions because it is the only iso-form that is capable of relaying full downstream signalling pathways after binding of leptin. Ob-R mRNA is expressed in several organs (such as brain, liver, BAT and WAT, skeletal muscle, pituitary, ovary, testis, and uterus) where leptin may directly exert regulatory actions (Krasnow & Steiner, 2006). Apart from this, short forms of leptin receptors

circulate in the blood stream and bind leptin. This mechanism is believed to play a role in activation and inactivation of leptin molecule (Kokta *et al.,* 2004; Houseknecht *et al.,* 1998; Macajova *et al.,* 2004).

Generally, the intricate influences of leptin on glucose homeostasis are exerted by the regulatory actions of leptin on feed intake, thermoregulation, lipid metabolism, and physical activity (Williams *et al.,* 2002; Zieba *et al.,* 2005; Liefers *et al.,* 2005). Increases in adipose depots reflect elevated circulating leptin and enhanced transduction of leptin signalling in the brain. This results in decreased food intake and increased energy expenditure (Krasnow & Steiner, 2006). Thus, the main central effect of leptin when it binds to Ob-R in the brain is suppression of food intake and potential body weight loss due to increased metabolic rate (Hill, 2004; Krasnow & Steiner, 2006). In cattle, expression of leptin mRNA and/or circulating leptin are rapidly decreased by restriction of energy intake, but are increased by refeeding (Chilliard *et al.,* 2005; Krasnow & Steiner, 2006). In the periphery, leptin: (1) stimulates free fatty acid (FFA) oxidation and increases glucose uptake and metabolism in skeletal muscles; (2) increases lipolysis in adipose tissue; (3) decreases glucose output and increases FFA oxidation in the liver (Yildiz & Haznedaroglu, 2006; Kokta *et al.,* 2004; Macajova *et al.,* 2004). Interestingly, leptin has been found in milk (Weyermann *et al.,* 2006; Martin *et al.,* 2006). Although some studies have demonstrated leptin transfer from blood to milk by involving leptin receptors expressed by mammary epithelial cells, other studies have shown mammary synthesis of leptin (Bonnet *et al.,* 2002). Whatever the mechanism, it is not known why leptin is secreted in milk or why its receptors exist in the mammary gland.Female *ob/ob* mice usually fail to lactate when leptin treatment is ceased before parturition. Additionally, female *ob/ob* mice treated with leptin for 14.5 days after successful mating were unable to lactate, even if leptin treatment was resumed after parturition. This implies a role of leptin in mammary gland development and lactation (Krasnow & Steiner, 2006) but it needs to be further investigated.

Leptin may be modulated by insulin and GH, and conversely (Casanueva & Dieguez, 1998; Williams *et al.,* 2002; Zieba *et al.,* 2005; Liefers *et al.,* 2005). Generally, insulin

stimulates circulating leptin in rodents and humans, but insulin effects on leptin in ruminants are unclear (Houseknecht *et al.,* 2000; Block *et al.,* 2001; Leury *et al.,* 2003; Thorn *et al.,* 2008). Insulin is a positive regulator of circulating leptin in lactating dairy cows in positive energy balance (Block *et al.,* 2003) and this may imply that cellular energy availability is the primary factor regulating leptin synthesis (Block *et al.,* 2001). However, the interaction between leptin and insulin could be characterized as quite complex and bidirectional (Yildiz & Haznedaroglu, 2006). On the one hand, increased plasma leptin promotes peripheral insulin sensitivity. On the other hand, the same levels of circulating leptin reduce insulin secretion from pancreatic β cells. This may suggest that there is a feedback loop between adipose tissue and pancreatic islets; the so-called ”Adipoinsular axis”. Any perturbation or deregulation of this axis is likely to contribute to development of certain pathologies such as insulin resistance, diabetes, and glucose intolerance (Yildiz & Haznedaroglu, 2006; Block *et al.,* 2003; Zieba *et al.,* 2005). A recent study suggests that hypoglycaemia (induced by hyperinsulinaemia) inhibits rises in leptin in rats. This finding may support the hypothesis that falling glucose levels during a prolonged fast, directly or indirectly, signal the adipocyte to reduce leptin secretion (Yildiz & Haznedaroglu, 2006).

Human obese patients showed decreased circulating GH and increased circulating leptin (Casanueva & Dieguez, 1998). In contrast, acromegalic and anorexia nervosa patients expressed decreased circulating leptin and increased circulating GH (Popovic *et al.,* 2001; Scacchi *et al.,* 1999). Leifers *et al.* (2005) suggested that leptin expression in adipose tissue is possibly regulated in early lactating cows by the negative impact of GH. Moreover, restriction of energy intake stimulates GH secretion but suppresses leptin expression in human and ruminant adipose tissue (Block *et al.,* 2001; Nagatani *et al.,* 2000). According to Scacchi *et al.* (1999) and Dieguez *et al.* (2000) increased circulating free fatty acids and adiposity are mainly responsible for the inhibition of GH secretion. However, it is possible that leptin is another hormonal signal controlling GH secretion by acting on somatostatin and growth hormone releasing hormone (GHRH) producing neurons (Scacchi *et al.*, 1999; Dieguez *et al.*, 2000). Furthermore, GH prevents insulin-induced production of leptin by bovine white adipose tissue (WAT) explants (Houseknecht *et al.* 2000). In contrast, GH

administration had no effect on circulating leptin during late pregnancy or early lactation in dairy cows (Leury *et al.,* 2003; Block *et al.,* 2003) and GH was potently stimulated by exogenous leptin administration in fasting sheep (Nagatani *et al.,* 2000). Although leptin can act directly at the bovine anterior pituitary to modulate GH release, this effect is greatly dependent on nutritional status of the cow (Zieba *et al.,* 2003). Moreover, GH treatment decreased circulating leptin only in pregnant dairy cows (Sauerwein *et al.,* 2004). Therefore, the regulatory roles of GH on leptin and *vice versa* are debatable and largely dependent on the endocrine and the metabolic status of animal.

Much attention has been given to whether reproductive function and nutritional status are bridged by leptin (Krasnow & Steiner, 2006). Leptin is a potent regulator of the hypothalamic pituitary ovary (HPO) axis and reproduction (Hill, 2004; Chilliard *et al.,* 2005). In the leptin knockout mouse model, males and females are sterile, and treatment with leptin supplementation restores fertility (Hill, 2004). *In vivo*, leptin stimulates secretion of GnRH and gonadotrophins (Williams *et al.,* 2002; Hill, 2004; Liefers *et al.,* 2005; Krasnow & Steiner, 2006), but *in vitro* inhibits steroidogenesis in the ovary and testis (Krasnow & Steiner, 2006; Williams *et al.,* 2002). *In vivo*, leptin is a potent stimulator of LH (Williams *et al.,* 2002; Krasnow & Steiner, 2006). In ruminants, central or peripheral supplementation of leptin to feed-deprived animals completely prevents the decreases in LH (Hill, 2004; Liefers *et al.,* 2005; Chilliard *et al.,* 2005). Leptin may serve as a trigger for sexual maturation or play a permissive role in initiation of the onset of puberty (Williams *et al.,* 2002; Chilliard *et al.,* 2005; Krasnow & Steiner, 2006). Age at onset of puberty is affected by mostly feed intake, growth rate and adiposity (Moran *et al.* 1989). Elevated circulating leptin precedes sexual maturation in many animals, and it has been demonstrated that leptin plays at least a permissive role in onset of puberty (Ahima *et al.,* 1997; Garcia *et al.,* 2002). Leptin enhances bovine oocyte maturation (Paula-Lopes *et al.,* 2007) and improves the ability of the bovine oocyte to sustain embryonic development (Boelhauve *et al.,* 2005).

Leptin roles are not restricted to regulation of reproductive function, but are extended to pregnancy and lactation (Hill, 2004; Chilliard *et al.,* 2005). In many animals, including

dairy cows, circulating leptin increases during pregnancy and reaches a peak just before parturition, at which point it starts to decrease rapidly (Chilliard *et al.,* 2005; Krasnow & Steiner, 2006). Normally, increased circulating leptin is accompanied by decreased food consumption, but because a sort of central leptin resistance is established, females of many species express hyperphagia (Hill, 2004; Krasnow & Steiner, 2006). In the bovine, leptin resistance may be considered a successful homeorhetic adaptation because it allows pregnant females to consume greater quantities of feed than normal in order to support the great energetic demands of the ongoing foetal growth and the imminent lactation (Krasnow & Steiner, 2006). Feed intake is already low 2 weeks prepartum and further decreases postpartum in dairy cows (Allen & Bradford, 2007). Therefore, modern periparturient dairy cows may be an exception displaying earlier hypophagia, although central leptin resistance has never been documented.

2.4.4. Adiponectin

Adiponectin, also known as Acrp30, apM1, AdipoQ, and GBP28, is an adipokine secreted by adipose tissue (Fruhbeck *et al.,* 2001; Kadowaki & Yamauchi, 2005; Berg *et al.,* 2002). Adiponectin protein form structurally belongs to the complement 1*q* family, it is multimeric, and it is constituted by four main domains. Serum human or mouse adiponectin forms a variety of multimers from trimers to high molecular weight (HMW) multimers. Oligomerization, proteolytic cleavage, hydroxylation, and glycosylation of multimers are shown to play important roles in adiponectin activation and deactivation (Kadowaki & Yamauchi, 2005). Adiponectin can exist as full-length or a smaller globular fragment, however, almost all circulating adiponectin appears to be as full-length adiponectin in plasma (Kadowaki & Yamauchi, 2005; Garaulet *et al.,* 2007; Giannessi *et al.,* 2007). It has been postulated that the different adiponectin multimers exert diverse effects in various tissues. Thus, adiponectin oligomerization is of high importance and it must be considered when adiponectin function is studied in a specific tissue (Garaulet *et al.,* 2007; Shetty *et al.,* 2009).

Binding of adiponectin to its receptors is the first step of its biological action. Two kinds of distinct but structurally related adiponectin receptors have been identified (AdipoR1 and AdipoR2) and they both bind globular and full-length adiponectin with different affinities (Ahima, 2006; Garaulet *et al.,* 2007). AdipoR1 and AdipoR2 are abundantly expressed in many tissues and organs (Kadowaki & Yamauchi, 2005; Dridi & Taouis, 2009) with AdipoR1 predominantly expressed in skeletal muscle and AdipoR2 in liver (Yamauchi *et al.,* 2003; Barb *et al.,* 2007). Ohtani *et al.* (2011) demonstrated the existence of an autocrine-paracrine system of adiponectin in bovine mammary gland and the presence of adiponectin receptors was shown in human breast cancer cell line MCF-7 (Dieudonne *et al*., 2006). It is known that AdipoR1 knock-out mice are obese and glucose-intolerant and they have decreased energy expenditure. In contrast, AdipoR2 knock-out mice are lean, with decreased plasma cholesterol levels, and resistant to high-fat-diet induced obesity and show increased energy expenditure (Bjursell *et al.,* 2007). This clearly demonstrates that both receptors are involved in energy metabolism, although they have opposing effects

(Bjursell *et al.,* 2007). According to Gonzalez *et al.* (2010) it is plausible to hypothesize that there is a different correlation between circulating adiponectin and AdipoR1 and AdipoR2 expressions. Moreover, AdipoR1 appears to mediate adiponectin effects via AMPK, whereas AdipoR2 via PPAR-*α* (Kadowaki *et al.*, 2008). Furthermore, nutritional status, pregnancy and circulating leptin seem to control circulating adiponectin and AdipoR2 expression in tissue specific manner in rats, whereas AdipoR2 expression is not correlated with circulating adiponectin (Gonzalez *et al.,* 2010).

The effects of adiponectin on liver and muscle tissue are well documented in human and mice (Yamauchi *et al.*, 2001,2002, 2003; Berg *et al.,* 2002; Kadowaki & Yamauchi, 2005), but they have not been explored in other domestic animal models and dairy cows. It has been reported that adiponectin-deficient mice are insulin-resistant and present lower plasma insulin levels after glucose loading than wild-type mice (Kharroubi *et al.,* 2003). Moreover, high circulating adiponectin levels were associated with a lower risk of development of type 2 diabetes and adiponectin was negatively correlated with circulating glucose (Hotta *et al.,* 2000; Weyer *et al.,* 2001; Spranger *et al.,* 2003). The main impacts of adiponectin on glucose homeostasis and nutrient partitioning can be summarized as: (1) adiponectin decreases glucose output, increases FFA oxidation, and increases influx of NEFA in the liver; (2) adiponectin increases FFA utilization and stimulates glucose usage in muscle tissue. For these reasons, it is believed that adiponectin generally improves the ratio of glucose to FFA and consequently has insulin sensitizing activity (Kershaw & Flier, 2004; Kadowaki & Yamauchi, 2005; Garaulet *et al.,* 2007; Shetty *et al.,* 2009).

Generally, the mechanism of insulin sensitizing of adiponectin involves activation of 5' AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor alpha (PPAR*α*), increased glucose uptake by GLUT4, increased glycolysis by phosphorylation of phosphofructokinase, and increased fatty acid oxidation by inactivation of acetyl CoA carboxylase and activation of carnitine palmitoyltransferase I (Yamauchi *et al.*, 2001, 2002; Ahima, 2006; Kadowaki *et al.,* 2006; Kamada *et al.*, 2008). According to Combs *et al.* (2001) even a moderate surge in circulating adiponectin can suspend both the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose

production. AMPK is a cellular energy sensor that is activated by a rise in the intracellular AMP/ ATP ratio. It is believed that adiponectin is a potent activator of AMPK and exerts its metabolic effects by activation of AMPK and subsequent deactivation of acetyl-coenzyme A carboxylase (Yamauchi *et al.*, 2002). This leads to decreased activation of malonyl CoA and increased activation of carnitine palmitoyltransferase I. Finally, activation of AMPK has been demonstrated to stimulate the recruitment of GLUT4 to the plasma membrane from cytoplasm, and to increase glucose uptake (Mullen *et al.,* 2007).

The impact of adiponectin on metabolism in dairy cows is almost unknown and few studies have measured adiponectin in cows. Komatsu *et al.* (2007) assessed adiponectin mRNA change in adipose tissue, in peak-, late-, and non-lactating cows. This study demonstrated that adiponectin concentrations reached a peak in non-lactating cows, and this value was greater than both peak- and late-lactation adiponectin values. Raddatz *et al.* (2008) measured adiponectin concentrations in lactating Holstein cows for the first 11 weeks of lactation. Adiponectin concentrations increased from week 1 to week 4 postpartum and then declined to remain at 12-13 *ng/ml* for the remainder of the study. Circulating adiponectin did not correlate with BCS or energy corrected milk yield, and circulating adiponectin did not affect resumption of oestrous cycles. Puntenney (2006) examined the effect of prepartum dietary treatment, breed (Jersey *versus* Holstein), and BCS in dairy cows. None of these factors had an effect on circulating adiponectin. Circulating Adiponectin was not correlated with NEFA, insulin, glucose, tumour necrosis factor-*alpha* (TNF-α), and BCS. Ohtani *et al.* (2011) examined mRNA expression levels of adiponectin and its receptors in various bovine tissues and mammary glands at different stages of lactation, and the effects of lactogenic hormones (insulin, dexamethasone and prolactin) and GH on mRNA expression of adiponectin receptors in cultured bovine mammary epithelial cells. AdipoR1 and AdipoR2 mRNAs were widely expressed in various bovine tissues, but adiponectin mRNA expression was significantly higher in adipose tissue than in other tissues. Although adiponectin mRNA expression was significantly decreased in the lactating mammary gland, AdipoR1 mRNA expression was significantly higher at peak lactation than at drying off. Moreover, in mammary epithelial cells lactogenic hormones and GH up-regulated AdipoR2 mRNA expression but did not change AdipoR1 mRNA.

Lemor *et al.* (2009) reported that AdipoR1 and AdipoR2 mRNA abundance in adipose tissue decreased as high yielding cows moved from pregnancy to lactation.

Some evidence suggests that adiponectin could directly regulate reproductive functions. Adiponectin and its receptors are present in theca and granulosa cells, oocytes and the corpus luteum (Lord *et al.,* 2005; Ledoux *et al.,* 2006; Ramachandran *et al.,* 2007; Chabrolle *et al.,* 2007a, 2007b). Adiponectin is also present in porcine and human follicular fluid (Ledoux *et al.,* 2006; Chabrolle *et al.,* 2009). Adiponectin may influence steroidogenesis, but contradictory results have been reported (Ledoux *et al.*, 2006; Lagaly *et al.*, 2008; Chabrolle *et al.,* 2009; Gutman *et al.,* 2009; Pierre *et al.*, 2009; Maillard *et al.*, 2010). The response of theca cells to adiponectin may be regulated by LH and IGF-I. Supplementation with LH during the late follicular phase may enhance follicular insulin sensitivity, resulting in decreased androgen levels through a pathway mediated by increased production of adiponectin by the human follicle (Gutman *et al.,* 2009). In the ovary, most of the adiponectin-induced modulations in gene and protein expression are mediated by AMPK (Chabrolle *et al.,* 2007b) or ERK 1/2- mitogen-activated protein kinase (MAPK) dependent pathway (Maillard *et al.,* 2010). In the ovary, the association between adiponectin and AMPK suggests that adiponectin may act as a key signal regulating the amount of energy required for growth of follicles and oocytes (Dupont *et al.,* 2008). AdipoR1 or AdipoR2 knock-out mice are fertile, and thus adiponectin is not essential for normal ovarian function (Brochu-Gaudreau *et al.,* 2010). It has been hypothesized that adiponectin effects on the ovary are mediated through its insulin-sensitizing traits (Mitchell *et al.*, 2005) and through its effect on IGF-I (Campos *et al.,* 2007; Dupont *et al.*, 2008; Michalakis & Segars, 2010). Chappaz *et al.* (2008) concluded that when adiponectin was added to media culture, meiotic maturation and embryo development of porcine oocytes were influenced positively. This indicates an effect of adiponectin on early embryonic development, but it needs to be elucidated further.

Weyermann *et al.* (2006) and Martin *et al.* (2006) examined adiponectin and leptin in human milk. Both of these studies confirmed the presence of adiponectin in human milk. On the one hand, Weyermann *et al.* (2006) reported that concentrations of adiponectin and

leptin varied strongly in maternal serum, cord blood, and breast milk, with only moderate correlations between both adipokines in maternal serum and breast milk. On the other hand, Martin *et al.* (2006) found that adiponectin concentrations in human milk are associated with stage of lactation, maternal adiposity (BMI), and ethnicity. In addition, Bronsky *et al.* (2006) measured adiponectin in human breast milk and found that milk adiponectin did not differ significantly in mothers who delivered boys *versus* girls. The authors also reported that there was a positive correlation between milk adiponectin and body weight of mothers before pregnancy, but no correlation between milk adiponectin and body weight at time of delivery.

Adiposity and circulating adiponectin are negatively correlated. According to Ishioka *et al.* (2006), canine adiponectin mRNA was detectable only in adipose tissue and obese dogs showed heavier body weights but lower circulating adiponectin. In the same study, circulating leptin was negatively correlated with circulating adiponectin. Kearns *et al.* (2006) reported that adiposity in horses was positively correlated with leptin and negatively correlated with adiponectin. Circulating adiponectin was negatively correlated with LWT, BMI, and insulin in humans (Berg *et al.,* 2002; Matsuzawa *et al.*, 2004; Ahima, 2006). No study has investigated the association of circulating adiponectin with LWT and BCS at calving in dairy cows. However, Raddatz *et al.* (2008) proposed that circulating adiponectin is not correlated with BCS in early lactating dairy cows.

Basu *et al.* (2009) investigated *in utero* the effect of gender dimorphism of adiponectin in humans. In this study it was reported that total adiponectin concentrations were higher in female compared with male foetuses. Furthermore, adiponectin was positively correlated with neonatal fat mass and percent body fat in female foetuses, and with lean mass in males. Many studies suggested higher circulating adiponectin in women compared to men (Kern *et al.,* 2003; Silha *et al.,* 2003; Tschritter *et al.,* 2003), but no sexual dimorphism was noted between mares and geldings (Gordon *et al.,* 2007). These findings implicate a complicacy of other factors, such as reproductive hormones, which possibly are involved in the regulation of adiponectin. Oestrogen was found to be negatively correlated with adiponectin in women, although pharmacologic doses given to women did not decrease

adiponectin levels (Mazaki-Tovi *et al.,* 2005). According to Barb *et al.* (2007) testosterone down-regulates adiponectin expression in adipose tissue, resulting in higher circulating adiponectin in females. The effect of testosterone on adiponectin is also unclear, with some studies reporting significant a suppressive effect on adiponectin and others reporting no effect (Mazaki-Tovi *et al.,* 2005; Mitchell *et al.,* 2005).

Dietary factors may modulate circulating adiponectin (Reis *et al.,* 2010). High consumption of magnesium (Qi *et al.,* 2005; Cassidy *et al.,* 2009), caffeine (Williams *et al.,* 2008), omega-3 polyunsaturated fatty acids (n-3 PUFA) (Duda *et al.,* 2007), and dietary salt (Lely *et al.,* 2007) were associated in humans with higher circulating adiponectin. According to Barnea *et al.* (2006) mice fed a High Fat (HF) diet exhibited significantly greater weight gain, abnormal oral glucose tolerance test curves, and elevated insulin resistance, although circulating adiponectin remained unchanged compared with controls. Jones *et al.* (2009) studied the effect of HF diet supplementation on a mouse model from a period around mating and throughout gestation. The HF diet increased maternal adiposity and circulating maternal leptin, and decreased serum adiponectin. Cassidy *et al.* (2009) observed that dietary intakes of carbohydrate, protein, and trans-fat were negatively associated with circulating adiponectin in human female twins. Pischon *et al.* (2005) showed that circulating adiponectin was negatively related to glycaemic load, but tended to be positively associated with total fat intake in men. Shimabukuro *et al.* (2007) studied the effects of consuming a high-carbohydrate meal, a HF meal, or a standard test meal on postprandial circulating adiponectin in healthy humans and found no association of circulating adiponectin with type of meal. Reis *et al.* (2010) reviewed the effects of diet components on adiponectin levels in rodent and human models. In this review it was concluded that diets rich in saturated fat reduce levels of adiponectin, whereas diets rich in PUFA and supplementation with n-3 PUFA increase both gene expression and circulating adiponectin. Gordon & McKeever (2005) showed that circulating adiponectin in horses did not alter throughout a 24-hour blood sampling period and neither dextrose challenge nor oral grain challenge changed circulating adiponectin. The effects of dietary components on circulating adiponectin have not yet been demonstrated in ruminants.

Many studies suggest an important role of adiponectin in regulation of insulin sensitivity, however, little is known about the regulatory role of this hormone during pregnancy (Mazaki-Tovi *et al.,* 2005). Catalano *et al.* (2006) investigated the roles of adiponectin in regulating glucose and lipid metabolism in human pregnancy. The authors reported that circulating adiponectin was lower in the third trimester than in the pregravid condition and this hypoadiponectinaemia was reflected by a 2.5-fold decrease in WAT adiponectin mRNA, and 25% increase in fat mass. In the same study, insulin infusion decreased high molecular weight adiponectin complexes in pregravid women and the suppressive effect of insulin on adiponectin was lost during pregnancy. Asai-Sato *et al.* (2006) studied the long term changes in circulating adiponectin during pregnancy and lactation along with its relations with prolactin in lean healthy women. They reported that circulating adiponectin declined slightly as pregnancy advanced and reached its lowest level during lactation. The authors also concluded that the lowest levels of adiponectin during lactation were possibly because prolactin influenced regulation of maternal metabolism by suppressing adiponectin. Prolactin suppresses circulating adiponectin in mice (Combs *et al.,* 2003), but in a transgenic mouse model increased circulating adiponectin and prolactin levels were observed (Combs *et al.,* 2004). Another study (Nien *et al.,* 2007), conducted to investigate the effect of different levels of adiposity on circulating adiponectin in pregnant women, concluded that there was no difference in circulating adiponectin between pregnant and non-pregnant women and circulating adiponectin was negatively correlated with gestational age only among pregnant women of normal weight. Sir-Petermann *et al.* (2007) reported that circulating adiponectin was lower in women with gestational diabetes mellitus (GDM) than in women with normal glucose tolerance. Gao *et al.* (2008) looked for possible associations of TNF-α, leptin, and adiponectin in mid-trimester women with gestational diabetes mellitus. Women with GDM have the highest circulating TNF-α and leptin and the lowest circulating adiponectin compared to those with gestational impaired glucose tolerance and to healthy controls at 14–20 weeks of gestation.

Adiponectin involvement in the control of glucose homeostasis in peripheral tissues via AdipoR1 and AdipoR2 receptors is beyond any doubt (Shetty *et al.,* 2009; Garaulet *et al.,* 2007; Giannessi *et al.,* 2007). However, recent studies have implied a central regulatory

role of adiponectin. Adiponectin intra-cerebro-ventricular (ICV) infusion in mice led to weight loss through increased energy expenditure (Qi *et al.,* 2004). AdipoR1 and AdipoR2 were discovered to be expressed by neurons (including pro-opiomelanocortin (POMC) and neuropeptide Y (NPY) neurons) and astrocytes in the rat hypothalamic nuclei, and adiponectin presence was ascertained in cerebrospinal fluid (Steinberg & Kemp, 2007). In addition, increased phosphorylation of AMPK in the rat hypothalamus was induced by adiponectin (Guillod-Maximin *et al.,* 2009). It has been demonstrated that AMPK activation and deactivation in hypothalamic neural cells play a key role in monitoring energy status and regulating food intake (Andersson *et al.,* 2004; Minokoshi *et al.,* 2008). Leptin and adiponectin control AMPK, with leptin to deactivate (Minokoshi *et al.*, 2008) and adiponectin to activate it (Kubota *et al.*, 2007) in hypothalamic neurons. Kubota *et al.,* 2007 showed that adiponectin binding to AdipoR1 in the murine arcuate hypothalamus enhanced AMPK activity and stimulated food intake. The discovery a role for adiponectin in stimulating appetite led Kubota *et al.* (2007) to propose the ‘‘fat-centric’’ hypothesis. According to this hypothesis, energy stores are maintained by the opposing central actions of leptin and adiponectin (Steinberg & Kemp, 2007). However, this hypothesis needs to be further investigated.

Some studies have demonstrated multiple regulatory interactions between adiponectin and GH at central and peripheral level. Engström *et al.* (2003) reported that GH-deficient women who followed a nine-month treatment with exogenous GH supplementation showed elevated circulating adiponectin compared to controls. Lam *et al.* (2004) reported that circulating adiponectin was low in patients suffering from acromegaly, and circulating adiponectin increased after GH-lowering therapies. Fasshauer *et al.* (2004) demonstrated that GH was a putative regulator of AdipoR2 in 3T3-L1 adipocytes and that GH stimulated AdipoR2 synthesis was increased up to 4.8-fold during differentiation of 3T3-L1 preadipocytes. Moreover, this positive effect of GH on AdipoR2 expression could be reversed by GH withdrawal for 24 hours. Nilsson *et al.* (2005) demonstrated that gene expression of AdipoR1 and AdipoR2 in human adipose tissue is differentially regulated by prolactin and GH. Also, prolactin and GH reduced adiponectin secretion by human adipose tissue *in vitro* and *in vivo* in mice. Rodriguez-Pacheco *et al.* (2007) investigated the central

role of adiponectin in regulating somatotroph and gonadotroph function. The authors showed that short-term adiponectin exposure abolished both basal and stimulated (by Ghrelin and GnRH) secretion of GH and LH by rat pituitary cells *in vitro*. Also, this study demonstrated the existence of a complete autocrine/ paracrine system of adiponectin in rat pituitary in which both adiponectin and its receptors are expressed. Thus, adiponectin seems to regulate energy expenditure, body reserves, and reproduction by playing a complicated central and peripheral role similar to leptin. Steyn *et al.* (2009) confirmed the regulatory effect of adiponectin on GH secretion in rat pituitary somatotrophs. However, this study demonstrated that adiponectin stimulated GH secretion in a dose-dependent manner through binding to either AdipoR1 or AdipoR2. Although these results are conflicting, they demonstrate interaction between adiponectin, GH, and their receptors at the central and peripheral level. The terms of this interaction needs to be further elucidated by both *in vitro* and *in vivo* experiments.

To conclude, adiponectin is a very interesting molecule but research has been mostly conducted in humans and rodents. There is only limited evidence for a role of adiponectin in nutrient partitioning, reproductive performance, and milk yield in dairy cattle. Thus, the interplay of adiponectin with other bovine hormonal and metabolic stimuli must be investigated further.

2.5. Working hypotheses and objectives

The literature review strongly suggested that reproductive performance of lactating dairy cows interacts with nutrition and BCS at calving and that the related hormonal and metabolic profile plays a role in cow fertility. This was the main hypothesis of the present thesis. This hypothesis has been developed in Chapter 3 in this P*h*D project, and has been tested in a series of specific objectives. More specifically the objectives were to:

- Examine the effect of two dietary treatments (high starch *versus* high fat diet) on metabolic profile and reproduction (Chapter 3).
- Explore the effect of BCS at calving on metabolic profile and reproductive performance (Chapter 3).
- Define optimum plasma insulin concentration in terms of reproduction, and address its impact on pregnancy rate and milk progesterone profile (Chapter 3).

The second hypothesis was that different BCS at calving, diets, milk yield potential and physiological stages will affect circulating and milk adiponectin values, and production traits, metabolic hormones and metabolites, and their relationships. This hypothesis has been developed in Chapter 4 in this P*h*D project, and has been broken down into more specific objectives. More specifically the objectives were to:

- Measure circulating adiponectin and milk adiponectin in dairy cows (Chapter 4).
- Investigate the effects of diet and BCS at calving on circulating adiponectin (Chapter 4).
- Demonstrate the effect of stage of lactation on circulating adiponectin (Chapter 4).
- Investigate associations between leptin, adiponectin, production traits, and metabolic and hormonal signals (Chapter 4).
- Assess the association of circulating adiponectin with DMI in lactating dairy cows (Chapter 4).

The third hypothesis was that high yielding cows have lower circulating adiponectin than low yielding cows due to increased circulating GH and its antagonistic relationship with adiponectin. This hypothesis has been developed in Chapter 4 in this P*h*D project.

The fourth hypothesis was that animals with elevated plasma adiponectin concentrations (up to three times) will express different hormonal, metabolic, productive, and/or reproductive pattern. This hypothesis has been developed in Chapter 5 in this P*h*D project.

The fifth hypothesis was that high yielding cows will regulate glucose homeostasis differently to low yielding cows, and glucose homeostasis in dairy cows will be regulated by adiponectin. This hypothesis has been developed in Chapter 5 in this P*h*D project.

A general discussion summarizing and integrating the results of these experiments has been presented in Chapter 6.

3. Effects of body condition score at calving and diet on circulating metabolic hormones, metabolites, and reproductive traits in dairy cows

3.1. INTRODUCTION

Body condition score (BCS) is a rapid, non-invasive, inexpensive, but subjective way of assessing body reserves of the cow. It is easy applicable at the farm level and might give a more realistic view of the lipid and muscle reserves than live weight (Garnsworthy, 2006). In recent decades, BCS has been proved a useful management tool for assessing nutritional status and EB during lactation in dairy cows. Estimates of BCS are significantly correlated to subcutaneous fat (Garnsworthy & Topps, 1982; Domecq *et al.,* 1995; Heuer *et al.,* 1999; Garnsworthy *et al.,* 2006). Ruegg & Milton (1995) reported that excessive loss of BCS during early lactation is related with metabolic diseases and Gearhart *et al.* (1990) described high BCS at parturition as a risk factor for reproductive performance in dairy cows. It is well known that body condition loss and especially BCS at certain points of the cow cycle impacts directly on DMI, NEB, fertility, milk yield and milk composition, and health of high yielding cow (Butler, 2003; Garnsworthy, 2006). According to Chagas *et al.* (2007) the perfect BCS fluctuation to minimize the effects of NEB on health and reproduction is BCS at calving 3.0-3.5 units with a nadir BCS of 2.5-3.0 units. Mulligan *et al.* (2006b) suggested BCS targets for drying-off point; 2.75 units, BCS at calving; 3.0 units, BCS at service ≥ 2.75 units, and nadir BCS; 2.75 units.

According to Mulligan *et al.* (2006a, 2006b), over-fat cows at calving have a greater possibility of expressing fatty liver, ketosis, retained placenta, calving difficulties and milk fever. Butler & Smith (1989) and Villa-Godoy *et al.* (1990) demonstrated that when NEB is accompanied by excess BCS loss then the result is declined fertility. Numerous studies showed the negative impact of excess or inadequate BCS at calving, BCS loss and nadir

BCS on postpartum reproductive performance (Garnsworthy & Topps, 1982; Heuer *et al.,* 1999; Moreira *et al.,* 2000; Yamada *et al.,* 2003; Lopez-Gatius *et al.,* 2003; Agenas *et al.,* 2003; Roche *et al.,* 2007b). The main outcome from these studies was that reproductive indices determining the reproductive efficiency of the herd, such as calving interval, days to first oestrus, days to first service, first service conception rate and number of services were related to BCS, which is considered a key point of reproductive management.

BCS has been considered an indirect measure of nutritional status (Short *et al.,* 1990; Garnsworthy, 2006; Bewley & Schutz, 2008). That implies that BCS is affected by diet composition and DMI (Short *et al.,* 1990; Garnsworthy, 2006). According to Short *et al.* (1990) the effect of nutrition on reproduction depends on whether nutritional differences exist before or after calving. In addition, BCS at calving is more important than postpartum BCS loss and consequently dietary and nutritional management of animals in the dry period is a crucial factor to ensure timely reproductive functionality after calving (Short *et al.,* 1990).

The interaction between nutrition and reproduction is complicated and variable (Boland *et al.,* 2001). This interaction involves both past and present nutritional status, but other factors such as genetic makeup, BCS, environmental influence, and physiological state are determinant modifiers of nutritional effects on reproductive performance (Short *et al.,* 1990; Garnsworthy & Webb, 1999; Lucy, 2001; Stevenson, 2001; Robinson *et al.,* 2006). High fat diets with dietary total fat concentration over 50 *g/kg* of DM depressed plasma insulin concentration in cows at the onset of the breeding period (Garnsworthy *et al.,* 2008b). Conversely, high starch diets induced high plasma insulin concentration and increased the proportion of cows ovulating within 50 days of calving and reduced the interval from calving to first ovulation, and tended to reduce the intervals from calving to first service and to conception (Gong *et al.,* 2002b). Moreover, Adamiak *et al.* (2005, 2006) in heifers (beef x dairy) and Fouladi-Nashta *et al.* (2005) in lactating dairy cows demonstrated that diets designed to increase plasma insulin concentration had negative effects on blastocyst rate in heifers and lactating cows.

For the purposes of the present study, data generated by Garnsworthy *et al.* (2009) were re-analysed. Garnsworthy *et al.* (2009) provided evidence to support the hypothesis that pregnancy rate will be enhanced by feeding a diet that increased plasma insulin (HS diet) until cows resume ovarian cycles, and then switching to a diet (HF diet) that decreased plasma insulin during the mating period. In the study of Garnsworthy *et al.* (2009), the impact of BCS on reproductive performance of lactating cows was not assessed. Thus, the first objective of this study was to determine the effects of BCS at calving and diet on reproductive performance in early lactating dairy cows. It was hypothesized that different BCS at calving and diet would result in different metabolic profiles and reproductive performance. Hormonal and metabolic profiles of the cows were used to explain differences in reproductive traits such as days to first oestrus, pregnancy rate, and milk progesterone profile. Gong *et al.* (2002b) demonstrated that feeding a high starch diet to dairy cows for the first 50 days postpartum increased circulating insulin concentrations, and reproductive performance was enhanced. In addition, Fouladi-Nashta *et al.* (2007) reported that diets with a high fat content had beneficial effects on blastocyst rate in lactating dairy cows although they decreased circulating insulin. These two results taken together may imply that there is an optimum insulin concentration necessary for normal reproductive performance in lactating dairy cows. Moreover, it is known that fat cows at calving are more insulin resistant than thin cows at calving (Holtenius & Holtenius, 2007; Sinclair, 2010). In the study of Garnsworthy *et al.* (2009) cows fed the high starch and high fat diets had normal to high concentrations of circulating insulin and expressed very low pregnancy rate (26.7%). The second objective of the study was to test the hypothesis that there is an optimum insulin concentration necessary for normal reproductive performance in lactating dairy cows, and that is dependent on BCS at calving.

3.2. MATERIALS AND METHODS

3.2.1. Data

This study utilized data generated by the study of Garnsworthy *et al.* (2009). Key points of materials and methods, further statistical analysis, and handle of the data are presented hereinafter.

3.2.2. Experimental Design

Thirty high-yielding multiparous Holstein dairy cows were blocked according to calving date and parity, and were allocated at random to two equal dietary treatment groups (HS and HF, 15 cows in each). Within dietary treatment groups, cows were selected on the basis of BCS, and divided into FAT and THIN cows. Cows with BCS at calving greater than 3.25 were the FAT group (19 cows in total; HS, 9 cows; HF, 10 cows) and cows with BCS at calving equal or less than 3.25 were the THIN group (11 cows in total; HS, 6 cows; HF, 5 cows). Prepartum, all cows were fed on the same diet. Blood samples were taken from each cow from 2nd week until 17th week postcalving. The data were obtained from this experiment were panel, longitudinal or cross-sectional data. Cows were the panels, and dietary (DIET) and condition (CONDITION) treatments were nested within week of experiment (WEEK). Two diets were formulated to have equal concentrations of DM, ME and CP, but to differ in starch, fat and NDF concentrations (Table A.1 in appendix). Diet HS was expected to induce relatively high plasma insulin concentrations because of its higher starch and lower fat contents; Diet HF was expected to induce relatively low insulin concentrations because of its lower starch and higher fat contents. These diets were equivalent to the high and low insulin diets used by Gong *et al.* (2002), Fouladi-Nashta *et al.* (2005), and Garnsworthy *et al. (*2009). Milk progesterone was measured twice each week (either Monday and Thursday or Tuesday and Friday mornings) and a rise in progesterone was defined as above 3 *ng/ml* for two consecutive samples (Lamming & Darwash, 1998; Garnsworthy *et al.,* 2009).

3.2.3. Feeding and milking

Cows were housed as one group in a freestall barn throughout the experiment, and were fed individually via electronic feeders (Roughage Intake Control feeders, Fullwood Ltd, Ellesmere, UK) that recorded feed intake automatically. Cows were milked by an automatic (robotic) milking system (AMS; Merlin, Fullwood Ltd.), which they entered voluntarily and were milked on average 2.65±0.09 times per day. In order to encourage cows to use the AMS, 4 kg fresh weight of each cow's daily concentrate allocation was dispensed automatically in the AMS during milking (Garnsworthy *et al.,* 2009).

3.2.4. Reproductive management

Cows were artificially inseminated at the first or second oestrus. Insemination was repeated at any subsequent oestrus until the end of the experiment at 120 days post partum. Oestrus was detected using a combination of behavioural observations, pedometer activity monitoring and milk progesterone profiles. Milk progesterone was monitored daily from 4 days before expected oestrus until signs of oestrus were detected; monitoring then returned to twice weekly until 4 days before the next expected oestrus (21 days later). Progesterone profiles were used subsequently to classify oestrous cycles as normal or abnormal (DOV1, DOV2, PCL1 or PCL2), following the definitions of Lamming & Darwash (1998) (Table A.2 in appendix).

3.2.5. Recording, sampling and analysis

Milk yield and feed intake were recorded daily throughout the experiment. Live weight and BCS (units; 1 to 5) were recorded weekly. Milk samples were taken twice each week and analyzed for progesterone by ELISA (Ridgeway Scientific, Alvington, UK). Feed samples were taken weekly and pooled on a monthly basis for analysis of DM, CP, NDF, starch, sugars, fat and ME, as detailed in Garnsworthy *et al.* (2008b). Blood samples were taken every Wednesday at 09:30 h throughout of the sampling period for measurement of hormones and metabolites. Blood samples were analyzed for the following hormones:

insulin, GH, IGF1, glucagon, and leptin, as detailed in Garnsworthy *et al.* (2009). Blood samples were analyzed for the following metabolites on a Bayer opera autoanalyzer (Bayer UK Ltd): urea-N, glucose, BOHB, and NEFA as detailed in Garnsworthy *et al.* (2009). All instances of ill health and veterinary treatments were recorded. The ultimate fate of each cow (subsequent calving or culling) was recorded to allow calculation of calving interval. For cows that were pregnant when culled, subsequent calving date was estimated as date of successful insemination plus 282 days; cows that were not pregnant when culled were omitted from statistical analysis of calving interval (Garnsworthy *et al.,* 2009).

3.3. STATISTICAL ANALYSIS

All data were analyzed using PASW© 18 Edition (SPSS Inc., Chicago, USA). Generalized Linear models (GLM) and Generalized Estimating Equations (GEE) were used to test the specific hypotheses (Lindsey, 1997; McCullagh & Nelder, 1989; Dobson, 2002; Horton & Lipsitz, 1999). Family distribution and link function were selected by running a null model including only the dependent variable and various combinations of family distribution and link function (Lindsey, 1997; McCullagh & Nelder, 1989; Dobson, 2002; Horton & Lipsitz, 1999). Models were compared in terms of values of Akaike's information criterion (AIC) criterion and the one with the lowest AIC value was chosen (Lindsey, 1997; McCullagh & Nelder, 1989; Dobson, 2002; Horton & Lipsitz, 1999). AIC is a statistical model fit index, defined as AIC= $-2L_m + 2m$ where L_m is the maximized *log-likelihood* and m is the number of parameters in the model. Lower values of AIC indicate better fit of the model.

The biological validity of each model was tested at the post-prediction level and the appropriate model was selected by using AIC and the quasi-likelihood under the independence model criterion (QIC) for choosing the best correlation structure. QIC is adaptation of AIC for repeated measures, where quasi log likelihood is used instead of log likelihood of AIC for better model fit. As with AIC, smaller values of QIC indicate better model fit (Dobson, 2002). Moreover, the GEE models were selected based on this simple principle; working correlation matrix (*R matrix*) must at least in part reflect the real correlation structure of the data (Horton & Lipsitz, 1999).

Missing or incomplete data are inherent in studies where repeated measurements are obtained from animals (because of ill health, culling, and funding limitation (planned missing data) some animals are excluded from the study) (Little & Rubin, 1987; Allison, 2001; Frees, 2004) and this was the greatest limitation of the present study. On the one hand, exclusion of missing data from analysis may result in inconsistent parameter estimates and possible loss of statistical power (considerable reduction of the sample and estimation of unacceptably large standard errors) (Frees, 2004). Alternatively, inclusion of

missing data in the analysis may lead to biased results (Allison, 2001). However, missing data in panel studies can be handled by the methods of *maximum likelihood* (*ML*), *restricted maximum likelihood* (*REML*), and *multiple imputation estimation* (*MI*) (Allison, 2001; Schafer, 1997; Frees 2004). According to Allison (2001) *ML* estimation is proven to be an excellent method for handling panel missing data if the data are missing at random (MAR). According to Enders (2010) patterns of data missingness are not that important and *ML* is well suited for any type of missing data. *ML* was the method of choice to deal with the missing values in the present study. Common repeated measurements ANOVA (Albert, 1999; Davis, 2002) and Mixed Linear Models (Cnaan *et al.,* 1997; Littell *et al.,* 1998; Littell, 2002; Wang & Goonewardene, 2004; Gueorguieva & Krystal, 2004), which are alternatives for panel data analysis, were not the preferred method for analyzing the data, mainly because the data were nonparametric and unbalanced. Generalized linear models (GLM), generalized estimating equations (GEE), and log-linear Poisson models utilize *ML* to calculate parameter estimates and were the choice for analyzing the data in the present study (McCullagh & Nelder, 1989; Lindsey, 1997; Dobson, 2002). GEEs use *quasi likelihood* estimation and developed to extend the GLM to repeated measurements (Davis, 2002). GEE models were selected based on the principle that the working correlation (*R matrix*) must at least in part reflect the real correlation structure of the data (Dobson, 2002). Also, inclusion of robust estimator of covariance matrix in GEE models may provide more accurate parameter estimates (Zorn, 2000; Harden, 2011). Finally, GEE and GLM do not assume normality, but allow the choice of error distribution and link function that better fit the data, leading possibly to more efficient parameter estimates (McCullagh & Nelder, 1989; Lindsey, 1997).

Models selected to test the effects of BCS at calving (CONDITION) and diet on reproductive and production traits and hormonal and metabolic profile are described in Tables 3.1 to 3.3.

Milk progesterone profile variable (Normal; 16 cows, DOV1; 3 cows, DOV2; 5 cows, PCL2; 5 cows, and PCL1; 1 cow) was transformed to a binominal variable with two levels (NORMAL; 16 cows and ABNORMAL; 14 cows); pregnancy rate is a binomial variable

with two categories (PREGNANT; 8 cows and NON PREGNANT; 22 cows). Milk progesterone profile and pregnancy rate were used as binomial response variables in Generalized linear models to ascertain if BCS at calving and diet affected them (Table 3.1).

The output of generalized logistic regression and common logistic regression models are presented as probabilities, unstandardized beta (*b*) coefficients, and odds ratio (exp (*b*)) with 95% confidence intervals (Peng *et al.,* 2002). In common logistic regression models, *Hosmer & Lemeshow goodness of fit test* and *Likelihood Ratio test* (*LR*) were carried out. The *LR* test compared the selected model with the null model (a model which included only the intercept) and significant *P* values ($P<0.05$) indicated the superiority of the selected model. The *Hosmer & Lemeshow* test was used to examine the fitness of selected models to the actual data and non-significant *P* values ($P>0.05$) indicated better fit of the model (Agresti, 1996; Hosmer & Lemeshow, 2000).

Receiver Operating Characteristic (ROC) analysis was conducted to evaluate the performance and the accuracy of logistic regression models (Hand, 2010; Zou *et al.,* 2007). ROC curve is the plot of model sensitivity (true positive) *versus* model 1-specificity (=false positive) (Liu & Li, 2005). Area under the ROC curve (AUC_{ROC}), which is an overall statistic summary of model accuracy and ranges from zero to one, was calculated. AUC_{ROC} equals 0.5 when the ROC curve corresponds to random chance and 1 for perfect accuracy (Zou *et al.,* 2007). According to Hosmer & Lemeshow (2000) $AUC_{ROC}=0.5$ corresponds to a model with bad performance and accuracy, whereas $0.7 < AUC_{ROC} < 0.8$, $0.8 < AUC_{ROC} < 0.9$, and $AUC_{ROC} \geq 0.9$ are considered as acceptable, excellent, and outstanding performance and accuracy of the model, respectively. AUC_{ROC} is presented as mean± SE.

Non-random associations between two categorical variables in 2X2 contingency tables (*e.g.* BCS at calving X Diet) were tested by *Fisher's exact test. P* value equal or less than 0.05 ($P\leq0.05$) indicated statistically significant association between the two categorical variables.

The effect of BCS at calving and diet on time from calving to pregnancy was examined by survival analysis (Klein & Moeschberger, 2005; Selvin, 2008). Cows not pregnant at the end of the experiment were censored at 120 days postpartum and *Kaplan–Meier* estimates of the survivor function were compared for FAT and THIN cows, and cows fed high starch (HS) and high fat (HF) diets by using *Log-rank test* (Landau & Everitt, 2004; Jenkins, 2005; Guo, 2010). The models selected for survival analysis are described in Table 3.4.

Parameter estimates of GEE models are presented as marginal means plus/minus standard error of the difference (SED) in tables. Estimated marginal means, SEs and SEDs were obtained by using *Least Significant Difference* (*LSD*) adjustment for multiple comparisons in PASW© 18 statistical program. *P* values were calculated by *Newton-Raphson Maximum Likelihood (ML)* method and effects were considered statistically significant when *P* value was less than 0.05 ($P<0.05$).

Table 3.1: Selected models for analysis of reproductive traits

REPRODUCTIVE TRAIT *(RESPONSE VARIABLE: Y)*	TYPE OF MODEL	PREDICTORS *(X)*	CONTINUOUS COVARIATES	ERROR DISTRIBUTION FUNCTION	LINK FUNCTION	COVARIANCE MATRIX	STATISTICS
●**Milk P4 Profile** *(binomial variable with two levels; 0=Normal 1= Abnormal)*	*Multiple Logistic regression*	●CONDITION ●DIET	Insulin IGF-I Glucose Parity	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=29; AIC=35; *LR*(χ^2 =13.79, *df*=6, *P*=0.032)
●**Milk P4 Profile** *(binomial variable with two levels; 0=Normal 1= Abnormal)*	*GLM*	●CONDITION ●DIET	PARITY	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=30; AIC=36; *LR*(χ^2 =10, *df*=3,*P*=0.018)
●**Pregnancy** *(binomial variable with two levels; 0= non pregnant 1= pregnant)*	*GLM*	●CONDITION ●DIET	PARITY	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=30; AIC=35; *LR*(χ^2=6.2, *df*=2,*P*=0.045)
●**Pregnancy** *(binomial variable with two levels; 0= non pregnant 1= pregnant)*	*GLM*	●MILK P4 PROFILE	-	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=30; AIC=33; *LR*(χ^2 =3.89, *df*=1, *P*=0.048)
●**Pregnancy** *(binomial variable with two levels; 0= non pregnant 1= pregnant)*	*Multiple Logistic regression*	●CONDITION ●DIET	Insulin PARITY	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=30; AIC=33; *LR*(χ^2=16.56, *df*=4, *P*=0.002)
●**Days to Oestrus**	*GLM*	●CONDITION ●DIET	PARITY	*Poisson*	*Log*	*Model Based*	n=28; AIC=267; *LR*(χ^2 =4, *df*=3, *P*=0.26)

Table 3.2: Selected models for analysis of plasma metabolic hormones and metabolites

Hormone *(RESPONSE VARIABLE: Y)*	TYPE OF MODEL	PREDICTORS *(X)*	CONTINUOUS COVARIATES	ERROR DISTRIBUTION FUNCTION	LINK FUNCTION	COVARIANCE MATRIX	WORKING CORRELATION MATRIX	STATISTICS
●Insulin **●Leptin** **●IGF-I** **●Glucagon**	*GEE*	●CONDITION ●DIET ●WEEK	PARITY	*Gaussian (Normal)*	*Identity (ID)*	*Robust*	*Exchangeable*	n=427; QIC=66 n= 424; QIC=395 n=224; QIC=424,623 n= 234; QIC=116,230
●GH	*GEE*	●CONDITION ●DIET ●WEEK	PARITY	*Inverse Gaussian*	*Log*	*Robust*	*Exchangeable*	n=222; QIC=67
●Glucose **●Urea**	*GEE*	●CONDITION ●DIET ●WEEK	PARITY	*Gaussian (Normal)*	*Identity (ID)*	*Robust*	*Exchangeable*	n= 225; QIC=100 n=231; QIC=131
●BOHB **●NEFA**	*GEE*	●CONDITION ●DIET ●WEEK	PARITY	*Inverse Gaussian*	*Log*	*Robust*	*Exchangeable*	n=233; QIC=134 n= 233; QIC=204

Table 3.3: Selected models for analysis of production traits

PRODUCTIVE TRAIT (*RESPONSE VARIABLE: Y*)	TYPE OF MODEL	PREDICTORS (*X*)	CONTINUOUS COVARIATES	ERROR DISTRIBUTION FUNCTION	LINK FUNCTION	COVARIANCE MATRIX	WORKING CORRELATION MATRIX	STATISTICS
●**DMI** ●**Milk Yield** ●**LWT** ●**BCS**	*GEE*	●CONDITION ●DIET ●WEEK	PARITY	*Gaussian (Normal)*	*Identity (ID)*	*Robust*	*Exchangeable*	n= 460; QIC=4,306 n= 460; QIC=29,954 n= 460; QIC=271,156 n= 473; QIC=135
●**Nadir BCS** ●**Nadir Week** ●**ΔBCS**	*GLM*	●CONDITION ●DIET	PARITY	*Gaussian (Normal)*	*Identity (ID)*	*Robust*	--	n=30; AIC=24; $LR(\chi^2=6.4, df=3, P=0.09)$ n=30; AIC=24; $LR(\chi^2=10.2, df=3, P=0.017)$ n=30; AIC=42; $LR(\chi^2=12, df=3, P=0.01)$

Table 3.4: Selected models for survival analysis (Kaplan- Meier models) of interval from calving to conception

TIME VARIABLE (*Survival time*)	TYPE OF MODEL	STATUS VARIABLE (*Event variable*)	FACTORS (*Group variables*)	SURVIVAL FUNCTIONS COMPARISON TEST	OBSERVATIONS	RIGHT CENSORED OBSERVATIONS	UNCENSORED OBSERVATIONS
Days post partum *(0- 120)*	*Kaplan-Meier*	Pregnancy *(binomial variable with two levels; 0=non pregnant 1=event= pregnant)*	●CONDITION ●DIET	*Log-Rank (Mantel- Cox)*	30	22	8

3.4. RESULTS

3.4.1. Consistency of BCS at calving tretments

There was no association between BCS at calving and diet, and thus the retrospective classification of cows as FAT and THIN was not biased by the original dietary treatments (*Fisher's exact test*, n=30, P=1.0, *two–sided*) (Table 3.5).

3.4.2. Effect of milk progesterone profile on pregnancy rate

Cows with NORMAL milk progesterone profile had higher probability to be pregnant (44% *versus* 7%, AUC_{ROC}=0.74±0.08) than cows with ABNORMAL milk progesterone profile (P<0.05) (Table 3.6).

3.4.3. Effects of BCS at calving and diet on reproductive performance

THIN cows at calving had higher probability to be pregnant (52% *versus* 14%, AUC_{ROC}=0.79±0.09) than FAT cows at calving (P<0.05) (Table 3.7). THIN cows at calving had lower probability to express ABNORMAL milk progesterone profile (18% *versus* 62%, AUC_{ROC}=0.85±0.07) than FAT cows at calving (P<0.05) (Table 3.7). There was no effect of diet on probability of cows to be pregnant within 120 days postpartum (Table 3.9). Cows fed the HS diet had higher probability to express ABNORMAL milk progesterone profile (65% *versus* 29%, AUC_{ROC}=0.85±0.07) than cows fed the HF diet (P<0.05) (Table 3.8). There was no effect of BCS at calving and diet on days to first oestrus (Table 3.9). Survival analysis showed that FAT cows had a shorter interval from calving to conception than THIN cows, but FAT cows had a lower probability to be pregnant within 120 days postpartum than THIN cows (P<0.05) (Figure 3.1). However, survival analysis demonstrated that there was no effect of diet on probability of cows to be

pregnant within 120 days postpartum and cows fed the HS diet did not have significantly different interval from calving to conception compared to cows fed the HS diet (Figure 3.2).

3.4.4. Effects of BCS at calving and diet on circulating metabolic hormones

THIN cows at calving had higher circulating IGF-I (147.5±10.12 *ng/ml versus* 104.9±9.30 *ng/ml*) than FAT cows at calving (P<0.05) (Table 3.10). There was no effect of BCS at calving on circulating insulin, leptin, glucagon, and GH (Table 3.10). Cows fed the HS diet had higher circulating insulin (0.49±0.03 *ng/ml versus* 0.41±0.03 *ng/ml*) than cows fed the HF diet (P<0.05) (Table 3.11). There was no effect of diet on circulating IGF-I, leptin, glucagon, and GH (Table 3.11).

3.4.5. Effects of BCS at calving and diet on circulating metabolites

THIN cows at calving had lower circulating NEFA (0.32±0.03 *mmol/l versus* 0.41±0.03 *mmol/l*) than FAT cows at calving (P<0.05) (Table 3.12). There was no effect of BCS at calving on circulating glucose, BOHB, and urea (Table 3.12). Cows fed the HS diet had higher circulating urea (2.83±0.14 *mmol/l versus* 2.32±0.14 *mmol/l*) than cows fed the HF diet (P<0.05) (Table 3.13). Circulating BOHB was lower (P<0.05) for cows fed the HS diet (0.485±0.040 *mmol/l*) than for cows fed the HF diet (0.650±0.040 *mmol/l*) (Table 3.13). There was no effect of diet on circulating glucose and NEFA (Table 3.13).

3.4.6. Effects of BCS at calving and diet on production traits

THIN cows at calving had lower milk yield (40.5±2.5 *kg/d versus* 47.7±1.6 *kg/d*) than FAT cows at calving (P<0.05) (Table 3.15). There was no effect of BCS at calving on LWT and DMI (Table 3.14). THIN cows at calving had lower BCS (2.36±0.06 units *versus*

2.66±0.08 units), ΔBCS (0.47±0.11 units *versus* 0.82±0.09 units) and nadir BCS (2.02±0.06 units *versus* 2.30±0.09 units), and shorter nadir week (4.5±0.8 weeks *versus* 6.5±0.8 weeks) than FAT cows at calving ($P<0.05$) (Table 3.14).

There was no effect of diet on LWT, DMI, milk yield, BCS, ΔBCS, nadir week, and nadir BCS (Table 3.15).

3.4.7. Effect of insulin on pregnancy rate

Multiple logistic regression analysis examined the effects of insulin, BCS at calving, diet, and parity on pregnancy rate. Insulin had a strong negative effect (*OR*=$1.11*10^{-07}$~0, 95% CI for *OR* (0, 0.03), *b*=-16.01) on the odds of cows to be pregnant (*P*=0.012), holding the other predictors of the model at a fixed value. Dietary treatments (*OR*=0.73, 95% CI for *OR* (0.10, 5.33), b=-0.31) had no effect on the odds of cows to be pregnant (*P*=0.76). BCS at calving (categorical variable with two levels; 0=FAT, 1=THIN) (*OR*=52.32, 95% CI for *OR* (5.79, 472.38), *b*=3.96, *P*=0.001) and parity (*OR*=0.21, 95% CI for *OR* (0.05, 0.85), *b*=-1.54, *P*=0.03) had a negative effect on the odds of cows to be pregnant, holding the other predictors of the model at a fixed value (Table 3.16).

The output of this model was expressed as probability of cows to be pregnant. The negative association of circulating insulin concentrations with the probability of cows to be pregnant is illustrated in Figure 3.3. This graph presents that the optimum insulin concentration that maximized the probability of cows to be pregnant ranged from 0.2 to 0.3 *ng/ml*, whereas insulin greater than 0.6 *ng/ml* tended to zero the probability of cows to be pregnant. Probability of cows to be pregnant was adjusted for different condition status at calving (FAT *versus* THIN), dietary treatments (HS *versus* HF), and circulating insulin concentrations (Figure 3.4, Figure 3.5, and Figure 3.6). According to Figure 3.4, THIN cows at calving had higher probability to be pregnant than FAT cows at calving ($P<0.05$), when insulin ranged from 0.3 to 0.6 *ng/ml*. Moreover, there was no effect of diet on probability of cows to be pregnant ($P>0.05$), when the animals had approximately the same

insulin concentration and insulin concentration ranged from 0.1 to 1.2 *ng/ml* (Figure 3.5). Also, there was no effect of (BCS at calving*diet) on probability of cows to be pregnant (P>0.05) (Figure 3.6).

3.4.8. Effects of insulin, IGF-I, glucose, and parity on milk progesterone profile

Multiple logistic regression analysis examined the effects of insulin, IGF-I, glucose, and parity on milk progesterone profile. Each exponentiated coefficient in this model is the change in odds for a unit increase in the corresponding predictor variable holding other variables at certain value. Odds ratio (*OR*) for insulin was very high and that means strong positive effect of insulin on the odds for cows to express abnormal milk progesterone profile (P<0.05) (Table 3.17). *OR* for circulating glucose was very low and that means strong negative effect of circulating glucose on the odds for cows to express abnormal milk progesterone profile (P<0.05) (Table 3.5). *OR* for parity was 8.59 (P<0.05) (Table 3.17). So holding insulin, IGF-I, BCS at calving, diet, and glucose at a fixed value, an increase of parity by 1 will increase the odds for cows to express atypical milk progesterone profile by 759 %. IGF-I (*OR*=0.97) also tended to have negative influence on the odds for cows to express abnormal milk progesterone profile ($P\leq$ 0.1), holding the other predictors of the model at a fixed value (Table 3.17).

The output of this model was expressed as probability for cows to express abnormal milk progesterone profile. The positive association of circulating insulin concentrations with the probability for cows to express abnormal milk progesterone profile is illustrated in Figure 3.7. This graph presents that the optimum insulin concentration that minimized the probability for cows to express abnormal milk progesterone profile ranged from 0.1 to 0.3 *ng/ml*, whereas insulin greater than 0.5 to 0.6 *ng/ml* tended to maximize the probability for cows to express abnormal milk progesterone profile. Probability for cows to express atypical milk progesterone profile was adjusted for different condition status at calving (FAT *versus* THIN), dietary treatments (HS *versus* HF), and circulating insulin concentrations (Figure 3.8, Figure 3.9, and Figure 3.10). According to Figure 3.8, THIN

cows at calving had lower probability to to express abnormal milk progesterone profile than FAT cows at calving (P<0.05), when the animals had approximately the same insulin concentration and insulin concentration ranged from 0.4 to 0.9 *ng/ml*. Moreover, there was no effect of diet on probability for cows to express abnormal milk progesterone profile (P>0.05), when the animals had approximately the same insulin concentration and insulin concentration ranged from 0.1 to 1.2 *ng/ml* (Figure 3.9). However, THIN cows at calving that fed the HF diet had lower probability to express abnormal milk progesterone profile than THIN cows at calving that fed the HS diet (P<0.05), when the animals had approximately the same insulin concentration and insulin concentration ranged from 0.1 to 0.6 *ng/ml*. (Figure 3.10). In addition, THIN cows at calving that fed the HF diet had lower probability to express abnormal milk progesterone profile than THIN cows at calving that fed the HS diet (P<0.05), when insulin concentration ranged from 0.1 to 0.3 *ng/ml*.

Table 3.5: Crosstabulation of BCS at calving with diet. Association between BCS at calving and diet was tested by performing *Fisher's exact test* (n= 30, *P*= 1.0, *two–sided*). There was no association between BCS at calving and diet.

	BCS at calving		
Diet	**FAT**	**THIN**	**Total**
HS	9	6	15
HF	10	5	15
Total	19	11	30

Table 3.6: Effect of milk progesterone profile on probability cows to be pregnant

Treatment:	MILK PROGESTERONE PROFILE				
Parameters:	**NORMAL**†	n_1 ∂	**ABNORMAL**†	n_2 ∂	***P***
Probability for cows to be pregnant	0.44 (0.22, 0.68)	15	0.07 (0, 0.20)	15	0.049

† **Columns are means with 95 % Confidence Interval (CI).**
∂ n_1 **and** n_2 **are the numbers of animals expressed NORNAL and ABNORMAL milk progesterone profile, respectively.**

Table 3.7: Effect of BCS at calving (CONDITION) on probability of cows to be pregnant and to express abnormal milk progesterone profiles

Treatment:	CONDITION				
Parameters:	**FAT**†	n_1 ∂	**THIN**†	n_2 ∂	***P***
Probability for cows to be pregnant	0.14 (0, 0.20)	15	0.52 (0.24, 0.81)	15	0.017
Probability for cows to express abnormal milk progesterone profile	0.62 (0.43, 0.80)	15	0.18 (0, 0.38)	15	0.02

† **Columns are means with 95 % Confidence Interval (CI).**
∂ n_1 **and** n_2 **are the numbers of animals assessed FAT and THIN at calving, respectively.**

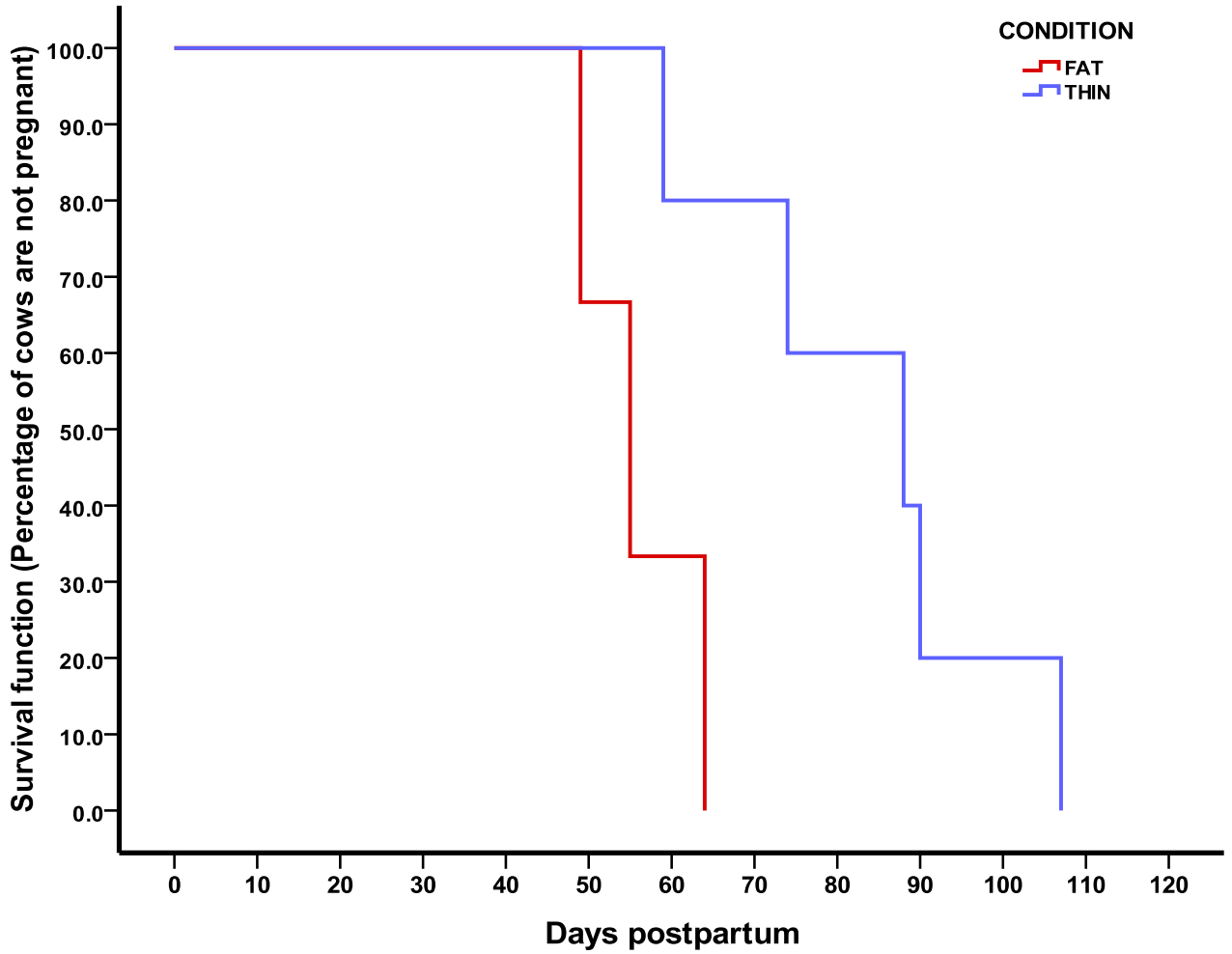


Figure 3.1: Calving-to-conception survival analysis curves for FAT and THIN cows at calving. The survival curve for THIN cows is higher than the curve for FAT cows (*n=30, Log-rank test P*=0.022). Vertical drop in the survival curves indicates an event (a cow became pregnant). FAT cows at calving had a shorter interval from calving to conception (55±4.9 days postpartum *versus* 88±15.3 days postpartum) than THIN cows at calving.

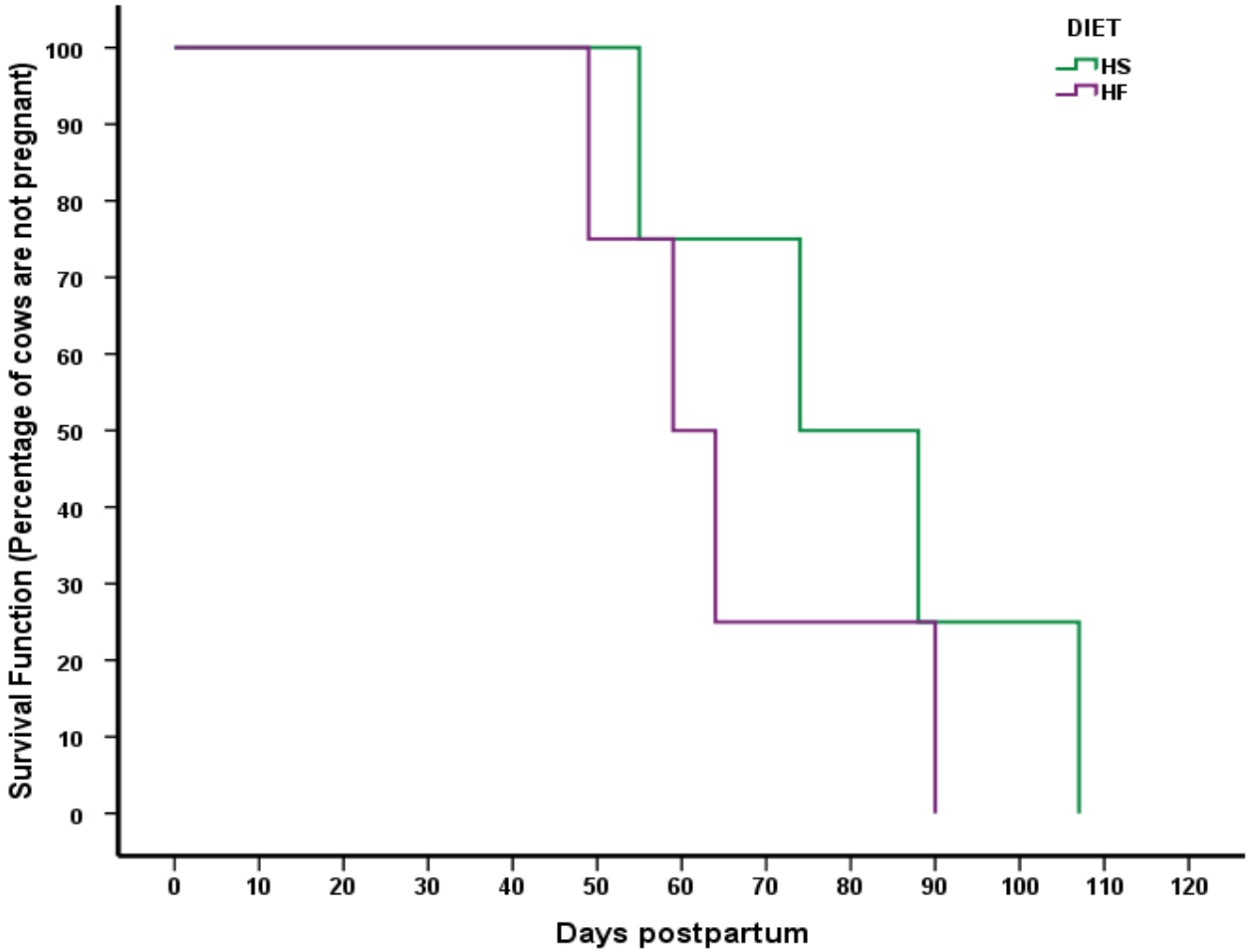


Figure 3.2: Calving-to-conception survival analysis curves for cows fed the high starch (HS) and the high fat (HF) diet. There are no differences in the survival curves for cows fed with HS and HF diet (*n=30*, *Log-rank test P*=0.40). Vertical drop in the survival curves indicates an event (a cow became pregnant). Cows fed the HS diet did not have significantly different interval from calving to conception (74±16.5 days postpartum *versus* 59±7.5 days postpartum) compared to cows fed the HS diet.

Table 3.8: Effect of diet on probability of cows to be pregnant and to express abnormal milk progesterone profiles

Treatment:	DIET				
Parameters:	**HS**[†]	n_1 [∂]	**HF**[†]	n_2 [∂]	***P***
Probability for cows to be pregnant	0.23 (0.07, 0.40)	15	0.31 (0.08, 0.53)	15	0.59
Probability for cows to express abnormal milk progesterone profile	0.65 (0.47, 0.86)	15	0.27 (0.09, 0.45)	15	0.03

[†] **Columns are means with 95 % Confidence Interval (CI).**
[∂] n_1 **and** n_2 **are the numbers of animals fed with HS and HF diets, respectively.**

Table 3.9: Effect of diet and BCS at calving (CONDITION) on days to first oestrus

Treatments:	DIET					CONDITION				
Parameters:	**HS**[†]	n_1 [∂]	**HF**[†]	n_2 [∂]	***P***	**FAT**[†]	n_3 [‡]	**THIN**[†]	n_4 [‡]	***P***
Days to first oestrus (*days postpartum*)	58.2 (50.2, 66.2)	13	51.2 (44.5, 57.9)	15	*0. 19*	53.3 (46.7, 59.9)	13	56.1 (47.6, 64.7)	15	*0.26*

[†] **Columns are means with 95 % Confidence Interval (CI).**
[∂] n_1 **and** n_2 **are the numbers of animals fed with HS and HF diets, respectively.**
[‡] n_3 **and** n_4 **are the numbers of animals assessed FAT and THIN at calving, respectively.**

Table 3.10: Effect of BCS at calving (CONDITION) on circulating insulin, glucagon, GH, IGF-I, and leptin

Treatment:	CONDITION					
Parameters:	**FAT**[†]	n_1 [∂]	**THIN**[†]	n_2 [∂]	**SED**[‡]	***P***
Insulin (*ng/ml*)	0.43	274	0.47	153	0.040	*0.19*
Glucagon (*pg/ml*)	93.4	151	95.3	83	5.97	*0.75*
GH (*ng/ml*)	4.40	141	4.27	81	0.670	*0.84*
IGF-I (*ng/ml*)	104.9	142	147.5	82	9.75	*0.03*
Leptin (*ng/ml*)	1.72	272	1.41	15	0.298	*0.30*

[†] **Columns are means.**
[∂] **n_1 and n_2 are the repeated observations of animals assessed FAT and THIN at calving, respectively.**
[‡] **SED = standard error of the difference between treatment means.**

Table 3.11: Effect of diet on circulating insulin, glucagon, GH, IGF-I, and leptin

Treatment:	DIET					
Parameters:	**HS**†	n_1 ∂	**HF**†	n_2 ∂	**SED**‡	***P***
Insulin (*ng/ml*)	0.49	211	0.41	216	0.034	*0.02*
Glucagon *(pg/ml)*	99.5	116	89.6	118	5.28	*0.06*
GH (*ng/ml*)	4.54	106	4.15	116	0.650	*0.54*
IGF-I (*ng/ml*)	132.0	105	122.7	119	13.16	*0.48*
Leptin (*ng/ml*)	1.73	208	1.43	216	0.312	*0.35*

† **Columns are means.**

∂ n_1 **and** n_2 **are the repeated observations of animals fed with HS and HF diets, respectively.**

‡ **SED = standard error of the difference between treatment means.**

Table 3.12: Effect of BCS at calving (CONDITION) on circulating glucose, urea, *β*-hydroxy butyrate (BOHB), and non-esterified fatty acids (NEFA).

Treatment:	CONDITION					
Parameters:	**FAT**[†]	n_1 [∂]	**THIN**[†]	n_2 [∂]	**SED**[‡]	***P***
Glucose (*mmol/l*)	3.65	138	3.55	87	0.142	*0.44*
Urea (*mmol/l*)	2.66	145	2.49	86	0.160	*0.29*
BOHB (*mmol/l*)	0.580	146	0.583	87	0.0383	*0.92*
NEFA (*mmol/l*)	0.41	146	0.32	87	0.032	*0.021*

[†] **Columns are means.**
[∂] ***n*$_1$ and *n*$_2$ are the repeated observations of animals assessed FAT and THIN at calving, respectively.**
[‡] **SED = standard error of the difference between treatment means.**

Table 3.13: Effect of diet on circulating glucose, urea, *β*-hydroxy butyrate (BOHB), and non-esterified fatty acids (NEFA).

Treatment:	DIET					
Parameters:	**HS**[†]	n_1 [∂]	**HF**[†]	n_2 [∂]	**SED**[‡]	***P***
Glucose (*mmol/l*)	3.60	115	3.61	110	0.114	*0.92*
Urea (*mmol/l*)	2.83	114	2.32	117	0.146	*0.01*
BOHB (*mmol/l*)	0.485	115	0.650	118	0.0404	*0.01*
NEFA (*mmol/l*)	0.306	115	0.309	118	0.0262	*0.90*

[†] **Columns are means.**
[∂] n_1 **and** n_2 **are the repeated observations of animals fed with HS and HF diets, respectively.**
[‡] **SED = standard error of the difference between treatment means.**

Table 3.14: Effect of BCS at calving (CONDITION) on dry matter intake (DMI), live weight (LWT), milk yield, BCS, nadir BCS, nadir week, and postpartum body condition loss (ΔBCS)

Treatment:	CONDITION					
Parameters:	**FAT**[†]	n_1 [∂]	**THIN**[†]	n_2 [∂]	**SED**[‡]	***P***
DMI (*kg/d*)	22.52	294	21.12	166	1.401	*0.18*
Milk Yield (*kg/d*)	47.7	294	40.5	166	2.06	*0.02*
LWT (*kg*)	658	294	664	166	7.1	*0.41*
BCS (*units 1-5*)	2.66	304	2.36	169	0.069	*0.003*
Nadir BCS (*units 1-5*)	2.30	15	2.02	15	0.076	*0.017*
Nadir Week (*weeks*)	6.5	15	4.5	15	0.59	*0.024*
ΔBCS (*units 1-5*)	0.82	15	0.47	15	0.103	*0.025*

[†] **Columns are means.**
[∂] **n_1 and n_2 are the observations of animals assessed FAT and THIN at calving, respectively.**
[‡] **SED = standard error of the difference between treatment means.**

Table 3.15: Effect of diet on dry matter intake (DMI), live weight (LWT), milk yield, BCS, nadir BCS, nadir week, and postpartum body condition loss (ΔBCS)

Treatment:	DIET					
Parameters:	**HS**[†]	n_1 [∂]	**HF**[†]	n_2 [∂]	**SED**[‡]	***P***
DMI (*kg/d*)	21.50	228	22.15	232	0.957	*0.50*
Milk Yield (*kg/d*)	45.4	228	42.8	232	2.56	*0.31*
LWT (*kg*)	658	228	664	232	7.2	*0.37*
BCS (*units 1-5*)	2.44	240	2.58	233	0.099	*0.17*
Nadir BCS (*units 1-5*)	2.11	15	2.27	15	0.084	*0.19*
Nadir Week (*weeks*)	4.9	15	6.6	15	0.74	*0.12*
ΔBCS (*units 1-5*)	0.76	15	0.63	15	0.112	*0.43*

[†] **Columns are means.**

[∂] **n_1 and n_2 are the observations of animals fed with HS and HF diets, respectively.**

[‡] **SED = standard error of the difference between treatment means.**

Table 3.16: Effects of insulin, parity, BCS at calving, and diet on probability of cows to be pregnant

Parameters in the model:	exp (*b*)[†]	95% CI for exp (*b*)		Beta (*b*) coefficient	*P*
		Lower	Upper		
Constant				8.13	0.01
Insulin	0	0	0.03	-16.01	0.01
Parity	0.21	0.05	0.85	-1.54	0.03
BCS at calving [∂]	52.32	5.79	472.38	3.96	0.001
Diet [✧]	0.73	0.10	5.34	-0.31	0.76

Likelihood ratio test **(χ^2=16.56, *df*=4, *P*=0.002);** ***Hosmer & Lemeshow test for fitness*** **(χ^2=5.97, *df*=8, *P*=0.65);** **AUC_{ROC}=0.85±0.08**

[†] **exp (*b*) is the odds ratio**

[∂] **BCS at calving is a variable with two levels; 0= FAT and 1= THIN**

[✧] **Diet is a variable with two levels; 0= HS and 1= HF**

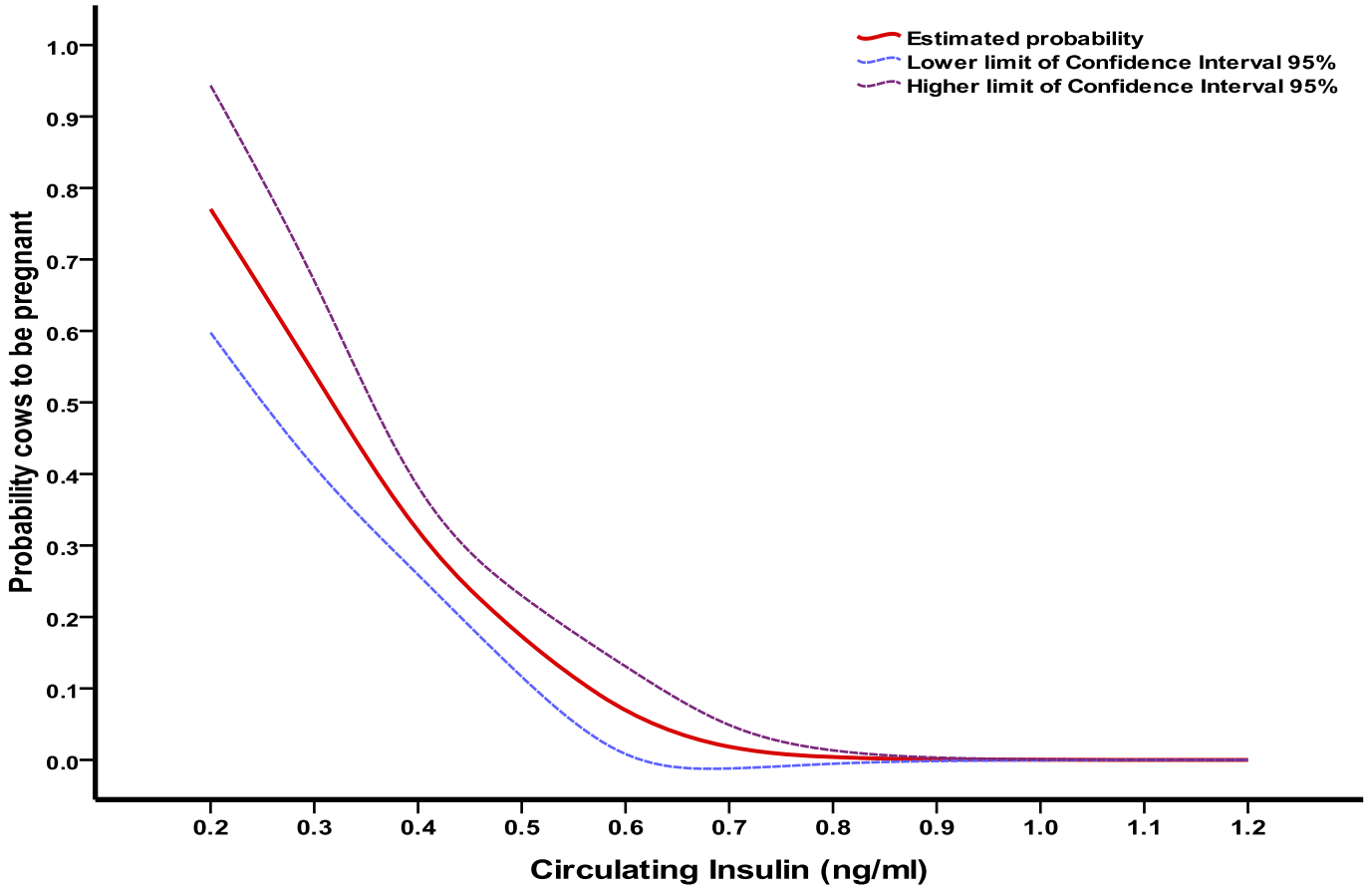


Figure 3.3: Effect of circulating insulin on probability of cows to be pregnant (n= 30, *P*=0.01, AUC_{ROC}=0.85±0.08, *LR* (χ^2=16.56, *df*=4, *P*=0.002), *Hosmer & Lemeshow test for fitness* (χ^2=5.97, *df*=8, *P*=0.65), multiple logistic regression model with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was pregnancy (0= non pregnant, 1= pregnant) whereas insulin and parity were added as continuous covariates, and BCS at calving and diet as factors. Red solid line represents the estimated probability whereas blue and purple dashed lines show the upper and lower 95 % Confidence Interval (CI). Optimum insulin concentration that maximized the probability of cows to be pregnant ranged from 0.2 to 0.3 *ng/ml*, whereas insulin greater than 0.6 *ng/ml* tended to zero the probability of cows to be pregnant. Model parameter estimates are presented in Table 3.16.

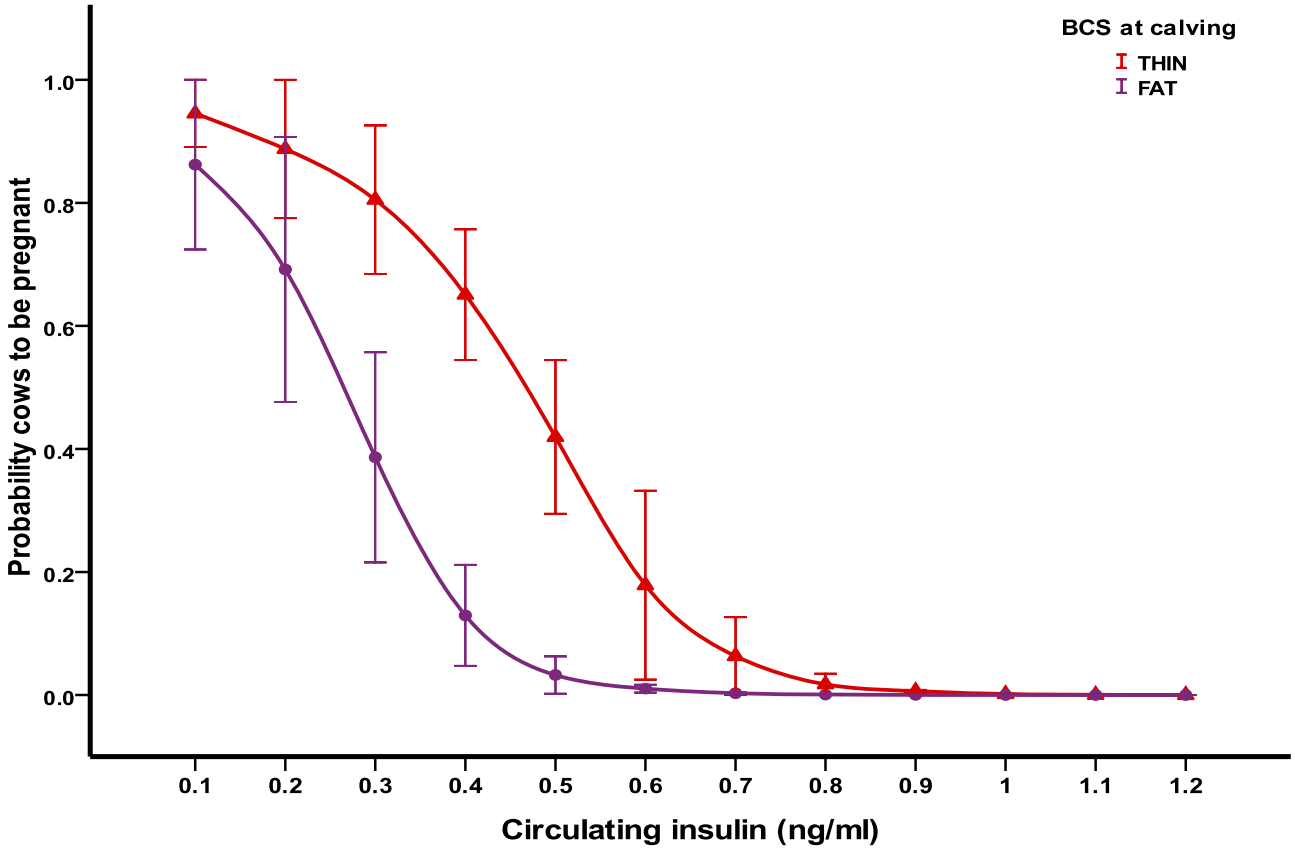


Figure 3.4: Probability of cows to be pregnant adjusted for different condition status at calving (FAT *versus* THIN) and circulating insulin concentrations (n= 30, *P*=0.01, AUC_{ROC}=0.85±0.08, *LR* (χ^2=16.56, *df*=4, *P*=0.002), *Hosmer & Lemeshow test for fitness* (χ^2=5.97, *df*=8, *P*=0.65), multiple logistic regression model with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was pregnancy (0= non pregnant, 1= pregnant) whereas insulin and parity were added as continuous covariates, and BCS at calving and diet as factors. Red triangles (▲) represent marginal means ± SE for THIN cows at calving, whereas purple dots (●) are marginal means ± SE for FAT cows at calving. THIN cows at calving had higher probability to be pregnant than FAT cows at calving (*P*<0.05), when the animals had approximately the same insulin concentration and insulin concentration ranged from 0.3 to 0.6 *ng/ml*. Model parameter estimates are presented in Table 3.16.

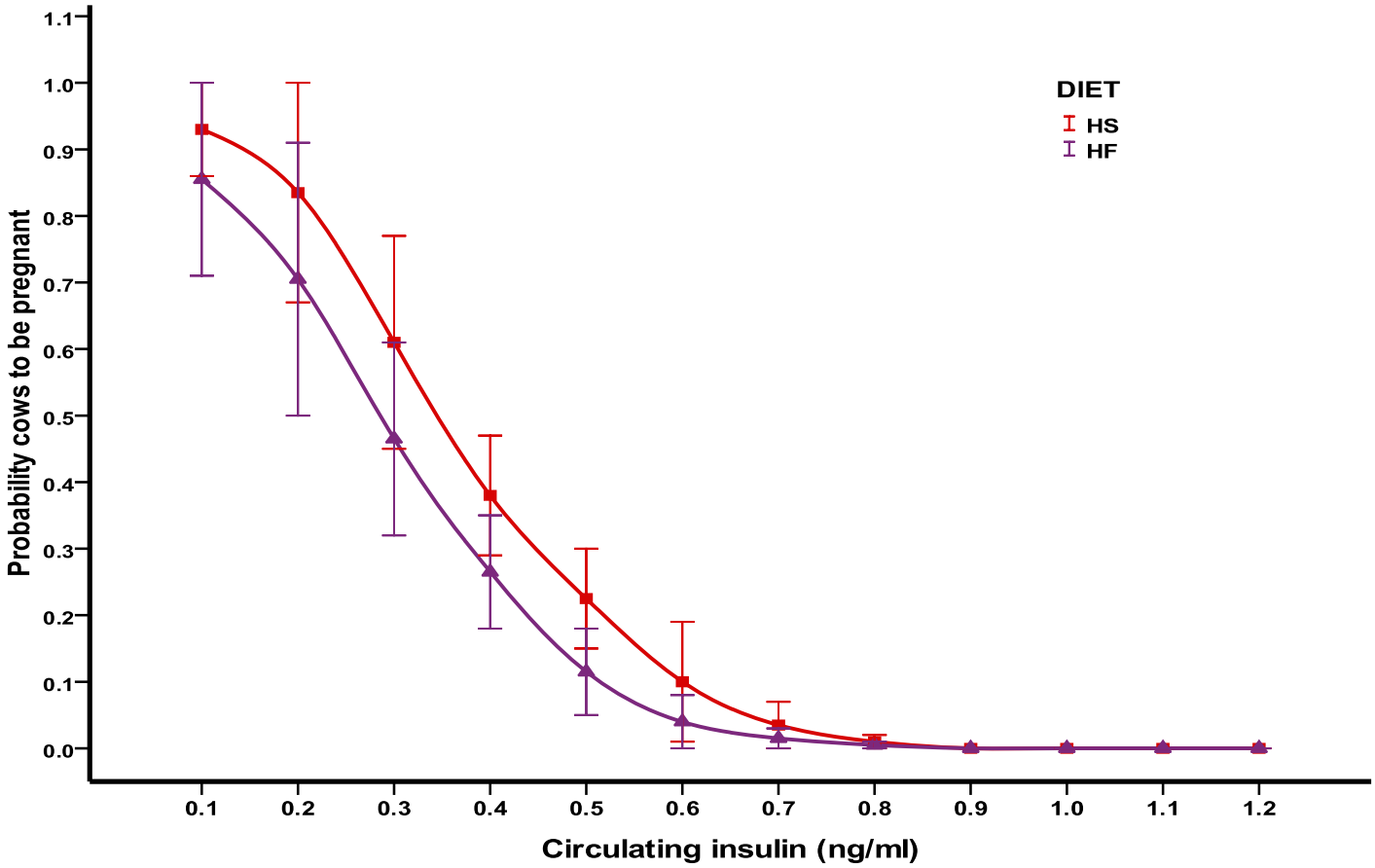


Figure 3.5: Probability of cows to be pregnant adjusted for different dietary treatments (HS *versus* HF) and circulating insulin concentrations (n= 30, P=0.76, AUC_{ROC}=0.85±0.08, *LR* (χ^2=16.56, *df*=4, P=0.002), *Hosmer & Lemeshow test for fitness* (χ^2=5.97, *df*=8, P=0.65), multiple logistic regression model with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was pregnancy (0= non pregnant, 1= pregnant) whereas insulin and parity were added as continuous covariates, and BCS at calving and diet as factors. Purple triangles (▲) represent marginal means ± SE for cows fed the HF diet, whereas red rectangles (■) are marginal means ± SE for cows fed the HS diet. There is no effect of diet on probability of cows to be pregnant (P>0.05), when the animals had approximately the same insulin concentration and insulin concentration ranged from 0.1 to 1.2 *ng/ml*. Model parameter estimates are presented in Table 3.16.

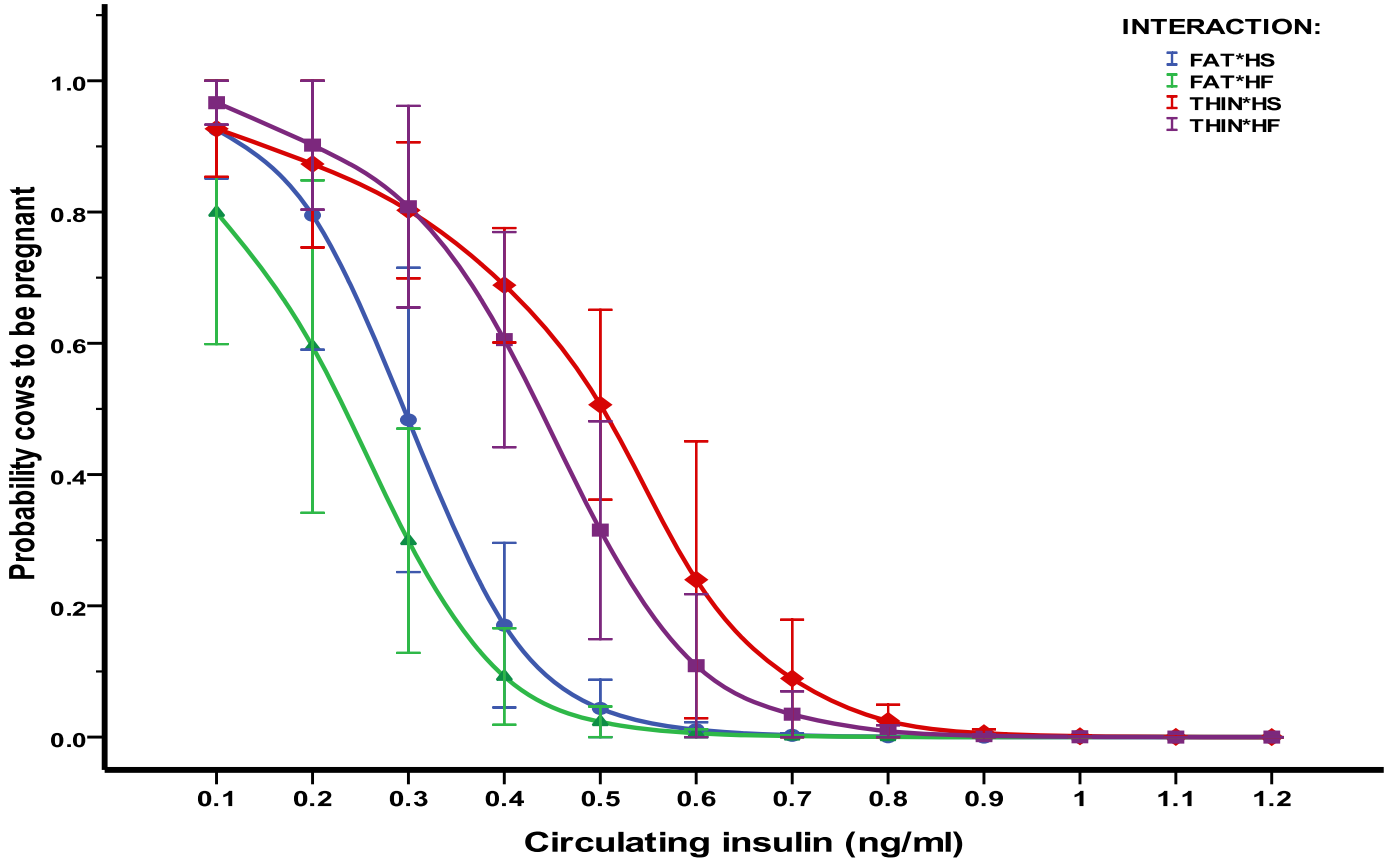


Figure 3.6: Probability of cows to be pregnant adjusted for different condition status at calving (FAT *versus* THIN), diets (HS *versus* HF), and circulating insulin concentrations (n= 30, P=0.76, AUC_{ROC}=0.85±0.08, *LR* (χ^2=16.56, *df*=4, P=0.002), *Hosmer & Lemeshow test for fitness* (χ^2=5.97, *df*=8, P=0.65), multiple logistic regression model with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was pregnancy (0= non pregnant, 1= pregnant) whereas insulin and parity were added as continuous covariates, and BCS at calving, diet, and interaction (BCS at calving*diet) as factors. Red diamonds (◆) are marginal means ± SE for THIN cows fed the HS diet. Blue dots (●) are marginal means ± SE for FAT cows fed the HS diet. Green triangles (▲) represent marginal means ± SE for FAT cows fed the HF diet. Purple rectangles (■) are marginal means ± SE for THIN cows fed the HS diet. There was no effect of (BCS at calving*diet) on probability of cows to be pregnant (P>0.05).

Table 3.17: Effects of insulin, IGF-I, glucose, parity, BCS at calving, and diet on milk progesterone profile

Parameters in the model:	exp (*b*)[†]	95% CI for exp (*b*)		Beta (*b*) coefficient	*P*
		Lower	Upper		
Constant				13.67	0.057
Insulin	30,517	1.30	$\sim+\infty(1.04*10^9)$	10.32	0.044
IGF-I	0.97	0.937	1.004	-0.03	0.087
Glucose	0.007	0.0001	0.311	-5.02	0.011
Parity	8.59	1.49	49.44	2.15	0.016
BCS at calving [∂]	0.014	0.0007	0.284	-4.23	0.005
Diet [✧]	0.079	0.004	1.778	-2.53	0.110

Likelihood ratio test **(χ^2 =13.79, *df*=6, *P*=0.032);** ***Hosmer & Lemeshow test for fitness*** **(χ^2=3, *df*=8, *P* =0.934); AUC_{ROC}=0.91±0.05**

[†] **exp (*b*) is the odds ratio**

[∂] **BCS at calving is a variable with two levels; 0= FAT, 1= THIN**

[✧] **Diet is a variable with two levels; 0= HS, 1= HF**

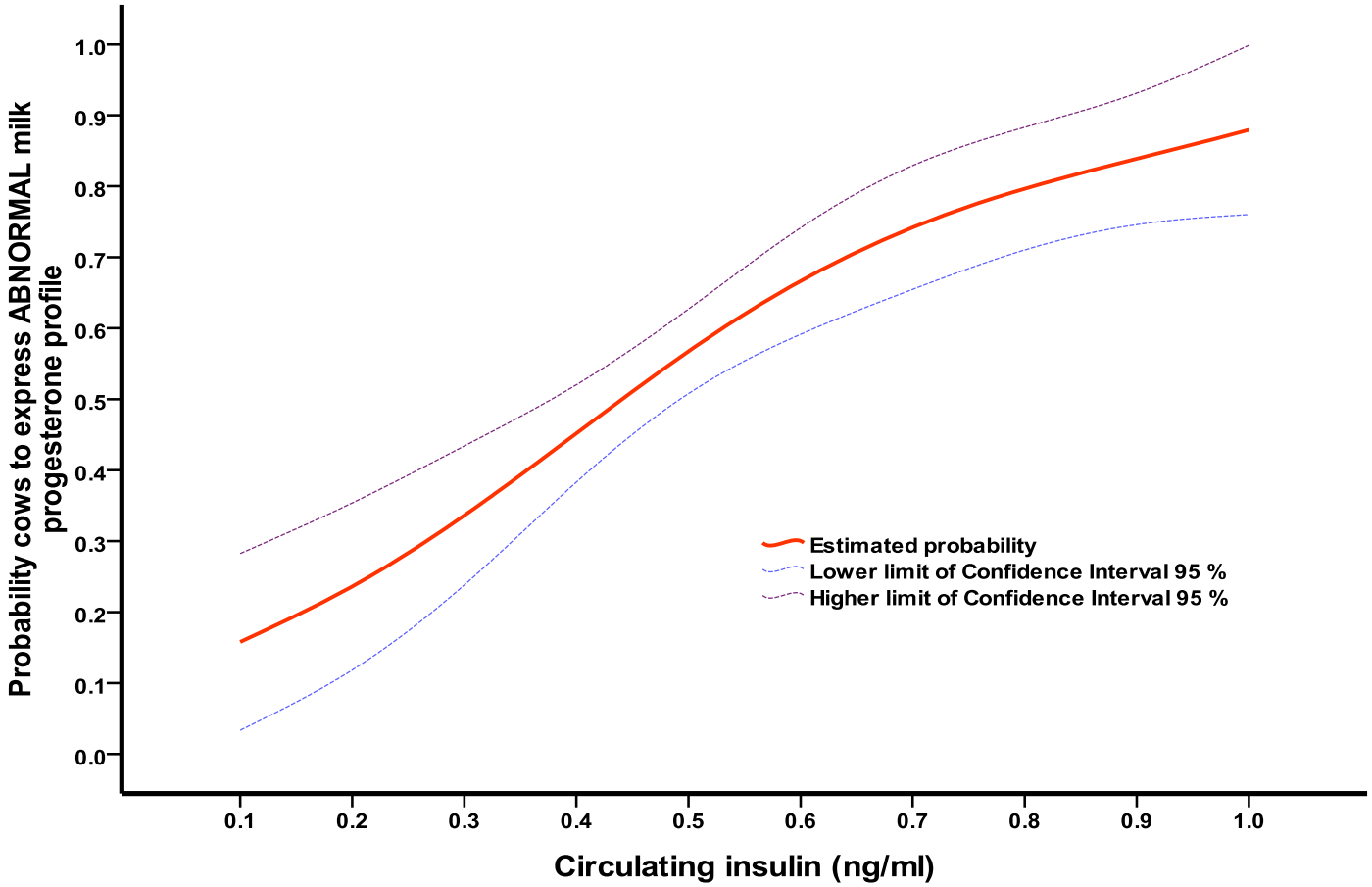


Figure 3.7: Effect of circulating insulin on probability for cows to express abnormal milk progesterone profile (n=30, *P*=0.044, AUC_{ROC}=0.91±0.05, *LR* (χ^2 =13.79, *df*=6, *P*=0.032), *Hosmer & Lemeshow test for fitness* (χ^2=3, *df*=8, *P* =0.934), multiple logistic regression model with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was milk progesterone profile (0= normal, 1= abnormal) whereas insulin, IGF-I, glucose, and parity were added as covariates, and BCS at calving and diet as factors. Red solid line represents the estimated probability whereas blue and purple dashed lines show the upper and lower 95 % Confidence Interval (CI). Optimum insulin concentration that minimized the probability for cows to express abnormal milk progesterone profile ranged from 0.1 to 0.3 *ng/ml*, whereas insulin greater than 0.5 to 0.6 *ng/ml* tended to maximize the probability for cows to express abnormal milk progesterone profile. Model parameter estimates are presented in Table 3.17.

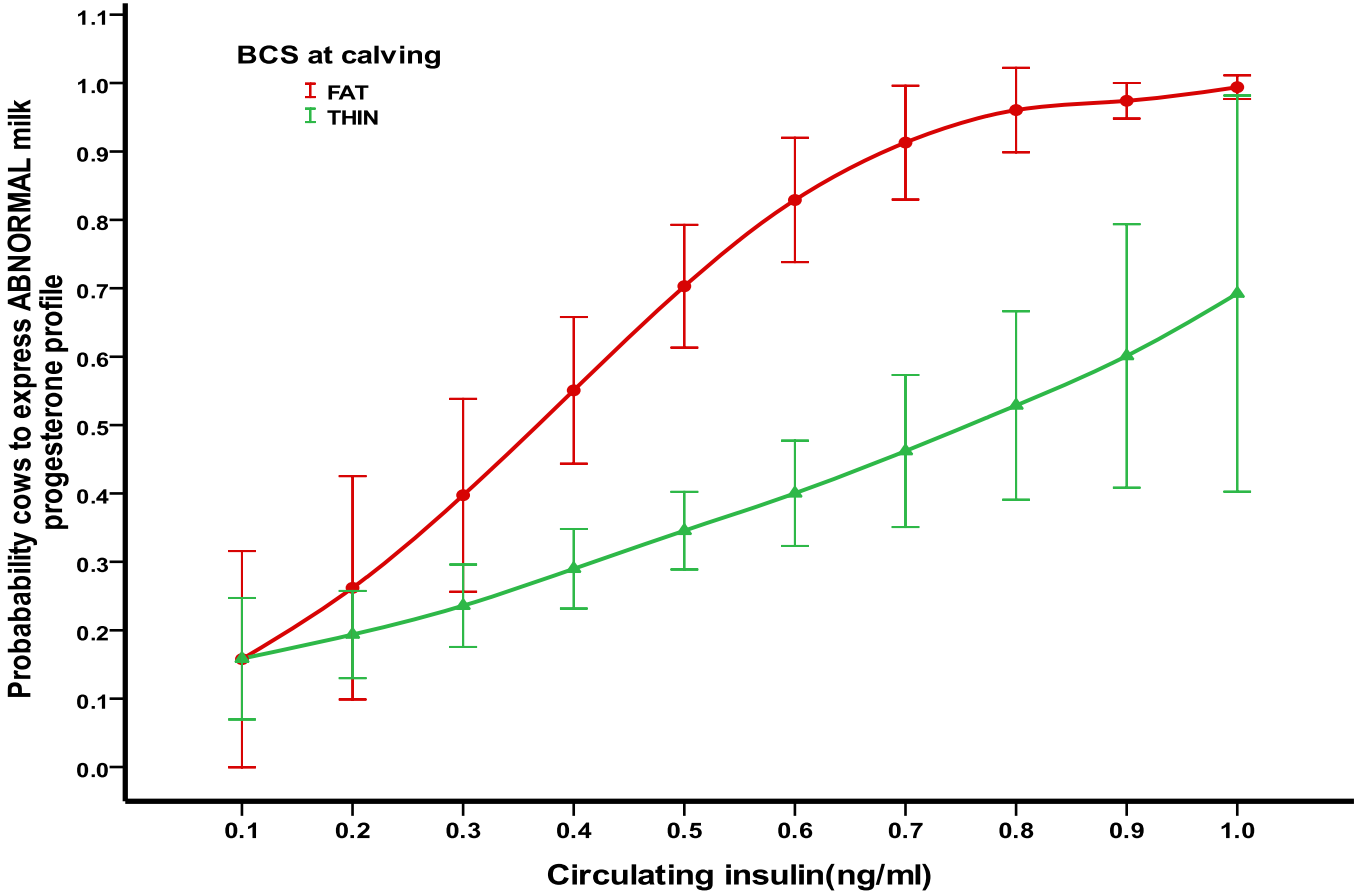


Figure 3.8: Probability for cows to express abnormal milk progesterone profile adjusted for different condition status at calving (FAT *versus* THIN) and circulating insulin concentrations (n=30, P=0.014, AUC_{ROC}=0.91±0.05, *LR* (χ^2 =13.79, *df*=6, P=0.032), *Hosmer & Lemeshow test for fitness* (χ^2=3, *df*=8, P =0.934), multiple logistic regression model with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was milk progesterone profile (0= normal, 1= abnormal) whereas insulin, IGF-I, glucose, and parity were added as covariates, and BCS at calving and diet as factors. Green triangles (▲) represent marginal means ± SE for THIN cows at calving, whereas red dots (●) are marginal means ± SE for FAT cows at calving. THIN cows at calving had lower probability to express abnormal milk progesterone profile than FAT cows at calving (P<0.05), when the animals had approximately the same insulin concentration and insulin concentration ranged from 0.4 to 0.9 *ng/ml*. Model parameter estimates are presented in Table 3.17.

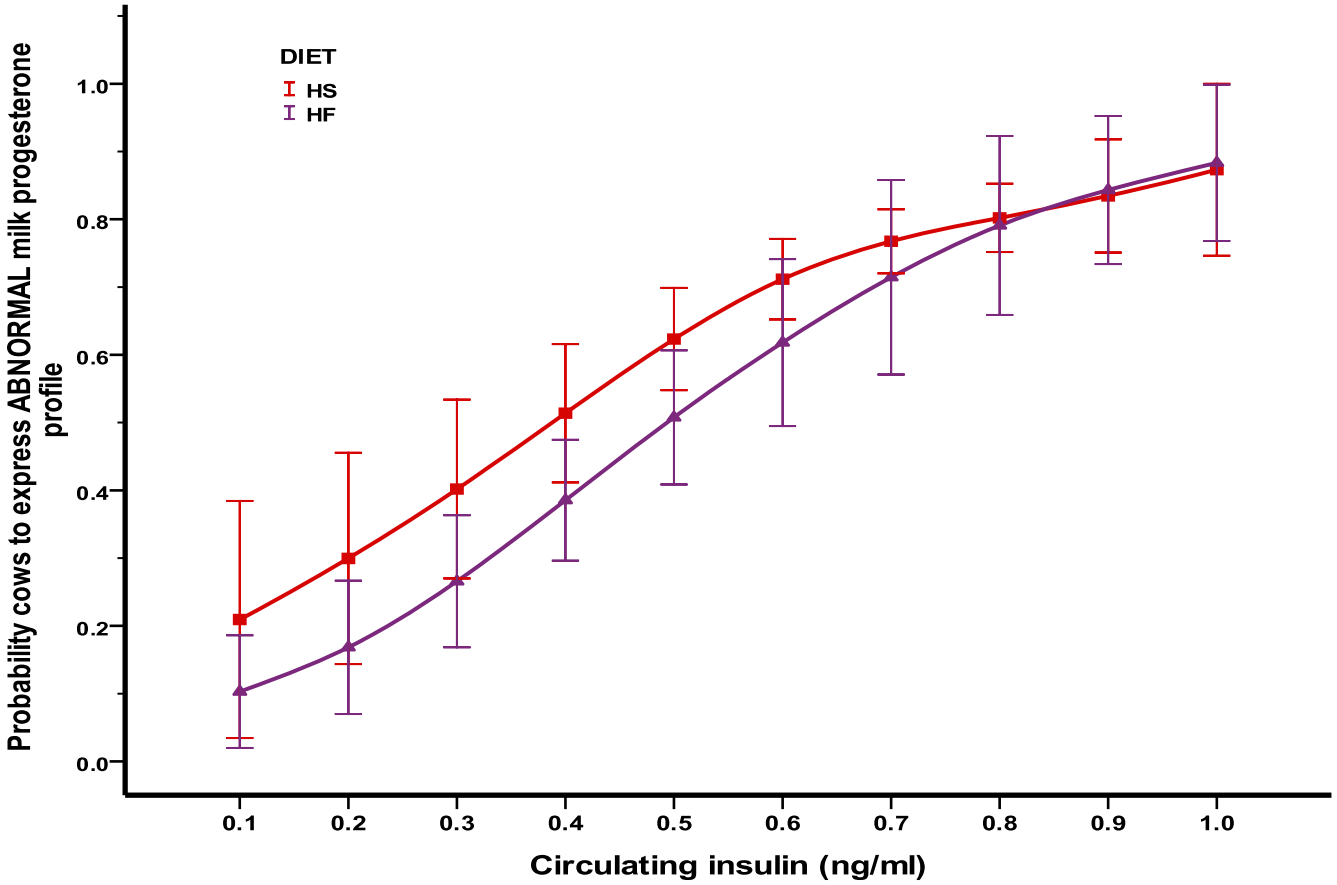


Figure 3.9: Probability for cows to express abnormal milk progesterone profile adjusted for different dietary treatments (HS *versus* HF) and circulating insulin concentrations (n=30, AUC_{ROC}=0.91±0.05, *LR* (χ^2 =13.79, *df*=6, *P*=0.032), *Hosmer & Lemeshow test for fitness* (χ^2=3, *df*=8, *P* =0.934), multiple logistic regression model with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was milk progesterone profile (0= normal, 1= abnormal) whereas insulin, IGF-I, glucose, and parity were added as covariates, and BCS at calving and diet as factors. Purple triangles (▲) represent marginal means ± SE for cows fed the HF diet, whereas red rectangles (■) are marginal means ± SE for cows fed the HS diet. There is no effect of diet on probability for cows to express abnormal milk progesterone profile (*P*>0.05), when the animals had approximately the same insulin concentration and insulin concentration ranged from 0.1 to 1.2 *ng/ml*. Model parameter estimates are presented in Table 3.17.

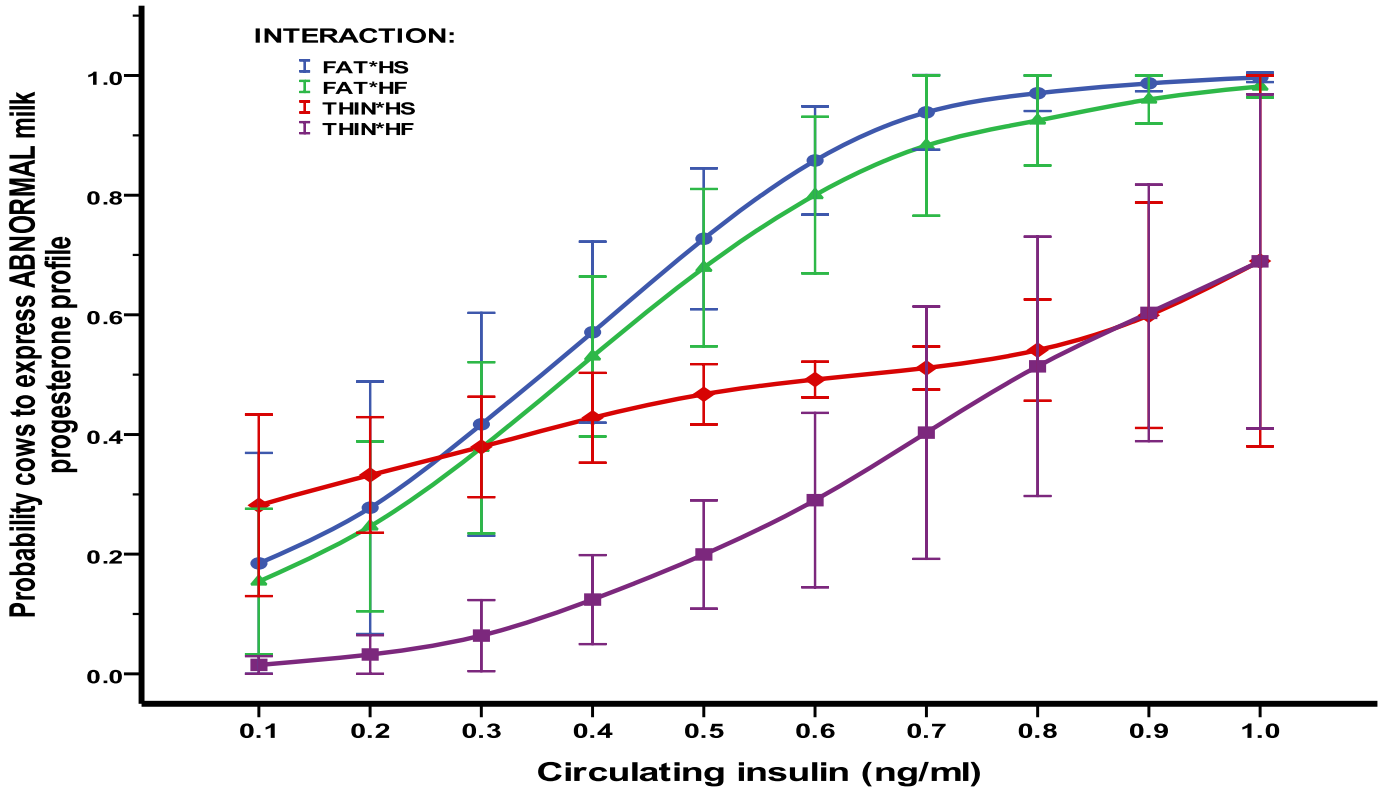


Figure 3.10: Probability for cows to express abnormal milk progesterone profile adjusted for different condition status at calving (FAT *versus* THIN), diets (HS *versus* HF), and circulating insulin concentrations (n=30, AUC_{ROC}=0.91±0.05, *LR* (χ^2 =13.79, *df*=6, *P*=0.032), *Hosmer & Lemeshow test for fitness* (χ^2=3, *df*=8, *P* =0.934), multiple logistic regression model with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was milk progesterone profile (0= normal, 1= abnormal) whereas insulin, IGF-I, glucose, and parity were added as covariates, and BCS at calving, diet, and interaction (BCS at calving*diet) as factors. Red diamonds (◆) are marginal means ± SE for THIN cows fed the HS diet. Blue dots (●) are marginal means ± SE for FAT cows fed the HS diet. Green triangles (▲) represent marginal means ± SE for FAT cows fed the HF diet. Purple rectangles (■) are marginal means ± SE for THIN cows fed the HS diet. THIN cows at calving that fed the HF diet had lower probability to express abnormal milk progesterone profile than THIN cows at calving that fed the HS diet (*P*<0.05), when the animals had approximately the same insulin concentration and insulin concentration ranged from 0.1 to 0.6 *ng/ml*. Moreover, THIN cows at calving that fed the HF diet had lower probability to express abnormal milk progesterone profile than THIN cows at calving that fed the HS diet (*P*<0.05), when insulin concentration ranged from 0.1 to 0.3 *ng/ml*.

3.5. DISCUSSION

3.5.1. Effect of BCS at calving on circulating metabolic hormones, metabolites, and reproductive traits in dairy cows

The present study utilized data generated by the study of Garnsworthy *et al.* (2009). Because the animals retrospectively allocated to BCS at calving groups, analysis performed to assess the consistency of BCS at calving treatments. This analysis clearly demonstrated that the retrospective classification of cows as FAT and THIN was not biased by the original dietary treatments (Table 3.5).

The present study showed that there was no effect of BCS at calving on days to first oestrus. In agreement with this result, Pedron *et al.* (1993), Ruegg *et al.* (1992b), and Ruegg & Milton (1995) did not find any influence of BCS at calving on days to first oestrus in dairy cows. Grainger *et al.* (1982) found that increasing BCS at calving resulted in fewer days to first oestrus in dairy cows. Also, Garnsworthy & Topps (1982) demonstrated that dairy cows calving at a medium BCS (2.5-3.0; 1-5 units) had significantly fewer days to first oestrus than cows with high or low BCS at calving. These latter results are not in line with the present study.

The present study showed that FAT cows at calving had shorter interval from calving to conception than THIN cows at calving. Primiparous fat dairy cows (BCS at calving≥3.0; 1-5 units) had a shorter interval from calving to conception than primiparous lean dairy cows (BCS at calving<3.0; 1-5 units) in the study of Meikle *et al.* (2004), which is in line with the current study. In contrast, other studies have failed to find any relationship between BCS at calving and calving interval (Garnsworthy & Jones, 1988; Ruegg & Milton, 1995; Pedron *et al.,* 1993).

The present study found that FAT cows at calving had lower probability to be pregnant within 120 days postpartum than THIN cows at calving. According to Buckley *et al.*

(2003) cows with precalving BCS>3.0 (1-5 units) had lower probability of pregnancy after 42 day of breeding, which is in agreement with the present study.

According to Lamming & Darwash (1998), cows with abnormal patterns in milk progesterone profile showed a significant deterioration in postpartum reproductive performance. Also, dairy cows with abnormal oestrous cycles had lower pregnancy rate compared to cows with normal oestrous cycles in the study of Garnsworthy *et al.* (2009) and postpartum BCS loss of Holstein cows was risk factor for a prolonged luteal phase in the study of Ledoux *et al.* (2011). The present study found that cows with normal milk progesterone profile had higher probability to be pregnant than cows with abnormal milk progesterone profile, and FAT cows had higher probability to express abnormal milk progesterone profile than THIN cows. Moreover, FAT cows had lower IGF-I than THIN cows and IGF-I had a negative impact (tendency) on the odds for cows to express abnormal milk progesterone profile. This finding may explain why FAT cows had higher probability to express abnormal milk progesterone profile than THIN cows. Finally, these results taken together may explain why in the present study FAT cows were found to have lower probability to be pregnant within 120 days postpartum than THIN cows.

In the present study, components of postpartum reproductive performance (probability for cows to be pregnant or express abnormal milk progesterone profile, and interval from calving to conception) were influenced by BCS at calving. Only days to first oestrus was unaffected by BCS at calving. This suggests that BCS at calving is a determinant factor for postpartum reproductive performance of dairy cows. Looking for the underlying explanation for the influence of BCS at calving on reproduction, the present study examined the effect of BCS at calving on metabolic hormones and metabolites, and productive traits. Of the metabolic hormones measured, only IGF-I was affected by BCS at calving. In agreement with this result, Meikle *et al.* (2004) and Lake *et al.* (2006) found that circulating insulin was unaffected by BCS at calving. Meikle *et al.* (2004) reported that circulating leptin was statistically higher in fat cows (BCS at calving ≥3; 1-5 units) than in thin cows (BCS at calving <3; 1-5 units), which is not in agreement with the present study. A likely reason for these discrepancies between studies is that cows in the

present study were over-conditioned at calving compared with the cows in the studies of Lake *et al.* (2006) and Meikle *et al.* (2004). Another explanation could be variation in breeds, diets, and production systems. It is known that circulating insulin and glucagon are modulated mostly by dietary starch intake and its interaction with blood sugar (Brockman 1978, 1979; Marieb & Hoehn, 2007; Aronoff *et al.* 2004), and that is possibly the reason for no effect of animal condition in this experiment. Circulating GH and leptin may be associated with BCS at parturition (Garnsworthy *et al.,* 2008a), but this is only when differences of BCS at calving are great (Ryan *et al.,* 1994). In the present study condition differences at calving were minimal, which could account for the lack of influence of condition at parturition on circulating GH and leptin. According to Hill (2004) the correlation between leptin and body condition appears to weaken postpartum when many other physiological influences are in action, and this is possibly an extra reason for no effect of BCS at parturition on circulating leptin.

FAT cows at calving had lower circulating IGF-I than THIN cows at calving throughout the experimental period, which could also explain why THIN cows showed superior reproductive performance compared with FAT cows. Lake *et al.* (2006) found that serum IGF-I was greater in fat cows (BCS at calving: 6.0; 1-10 units) than in thin cows (BCS at calving: 4.0; 1-10 units) and Meikle *et al.* (2004) noted that lean dairy cows at calving (BCS<3; 1-5 units) had lower circulating IGF-I than fat cows (BCS≥3; 1-5 units). These two results appear to contradict the finding of the present study. However, the majority of cows in the present study were over-conditioned at calving (BCS=3.38±0.2); and THIN cows at calving (BCS=3.00±0.08) were actually fat to moderate fat whereas FAT cows at calving (BCS=3.59±0.03) were over-fat. Therefore, the results of Lake *et al.* (2006) and Meikle *et al.* (2004) are, at least in part, in agreement with the result of the present study.

Of the metabolites examined in this experiment, only circulating NEFA was affected by BCS at calving. Busato *et al.* (2002) found that circulating NEFA was higher in fat cows (BCS one week ante partum ≥3.25; 1-5 units) that lost greater than 0.75 units of BCS throughout the experimental period. Furthermore, the same study showed that circulating glucose and insulin were not influenced by BCS one week ante partum combined with

different levels of BCS loss. In the study of Lake *et al.* (2006), circulating glucose and BOHB was not affected by BCS at calving, which is in agreement with the current study; however, they observed a trend for circulating NEFA to be higher in fat cows compared to thin cows, in contrast to the current results. Also, Meikle *et al.* (2004) in agreement with the current results did not find any effect of BCS at calving on circulating BOHB but there was a trend for circulating NEFA to be higher in fat cows. Pedron *et al.* (1993) noted that circulating glucose was unchanged among classes of cows with different BCS at calving, but circulating FFA tended to be higher only in over-conditioned cows at parturition (BCS=4; 1-5 units).

The present study showed that there was no effect of BCS at calving on DMI and LWT, but milk yield, BCS, ΔBCS, nadir BCS, and nadir week were higher in FAT than THIN cows at calving. In line with these results, Meikle *et al.* (2004) found that lean cows at calving (BCS<3.0) had lower mean BCS during the experimental period, and fat cows at calving (BCS ≥3.0) tended to lose more BCS. Also, Lake *et al.* (2005) demonstrated that thin beef cows at parturition maintained condition over the course of the study, whereas fat beef cows lost condition, but without any effect of BCS at parturition on milk yield. In agreement with the results of the present study Ruegg & Milton (1995) showed that duration and magnitude of condition loss depended primarily on BCS at calving and was greater for cows that calved with higher BCS. Pedron *et al.* (1993) found that class of BCS at parturition (BCS at calving; 3.0, 3.5, and 4.0; 1-5 units) did not influence milk yield, but Waltner *et al.* (1993) showed exactly the opposite. According to Garnsworthy & Jones (1987) quality of diet postcalving may influence the association between BCS at calving and milk production, and it is likely to be a reason for the variation in the results reported from different studies. Many studies demonstrated that higher postpartum BCS loss (ΔBCS) accompanied higher milk production (Ruegg & Milton, 1995; Domecq *et al.,* 1997), in agreement with results of the current study.

In the present study, FAT cows at calving showed higher nadir BCS, longer nadir week, and smaller BCS change. Roche *et al.* (2007c, 2009) proposed that the greater the calving and nadir BCS, and the smaller the BCS loss between calving and nadir, the shorter the

postpartum anoestrous interval. According to Houghton *et al.* (1990) fatter beef cows at calving exhibited a shorter postpartum anoestrous interval. In line with these results FAT cows at calving in the present study had shorter interval from calving to conception than THIN cows at calving.

It is known that postpartum DMI decreases with increasing BCS at calving (Broster & Broster, 1998; Garnsworthy, 2006). In the current study, however, DMI was unaffected by BCS at calving. It could be that the differences in BCS at calving between THIN and FAT cows were not large enough and the majority of the cows were over-conditioned at calving (mean BCS at calving=3.38±0.20). Thus, the difference in DMI between FAT and THIN cows at calving was smaller.

The results of the present study clearly indicate that THIN cows at calving (BCS≤3.25) had better reproductive performance than FAT cows at calving (BCS>3.25). However, BCS at calving had no effect on days to first oestrus and FAT cows at calving showed shorter interval from calving to conception than THIN cows at calving. Although, reproductive hormones were not measured by the present study, metabolic hormones, metabolites, and productive traits convincingly explain why THIN cows at calving expressed better reproductive performance than FAT cows at calving. Circulating NEFA was higher in FAT cows at calving. NEFA are used as an alternative energy source by the liver to preserve glucose (Hayirli, 2006), and the majority of circulating glucose is used preferentially by the mammary gland to form lactose (Bauman & Currie, 1980). In the present study, FAT cows at calving lost more condition to support milk production and without increasing DMI. The depressed DMI in FAT cows at calving could be the direct effect of elevated circulating NEFA on hypothalamic neural centres controlling feeding behaviour (Ingvartsen & Andersen, 2000; Krasnow & Steiner, 2006). Also, elevated circulating NEFA may detrimentally affect follicular growth and development by acting directly on follicle cells (Leroy *et al.,* 2008, 2010), which may explain the inferior reproductive performance of FAT cows at calving. Circulating IGF-I was higher in THIN cows at calving. The IGF-system plays an important role in follicle growth and development by acting directly on ovarian cells (Webb *et al.,* 2004). Moreover, low circulating IGF-I

negatively influences postpartum reproductive performance (Butler *et al.,* 2000; Huszenicza *et al.,* 2001; Meikle *et al.,* 2004). To conclude, the present study demonstrated that THIN cows at calving (BCS≤3.25), which lost less than 0.5 units of BCS (ΔBCS) during the first 4 months of lactation, had superior reproductive performance, and that was because of increased circulating IGF-I and decreased circulating NEFA. However, further work is required to determine the effects of BCS at calving on fertility.

3.5.2 Effect of diet on circulating metabolic hormones, metabolites, and reproductive traits in dairy cows

A secondary objective of the present study was to determine whether feeding a HS diet (designed to induce increasing circulating insulin) or a HF (designed to reduce circulating insulin) for the first 4 months postpartum could result in changing reproductive performance of dairy cows. The present study showed that cows fed HS diet had higher probability to express abnormal milk progesterone profile than cows fed HF diet, but there was no effect of diet on the probability of cows to be pregnant, days to first oestrus, and interval from calving to conception. Additionally, high circulating insulin was associated with higher odds for cows to express abnormal milk progesterone profile, and that may explain why cows fed the HS diet had higher probability to express abnormal milk progesterone profile than cows fed the HF diet. Moreover, cows fed the HS diet had insulin concentrations tended to be 0.5 *ng/ml*, whereas cows fed the HF diet had insulin concentrations tended to be 0.4 *ng/ml* in the present study (Table 3.11). This difference in insulin concentrations between animals fed the HS and HF diet may explain why cows fed the HS diet had higher probability to express abnormal milk progesterone profile than cows fed the HF diet (Table 3.8 and Figure 3.7).

Cows fed the HS diet had higher circulating insulin than cows fed the HF diet, but there was no effect of diet on circulating GH, leptin, and IGF-I. This finding is in agreement with the results of Gong *et al.* (2002b) and Garnsworthy *et al.* (2008b). According to Garnsworthy *et al.* (2008a) and Lucy (2000), circulating GH and IGF-I are associated

more to productive traits (such as milk yield, live weight and energy balance) than to diet composition, whereas leptin is mainly linked with BCS (Hill, 2004), and it is conflicting if it can be affected by diet (Chilliard *et al.,* 2005). Also, glucose and insulin seem to play a key role in regulating leptin expression in ruminants (Chilliard *et al.,* 2005). Milk yield, BCS, ΔBCS, LWT, and circulating glucose were not affected by diet in this experiment and maybe that is why circulating GH, leptin, and IGF-I were not influenced by diet. Failure of diet to influence circulating metabolic hormones (except insulin), may explain why reproductive performance was the same for the two dietary treatments, as it is known that circulating GH, leptin, and IGF-I directly affect reproductive performance (Garnsworthy *et al.,* 2008a).

The study of Gong *et al.* (2002b) used similar diets to the current study, but for a period of 50 days postpartum. Their study clearly demonstrated a beneficial effect of cows fed HS diet on early reproductive indices (*i.e.* increased proportion of cows ovulating within 50 days postpartum, decreased intervals from calving to first ovulation, and decreased intervals from calving to first service and to conception). However, in the study of Gong *et al.* (2002b) the subsequent fertility parameters (*i.e.* conception rate to first service and number of services required per conception) were not affected by diet and that finding is at least in part in agreement with the current study. Moreover, this finding may suggest that the beneficial effect of HS diet on cow fertility is constrained to the first 50 days postpartum. In general, the results of the present study concerning early reproductive indices are not in line with those of the study of Gong *et al.* (2002b). Cows fed the HS diet did not show shorter days to first oestrus or intervals from calving to conception and they had higher probability to express abnormal milk progesterone profile than cows fed HF diet. This latter result combined with the finding that cows with normal milk progesterone profile had higher probability to be pregnant than cows with abnormal milk progesterone profile may indicate that cows fed the HS diet are likely to have lower probability to be pregnant than cows fed the HF diet. However, this latter conclusion was not confirmed by the present study as diet had no effect on the probability cows to be pregnant. An explanation for the discrepancies between the two studies could be that in the present study

the animals were dietary manipulated for longer (up to 2 times) than the animals of the study of Gong *et al.* (2002b).

In the study of Gong *et al.* (2002b) circulating insulin was 0.32 ± 0.015 *ng/ml* and 0.21 ± 0.011 *ng/ml* for the high yielding cows fed HS and HF diets, respectively. In the present study, mean insulin was 0.44±0.02 *ng/ml* and animals fed the HS diet had higher mean circulating insulin (0.49±0.03 *ng/ml*) than cows fed the HF diet 0.41±0.03 *ng/ml*. According to Kaneko *et al.* (2008b), reference range of circulating insulin for cows varies from zero (undetectable) to 0.23 *ng/ml*. Moreover, basal circulating insulin varied from 0.14±0.02 *ng/ml* to 0.15±0.03 *ng/ml*, independently from diet and genetic merit for milk yield in the study of Chagas *et al.* (2009). That means circulating insulin was approximately two times higher than the reference range in the current study (or about three times higher than the mean insulin in the study of Chagas *et al.* (2009)), independently from the dietary treatments. Thus, the animals in the present study exceeded an optimum insulin concentration which was not surpassed by the animals in the study of Gong *et al.* (2002b), and this is another explanation for the discrepancies between the two studies.

Butler (2000) reported that increased circulating urea may lead to impaired fertility of cows as high plasma urea concentrations interfere with the normal inductive actions of progesterone on the microenvironment of the uterus, and therefore cause suboptimal conditions for supporting embryo development. Sinclair *et al.* (2000) suggested that exposure of oocytes in antral follicles to high levels of ammonia, prevents cleavage and blastocyst formation. In the current study, cows fed the HS diet had higher circulating urea than cows fed the HF diet and this may explain the higher probability to express abnormal milk progesterone profile observed in cows fed the HS diet.

BOHB was influenced by diet in the present study, and cows fed the HS diet had lower circulating BOHB than cows fed the HF diet. Increased BOHB is associated with NEB (Bell, 1995; Grummer, 1995), but there was no effect of diet on other metabolites or productive traits (milk yield, BCS, ΔBCS, and nadir BCS) to indicate higher body fat

mobilization of cows fed the HF diet in this experiment. The reason why circulating BOHB was elevated in this study is not known. BOHB has no direct effect in reproductive performance (Leroy *et al.,* 2008), and its higher concentration in cows fed HF diet cannot explain why these cows had lower probability to express ABNORMAL milk progesterone profile.

To conclude, the present study demonstrated that feeding dairy cows with a HS diet or a HF diet for the first 4 months of lactation would not result in changing reproductive performance (probability of cows to be pregnant, interval from calving to conception, and days to first oestrus were not affected by dietary treatment). This was mainly because important metabolic hormones (*i.e.* leptin, IGF-I, and GH), productive traits (milk yield, BCS, ΔBCS, nadir week, and nadir BCS), and metabolites (*i.e.* NEFA and glucose) were not influenced by diet. In addition, long-term moderately elevated insulin concentration induced by feeding dairy cows the HS diet might account for the higher probability to express abnormal milk progesterone profile. However, further work is required to determine the effects of HS and HF diets on fertility, and especially the association of chronic moderately elevated insulin concentration with reproductive performance.

3.5.3 Reproductive performance and optimum insulin concentration

The present study showed that pregnancy rate was negatively affected by insulin (Figure 3.3) but this effect was dependent on BCS at calving (Figure 3.4). Also, cows with high insulin had higher probabilities to express abnormal milk progesterone profile (Figure 3.7) and this effect was dependent on BCS at calving (Figure 3.8). These two results taken together may imply that there was an optimum insulin concentration necessary for normal reproductive performance in lactating dairy cows which was dependent on BCS at calving. According to Garnsworthy *et al.* (2009) there is a minimum insulin concentration (from 0.21 to 0.32 *ng/ml*) that is necessary for commencement of normal ovarian activity in early lactating cows. In accordance with this observation, the present study presented that; (1) optimum insulin concentration that maximized the probability of cows to be pregnant

varied from 0.2 to 0.3 *ng/ml*, whereas insulin greater than 0.6 *ng/ml* tended to zero the probability of cows to be pregnant (Figure 3.3); and (2) optimum insulin concentration that minimized the probability for cows to express abnormal milk progesterone profile varied from 0.1 to 0.3 *ng/ml*, whereas insulin greater than 0.4 to 0.6 *ng/ml* tended to maximize the probability for cows to express abnormal milk progesterone profile (Figure 3.7). Thus, insulin concentration that optimized fertility in lactating cows varied from 0.2 to 0.3 *ng/ml* while insulin concentration greater than 0.6 *ng/ml* impaired reproductive performance in lactating dairy cows. Moreover, the current study found that BCS at calving was a critical modulator of fertility in lactating dairy cows (Figure 3.4 and Figure 3.8), whereas reproductive performance (pregnancy rate and milk progesterone profile) of dairy cows was not affected by dietary treatments (Figure 3.5 and Figure 3.9**)** when insulin concentrations were about the same. Therefore*,* achieving a BCS of less than 3.25 units at calving is essential to ensure optimum cow fertility.

In the present study, mean insulin was 0.44±0.02 *ng/ml* whereas the actual percentage of pregnant cows was 26.7% and the actual percentage of cows expressed irregular oestrous cycles was 46.7%. According to Figure 3.3 and Figure 3.7, the model estimated percentage of pregnant cows and the model estimated percentage of cows expressed irregular oestrous cycles was (25.5±5) % and (49.9±6) %, respectively. Thus, the low percentage of pregnant cows and the high percentage of irregular oestrous cycles in the animals of the present study were due to insulin concentration exceeded the optimum (0.2-0.3 *ng/ml*) in terms of reproduction.

Another finding of the present study was that THIN cows at calving that fed the HF diet had lower probability to express abnormal milk progesterone profile than THIN cows at calving that fed the HS diet, when insulin concentration ranged from 0.1 to 0.6 *ng/ml* (Figure 3.10). Therefore, HF diet must be the preferable feeding strategy to maximaze reproductive performance of dairy cows when insulin concentration ranges from 0.1 to 0.6 *ng/ml* and BCS at calving is equal or lower than 3.25 units. Moreover, because THIN cows at calving had lower probability to express abnormal milk progesterone profile than FAT cows at calving when circulating insulin was relatively high (0.4 to 0.9 *ng/ml*) (Figure 3.8),

body condition management must be aimed at a BCS of less than 3.25 units at calving. Alternatively, if the animals are FAT at calving, the best feeding strategy to ensure optimum fertility is the animals to be fed a diet that induces an optimum circulating insulin concentration (0.2 - 0.3 *ng/ml*). However, this approach is not easy to be achieved in the long term, and consequently obtaining a BCS of less than 3.25 units at calving must be considered the most realistic strategy. Thus, BCS at calving management has greater impact on cow fertility than postpartum dietary treatments such as HS and HF diets. This latter may imply that prepartum feeding strategy and BCS management are essential for optimal reproduction performance in lactating dairy cows.

3.5.4. Conclusions

In conclusion, results of this study support the concept that BCS at calving is a critical modulator of fertility in lactating dairy cows. THIN cows at calving (BCS≤3.25), which lost less than 0.5 units of BCS (ΔBCS) during the first 4 months of lactation, had superior reproductive performance, and that was because of increased circulating IGF-I and decreased circulating NEFA. Moreover, this study clearly shows that there is an optimum insulin concentration (0.2 - 0.3 *ng/ml*) necessary for normal reproductive performance, while insulin concentration greater than 0.6 *ng/ml* impairs reproductive performance in lactating dairy cows. The results strongly suggest that optimal reproductive performance of dairy cow is dependent on insulin concentration and BCS at calving. Cow fertility was not affected by dietary treatments, mainly because optimum insulin concentration was exceeded by the animals of the present study. However, HF diet must be the preferable feeding strategy to maximize reproductive performance of dairy cows when insulin concentration ranges from 0.1 to 0.3 *ng/ml* and BCS at calving is equal or lower than 3.25 units. Finally, moderately elevated insulin concentration that exceeded optimum insulin concentration may explain the low percentage of pregnant cows and the high percentage of cows expressed abnormal oestrous cycles in this experiment. However, these results need to be investigated further.

4. Measurement of plasma and milk adiponectin, and the impact of lactation stage, diet, and body condition at calving on plasma adiponectin concentrations in dairy cows

4.1. INTRODUCTION

Adiponectin, also known as Acrp30, apM1, and AdipoQ, is an adipokine mainly secreted by adipocytes (Berg *et al.,* 2002; Kadowaki & Yamauchi, 2005; Shetty *et al.,* 2009). Adipokines are highly bioactive molecules and may be implicated in regulation of energy expenditure and homeostasis, immunity, and reproduction (Yokota *et al.*, 2000; Mitchell *et al.,* 2005; Antuna-Puente *et al.*, 2008; Guzic *et al.*, 2006; Kamada *et al.*, 2008; Dridi & Taouis, 2009). Although leptin and adiponectin are the main adipokines expressed in adipose tissue (Guzic *et al.*, 2006; Ahima, 2006), adiponectin concentration in plasma is two to three times higher than other hormones (Ahima, 2006). Circulating adiponectin varies in different species (human; 3-30 *μg/ml*, rat; 10-30 *μg/ml*, horses; 1.3-4.8 *μg/ml*, dog; 20-40 *μg/ml*, cats; 1-4 *ng/ml*) (Kamada *et al.*, 2008; Mousavinasab *et al.*, 2005; Combs *et al.*, 2004; Goldstein & Scalia, 2007; Ishioka *et al.*, 2006; Kearns *et al.,* 2006; Radin *et al.,* 2009; Hoenig *et al.*, 2007).

It is known that periparturient cows enter a period of negative energy balance accompanied by decreased insulin sensitivity (Bell, 1995; Veerkamp *et al.,* 2003; Hayirli, 2006), and adiponectin is implicated in murine and human insulin resistance (Kadowaki & Yamauchi, 2005). Lemor *et al.* (2009) reported that AdipoR1 and AdipoR2 mRNA abundance in adipose tissue decreased as high yielding cows moved from pregnancy to lactation. Raddatz *et al.,* (2008) reported that plasma adiponectin concentration varied from 8.3±1.4 *ng/ml* to 16.0±2.7 *ng/ml* in early lactating cows and circulating adiponectin was

significantly increased from week 1 to week 4 post partum, and declined to remain at 12-13 *ng/ml* until week 11 postpartum. Komatsu *et al.* (2007) showed that adiponectin mRNA expression in adipose tissue was higher in non-lactating cows than peak-lactation cows, but it was similar in early-lactation and late-lactation cows. The pattern of change of circulating adiponectin throughout the lactation cycle of dairy cows has not yet been determined.

Contrary to what happens for most adipokines, circulating values of adiponectin are higher in lean than in obese individuals (Berg *et al.,* 2002; Matsuzawa *et al.*, 2004; Kadowaki & Yamauchi, 2005; Kadowaki *et al.*, 2006; Shetty *et al.,* 2009; Goldstain & Scalia, 2007). Kearns *et al.* (2006) reported that adiponectin is inversely proportional to adiposity in horses. According to Ishioka *et al.* (2006) obese dogs showed low circulating adiponectin. Raddatz *et al.* (2008) proposed that circulating adiponectin is not correlated with BCS in early lactating cows. Circulating adiponectin was negatively correlated with body weight, body mass index, and insulin in humans (Berg *et al ,* 2002; Matsuzawa *et al.*, 2004; Guzic *et al.*, 2006; Ahima, 2006), but the association of circulating adiponectin with body weight, BCS at calving, and metabolic profile in cattle or other ruminants have not yet been investigated.

Dietary factors may modulate circulating adiponectin (Reis *et al.,* 2010). High consumption of magnesium (Qi *et al.,* 2005; Cassidy *et al.,* 2009), caffeine (Williams *et al.,* 2008), n-3 PUFA (Duda *et al.,* 2007), and dietary salt (Lely *et al.,* 2007) are associated in humans with higher circulating adiponectin. According to Barnea *et al.* (2006) mice fed a High Fat diet exhibited elevated insulin resistance, although circulating adiponectin remained unchanged compared to controls. Jones *et al.* (2009) demonstrated that a high-fat diet increased maternal adiposity and circulating maternal leptin, but murine serum adiponectin was decreased from a period around the mating and throughout gestation. Pischon *et al.* (2005) showed that circulating adiponectin was negatively related to glycaemic load in men. It is known that ruminants regulate glucose homeostasis and lipid metabolism in different ways than other monogastric animals (Allen *et al.,* 2005; Nafikov & Beitz, 2007). For example, many ruminant tissues preferentially utilize acetate rather

than glucose, and VFA are the main products of organic matter fermentation (Lewis & Hill 1983; Nafikov & Beitz, 2007). The effect of dietary components on circulating adiponectin has not yet been demonstrated for any ruminant.

Adiponectin is assessed in human milk (Weyermann *et al.,* 2006; Martin *et al.*, 2006; Bronsky *et al.*, 2006), and bovine mammary epithelial cells are expressed AdipoR1 (Ohtani *et al.*, 2011). However, the presence of adiponectin in bovine milk has not yet been reported.

In this project, circulating and milk adiponectin were measured by using a commercially available human adiponectin kit (HADP-61 HK, Linco Research, Millipore, St. Charles, MO, USA). The presence of adiponectin in bovine milk was confirmed for first time. The main objective of the study was to test the hypothesis that as physiological state of cow dramatically changes by moving from pregnancy to calving and lactation, major physiological changes occur, which may be partially reflected in circulating adiponectin. Additionally, the influences of BCS at calving and diet on circulating adiponectin, and the relationships of circulating adiponectin with other metabolic hormones, metabolites, and productive traits were investigated. Kubota *et al.,* (2007) showed that adiponectin stimulate food intake in mouse. A second hypothesis was that circulating adiponectin would be associated with DMI in lactating dairy cows. Evidence may indicate that the relationship between GH and adiponectin is negative in human and rat (Lam *et al.,* 2004; Nilsson *et al.,* 2005; Rodriguez-Pacheco *et al.,* 2007). It is known that genetic selection for milk yield led to higher circulating GH in dairy cows (Veerkamp *et al.*, 2003). A third hypothesis was that high yielding cows would have lower circulating adiponectin than low yielding cows due to increased circulating GH and its antagonistic relationship with adiponectin.

4.2. MATERIALS AND METHODS

4.2.1. Measurement of adiponectin in bovine milk (Experiment 1)

Milk samples were collected from 6 cows fed on the same diet, but at different stages of lactation (from 5 to 50 days post partum). Cows were milked through a robotic milking system (AMS; Merlin, Fullwood Ltd.). All milk samples were collected between 15:00 and 17:00 in the afternoon and left in the fridge (0 to 4 °C) overnight. Because lipids interfere with radioimmunoassays (RIA), skim milk was used (Martin *et al.,* 2006). Milk samples (4ml) were mixed by vortex and skim milk (aqueous phase) was prepared by centrifugation (1500 ×*g*, 20 min, 4 °C) and removal of the fat layer. Adiponectin was determined in duplicate by using a commercial RIA kit (Linco Research, St Charles, MO, USA), as described in the protocol (A.4 in appendix).

4.2.2. Measurement of circulating adiponectin (Experiment 2)

4.2.2.1. Data

This study utilized the dataset generated by the study of Garnsworthy *et al.* (2009). In the current study, circulating adiponectin values were measured and added. Key points of materials and methods, further statistical analysis, and handling of the data are presented hereinafter.

4.2.2.2. Experimental design

Sixty high-yielding multiparous Holstein dairy cows were blocked according to calving date and parity, and were allocated at random to two dietary treatment groups (HS and HF, 30 cows in each). Two diets were formulated to have equal concentrations of DM, ME and CP, but to differ in starch, fat and NDF concentrations (Table A.1 in appendix). Diet HS

had higher starch content and was expected to induce relatively high plasma insulin concentrations; Diet HF had a higher fat content and was expected to induce relatively low plasma insulin concentrations. These diets were equivalent to the high and low insulin diets used by Gong *et al.* (2002b), Fouladi-Nashta *et al.* (2005), and Garnsworthy *et al.* (2009).

Within dietary treatment groups, cows were allocated on the basis of BCS at calving into FAT (BCS >3.25 units) and THIN (BCS ≤3.25 units) cows. The FAT group comprised 40 cows (19 HS; 21 HF); the THIN group comprised 20 cows (11 HS; 9 HF). Prepartum, all cows were fed on the same diet.

4.2.2.3. Feeding and milking

These are described in § 3.2.3.

4.2.2.4. Recording, sampling and analysis

Milk yield and feed intake were recorded daily throughout the experiment. Previous milk yield (PMY) was obtained by dairy records while aggregated milk yield (AMY) was calculated by summarizing the milk yield for each cow at the end of the experiment. Live weight and BCS (units; 1 to 5) were recorded weekly. Milk samples were taken twice in a week (for the weeks; +2, +4, +6, +8, +12, + 15 postpartum) and analyzed for fat, protein and lactose contents by infrared analysis at the National Milk Records Laboratory, Harrogate, UK, using AOAC reference method No. 972.16 (AOAC, 1990). Metabolizable energy balance was calculated for each cow from LWT, milk energy output, and metabolizable energy intake, using UPWin software (AGM Systems, Exeter, UK) and Feed Into Milk equations (Thomas *et al.,* 2004).

Blood samples were taken every Wednesday at 09:30 h from weeks -2 prepartum and +2, +4, +6, +8, +12, + 15 postpartum for measurement of hormones and metabolites. Blood

samples were analyzed for the following hormones: insulin, GH, glucagon, adiponectin, and leptin, as detailed in Garnsworthy *et al.* (2009). Circulating and milk adiponectin were measured by using a commercially available human adiponectin kit (HADP-61 HK, Linco Research, Millipore, St. Charles, MO, USA) as described in the protocol (A.3 in appendix). The sensitivity of the method (expressed as ED_{80}) was 2.33 ng/ml. Blood samples were analyzed for the following metabolites on a Bayer opera autoanalyzer (Bayer UK Ltd): urea-N, glucose, BOHB and NEFA, as detailed in Garnsworthy *et al.* (2009). All instances of ill health and veterinary treatments were recorded. All analytical methods used to measure metabolites, and metabolic hormones are described in the appendices.

4.3. STATISTICAL ANALYSIS

4.3.1. Adiponectin in bovine milk (Experiment 1)

Cows were divided into two groups according to daily milk yield; cows yielding >30 *kg/d* were the HIGH group (3 cows); cows yielding <30 *kg/d* were the LOW group (3 cows). The difference between groups in milk adiponectin concentration was tested by using *Student's t-test* within the PASW© 18 Edition (SPSS Inc., Chicago, USA) statistical software package.

4.3.2. Circulating adiponectin (Experiment 2)

All data were analyzed using PASW© 18 Edition (SPSS Inc., Chicago, USA). Generalized Estimating Equations (GEE) were used to test specific hypotheses (Lindsey, 1997; McCullagh & Nelder, 1989; Dobson, 2002; Horton & Lipsitz, 1999) because adiponectin values were not normally distributed (Shapiro-Wilk W value=0.847, Shapiro-Wilk *P* value=0.001), and all known transformations failed to normalize the data. Family distribution and link function were selected as detailed in § 3.3. Selection of GEE models was performed based on QIC criterion, as detailed in § 3.3.

Models selected to test effects of lactational stage (WEEK), BCS at calving (CONDITION) and diet on production traits and hormonal and metabolic profile are illustrated in Table 4.1 and 4.2.

Parameter estimates of GEE models are presented as marginal means plus/minus standard error of the difference (SED) in tables. Estimated marginal means, SEs and SEDs were obtained by using *Least Significant Difference* (*LSD*) adjustment for multiple comparisons in PASW© 18 statistical program. *P* values were calculated by *Newton-Raphson Maximum Likelihood (ML)* method and effects were considered statistically significant when *P* value was less than 0.05 ($P<0.05$).

Shapiro-Wilk test was used to assess whether the distribution of variables followed a normal distribution. Linear relationships between quantitative variables were assessed with the Spearman correlation coefficients for nonparametric data. *Spearman* correlation coefficients (*rho*) were calculated and considered statistically significant when *P* value was less than 0.05 ($P<0.05$) whereas *P* value equal or less than 0.1 ($P \leq 0.1$) were considered as a trend.

Regression analysis was performed for DMI, adiponectin, leptin, glucose, milk yield, and MEBAL in FAT and THIN cows at calving (Table 4.3). In this way, two regression models for DMI were constructed. The model fitness was inspected by graphing observed values of DMI against predicted values of DMI and R-squared coefficient. *Raw residuals* and *Pearson residuals* were calculated, and the validity and the stability of the models were examined by exploring their normality. Beta (unstandardized) coefficients were estimated and they were used to build up regression equations to predict DMI. Beta coefficients were considered statistically significant and entered in the regression equations when *P* value was less than 0.1 ($P<0.1$).

Table 4.1: Selected models for analysis of the effect of lactational stage on circulating adiponectin, metabolites, metabolic hormones, and production traits

RESPONSE VARIABLES *(Y)*	TYPE OF MODEL	PREDICTORS *(X)*	SUBJECT (ID) VARIABLE	WITHIN-SUBJECT VARIABLE	CONTINUOUS COVARIATES	ERROR DISTRIBUTION FUNCTION	LINK FUNCTION	COVARIANCE MATRIX	WORKING CORRELATION MATRIX	STATISTICS
●Adiponectin	*GEE*	●WEEK	COW (60)	WEEK	PARITY	*Gamma*	*log*	*Robust*	*Exchangeable*	n=378; QIC=230
●Insulin	*GEE*	●WEEK	COW (60)	WEEK	PARITY	*Gaussian (Normal)*	*Identity (ID)*	*Robust*	*Exchangeable*	n=377; QIC=28
●Leptin										n= 378; QIC=474
●Glucagon										n= 216; QIC=123,651
●Glucose										n=246; QIC=75
●Urea										n=249; QIC=130
●DMI										n=352; QIC=3,242
●Milk yield										n=352; QIC=23,775
●LWT										n=352; QIC=228,742
●BCS										n=418; QIC=79
●MEBAL										n=352; QIC=50,678
●GH	*GEE*	●WEEK	COW (60)	WEEK	PARITY	*Inverse Gaussian*	*Log*	*Robust*	*Exchangeable*	n=206; QIC=37
●BOHB										n=248; QIC=122
●NEFA										n=249; QIC=224

Table 4.2: Selected models for analysis of the effects of diet and BCS at calving (CONDITION) on circulating adiponectin, metabolites, metabolic hormones, and production traits

RESPONSE VARIABLES *(Y)*	TYPE OF MODEL	PREDICTORS *(X)*	SUBJ. VAR.	WITHIN-SUBJECT VARIABLE	CONTINUOUS COVARIATES	ERROR DISTRIBUTION FUNCTION	LINK FUNCTION	COVARIANCE MATRIX	WORKING CORRELATION MATRIX	STATISTICS
●Adiponectin	*GEE*	●CONDITION ●DIET*	COW (60)	WEEK	PARITY	*Gamma*	*log*	*Robust*	*Exchangeable*	n=342; QIC=214
●Insulin **●Leptin** **●Glucagon** **●Glucose** **●Urea** **●DMI** **●Milk yield** **●LWT** **●BCS** **●MEBAL**	*GEE*	●CONDITION ●DIET*	COW (60)	WEEK	PARITY	*Gaussian (Normal)*	*Identity (ID)*	*Robust*	*Exchangeable*	n= 33O; QIC=21 n= 332; QIC=356 n= 216; QIC=121,477 n=202; QIC=69 n=205; QIC=114 n=352; QIC=3,969 n=352; QIC=26,606 n=352; QIC=256,097 n=358; QIC=72 n=352; QIC=86,330
●ΔBCS	*GLM*	●CONDITION ●DIET	-	-	PARITY	*Gaussian (Normal)*	*Identity (ID)*	*Robust*	*Independent*	n=59; AIC=77; *LR*(χ^2=8.81, df=2, *P*=0.032)
●GH **●BOHB** **●NEFA**	*GEE*	●CONDITION ●DIET*	COW (60)	WEEK	PARITY	*Inverse Gaussian*	*Log*	*Robust*	*Exchangeable*	n=206; QIC=41 n=205; QIC=93 n=205; QIC=273

*** Precalving data were excluded from the analysis**

Table 4.3: Selected model for regression analysis of DMI

RESPONSE VARIABLES *(Y)*	TYPE OF MODEL	PREDICTORS *(X)*	SUBJECT (ID) VARIABLE	WITHIN-SUBJECT VARIABLE	CONTINUOUS COVARIATES	ERROR DISTRIBUTION FUNCTION	LINK FUNCTION	COVARIANCE MATRIX	WORKING CORRELATION MATRIX	OBSERVATIONS
●**DMI***	*GEE*	-	COW (60 cows)	WEEK	Adiponectin Milk yield Glucose Leptin MEBAL	*Gaussian (Normal)*	*Identity (ID)*	*Robust Estimator*	*Exchangeable*	111 (each time)*

***The same model was run twice, for FAT and THIN cows at calving.**

4.4. RESULTS

4.4.1. Adiponectin in bovine milk (Experiment 1)

Mean milk adiponectin concentration was 11.5 ±3.49 *ng/ml*. Milk adiponectin varied with day in milk, from low (<5 *ng/ml*; 10 and 46 day in milk) to moderate (10 *ng/ml*; 6 day in milk) and high (20-25 *ng/ml*; 15-36 day in milk). Cows with HIGH daily milk yield did not have higher (*P*= 0.31) milk adiponectin (8. 8±4.01 *ng/ml versus* 17.0± 6.40 *ng/ml*) than cows with LOW daily milk yield.

4.4.2. Circulating adiponectin (Experiment 2)

Mean circulating adiponectin was 8.6 ±0.33 *ng/ml* and this was greater than the mean of other metabolic hormone (insulin; 0.4±0.01 *ng/ml*, leptin; 1.7±0.06 *ng/ml*, GH; 4.6±0.22 *ng/ml*, glucagon; 95.9±0.63 pg/ml). Circulating adiponectin values showed high correlation (average correlation; ρ= 0.844) by week of experiment. They were not normally distributed (*Shapiro-Wilk W* value=0.847, *Shapiro-Wilk P* value=0.001), left skewed (median=7.21; skewness=1.80), and highly peaked (leptokurtotic probability distribution; kurtosis=7.49). Individual cows showed consistently high or normal circulating adiponectin values throughout the experimental period (Figure 4.1).

4.4.2.1. Lactational stage

There was an effect of lactational stage on circulating adiponectin, insulin, leptin, and GH (*P*<0.01) (Table 4.4). Circulating adiponectin was elevated 2 weeks prepartum (11.1 ±1.06 *ng/ml*), decreased to week 2 postpartum (7.1±0.90 *ng/ml*), increased to week 4 postpartum (8.9±0.76 *ng/ml*), remained stable until week 12 postpartum (9.1±0.86 *ng/ml*), and then declined to 6.5±0.75 *ng/ml* at the end of the study (Figure 4.2). Circulating insulin

decreased from week 2 prepartum (0.4±0.03 *ng/ml*) to week 2 postpartum (0.3±0.02 *ng/ml*), and then increased constantly between 0.33 and 0.54 *ng/ml* for the remainder of the study. Circulating leptin was elevated 2 weeks prepartum (2.2±0.24 *ng/ml*), decreased until week 2 postpartum (1.5±0.10 *ng/ml*), remained at 1.4- 1.64 *ng/ml* until the end of week 8 postpartum, and then increased constantly for the remainder of the study (Table 4.4). Circulating GH was elevated 2 weeks postpartum (6.9±0.53 *ng/ml*), decreased steadily until week 12 (3.2±0.53 *ng/ml*), and remained unchanged after week 12 postpartum (Table 4.4). There was no effect of lactational stage on circulating glucagon (Table 4.4).

There was an effect of lactational stage on circulating glucose, NEFA, and urea ($P< 0.01$) (Table 4.5). Circulating glucose was (3.0±0.07 *mmol/l*) in week 2 prepartum and week 2 postpartum, increased rapidly to week 8 postpartum (3.6±0.08), and then remained at the same level until the end of the experiment (Table 4.5). NEFA increased from 2 weeks prepartum (0.4±0.05 *mmol/l*) to week 2 postpartum (0.9±0.16 *mmol/l*), then declined substantially and remained between 0.54 and 0.24 *mmol/l* for the remainder of the study (Table 4.5). Circulating urea was lowest 2 weeks prepartum (1.6±0.10 *mmol/l*) and increased steadily throughout the study. There was no effect of lactational stage on circulating BOHB (Table 4.5).

There was an effect of lactational stage on DMI, LWT, milk yield, BCS, and MEBAL ($P<0.01$) (Table 4.6). DMI (17.9±0.23 *kg/d*) and milk yield (35.1±0.52 *kg/d*) were low 2 weeks postpartum, increased until week 8 postpartum (DMI 22.8 ±0.23 *kg/d*; milk yield 46.8 ±0.52 *kg/d*) and decreased after this point to be 22.3±0.23 *kg/d* and 41.1±0.51 *kg/d* respectively, at the end of the study (Table 4.6). BCS was high 2 weeks postpartum (2.9±0.04) and decreased rapidly until week 6 postpartum (2.4±0.04). After week 6 postpartum, cows gained condition for the remainder of the study (Table 4.6). Metabolizable energy balance (MEBAL) was negative from week 2 to week 6 postpartum and remained positive after week 8. LWT remained between 650 and 654 kg from week 2 to week 6 postpartum and then increased steadily to reach 669 kg at the end of the study (Table 4.6).

4.4.2.2. Diet and BCS at calving

Circulating adiponectin was not different (P>0.05) for cows fed the HS diet (8.3±0.68 *ng/ml*) than for cows fed the HF diet (8.8±0.81 *ng/ml*). Cows fed the HS diet had higher circulating insulin, NEFA, and BOHB (P<0.05) than cows fed the HF diet (Tables 4.7 & 4.8). There was no effect of DIET on DMI, LWT, milk yield, BCS, ΔBCS, and MEBAL (Table 4.9).

Circulating adiponectin was not different (P>0.05) for FAT cows at calving (8.3±0.87 *ng/ml*) than for THIN cows at calving (8.91.39 *ng/ml*) (Table 4.7). There was no effect of BCS at calving on circulating insulin, leptin, GH, glucagon, glucose, NEFA, BOHB, and urea (Tables 4.7 & 4.8). FAT cows at calving had higher DMI, milk yield, BCS, and ΔBCS than THIN cows at calving (P<0.05). There was no effect of BCS at calving on LWT and MEBAL (Table 4.9).

4.4.2.3. Associations of circulating adiponectin with circulating metabolites, metabolic hormones, and production traits

Plasma adiponectin was negatively correlated with GH concentration (*rho*= -0.144, P=0.042) and tended to be correlated negatively with plasma glucose (*rho*= -0.110, P=0.10). Circulating GH was correlated negatively with circulating insulin (*rho* = -0.287, P=0.001) and leptin (*rho*= -0.319, P=0.001). Circulating insulin was correlated negatively with ΔBCS (*rho*= -0.158, P=0.002)

Circulating adiponectin was correlated negatively with previous milk yield (PMY; *rho*= -0.256, P=0.001) and aggregated milk yield (AMY; *rho*= -0.119, P=0.02). AMY was correlated positively with PMY (*rho*= 0.430, P=0.001). GH was correlated positively with AMY (*rho*= 0.216, P=0.002) and PMY (*rho*= 0.127, P=0.05). Leptin was correlated negatively with PMY (*rho*= -0.122, P=0.018) and positively with circulating insulin (*rho*= 0.356, P=0.001).

Adiponectin concentration was correlated negatively with ΔBCS (*rho*= -0.173, *P*=0.001). ΔBCS was correlated positively with GH (*rho*=0.253, *P*=0.001), and negatively with circulating leptin (*rho*= -0.261, *P*=0.001). ΔBCS was correlated positively with PMY (*rho*= 0.281, *P*=0.001), AMY (*rho*= 0.434, *P*=0.001), milk yield (*rho*= 0.388, *P*=0.001), DMI (*rho*= 0.382, *P*=0.001) and BOHB (*rho*= -0.193, *P*=0.002).

Milk yield was correlated positively with PMY (*rho*= 0.473, *P*=0.001) and AMY (*rho*= 0.753, *P*=0.001). AMY was correlated positively with PMY (*rho*= 0.513, *P*=0.001) and DMI (*rho*=0.595, *P*=0.001). PMY was correlated positively with DMI (*rho*= 0.365, *P*=0.001).

4.4.2.4. Relationships between DMI and circulating adiponectin, glucose, leptin, MEBAL, and milk yield in FAT and THIN cows at calving

DMI for THIN cows at calving can be predicted, with high precision (R^2= 0.88), by the regression equation; DMI=0.318 * MY (*kg/d*) + 0.057 * MEBAL (*MJ/d*) - 0.043 * ADIPO (*ng/ml*)+ 8.74, whereas DMI for FAT cows at calving can be predicted by the regression equation; DMI=0.293 * MY (*kg/d*) + 0.055 * MEBAL (*MJ/d*) + 0.047*ADIPO (*ng/ml*)- 0.54* GLU (*mmol/l*) +10.71 (R^2= 0.78) (Figure 4.3). Adiponectin *beta* coefficient in the regression equation for THIN cows was negative (-0.043), whereas it was positive (0.047) for FAT cows at calving (Table 4.10 & 4.11). Glucose featured only in the model for FAT cows at calving and its beta coefficient was negative (-0.54) (Table 4.11).

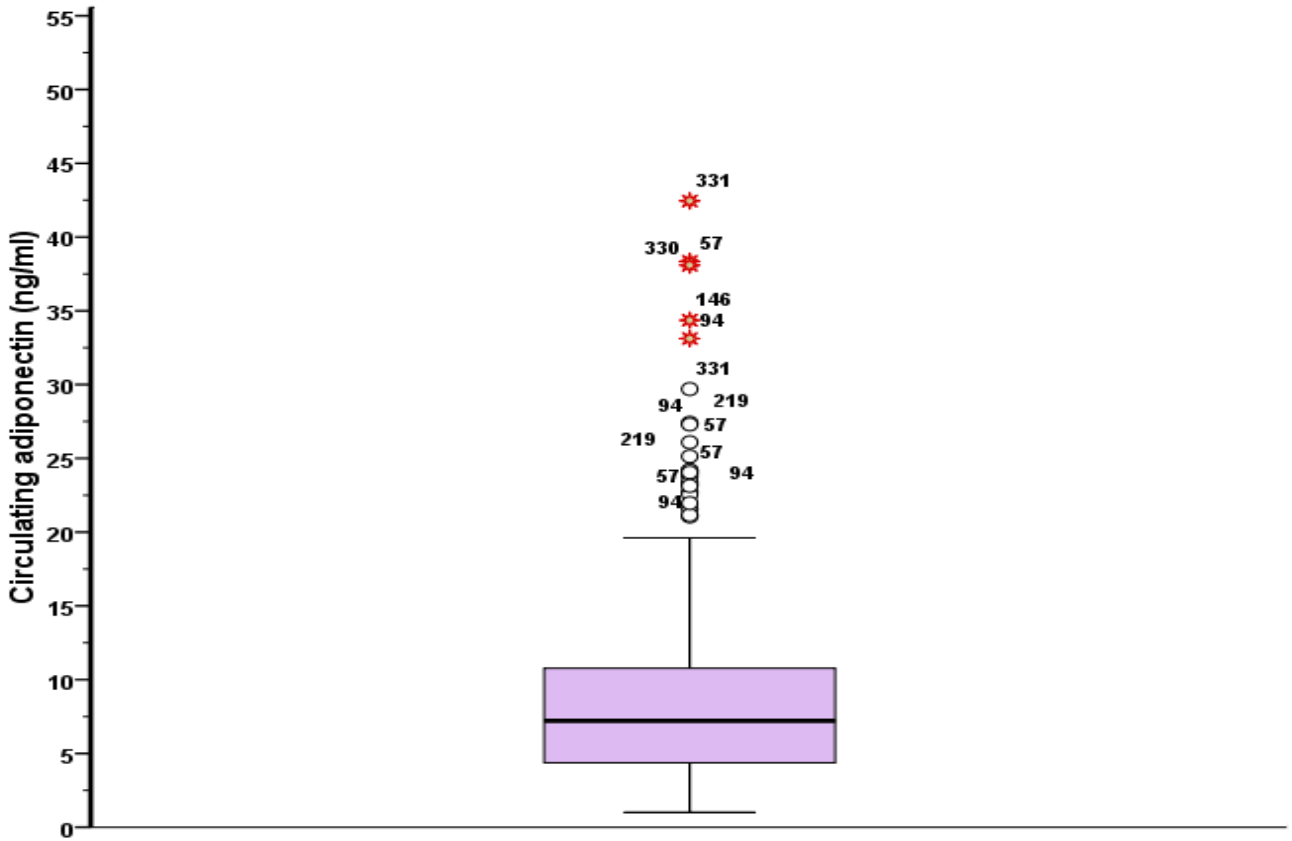


Figure 4.1: Box –Whisker plot of circulating adiponectin values (*n*= 387). Box shows median value of circulating adiponectin, and the size of the first and third quartile. Outliers appear as individual points ○ or ✶ outside the box (marked by cow id number). The ○ outlier values are known as outside values, and the ✶ outlier values as far outside values. Outliers are at the upper range of the data (above the box); the mean value is above the median (the centre line in the box); the median line does not evenly divide the box; and the upper tail of the box plot is longer than the lower tail; thus, the population distribution from which the data were sampled is skewed to the right. Interestingly, outlier values come consistently from 6 cows (id; 57, 94, 146, 219, 330, and 331) which showed the same trend for higher adiponectin values throughout the experiment.

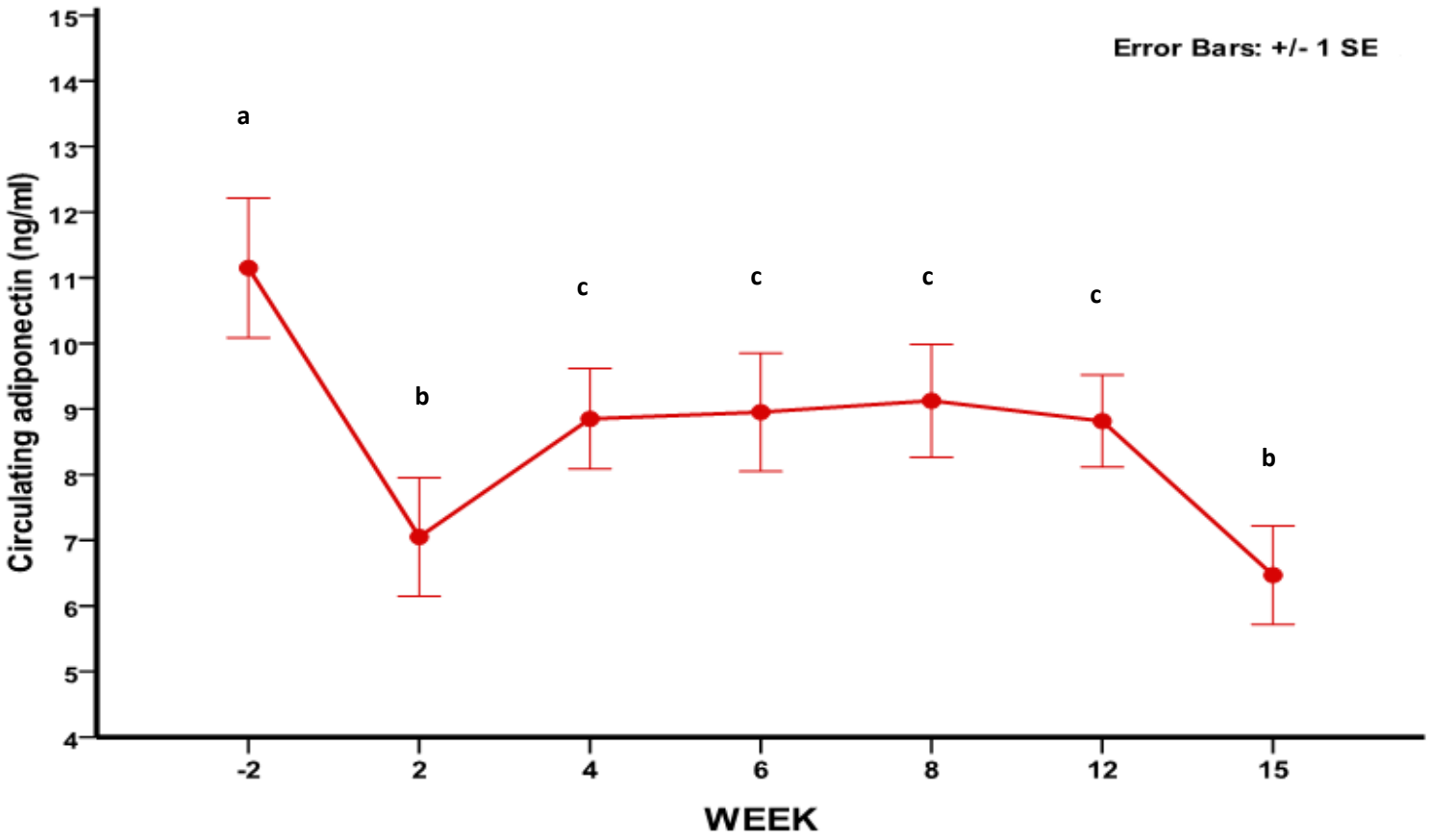


Figure 4.2: Effect of lactational stage (WEEK) on circulating adiponectin (n = 387, P value = 0.001). GEE model with *Gamma* error distribution, *log* link function, and *exchangeable R matrix* structure with robust estimator of covariance matrix. Dependent variable was adiponectin, subject variable was cows (60 cows), and within subject variable was WEEK (7 weeks).WEEK was added as factorial independent, and parity as continuous covariate in the model). Red dots (●) represent marginal means ± SE. -2 point in *x-axis* represents two weeks prepartum whereas 15 is fifteen week postpartum. Means marked with different letters are significant different (P<0.05).

Table 4.4: Effect of lactational stage (WEEK) on circulating metabolic hormones

Factors:	WEEK									Contrasts✧ *P val.*					
Parameters:	**-2†**	**2†**	**4†**	**6†**	**8†**	**12†**	**15†**	**SED‡**	***n*∂**	***c1***	***c2***	***c3***	***c4***	***c5***	***c6***
Adiponectin *(ng/ml)*	11.19	7.01	8.83	8.90	9.09	8.82	6.48	0.842	387	<0.01	<0.01	NS*	NS	NS	<0.01
Insulin *(ng/ml)*	0.36	0.26	0.33	0.40	0.45	0.48	0.54	0.026	377	<0.01	NS	<0.01	<0.01	<0.01	<0.01
GH *(ng/ml)*	-	6.9	5.1	4.1	3.9	3.2	3.7	0.53	206	-	<0.01	<0.01	<0.01	<0.01	NS
Leptin *(ng/ml)*	2.21	1.46	1.43	1.50	1.65	1.84	2.15	0.110	378	<0.01	NS	NS	<0.05	NS	<0.05
Glucagon *(pg/ml)*	-	96.3	93.5	91.7	92.1	96.5	100.4	4.30	216	NS	NS	NS	NS	NS	NS

† **Columns are means.**
‡ **SED = standard error of the difference between treatment means.**
✧ **c1 (-2 week *versus* 2 week); c2 (2 week *versus* 4 week); c3 (4 week *versus* 6 week); c4 (6 week *versus* 8 week); c5 (8 week *versus* 12 week); c6 (12 week *versus* 15 week).**
∂ ***n is* the total repeated observations of animals**
* **NS = non-significant**

Table 4.5: Effect of lactational stage (WEEK) on circulating metabolites

Factors:	WEEK									Contrasts✧ *P val.*					
Parameters:	**-2†**	**2†**	**4†**	**6†**	**8†**	**12†**	**15†**	**SED‡**	***n*∂**	***c1***	***c2***	***c3***	***c4***	***c5***	***c6***
Glucose *(mmol/l)*	3.03	3.07	3.27	3.44	3.60	3.74	3.75	0.090	246	NS*	<0.05	<0.05	<0.05	NS	NS
BOHB *(mmol/l)*	0.70	0.73	0.68	0.59	0.60	0.61	0.62	0.074	248	NS	NS	NS	NS	NS	NS
NEFA *(mmol/l)*	0.45	0.88	0.54	0.47	0.28	0.24	0.23	0.085	249	<0.01	<0.05	NS	<0.05	NS	NS
Urea *(mmol/l)*	1.63	2.11	2.26	2.51	2.67	2.71	2.90	0.124	249	<0.01	NS	<0.05	NS	NS	NS

† **Columns are means.**
‡ **SED = standard error of the difference between treatment means.**
✧ **c1 (-2 week *versus* 2 week); c2 (2 week *versus* 4 week); c3 (4 week *versus* 6 week); c4 (6 week *versus* 8 week); c5 (8 week *versus* 12 week); c6 (12 week *versus* 15 week).**
∂ ***n is* the total repeated observations of animals**
* **NS = non-significant**

Table 4.6: Effect of lactational stage (WEEK) on productive traits

Factors:	WEEK								Contrasts✧ *P val.*				
Parameters:	**2†**	**4†**	**6†**	**8†**	**12†**	**15**	**SED‡**	***n*∂**	***c2***	***c3***	***c4***	***c5***	***c6***
DMI *(kg/d)*	17.9	20.8	22.2	22.8	22.7	22.3	0.23	352	<0.01	<0.01	<0.01	NS	NS
LWT *(kg)*	654.7	645.2	650.0	657.4	667.6	669.3	3.00	352	<0.01	NS*	<0.01	<0.01	NS
Milk yield *(kg/d)*	35.1	43.2	45.9	46.9	45.6	44.1	0.51	352	<0.01	<0.01	<0.05	<0.05	<0.01
BCS *(units 1-5)*	2.95	2.60	2.45	2.51	2.64	2.77	0.037	418	<0.01	<0.01	<0.05	<0.01	<0.01
MEBAL *(MJ/d)*	-24.3	-12.6	-5.4	1.1	5.7	2.7	1.70	352	<0.01	<0.01	<0.01	<0.05	NS

† **Columns are means.**
‡ **SED = standard error of the difference between treatment means.**
✧ **Contrasts: c2 (2 week *versus* 4 week); c3 (4 week *versus* 6 week); c4 (6 week *versus* 8 week); c5 (8 week *versus* 12 week); c6 (12 week *versus* 15 week).**
∂ ***n is* the total repeated observations of animals**
* **NS = non-significant**

Table 4.7: Effect of diet and BCS at calving (CONDITION) on circulating metabolic hormones

Factors:	DIET						CONDITION					
Parameters:	**HS**†	n_1✧	**HF**†	n_2✧	**SED**‡	***P***	**FAT**†	n_3 ∂	**THIN**†	n_4 ∂	**SED**‡	***P***
Adiponectin *(ng/ml)*	8.29	170	8.77	172	0.745	0.23	8.34	229	8.92	113	1.130	0.73
Insulin *(ng/ml)*	0.46	168	0.38	162	0.027	0.01	0.40	223	0.43	107	0.029	0.37
GH *(ng/ml)*	4.7	94	4.2	112	0.32	0.31	4.7	126	4.2	80	0.54	0.30
Leptin *(ng/ml)*	1.75	165	1.67	167	0.183	0.66	1.59	224	1.82	108	0.265	0.41
Glucagon *(pg/ml)*	96.6	105	93.0	111	2.61	0.16	96.8	140	92.8	76	5.41	0.46

† **Means for high-starch (HS) and high-fat (HF) diet groups, and FAT (>3.25) and THIN (≤3.25) BCS at calving groups.**
✧ **n_1 and n_2 are the repeated observations of animals fed with HS and HF diets, respectively.**
∂ **n_3 and n_4 are the repeated observations of animals assessed FAT and THIN at calving, respectively.**
‡ **SED = standard error of the difference between treatment means.**

Table 4.8: Effect of diet and BCS at calving (CONDITION) on circulating metabolites

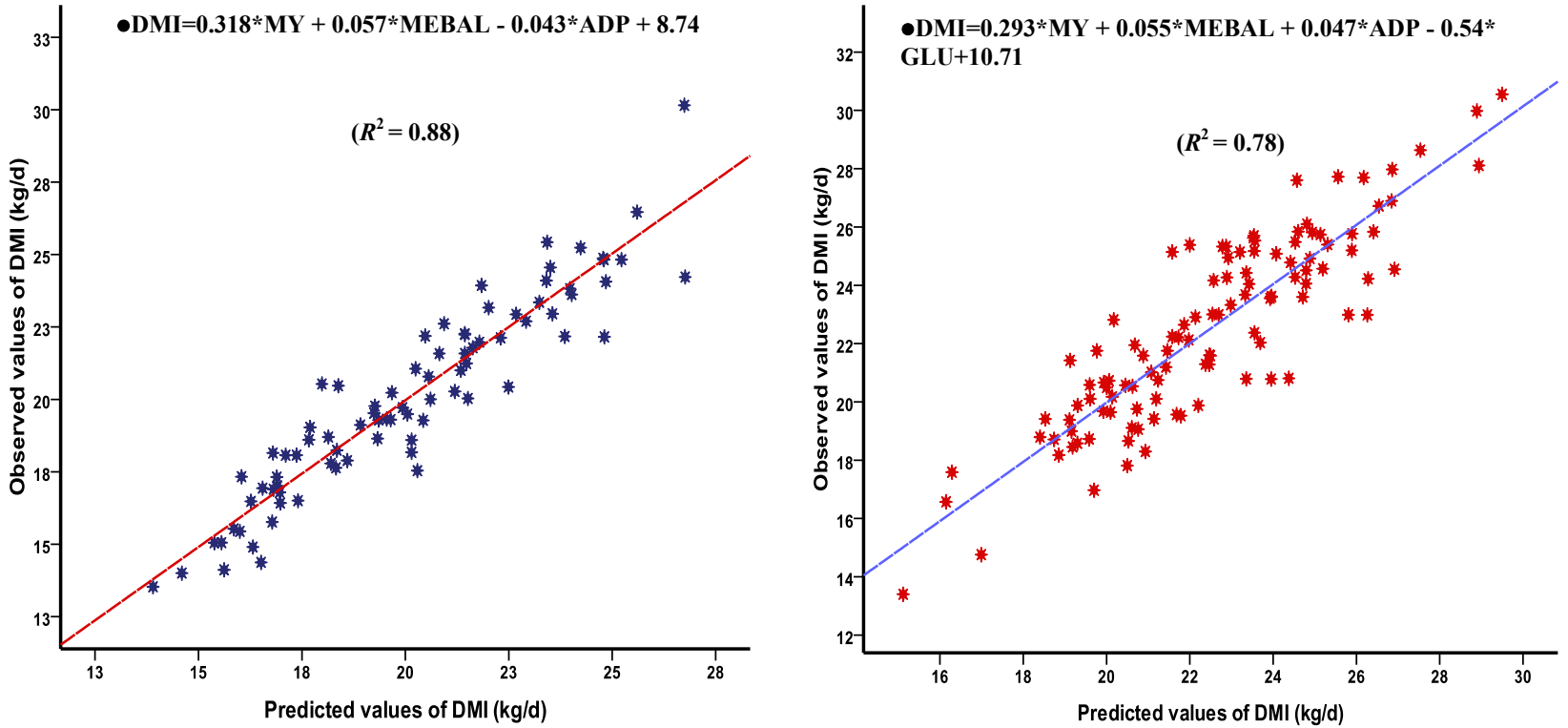
Factors:	DIET						CONDITION					
Parameters:	**HS**[†]	n_1[✧]	**HF**[†]	n_2[✧]	**SED**[‡]	***P***	**FAT**[†]	n_3[∂]	**THIN**[†]	n_4[∂]	**SED**	***P***
Glucose *(mmol/l)*	3.56	98	3.44	104	0.096	0.20	3.44	117	3.56	85	0.113	0.28
BOHB *(mmol/l)*	0.54	98	0.70	107	0.051	0.002	0.66	120	0.58	85	0.052	0.13
NEFA *(mmol/l)*	0.36	98	0.49	107	0.065	0.044	0.48	123	0.37	82	0.066	0.08
Urea *(mmol/l)*	2.65	96	2.41	109	0.119	0.047	2.63	121	2.44	84	0.163	0.23

[†] Means for high-starch (HS) and high-fat (HF) diet groups, and FAT (>3.25) and THIN (≤3.25) BCS at calving groups.
[✧] n_1 and n_2 are the repeated observations of animals fed with HS and HF diets, respectively.
[∂] n_3 and n_4 are the repeated observations of animals assessed FAT and THIN at calving, respectively.
[‡] SED = standard error of the difference between treatment means.

Table 4.9: Effect of diet and BCS at calving (CONDITION) on production traits

Factors:	DIET						CONDITION					
Parameters:	**HS**[†]	n_1[✧]	**HF**[†]	n_2[✧]	**SED**[‡]	***P***	**FAT**[†]	n_3[∂]	**THIN**[†]	n_4[∂]	**SED**[‡]	***P***
DMI *(kg/d)*	21.0	176	21.4	176	0.35	0.23	22.1	237	20.3	115	0.72	0.02
LWT *(kg)*	657.7	176	657.6	176	4.21	0.99	656.5	237	658.2	115	5.53	0.68
Milk yield *(kg/d)*	42.8	176	42.5	176	0.80	0.75	45.4	237	39.9	115	1.99	0.01
BCS *(units 1-5)*	2.61	180	2.60	178	0.046	0.92	2.75	240	2.47	118	0.075	0.01
ΔBCS *(units 1-5)*	0.60	30	0.58	29	0.083	0.81	0.77	40	0.41	19	0.122	0.01
MEBAL *(MJ/d)*	-4.7	176	-6.2	176	2.05	0.46	-5.6	237	-5.3	115	1.59	0.87

[†] **Means for high-starch (HS) and high-fat (HF) diet groups, and FAT (>3.25) and THIN (≤3.25) BCS at calving groups.**
[✧] n_1 **and** n_2 **are the repeated observations of animals fed with HS and HF diets, respectively.**
[∂] n_3 **and** n_4 **are the repeated observations of animals assessed FAT and THIN at calving, respectively.**
[‡] **SED = standard error of the difference between treatment means.**



(A) THIN cows at calving **(B) FAT cows at calving**

Figure 4.3: Relationships among DMI and circulating adiponectin, glucose, leptin, MEBAL, and milk yield in FAT and THIN cows at calving. GEE regression was performed for FAT and THIN cows (Table 4.3). In this model, DMI was the dependent variable and adiponectin, glucose, leptin, MEBAL, and milk yield were independent. Parameter estimates for regression models are presented in Table 4.10 for THIN cows at calving and Table 4.11 for FAT cows at calving. Model fitness, regression equations to predict DMI, and R-squared coefficients were presented to (A) for THIN cows at calving and (B) for FAT cows at calving.

Table 4.10: Regression parameter estimates for prediction of DMI in THIN cows at calving

Parameters in the model	Beta (*b*) Coefficient	SE†	*P*
Constant	8.74	1.24	0.001
Adiponectin (ADP)	-.043	0.010	0.001
Milk yield (MY)	.318	0.022	0.001
MEBAL	.057	0.011	0.001
Leptin	.031	0.086	0.72
Glucose (GLU)	-.156	0.271	0.56

†**Standard error of beta coefficient**

Table 4.11: Regression parameter estimates for prediction of DMI in FAT cows at calving

Parameters in the model	Beta (*b*) Coefficient	SE†	*P*
Constant	10.71	1.32	0.001
Adiponectin (ADP)	.047	0.028	0.097
Milk yield (MY)	.291	0.020	0.001
MEBAL	.055	0.011	0.001
Leptin	.014	0.145	0.92
Glucose (GLU)	-.539	0.299	0.07

†**Standard error of beta coefficient**

4.5. DISCUSSION

Transition from late pregnancy to early lactation is accompanied by substantial homeostatic and homeorhetic adaptations in energy and nutrient partitioning (Bauman & Currie, 1980; Beever, 2006). In early lactating cows, increased circulating GH, PRL, NEFA, BOHB, and glucocorticoids (GC) and decreased circulating insulin and leptin, induce metabolic adaptations in liver and insulin dependent tissues that prioritise glucose partitioning to the mammary gland (Jorritsma *et al.,* 2003; Leroy *et al.,* 2008; Leroy *et al.,* 2010). Adiponectin, because of its insulin sensitising actions (Berg *et al.,* 2002; Matsuzawa *et al.*, 2004; Kadowaki & Yamauchi, 2005), could be another putative regulator of metabolism during the transition from pregnancy to lactation (Mazaki-Tovi *et al.,* 2005; Mitchell *et al.,* 2005) . The purposes of this study were to measure circulating and milk adiponectin and to assess the impact of diet and body condition at calving on circulating adiponectin values in dairy cows.

4.5.1. Measurement of plasma and milk adiponectin

Circulating and milk adiponectin were measured in this study by using a commercially available human adiponectin kit. There is no validated RIA kit for measuring bovine adiponectin, but the Linco kit has been used for cows (Raddatz *et al.*, 2008), horses (Gordon & McKeever, 2005; Gordon *et al.*, 2006; Kearns et. al, 2006; Pratt *et al.*, 2005), and dogs (Gayet *et al.*, 2007; Brunson *et al.*, 2007). Bovine adiponectin protein sequence shares about 91% homology with mouse, and murine adiponectin protein sequence is similar up to 85% with human, rat, and monkey adiponectin protein sequences (Berg *et al.,* 2002). This high conservation and homology of adiponectin molecule among species and the fact that Linco kit measured both bovine circulating adiponectin and milk adiponectin in this study, plasma adiponectin in another experiment (Raddatz *et al.*, 2008), and circulating adiponectin in other species (Gordon & McKeever, 2005; Gordon *et al.*, 2006;

Kearns *et. al*, 2006; Pratt *et al.*, 2005; Gayet *et al.*, 2007; Brunson *et al.*, 2007), suggest that Linco kit can be used to assess bovine adiponectin.

In this experiment, mean plasma adiponectin varied from 6.5±0.75 *ng/ml* to 11.1±1.06 *ng/ml*, and some cows had constantly elevated plasma adiponectin throughout the experiment. Raddatz *et al.,* (2008) reported that plasma adiponectin values varied from 8.3±1.4 *ng/ml* to 16.0±2.7 *ng/ml* in early lactating cows and individual cows had consistently high or low adiponectin levels throughout the sampling period, in agreement with the present study. Circulating adiponectin values were not normally distributed, but were left skewed and highly peaked; this agrees with distributions reported for plasma adiponectin by Cassidy *et al.* (2009) and Pischon *et al.* (2005) in humans and Barnea *et al.* (2006) in mice. Adiponectin concentration in plasma has been reported to be two to three times higher than other hormones (Ahima, 2006); in the present study, mean circulating adiponectin was significantly greater than the mean of insulin, leptin, GH and glucagon. The high autocorrelation of circulating adiponectin values might indicate that plasma adiponectin concentration was tightly genetically controlled and other factors had only minimal impact, but this needs to be further elucidated.

Mean plasma adiponectin was 8.6±0.33 *ng/ml* whereas milk adiponectin was 11.5±2.60 *ng/ml*. Adiponectin concentrations in human breast milk ranged from 3.9 to 30.4 *ng/ml* (Bronsky *et al.,* 2006), and from 4.2 to 87.9 *ng/ml* (Martin *et al.,* 2006). Ahima (2006) reported that normal concentrations in human serum ranged from 5 to 30 *μg/ml*. No study directly compared circulating and milk adiponectin. In the present study, milk and plasma adiponectin concentrations were similar, which may imply that milk adiponectin is excreted at concentrations similar to blood, but this hypothesis needs to be investigated further.

In mice and human milk, adiponectin decreases over the course of lactation. Human milk adiponectin concentrations decrease approximately 5% – 6% with each month of lactation (Newburg *et al.,* 2010; Savino & Liguori, 2008). In the present study, milk adiponectin concentrations did not show the same trend, although samples were collected only for the

first two months of lactation; nevertheless, adiponectin varied with day in milk and the pattern needs to be investigated further. Another finding of this study was that milk adiponectin was not different for cows with high or low milk yield, which also needs to be investigated further.

4.5.2. The impact of lactational stage on circulating adiponectin

This study showed that, as in humans (Asai-Sato *et al.*, 2006; Ritterath *et al.*, 2008), the transition from pregnancy to lactation in dairy cows is associated with a reduction in the plasma concentration of adiponectin. The same pattern of decreased AdipoR1 and AdipoR2 mRNA expression in subcutaneous fat tissue during transition from pregnancy (1 week precalving) to lactation (3 weeks postcalving) was reported by Lemor *et al.* (2009) in high yielding dairy cows. The result of this study is generally in agreement with Komatsu *et al.* (2007) who reported that adiponectin mRNA expression in adipose tissue was higher in non-lactating cows (dried-off for 3–10 weeks) than cows at peak lactation (8–11 weeks after parturition). The present study generally agrees with Ohtani *et al.* (2011) who reported that mammary gland adiponectin mRNA expression was lower in early and late lactation compared with non-pregnant or dry cows. Moreover, the present study found that after the 4th week postpartum circulating adiponectin is significantly increased and remains at the same level until the 12th week postpartum. Another study measured circulating adiponectin in cows by using the same RIA kit (Raddatz *et al.*, 2008) reported that circulating adiponectin was significantly increased from (8.3 ± 1.4 *ng/ml*) in the first week to (16.0 ± 2.7 *ng/ml*) at week 4 postpartum and then declined to remain at 12-13 *ng/ml* until the 11th week postpartum. This pattern of circulating adiponectin is also in agreement with the pattern found in the present study. Furthermore, this study suggests that after 12th week postpartum there is a significant reduction in circulating adiponectin, whereas plasma adiponectin values do not change during the period from 4 to 12 week postpartum. In the study of Komatsu *et al.* (2007), adiponectin mRNA expression in adipose tissue from four cows in early lactation (8–11 weeks after parturition) did not differ from that of four cows in late lactation (40–50 weeks after parturition). This result does not directly contradict the

finding of the present study because Komatsu *et al.* (2007) measured expression of mRNA in adipose tissue at a much later stage of lactation.

In the present study, stage of lactation affected the majority of circulating metabolic hormones, metabolites, and production traits, but circulating adiponectin showed correlations only with circulating GH and ΔBCS, although there was a weak negative association between plasma adiponectin and glucose. The weekly changing pattern of circulating adiponectin can be partially explained by the negative relationship between GH and adiponectin; GH was elevated 2 weeks postpartum and adiponectin reached a nadir at the same point of time. Subsequently, GH decreased steadily until week 12 and then increased slightly, while adiponectin increased until week 4 and then remained stable until week 12and then decreased. ΔBCS was correlated negatively with plasma adiponectin and positively with circulating GH, and cows gained BCS after week 8 postpartum in this experiment, which might be another reason for the decrease in adiponectin between weeks 12 and 15. This is the first study to demonstrate a negative relationship between GH and adiponectin in dairy cows. However, this relationship needs to be investigated further.

In the present study, milk yield was correlated positively with GH and negatively with adiponectin. This may indicate that GH is the major regulator of circulating adiponectin postpartum in dairy cows. It is known that genetic selection for milk yield led to higher circulating GH in dairy cows (Veerkamp *et al.*, 2003), which makes the dairy cow the most suitable animal model to study relations between GH and adiponectin. Lam *et al.* (2004) reported that circulating adiponectin was low in patients who suffered from acromegaly, and that circulating adiponectin increased after GH-lowering therapies. Nilsson *et al.* (2005) demonstrated that gene expressions of AdipoR1 and AdipoR2 in human adipose tissue are differentially regulated by PRL and GH. Also, PRL and GH reduced adiponectin secretion by human adipose tissue *in vitro* and *in vivo* in mice. Rodriguez-Pacheco *et al.* (2007) investigated if adiponectin plays a central role, similar to leptin, in regulating somatotroph and gonadotroph function. They showed that short-term Adiponectin exposure abolishes both basal and stimulated (by Ghrelin and GnRH) secretion of GH and LH by rat pituitary cells *in vitro*. According to López-Siguero *et al.* (2010) GH deficient

children had higher circulating adiponectin than healthy controls. These studies are in line with the result of the present study as they imply negative correlation between circulating GH and adiponectin.

Other hormonal signals may be associated with changes in circulating adiponectin by week. According to Asai-Sato *et al.* (2006) circulating adiponectin declined slightly as pregnancy advanced and reached its lowest level during lactation, possibly because PRL affects regulation of maternal metabolism through suppression of adiponectin. Shi *et al.* (2010) demonstrated that GC decreased adiponectin in rat. TNF-*α* is another adipokine which is potent negative regulator of systemic insulin sensitivity, which has also been demonstrated to stimulate leptin (Zumbach *et al.,* 1997) but inhibits adiponectin production (Fasshauer *et al.,* 2002; Ruan *et al.,* 2002). It is possibly that elevated GH, PRL, and TNF-α, and GC could account for diminished circulating adiponectin 2 weeks postpartum, although TNF-α, PRL and Glucocorticoids were not measured in the present study.

The role of adiponectin is closely connected to glucose metabolism through enhancing insulin sensitivity and increasing glucose uptake via the GLUT4 transporter (Dridis & Taouis, 2009). Adiponectin increases insulin sensitivity in isolated primary hepatocytes, resulting in decreased glucose production (Berg *et al.,* 2001). NEFA and glucose are negatively correlated with circulating adiponectin in rodents and humans (Fruebis *et al.*, 2001; Yang *et al.*, 2004; Pischon *et al.*, 2005; Qi *et al.*, 2005; Cassidy *et al.*, 2008) but, in the present study, although circulating adiponectin tended to be negatively correlated with circulating glucose, adiponectin was not correlated with NEFA.

4.5.3. The impact of diet and BCS at calving on circulating adiponectin

There was no effect of diet and BCS at calving on circulating adiponectin, even though cows fed on the HS diet had higher insulin but lower BOHB and NEFA than cows fed on the HF diet. Also, diet had no effect on production traits. Circulating insulin, BOHB, and NEFA were not correlated with adiponectin. Yang *et al.* (2004) observed that rats fed on a

HF diet showed significant increases in postprandial serum triglycerides, FFA, and insulin but no change in serum glucose and adiponectin. Xu *et al.* (2007) explored the effect of dietary supplementation of cysteamine on expression of adiponectin and adiponectin receptors in various tissues of rat. They observed no significant change in adiponectin mRNA expression in adipose tissue whereas circulating GH remained unchanged and insulin was higher in the treatment group than in the control. Muhlhausler *et al.* (2007) reported that maternal plasma glucose and insulin and foetal plasma glucose and insulin were significantly increased in well-fed ewes and their foetuses when compared with control ewes and their foetuses, and this rise was followed by significantly increased circulating adiponectin in both maternal and foetal plasma of well-fed animals. These three studies may suggest that plasma adiponectin concentrations change only when circulating GH or/and glucose are changed, in agreement with the present study. Circulating GH and glucose were not altered by dietary treatments in this experiment, which was possibly the reason diet had no effect on circulating adiponectin.

Body condition score at calving had no effect on metabolic hormones and metabolites, which could be the reason it did not influence circulating adiponectin in this experiment. Plasma GH and glucose concentrations were not different in THIN and FAT cows at calving. Thus, it is possible that circulating adiponectin was unchanged by BCS at calving because this treatment did not influence circulating GH and glucose which affect circulating adiponectin. However, DMI, milk yield, and ΔBCS were higher in FAT cows at calving than THIN cows at calving. ΔBCS was correlated positively with GH and negatively with plasma adiponectin concentrations in this experiment. This means that FAT cows at calving should have had lower circulating adiponectin and higher circulating GH than THIN cows at calving. Unfortunately, this was not the case in the present study. GH was substantially reduced in human obesity (Williams *et al.,* 1984), but in dairy cows genetic improvement has led to animals with higher BCS and circulating GH (Veerkamp *et al.*, 2003). In the present study, BCS at calving was generally high in both condition groups, so the groups consisted of fat to over-fat cows, which might explain the lack of effect on circulating GH.

A metabolic explanation why in the present study BCS at calving and BCS did not influence adiponectin could be the following. The present study was carried out in pregnant and lactating animals. It is known that pregnancy and lactation require considerable energy uptake and mobilization by the mother (Bauman, 2000). Furthermore, the first half of pregnancy is associated with acute fat mass deposition to ensure proper embryonic development, and the second half of pregnancy and early lactation involve the development of an insulin-resistant state in the mother in order to increase hepatic gluconeogenesis and decrease glucose uptake in maternal muscle and adipose tissue (Bauman & Currie, 1980; Leroy *et al.,* 2010). This latter adaptation results in maximum supply of maternal glucose to the foetus (in late pregnancy) or mammary gland (in early lactation) and adiponectin, due to its insulin sensitizing effects, will preferably channel nutrients to maternal tissues rather than to foetus or mammary gland. So, it is possible that mammals have developed an alternative mechanism that results in uncoupling of maternal body condition with circulating adiponectin levels. In humans, there is a negative relationship between circulating adiponectin and pre-gestational BMI (Lopez-Bermejo *et al.,* 2004; Retnakaran *et al.*, 2004; Williams *et al.*, 2004), but adiponectin levels throughout pregnancy are not correlated with maternal BMI (Mazaki-Tovi *et al.*, 2005; Jansson *et al.*, 2008). According to Mazaki-Tovi *et al.* (2005) the disruption of the negative correlation between adiponectin and maternal weight can result from several factors, but dramatic hormonal changes during pregnancy, such as increased levels of oestrogen, prolactin, cortisol, and testosterone that characterize normal human pregnancy, must be the main reason. Moreover, circulating adiponectin showed a strong negative relationship with visceral body fat and visceral body fat is strongly related with BMI in humans (Matsubara *et al.,* 2002; Cnop *et al.,* 2003; Lara-Castro *et al.,* 2006). It is doubtful if BCS is related to visceral body fat of the cow, except for a possible negative correlation in thin cows (Garnsworthy, 2006), and that is possibly another reason why circulating adiponectin was not influenced by BCS at calving. Lemor *et al.* (2010) showed that mRNAs of both adiponectin receptors were more highly expressed in ovine visceral than in ovine subcutaneous fat tissue, whereas mRNA of adiponectin did not differ. The other adipokine, leptin, measured in the present study was also not affected by BCS at calving, which supports the above suggestion. Also, according to Mousavinasab *et al.* (2005) only in

severely obese cases (BMI≥30 kg/m^2) with more than 10% decrease in BMI circulating adiponectin tended to increase in humans, although not statistically significantly.

In the present study, circulating insulin, glucagon, leptin, NEFA, BOHB, and urea were not correlated with circulating adiponectin. Some studies suggest that the relationship between adiponectin and leptin is negative (Matsubara *et al.,* 2002; Huypens, 2007). However, other studies reported positive correlation between leptin and adiponectin (Pardo *et al.,* 2004; Rossi *et al.,* 2005) or no correlation (Park *et al.,* 2004) which was the case in this experiment. In line with this study, Giahi *et al.* (2008) in overweight diabetic and non-diabetic men and Raddatz *et al.*, (2008) in dairy cows showed that circulating insulin was not correlated with circulating adiponectin. Yamauchi *et al.* (2001) showed that adiponectin is a putative stimulator of protein synthesis and inhibitor of protein degradation through activation of the insulin signalling pathway, but circulating adiponectin was uncorrelated with plasma urea in this experiment. Also, BCS, milk yield, MEBAL, and LWT were not correlated with circulating adiponectin in this experiment. Raddatz *et al.* (2008) found no correlation of BCS, insulin, milk yield, DMI, and energy corrected milk yield with adiponectin, in agreement with the present study.

It has been demonstrated that AMPK activation and deactivation in hypothalamic neural cells play a key role in monitoring energy status and regulating food intake (Andersson *et al.,* 2004; Minokoshi *et al.,* 2004; Claret *et al.,* 2007; Lim *et al.,* 2010). Leptin and adiponectin control AMPK with leptin to deactivate (Minokoshi *et al.*, 2008) and adiponectin to activate it (Kubota *et al.*, 2007) in hypothalamic neurons. Receptors of leptin and adiponectin have been found in various hypothalamic regions and presence of leptin and adiponectin have been ascertained in cerebrospinal fluid (Steinberg & Kemp, 2007). Also, other stimuli, such as insulin and glucose, deactivate AMPK (Claret *et al.,* 2007). Kubota *et al.,* (2007) showed that adiponectin enhances AMPK activity in the murine arcuate hypothalamus through its receptor AdipoR1 to stimulate food intake. In the present study, regression analysis demonstrated that adiponectin is likely to be related with DMI in dairy cows, but this relation is minimal and dependent on BCS at calving. The equations predict a negative relationship between circulating adiponectin and DMI for

THIN cows at calving, but a positive relationship for FAT cows at calving. In both equations, adiponectin beta coefficients are small compared with beta coefficients of other terms in the models, which suggests a minimal influence of circulating adiponectin on feed intake. Removal of adiponectin from the model reduced R^2 by 1% for THIN cows at calving and 0.2% for FAT cows. Thus, adiponectin has only a marginal positive or negative effect on DMI depending on BCS at calving. However, this finding needs to be investigated further.

AMY (aggregated milk yield at the end of the experiment) and PMY (previous milk yield) positively correlated with milk yield (AMY; *rho*= 0.753, PMY; *rho*= 0.473), DMI (AMY; *rho*= 0.595, PMY; *rho*= 0.365), and ΔBCS (AMY; *rho*= 0.434, PMY; *rho*= 0.281) Also, AMY positively correlated with PMY (*rho*= 0.513). Thus, milk yield, DMI, and ΔBCS were all correlated positively in the present study. This means that high yielding cows gave more milk, eat more feed, and lost more condition than low yielding cows. Moreover, ΔBCS negatively correlated with circulating adiponectin (*rho*= -0.173) and leptin (*rho*= -0.261). Therefore, high yielding cows had lower circulating adiponectin and leptin than low yielding cows. In support of this, AMY (*rho*= -0.117) and PMY (*rho*= -0.256) negatively correlated with circulating adiponectin, whereas PMY negatively correlated with circulating leptin (*rho*= -0.122). ΔBCS positively correlated with circulating GH (*rho*= 0.253) in this study. This means that high yielding cows lost more condition postpartum, and they had higher circulating GH but lower circulating adiponectin and leptin than low yielding cows. In congruence with this, circulating adiponectin (*rho*= -0.144) and leptin (*rho*= -0.319) were negatively correlated with circulating GH. Furthermore, ΔBCS positively correlated with milk yield (*rho*= 0.388), DMI (*rho*=0.382), and negatively correlated with circulating insulin (*rho*= -0.158). Additionaly, insulin positively correlated with leptin (*rho*= 0.356) and negatively correlated with circulating GH (*rho*= -0.287) in the present study. To sum up, high yielding cows in this experiment lost more condition postpartum, ate more feed and had higher circulating GH, milk yield and DMI but lower circulating adiponectin, leptin, and insulin than low yielding cows. It is known that selection for a higher yield increases DMI, GH, and BOHB (Veerkamp *et al.,* 2003; Leroy *et al.,* 2010) and this is in agreement with the findings of this study.

Moreover, high yielding cows lose more condition postpartum than low yielding cows (Roche *et al.,* 2009) and that is in line with the results. Leifers *et al.* (2005) suggested that leptin expression in adipose tissue is possibly regulated in early lactating cows by the double impact of insulin (positive) and GH (negative), which might be the case in the present study. This is the first study to suggest that due to increased circulating GH and its antagonistic relationship with adiponectin (and leptin), high yielding cows may have lower circulating leptin and adiponectin than low yielding cows. However, this result needs to be elucidated further.

4.5.4. Conclusions

To conclude, circulating adiponectin and milk adiponectin were measured in dairy cows in the present study. Milk adiponectin varied by day in milk and it was unaffected by average daily milk yield. Circulating adiponectin was influenced by lactational stage, but was not influenced by diet and BCS at calving. The negative correlation between circulating GH and adiponectin may explain the effect of lactational stage on plasma adiponectin concentrations. Diet and BCS at calving did not influence circulating adiponectin values, mainly because they did not change circulating GH. ΔBCS was correlated negatively with leptin and adiponectin and may be a useful tool to study circulating adipokines in periparturient and early lactating cows. Regression analysis showed that adiponectin is likely to be related with DMI in dairy cows, but this relationship is minimal and dependent on BCS at calving. High yielding cows may have lower circulating leptin and adiponectin, and higher circulating GH than low yielding cows. However, these results need to be investigated further.

5. Interrelationship of adiponectin with glucose homeostasis, and the effect of circulating adiponectin levels on reproductive performance in dairy cows

5.1. INTRODUCTION

Adiponectin is an adipokine secreted by adipose tissue, which improves the ratio of glucose to FFA and consequently has insulin sensitizing activity (Kadowaki & Yamauchi, 2005; Ahima, 2006). In the liver, adiponectin decreases glucose output, increases FFA oxidation, and increases influx of NEFA. In muscle tissue, adiponectin increases FFA utilization and stimulates glucose usage (Kadowaki & Yamauchi, 2005; Garaulet *et al.,* 2007; Shetty *et al.,* 2009).

Lam *et al.* (2004) reported that circulating adiponectin was low in patients suffered from acromegaly and circulating adiponectin increased after GH-lowering therapies. Nilsson *et al.* (2005) demonstrated that GH declined adiponectin secretion by human adipose tissue *in vitro* and *in vivo* in mice. Rodriguez-Pacheco *et al.* (2007) presented that short-term adiponectin exposure abolished both basal and stimulated (by Ghrelin and GnRH) secretion of GH by rat pituitary cells *in vitro*. According to López-Siguero *et al.* (2010) GH deficient children had higher circulating adiponectin than healthy controls. It is known that genetic selection for milk yield led to higher circulating GH in dairy cows (Veerkamp *et al.*, 2003) and that makes dairy cow a suitable animal model to study relations between GH and adiponectin. However, the relationship between GH and adiponectin in dairy cows has not yet been investigated.

Adiponectin and its receptors are present in theca and granulosa cells, oocytes and the corpus luteum (Lord *et al.,* 2005; Ledoux *et al.,* 2006; Ramachandran *et al.,* 2007; Chabrolle *et al.,* 2007a, 2007b). In sows, adiponectin is also present in follicular fluid at a concentration that is estimated to be 80–90% of the concentration in serum (Ledoux *et al.,*

2006). Adiponectin may influence steroidogenesis but contradictory results were reported (Ledoux *et al.*, 2006; Lagaly *et al.*, 2008; Chabrolle *et al.,* 2009; Gutman *et al.,* 2009; Pierre *et al.*, 2009; Maillard *et al.*, 2010). It has been hypothesized that adiponectin effects on the ovary are mediated through its insulin-sensitizing traits (Mitchell *et al.*, 2005) and through its effect on the action of IGF-I (Dupont *et al.*, 2008; Michalakis & Segars, 2010).

The present study utilized the data generated by the previous experiment (data generated by Garnsworthy *et al.* (2009), in which circulating adiponectin values were measured and added in the working dataset as detailed in Chapter 4). In this dataset, some cows had persistently elevated plasma adiponectin concentrations throughout the experimental period. In line with this, Raddatz *et al.,* (2008) measured circulating adiponectin in twenty-six (26) lactating Holstein cows for the first 11 weeks of lactation, and they reported that individual cows had consistently high or low adiponectin levels throughout the sampling period. In the present study, cows were categorized according to high or low circulating adiponectin level. The main hypothesis was that elevated plasma adiponectin concentrations (up to three times) may result in changes in hormonal and metabolic profile, and/or reproductive performance. This hypothesis has not previously been tested in dairy cows. Also, using a multivariate approach, the association of circulating adiponectin with other metabolic hormones and metabolites, and glucose homeostasis were explored in high yielding and low yielding cows. The interrelationships of adiponectin with the metabolic and hormonal components of glucose homeostasis have not been examined previously in dairy cows.

5.2. MATERIALS AND METHODS

5.2.1. Data

This study utilized the dataset generated by the study of Garnsworthy *et al.* (2009). In the current study, circulating adiponectin values were measured and added (Chapter 4). Key points of materials and methods, further statistical analysis, and handling of the data are presented hereinafter.

5.2.2. Experimental design

Data generated by the previous experiment (Chapter 4) were used in the present study. In this dataset, diet and BCS at calving had no effect on circulating adiponectin, whereas lactational stage affected plasma adiponectin concentrations. Moreover, some cows had persistently elevated plasma adiponectin concentrations (up to 3 times the average) throughout the experimental period. Scatter plot graphical representation of circulating adiponectin values (Figure 5.1) illustrated that cows with elevated plasma adiponectin concentrations had a tendency to express characteristic peaks of circulating adiponectin for more than two weeks throughout the experimental period. Based on this observation, cows were grouped into a HIGH adiponectin subgroup (6 cows; 57, 94, 146, 219, 330, and 331, average circulating adiponectin; 21.2±1.32 *ng/ml*) and a NORMAL subgroup (54 cows, average circulating adiponectin; 7.1±0.23 *ng/ml*). Thus, the main factor studied in this experiment was ADIPONECTIN LEVEL (HIGH *versus* NORMAL).

The experimental design was a retrospective case-control epidemiological study. This design is highly susceptible to bias error, sources of which are not always obvious. However, there are occasions in which this design is the best choice in practice. This is where the examined trait is rare or takes a long time to develop (Woodward, 1999).

Multivariate analysis was performed to examine the associations of adiponectin with other metabolic hormones and metabolites, and central glucose homeostasis. This analysis and data arrangement are explained in § 5.3.2.

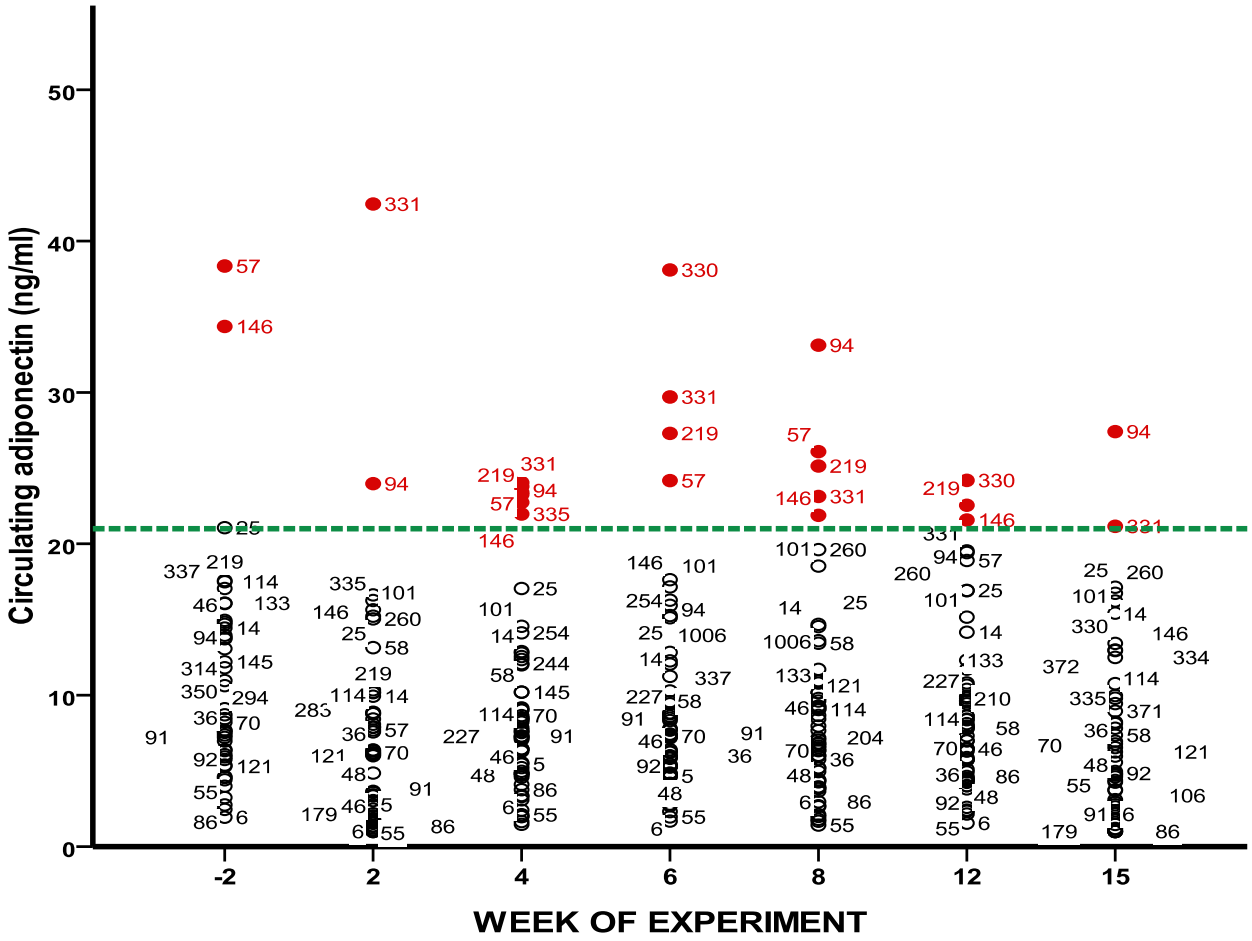


Figure 5.1: Scatter plot graphical representation of circulating adiponectin values (*n*= 387) by week of experiment. Outlier adiponectin values came consistently from 6 cows (id; 57, 94, 146, 219, 330, and 331) which showed the same trend (more than two weeks throughout the experimental period) for higher adiponectin values.

5.2.3. Feeding and milking

These are described in § 3.2.3.

5.2.4. Reproductive management

Cows were artificially inseminated at the first or second oestrus. Insemination was repeated at any subsequent oestrus until the end of the experiment at 120 days post partum. Oestrus was detected using a combination of behavioural observations, pedometer activity monitoring and milk progesterone profiles. Milk progesterone was monitored daily from 4 days before expected oestrus until signs of oestrus were detected; monitoring then returned to twice in a week until 4 days before the next expected oestrus (21 days later). Progesterone profiles were used subsequently to classify oestrous cycles as normal or abnormal (DOV1, DOV2, PCL1 or PCL2), following the definitions of Lamming & Darwash (1998) (Table A.2 in appendix).

Cows that had not been inseminated, or were not cycling normally, by 90 days post partum were scanned by trans-rectal ultrasound (Aloka SSD-500 scanner equipped with a 5-MHz linear array transducer, Aloka Co., Ltd., Tokyo, Japan) to investigate the existence of cystic body.

5.2.5. Recording, sampling and analysis

Milk yield and feed intake were recorded daily throughout the experiment whereas previous milk yield obtained from dairy records. Live weight and BCS (units; 0-5) were recorded weekly whereas ΔBCS calculated as BCS at calving minus BCS at 15 week postpartum. Collection and preparation of the samples, and all methods and approaches were used to measure animal productive traits, metabolites, and metabolic hormones are analytically described in the appendices and § 4.2.2.4.

5.3. STATISTICAL ANALYSIS

5.3.1 Effect of Adiponectin levels on metabolic and hormonal profiles, and reproductive performance

Data were analyzed by using PASW© 18 Edition (SPSS Inc., Chicago, USA). Generalized Estimating Equations (GEE) and Generalized Linear Models (GLM) were used to test specific hypotheses (Lindsey, 1997; McCullagh & Nelder, 1989; Dobson, 2002; Horton & Lipsitz, 1999).

Family distribution and link function were selected as detailed in § 3.3. Selection of GLM and GEE models was performed based on AIC and QIC criterion, as detailed in § 3.3.

Parameter estimates of GEE models are presented as marginal means plus/minus standard error of the difference (SED) in tables. Estimated marginal means, SEs and SEDs were obtained by using *Least Significant Difference* (*LSD*) adjustment for multiple comparisons in PASW© 18 statistical program. *P* values were calculated by *Newton-Raphson Maximum Likelihood (ML)* method and effects were considered statistically significant when *P* value was less than 0.05. *P* values equal or less than 0.1 were considered to show a trend.

The GEE and GLM models selected to test the effects of ADIPONECTIN LEVEL on productive traits, reproductive performance, and hormonal and metabolic profile are illustrated by Tables 5.1, 5.2, & 5.3. The effects of ADIPONECTIN LEVEL and leptin on days to first oestrus (days to oestrus were count data and in such data *Poisson* distribution function with *log* link function fits better) were assessed by fitting GLMs (Tables 5.1 & 5.2). The effect of ADIPONECTIN LEVEL on the probability for cows to be pregnant was examined by fitting a generalized logistic regression model (Table 5.2). Cows non pregnant at the end of the experiment were censored at 120 days post partum and *Kaplan–Meier* estimates of the survivor function were compared for cows with HIGH and

NORMAL adiponectin levels by using *Log-rank test* (Landau & Everitt, 2004; Jenkins, 2005; Guo, 2010). The models selected for survival analysis are illustrated in Table 5.4.

Data from milk progesterone profiles were pooled into two groups; cows with NORMAL milk progesterone profile (37 cows) and cows with ABNORMAL milk progesterone profile (23 cows; 5cows with DOV1, 8 cows with DOV2, 1 cow with PCL1, and 9 cows PCL2). In this way, milk progesterone profile was transformed to a binary variable, and causal effects of other continuous and binary variables on it were examined by common logistic regression (Table 5.3). Presence of a follicular cyst at day 90 (CYSTIC BODY) was binary output (8 cows were found with follicular cysts at 90 days postpartum; the remaining 52 cows had no cyst) and causal effects were examined by multiple logistic regression models (Table 5.3). The outputs of generalized logistic regression and multiple logistic regression models are presented as probabilities, unstandardized beta (*b*) coefficients, and odds ratio (exp (*b*)) with 95% confidence intervals. Fitness of multiple logistic regression models was assessed as detailed in § 3.3.

Receiver Operating Characteristic (ROC) analysis was conducted to evaluate the performance and the accuracy of logistic regression models, as detailed in § 3.3.

A *Shapiro-Wilk test* was used to assess whether the distribution of variables was normal. Non-random association between two categorical variables was tested by cross-tabulation and Fischer's exact test. *P* values equal or less than 0.05 were considered statistically significant whereas *P* values equal or less than 0.1 were considered to show a trend.

Table 5.1: Selected models for analysis of the effect of circulating adiponectin levels on metabolites, metabolic hormones, and production traits

RESPONSE VARIABLE *(Y)*	TYPE OF MODEL	PREDICTORS *(X)*	SUBJECT (ID) VARIABLE	WITHIN-SUBJECT VARIABLE	CONTINUOUS COVARIATES	ERROR DISTRIBUTION FUNCTION	LINK FUNCTION	COVARIANCE MATRIX	WORKING CORRELATION MATRIX	STATISTICS
●Insulin **●Glucagon** **●Urea** **●DMI** **●Milk yield** **●LW** **●MEBAL**	*GEE*	●ADP LEVELS	COW (60)	WEEK	PARITY	*Gaussian (Normal)*	*Identity (ID)*	*Robust*	*Exchangeable*	n=377; QIC=23 n= 216; QIC=126,067 n=249; QIC=167 n=352; QIC=4,041 n=352; QIC=27,836 n=352; QIC=255,866 n=352; QIC=85,754
●Adiponectin	*GEE*	●ADP LEVELS	COW (60)	WEEK	PARITY	*Gamma*	*log*	*Robust*	*Exchangeable*	n=387; QIC=166
●Leptin	*GEE*	●ADP LEVELS	COW (60)	WEEK	PARITY GH	*Gaussian (Normal)*	*Identity (ID)*	*Robust*	*Exchangeable*	n=204; QIC=232
●ΔBCS **●BCS at calving**	*GLM*	●ADP LEVELS	-	-	PARITY	*Gaussian (Normal)*	*Identity (ID)*	*Robust*	-	n= 59; AIC=69; *LR*(χ^2=5.9, *df*=1, *P*=0.01) n= 60; AIC=35; *LR*(χ^2=4.1, *df*=1, *P*=0.04)
●IGF-I	*GEE*	●ADP LEVELS	COW (60)	WEEK	PARITY	*Gaussian (Normal)*	*Identity (ID)*	*Robust*	*Exchangeable*	n=197;; QIC=516,092
●Glucose	*GEE*	●ADP LEVELS	COW (60)	WEEK	PARITY GH Leptin	*Gaussian (Normal)*	*Identity (ID)*	*Robust*	*Exchangeable*	n=188; QIC=64
●GH **●BOHB** **●NEFA**	*GEE*	●ADP LEVELS	COW (60)	WEEK	PARITY	*Inverse Gaussian*	*Log*	*Robust*	*Exchangeable*	n=206; QIC=35 n=248; QIC=113 n=249; QIC=333

Table 5.2: Selected models for analysis of reproductive traits

RESPONSE VARIABLE *(Y)*	TYPE OF MODEL	PREDICTORS *(X)*	CONTINUOUS COVARIATES	ERROR DISTRIBUTION FUNCTION	LINK FUNCTION	COVARIANCE MATRIX	STATISTICS
●**Pregnancy** *(binomial variable; 0= non pregnant,1= pregnant)*	*GLM*	●ADP LEVELS	PARITY	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=60; AIC=75; *LR*(χ^2=4.7, *df*=1, *P*=0.03)
●**Pregnancy** *(binomial variable; 0= non pregnant,1= pregnant)*	*GLM*	●ΔBCS	PARITY	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=59; AIC=73; *LR*(χ^2=10.9, *df*=2, *P*=0.004)
●**Pregnancy** *(binomial variable; 0= non pregnant,1= pregnant)*	*GLM*	●IGF-I	PARITY	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=60; AIC=73; *LR*(χ^2=4.9, *df*=1, *P*=0.02)
●**Pregnancy** *(binomial variable; 0= non pregnant,1= pregnant)*	*GLM*	●Milk P4 Profile	PARITY	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=60; AIC=75; *LR*(χ^2=7.5, *df*=2, *P*=0.024)
●**Pregnancy** *(binomial variable; 0= non pregnant,1= pregnant)*	*GLM*	●Milk Yield	PARITY	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=60; AIC=78; *LR*(χ^2=4.3, *df*=1, *P*=0.038)
●**Days to first oestrus**	*GLM*	●Leptin	PARITY	*Poisson*	*log*	*Model based*	n=58; AIC=587; *LR*(χ^2=29.3, *df*=2, *P*=0.001)
●**Days to first oestrus**	*GLM*	● ΔBCS	PARITY	*Poisson*	*log*	*Robust*	n=57; AIC=571; *LR*(χ^2=11.2, *df*=2, *P*=0.003)
●**Days to first oestrus**	*GLM*	●ADP LEVELS	PARITY	*Poisson*	*log*	*Model based*	n=58; AIC=595; *LR*(χ^2=21.1, *df*=2, *P*=0.001)
●**Days to conception**	*GLM*	-	Insulin Milk yield IGF-I	*Poisson*	*log*	*Robust*	n=21; AIC=207; *LR*(χ^2=31.5, *df*=3, *P*=0.001)
●**Days to conception**	*GLM*	●Days to first oestrus	PARITY	*Poisson*	*log*	*Robust*	n=21; AIC=213; *LR*(χ^2=8.5, *df*=2, *P*=0.01)

Table 5.3: Selected models for analysis of milk progesterone profile and cystic body

RESPONSE VARIABLE *(Y)*	TYPE OF MODEL	PREDICTORS *(X)*	CONTINUOUS COVARIATES	ERROR DISTRIBUTION FUNCTION	LINK FUNCTION	COVARIANCE MATRIX	STATISTICS
● **MILK P4 PROFILE** *(binomial variable; 0= NORMAL, 1= ABNORMAL)*	*GLM*	● Days to oestrus	PARITY	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=58; AIC=74; *LR*(χ^2=9.4, *df*=2, *P*=0.01)
● **MILK P4 PROFILE** *(binomial variable; 0= NORMAL, 1= ABNORMAL)*	*GLM*	● Adiponectin	PARITY	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=60; AIC=74; *LR*(χ^2=6, *df*=2, *P*=0.05)
● **MILK P4 PROFILE** *(binomial variable; 0= NORMAL, 1= ABNORMAL)*	*Multiple logistic regression*	● Milk yield ●DMI ●GH	PARITY	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=59; AIC=78; LR(χ^2=10.6, *df*=4, *P*=0.031)
●**CYSTIC BODY** *(binomial variable; 0= no cyst, 1= follicular cyst)*	*GLM*	● Days to oestrus	PARITY	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=58; AIC=45; LR(χ^2=7.1, *df*=2, *P*=0.03)
●**CYSTIC BODY** *(binomial variable; 0= no cyst, 1= follicular cyst)*	*Multiple logistic regression*	●Insulin ●GH ●Glucose ●NEFA	PARITY	*Bernoulli (Binomial)*	*Logit*	*Robust*	n=57; AIC=32; LR *(*χ^2=25.3, *df*=5, *P*=0.001)

Table 5.4: Selected models for survival analysis (Kaplan- Meier models) of interval from calving to conception

TIME VARIABLE (*Survival time*)	TYPE OF MODEL	STATUS VARIABLE (*Event variable*)	FACTORS *(Group variables*)	SURVIVAL FUNCTIONS COMPARISON TEST	OBSERVATIONS	RIGHT CENCORED OBSERVATIONS	UNCENCORED OBSERVATIONS
Days post partum *(0- 120 days post partum)*	*Kaplan-Meier*	Pregnancy *(binomial variable; 0=non pregnant 1=event= pregnant)*	●ADP LEVELS	*Log-Rank (Mantel- Cox)*	60	39	21

5.3.2 Exploration of adiponectin interrelationships with glucose, BCS, metabolic hormones, and metabolites: a multivariate approach

Data were analyzed by using AMOS© (AMOS Development Corporation, Spring House, PA, USA). AMOS© incorporates structural equation modelling (SEM) and path diagrams analysis (PDA). AMOS© is a relatively new graphical SEM analysis tool that can fit multiple models in a single analysis by constraining parameters within the models (Cunningham & Wang, 2005; Arbuckle, 2007). SEM and PDA enable researchers to answer a set of interrelated research questions in a single, systematic, and comprehensive analysis by modelling the relationships among multiple independent and dependent variables simultaneously (Gefen *et al.*, 2000; Ahn, 2002; Kline, 2005). The SEM approach is asymptotically the same as classical regression when its assumptions are satisfied. Regression works by minimizing variance but SEM is based on methods of maximization of likelihood (Gefen *et al.,* 2000). The main assumption of SEM is achievement of multivariate normality, but deviations from multivariate normality may or may not affect the result of analysis (Kline, 2005; Cunningham & Wang, 2005; Arbuckle, 2007).

The data used for this analysis were averaged by cow for each measured trait, metabolite, and metabolic hormone (*i.e.* one value per cow for adiponectin, leptin, etc) across the whole experimental period. Using this "main data set", a basic model was constructed for circulating glucose and its interrelationships with adiponectin, insulin, leptin, glucagon, GH, BOHB, NEFA, and BCS. The fitness of this basic model was ascertained by using fit indices and chi-square statistics and the data were imputed by using *Bayesian imputation* (*MCMC algorithm*, for 10,000 observations assuming 0.1 autocorrelation) (Jackman, 2004; Dunson *et al.*, 2005; Carter, 2006; Arbuckle, 2007). In this way, ten complete data sets plus the original were generated. The data sets were appended to the main data set and averaged by cow. Finally, a complete data set was created, containing one value for each measured trait, metabolite, and metabolic hormone for each individual cow. In this complete data set, the validity of the basic model was tested again using fit indices and chi-square statistics and a 1,000 samples *Bollen-Stine* bootstrap for nonparametric data. For

non-normal data, *Bollen-Stine* bootstrap can provide the correct P values for the chi-square statistic to assess overall model fit. *Bollen-Stine* bootstrap uses the same chi-square hypothesis structure, at the conventional significance level of 0.05, to test the fitness of the model. If the *P* value is larger than 0.05 the model is accepted, and thus conclude that the model fits the data well, whereas the model is rejected if *P* value is smaller than 0.05 (Arbuckle, 2007). The basic model showed excellent fitness to the data, so it was used as the source model for constructing further models.

Cows were classified according to milk yield in the previous lactation (PMY) with a cut-off point between high and low yielding cows of 10,000 kg/cow/lactation. PMY was modelled as a factor with two levels; LOW yielders (30 cows, 8,044 ±395 kg/lactation) and HIGH yielders (30 cows, 12,726±366 kg/lactation). DMI, milk yield, ΔBCS, and BCS at calving were assessed by multivariate analysis of variance (MANOVA) for statistically significant differences between PMY groups in the present lactation. *Wilks' lambda (λ) test* was used to examine the overall significance of the model. When the overall model was significant ($P<0.05$), then simultaneous effects of the independent variables on the dependent variable existed. *Box's test was* used to test the equality of covariance between the groups with insignificant *P* values ($P\geq0.05$) to denote that covariance matrices of the dependent variable were equal across groups. *Levene's test* examined whether or not the variance between independent variable groups was equal. Non-significant *P* values ($P\geq0.05$) of *Levene's test* denoted equal variance between groups. Power was the probability *of* correctly accepting or rejecting hypotheses based on the results of the test (Field, 2005; PASW© 18 Users guide, 2009). Differences in parity between PMY groups were assessed by Student's t-test (*two-sided*). This analysis was performed to confirm LOW PMY cows and HIGH PMY cows were low yielding and high yielding cows, respectively, in the present lactation. Because the animals retrospectively allocated to PMY groups, a *Fisher's exact test* performed to assess if PMY blocking of animals was biased by the original dietary treatments.

Glucose interrelationships with adiponectin, metabolites, and metabolic hormones in cows with HIGH and LOW previous milk yield were examined by applying and modifying the

basic model in the "main data set", so that the maximum fitness to be achieved, and under the condition that previous milk yield was HIGH or LOW. The validity and the fit of the models derived from the basic model were tested by model fit indices (Table 5.5) and comparison of the original correlation matrices with the model-based correlation matrices (Schreiber *et al.*, 2006; Arbuckle, 2007). Also, models were run by *Bayesian Estimation method* and the Deviance Information Criterion (DIC) was used to determine the fit of the models to the data.

Model parameter estimates (mean±SE) are illustrated in Table B.1 in appendix.

Table 5.5: Model fit criteria and acceptable fit interpretation

Model Fit Criterion	Acceptable level	Interpretation
●*Chi-square statistics* ***(χ^2)***	*P*>0.05 indicates good fit	Compares obtained chi-square value with table value for given degrees of freedom
●*Bollen-Stine nonparametric bootstrap*	*P*>0.05 indicates good fit	Provide the correct *P* value for chi-square statistic when data are non-normal.
●*Comparative fitness index (**CFI**)*	0 (no fit) to 1 (perfect fit)	Value close to 0.95 reflects a good model fit
*Normed fit index (**NFI**)*	0 (no fit) to 1 (perfect fit)	Value close to 0.95 reflects a good model fit
●*Root-mean-square error of approximation (**RMSEA**)*	<0.05 (good fit model) >1.0 (not acceptable)	Value less than 0.05 or 0.08 indicates a good model fit Value more than 1.0 indicates the model is not acceptable
●*Akaike's information criterion(**AIC**)*	The smaller value is the better	Compares values in alternative models
●*Deviance Information Criterion (**DIC**)*	Posterior predictive *P* value should be near 0.5 for a correct model, with values toward the extremes of 0 or 1 indicating that a model is not plausible	DIC is for comparing the fit of alternative models, with smaller values being better. DIC cannot be used to evaluate a single model in absolute terms.

Source: Arbuckle, 2007; Kline, 2005; Bollen & Curran, 2006

5.4. RESULTS

5.4.1. The effect of circulating adiponectin levels on metabolic and hormonal profile, production traits, and reproduction

5.4.1.1. Reproductive performance

Interval from calving to conception between cows with HIGH adiponectin levels and cows with NORMAL adiponectin levels were not statistically significant different (Figure 5.2). Cows with NORMAL milk progesterone profiles were more likely to become pregnant (48% *versus* 10%, AUC_{ROC}=0.73±0.06) during the experimental period than cows with ABNORMAL milk progesterone profiles (P<0.05) (Table 5.6). Cows with HIGH adiponectin levels had a higher probability to be pregnant (83% *versus* 30%, AUC_{ROC}=0.67±0.07) during the experimental period than cows with NORMAL adiponectin levels (P<0.05) (Table 5.7). Cows with HIGH adiponectin levels had lower days to first oestrus (43.1 *versus* 56) than cows with NORMAL adiponectin levels (P<0.05) (Table 5.7). There was a tendency for non-random association between cows with HIGH adiponectin levels and cows with NORMAL milk progesterone profile (P<0.1) because no cow with HIGH adiponectin had ABNORMAL profiles (Table 5.8). There was no association between adiponectin level and presence of follicular cysts at day 90 of lactation (Table 5.9).

Logistic regression analysis showed a significant association between change in BCS (ΔBCS) and the probability for a cow to be pregnant. Cows losing 0.25 units of BCS or less were more likely to become pregnant (56% *versus* 30%, AUC_{ROC}=0.72±0.06) than cows losing 0.70 units of BCS or more (Figure 5.3). The association between circulating IGF-I and probability to be pregnant was significant. Cows with average circulating IGF-I equal or greater than 155 *ng/ml* were more likely to be pregnant (46% *versus* 33%, AUC_{ROC}=0.70±0.07) than cows with average circulating IGF-I equal or less than 120 *ng/ml*

throughout the experimental period (Figure 5.4). Days to first oestrus were significantly associated with the probability of cows to express ABNORMAL milk progesterone profiles. Cows with average 43.1 days to first oestrus were less likely (25% *versus* 36%, AUC_{ROC}=0.72±0.07) to express ABNORMAL milk progesterone profiles than cows with average 56 days to first oestrus (Figure 5.5), whereas days to first oestrus were significantly associated with the probability for cows to express cystic body at day 90 postpartum (Figure 5.6). Moreover, ΔBCS significantly associated with days to first oestrus (Figure 5.7) and days to first oestrus positively associated with days to conception (Figure 5.8). Circulating adiponectin was significantly associated with the probability for cows to express ABNORMAL milk progesterone profiles (Figure 5.9), whereas circulating leptin was significantly associated with the days to first oestrus (Figure 5.10). Milk yield was significantly associated with the probability of cows to be pregnant (Figure 5.11).

Multiple logistic regression analysis examined the effects of milk yield, DMI, GH, and parity on milk progesterone profile. Each exponentiated coefficient in this model is the change in odds for a unit increase in the corresponding predictor variable holding other variables at certain value. Odds ratio (exp (*b*)) for milk yield was 1.21 (P<0.05) (Table 5.10). So holding GH, DMI and parity at a fixed value, an increase of milk yield by 1 *kg/d* will increase the odds for cows to express ABNORMAL milk progesterone profile by 21%. GH and DMI tended to influence milk progesterone profile (P<0.1). GH odds ratio was 1.31 (P=0.07); DMI odds ratio was 0.68 (P =0.07) (Table 5.10). The odds ratio for GH and DMI say that, holding the other predictors of the model at a fixed value, a one-unit increase in GH was expected to result in a 31% increase, and a one-unit increase in DMI a 32% decrease, in the odds of cows to express ABNORMAL milk progesterone profiles. Parity also tended to influence milk progesterone profile (P<0.1). The odds ratio for parity says that, holding the other predictors of the model at a fixed value, an increase of parity by one year resulting in an 86% rise in the odds of cows to express ABNORMAL milk progesterone profiles.

Multiple logistic regression analysis examined the effects of insulin, glucose, GH, NEFA, and parity on presence of follicular cysts at 90 days postpartum (Table 5.11). Odds ratios

for insulin and glucose were close to zero and that means strong negative effects on follicular cyst formation (P<0.05). Odds ratios for GH and NEFA were 2.1 and 201.6 respectively, indicating high positive effects of GH and NEFA on follicular cyst formation (P<0.05). Parity had no effect on follicular cyst formation.

Multiple poisson regression analysis examined the effects of insulin, milk yield, and IGF-I on days to conception (Table 5.12). Insulin, milk yield, and IGF-I *beta* coefficients were 0.98, 0.014, and -0.002, respectively (P<0.05). Beta coefficient of insulin is bigger than the *beta* coefficients of milk yield and IGF-I and that means the insulin effect on days to conception is bigger than the effect of milk yield and IGF-I. Insulin concentration greater than 0.7 *ng/ml* resulted in calving to conception interval greater than 100 days, whereas animals with insulin concentration within the range 0.2-0.3 *ng/ml* had a calving to conception interval of less than 70 days (Table 5.13).

5.4.1.2. Metabolic profile, hormonal profile, and productive traits

Cows with HIGH adiponectin levels had higher circulating adiponectin and IGF-I, but lower circulating GH and glucose than cows with NORMAL adiponectin levels (P<0.05) (Table 5.14). Cows with HIGH adiponectin levels showed a tendency for higher plasma concentrations of leptin, but lower circulating BOHB than cows with NORMAL adiponectin levels (P<0.1). Adiponectin levels had no effect on plasma concentrations of insulin, glucagon, NEFA, and blood urea (Table 5.14).

Cows with HIGH adiponectin levels had lower DMI, milk yield, BCS at calving, and ΔBCS than cows with NORMAL adiponectin levels (P<0.05) (Table 5.15). Adiponectin levels had no effect on LWT and MEBAL (Table 5.15).

5.4.2. Adiponectin interrelationships with glucose, BCS, metabolic hormones, and metabolites

There was no association between PMY and diet, and thus the retrospective classification of cows as PMY LOW and PMY HIGH was not biased by the original dietary treatments (*Fisher's exact test*, n=60, P=0.325, *two–sided*).

Multivariate analysis revealed that glucose balance in dairy cows was regulated by the negative influences of circulating adiponectin (-0.26, *P*=0.014), BOHB (-0.36, *P*=0.001), and glucagon (-0.23, *P*=0.036) on circulating glucose, and the positive influence of circulating insulin (0.24, *P*=0.036) on circulating glucose (Figure 5.12). GH, NEFA, leptin, and BCS had no direct effect on circulating glucose, but GH was negatively correlated with adiponectin (-0.24, *P*=0.042) and leptin (-0.35, *P*=0.001) whereas leptin was positively correlated with insulin (0.34, *P*=0.012) (Figure 5.12).

In low yielding cows, glucose balance was regulated by the negative influences of circulating adiponectin (-0.31, *P*=0.041) and glucagon (-0.31, *P*=0.043) on circulating glucose, and the positive influence of circulating insulin (0.44, *P*=0.008) on circulating glucose (Figure 5.13). GH, NEFA, leptin, and BCS had no direct effect on circulating glucose, but leptin was positively correlated with insulin (0.42, *P*=0.022).

In high yielding cows, glucose balance was regulated by the negative influences of BCS (-0.51, *P*=0.01) and BOHB (-0.48, *P*=0.001), and the positive influence of circulating insulin (0.45, *P*=0.035) on circulating glucose (Figure 5.14). GH, NEFA, leptin, and glucagon had no direct effect on circulating glucose. However, a multitude of interrelations between metabolites and hormones was revealed. Glucagon was positively correlated with leptin (0.48, *P*=0.004), but negatively correlated with BCS (-0.28, *P*=0.049). Adiponectin was positively correlated with BCS (0.34, *P*=0.031), but negatively correlated with GH (-0.34, *P*=0.031). GH was negatively correlated with BOHB (-0.31, *P*=0.05), BCS (-0.40, *P*=0.027), and leptin (-0.44, *P*=0.01). Leptin was positively correlated with NEFA (0.29,

P=0.003). Insulin was positively correlated with NEFA (0.45, *P*=0.02), BCS (0.58, *P*=0.027), and leptin (0.39, *P*=0.02).

One-way MANOVA revealed a significant multivariate main effect for PMY (Wilks' λ = 0.787, F=3.66, *P*=0.01, *df*=54, *Box's test P*=0.112, Power =0.850). Given the significance of the overall MANOVA model, the univariate main effects were examined. High PMY cows had higher milk yield (47.1±1.27 *kg/d versus* 40.3±1.25 *kg/d*, F=14.4, *df*=57, *P*=0.001, *Levene's test P*=0.937, Power=0.962), DMI (22.5±0.47 *kg/d versus* 20.5±0.46 *kg/d*, F=8.7, *df*=57, *P*=0.006, *Levene's test P*=0.466, Power=0.827), and BCS at calving (3.52±0.050 units *versus* 3.35±0.060 units, F=4.6, *df*=57, *P*=0.035, *Levene's test P*=0.005, Power=0.563), and a trend for higher ΔBCS (0.76±0.082 units *versus* 0.56±0.082 units, F=2.54, *df*=57, *P*=0.1, *Levene's test P*=0.662, Power=0.347) than low PMY cows in the present lactation.

Parity was not statistical different for high PMY cows compared to low PMY cows (2.77±0.190 lactations *versus* 2.40±0.190 lactations, *t* = 1.39, *df*=58, *P*=0.17).

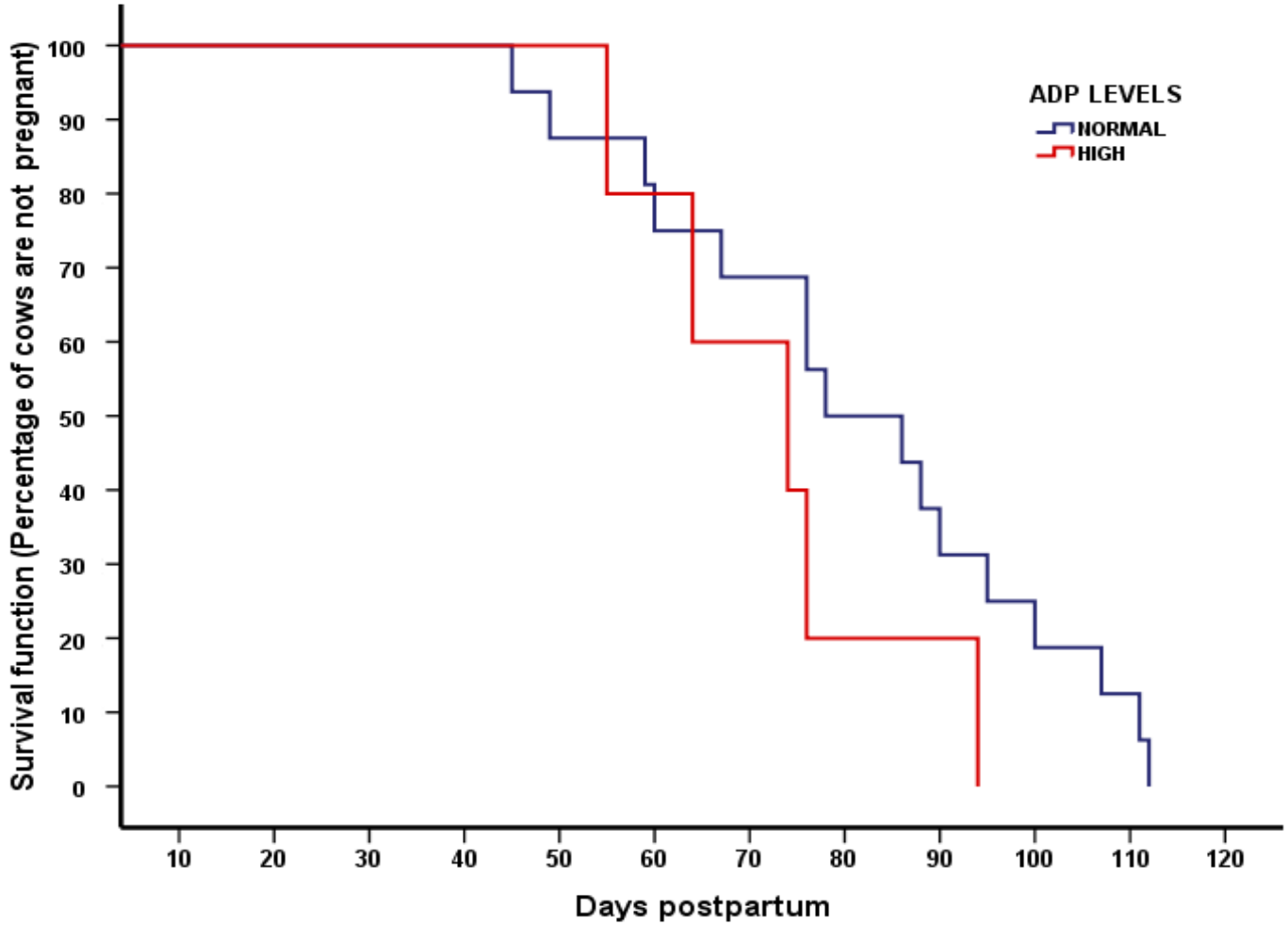


Figure 5.2: Calving-to-conception survival analysis curves for cows with HIGH and NORMAL adiponectin (ADP) levels. There was no difference in the survival curves for cows with HIGH and NORMAL adiponectin levels (*n=60, Log-rank,* P = 0.18). Vertical drop in the survival curves indicates an event (a cow became pregnant). Interval from calving to conception between cows with HIGH (74± 10.9 days) and NORMAL (78±10.0 days) adiponectin levels were not significantly different.

Table 5.6: Effect of milk progesterone profile on probability of cows to be pregnant

Treatment:	MILK PROGESTERONE PROFILE			
Parameters:	**NORMAL**[†]	**ABNORMAL**[†]	***n***	***P***
Probability for cows to be pregnant	0.48 (0.32, 0.65)	0.01 (0.00, 0.27)	60	0.001

[†] **Columns are means with 95 % Confidence Interval (CI).**

Table 5.7: Effect of circulating adiponectin levels (ADP LEVELS) on probability of cows to be pregnant

Treatment:	ADP LEVELS			
Parameters:	**NORMAL**[†]	**HIGH**[†]	***n***	***P***
Days to first oestrus (*days postpartum*)	56.0 (51.6, 60.4)	43.1 (36.8, 49.3)	56	0.001
Probability for cows to be pregnant	0.30 (0.17, 0.42)	0.83 (0.53, 0.99)	60	0.031

[†] **Columns are means with 95 % Confidence Interval (CI).**

Table 5.8: Crosstabulation of circulating adiponectin levels (ADP LEVELS) with milk progesterone profile. Association between ADP LEVELS and milk progesterone profile was tested by performing *Fisher's exact test* (n= 60, *P*=0.073, *two-sided*). There was a tendency for non-random association between cows expressing HIGH circulating adiponectin levels and NORMAL milk progesterone profile, and between cows expressing NORMAL adiponectin levels and ABNORMAL milk progesterone profile.

	ADP LEVELS		
Milk progesterone profile	**NORMAL**	**HIGH**	**Total**
NORMAL	31	6	37
ABNORMAL	23	0	23
Total	54	6	60

Table 5.9: Crosstabulation of circulating adiponectin levels (ADP LEVELS) with follicular cysts found at day 90 postpartum (CYSTIC BODY). Association between ADP LEVELS and CYSTIC BODY was tested by performing *Fisher's exact test* (n= 60, *P*= 0.585, *two–sided*). There was no association between adiponectin level and cystic body.

	ADP LEVELS		
CYSTIC BODY	**NORMAL**	**HIGH**	**Total**
NO	46	6	52
YES	8	0	8
Total	54	0	60

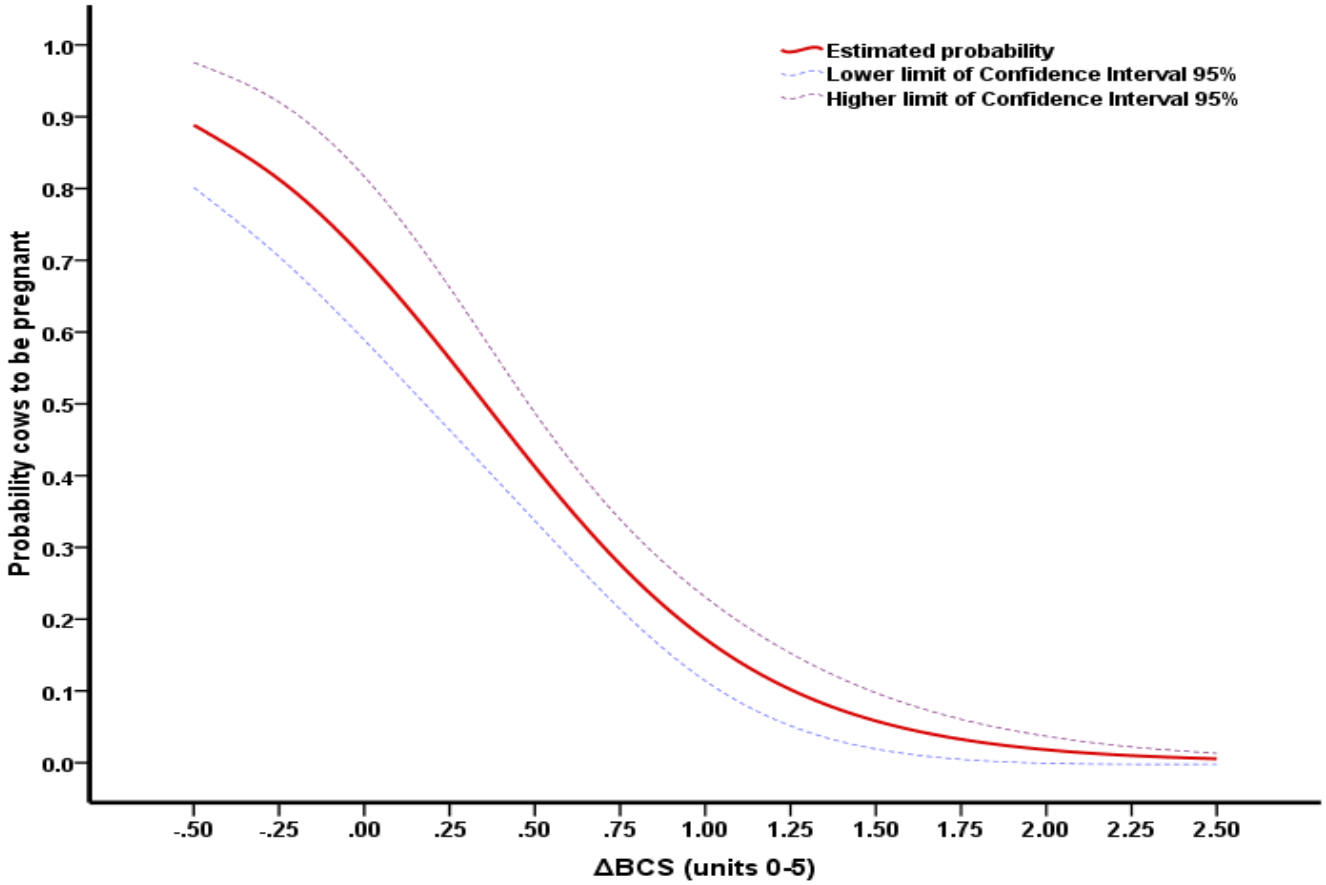


Figure 5.3: Effect of postpartum change in body condition score (ΔBCS) on probability of cows to be pregnant (n= 59, *P*=0.001, exp (*b*) =0.09, CI for exp (*b*); [0.02, 0.37], *beta* coefficient (*b*) = -2.43, AUC_{ROC}=0.72±0.06, GLM with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was pregnancy (0= non pregnant, 1= pregnant) whereas ΔBCS and parity were added as continuous covariates. Red solid line represents the estimated probability whereas blue and purple dashed lines show the upper and lower 95 % Confidence Interval (CI). ΔBCS values with negative sign indicate gain of BCS whereas positive ΔBCS values denote loss of BCS.

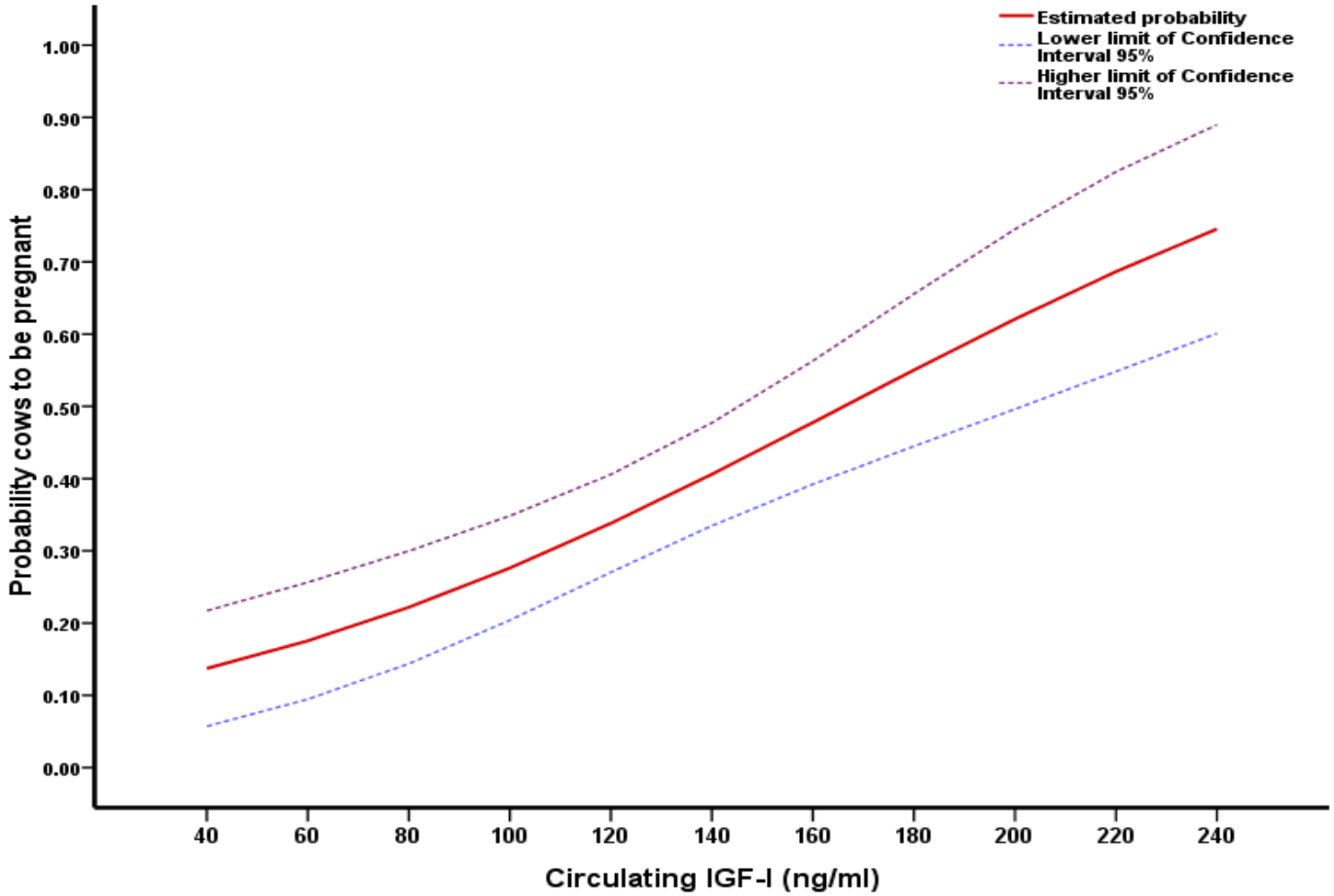


Figure 5.4: Effect of circulating IGF-I on probability of cows to be pregnant (n= 60, *P*=0.027, exp (*b*) =1.01, CI for exp (*b*); [1.002, 1.028], *beta* coefficient (*b*) = 0.015, AUC_{ROC}=0.70±0.07, GLM with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was pregnancy (0= non pregnant, 1= pregnant) whereas circulating IGF-I and parity were added as continuous covariates. Red solid line represents the estimated probability whereas blue and purple dashed lines show the upper and lower 95 % Confidence Interval (CI).

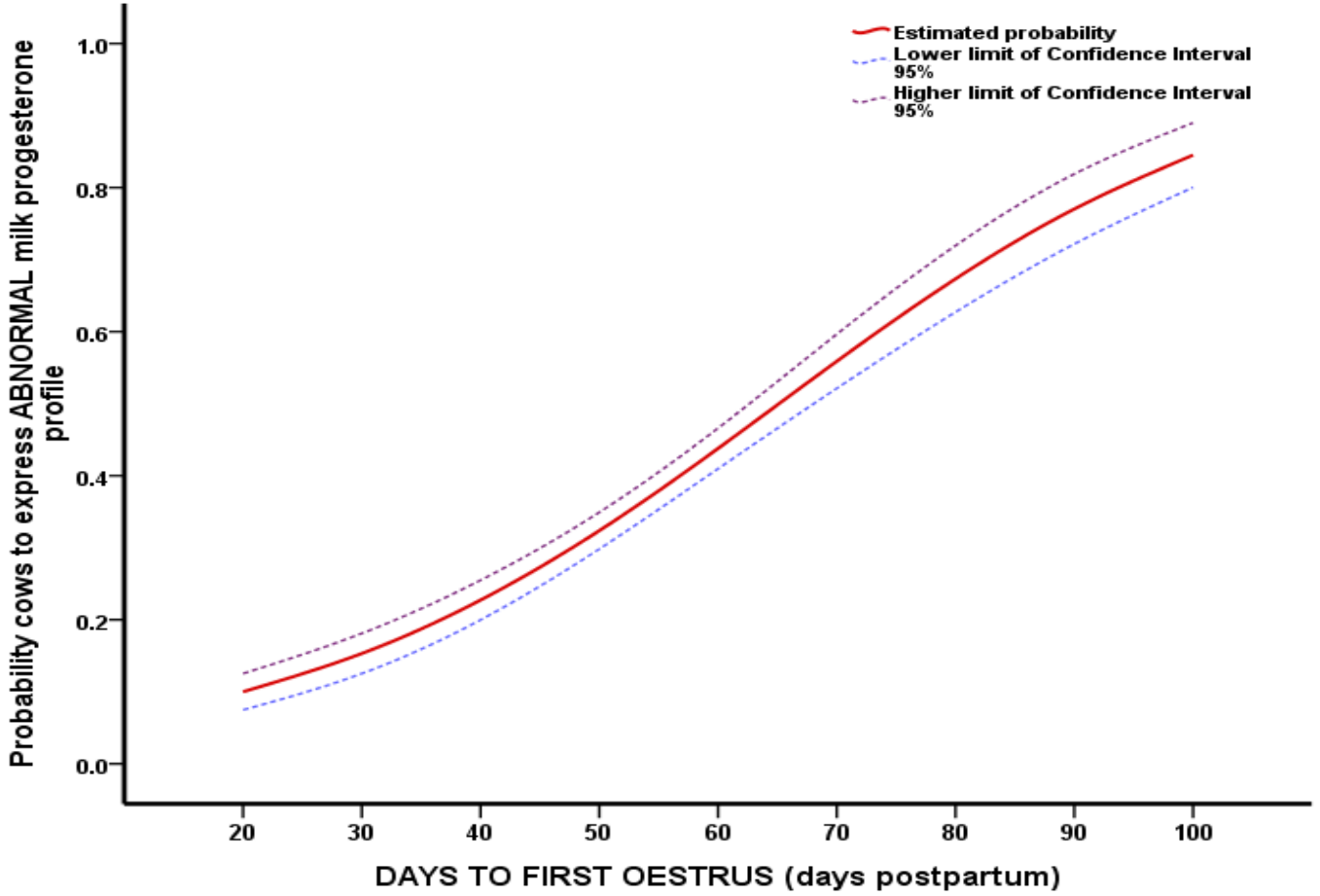


Figure 5.5: Effect of days to first oestrus on probability for cows to express abnormal milk progesterone profiles (n= 56, *P*= 0.016, exp (*b*) =1.011, CI for exp (*b*); [1.01, 1.09], *beta* coefficient (*b*) = 0.045, AUC_{ROC}=0.72±0.07, GLM with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was progesterone profile (0= normal, 1= abnormal) whereas days to oestrus and parity were added as continuous covariates. Red solid line represents the estimated probability whereas blue and purple dashed lines show the upper and lower 95 % Confidence Interval (CI).

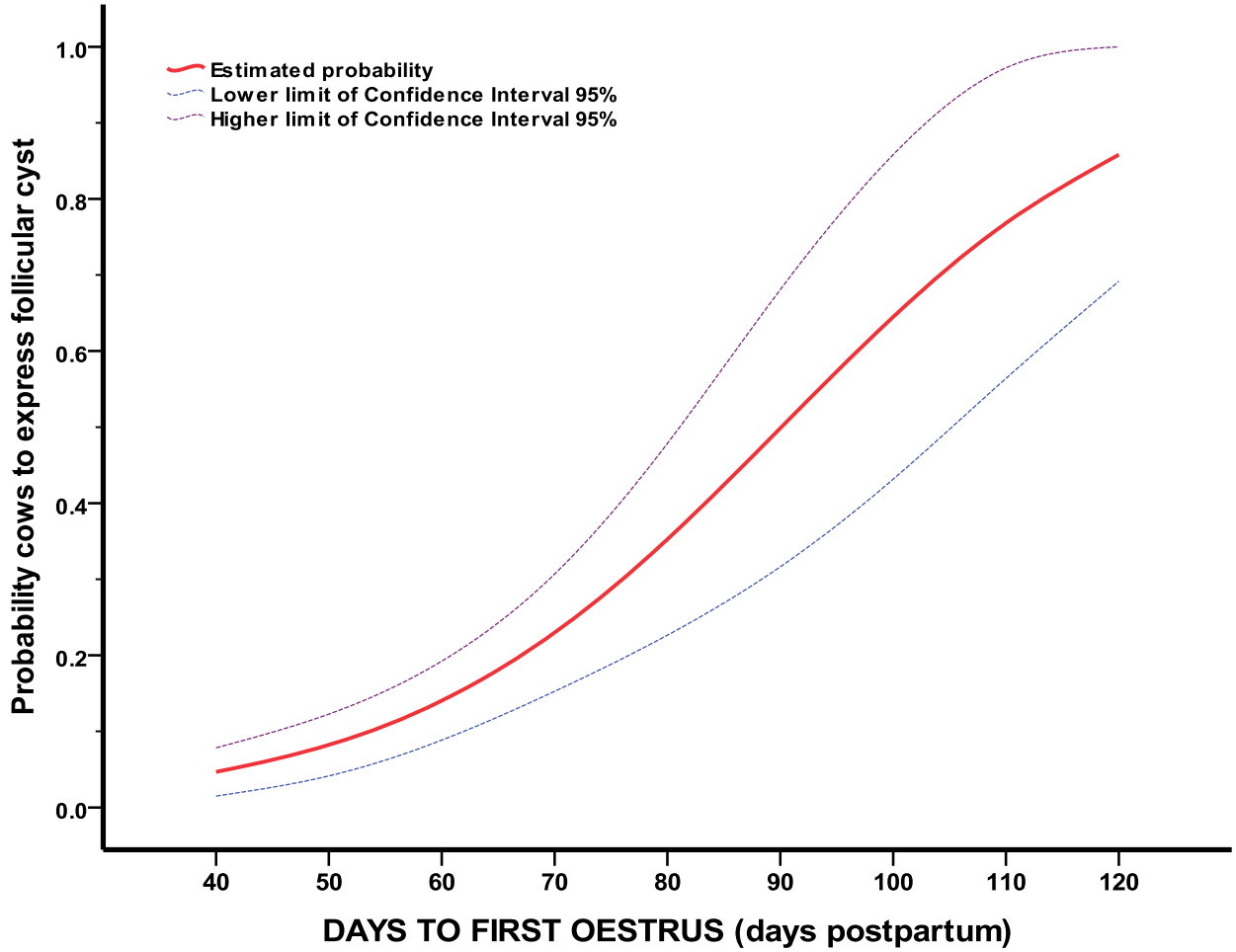


Figure 5.6: Effect of days to first oestrus on probability for cows to express cystic body (n= 58, *P*= 0.01, exp (*b*) =1.06, CI for exp (*b*); [1.01, 1.11], *beta* coefficient (*b*) = 0.06, AUC_{ROC}=0.76±0.09, GLM with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was cystic body (0=cystic body was not expressed, 1= cystic body was expressed) whereas days to oestrus and parity were added as continuous covariates. Red solid line represents the estimated probability whereas blue and purple dashed lines show the upper and lower 95 % Confidence Interval (CI).

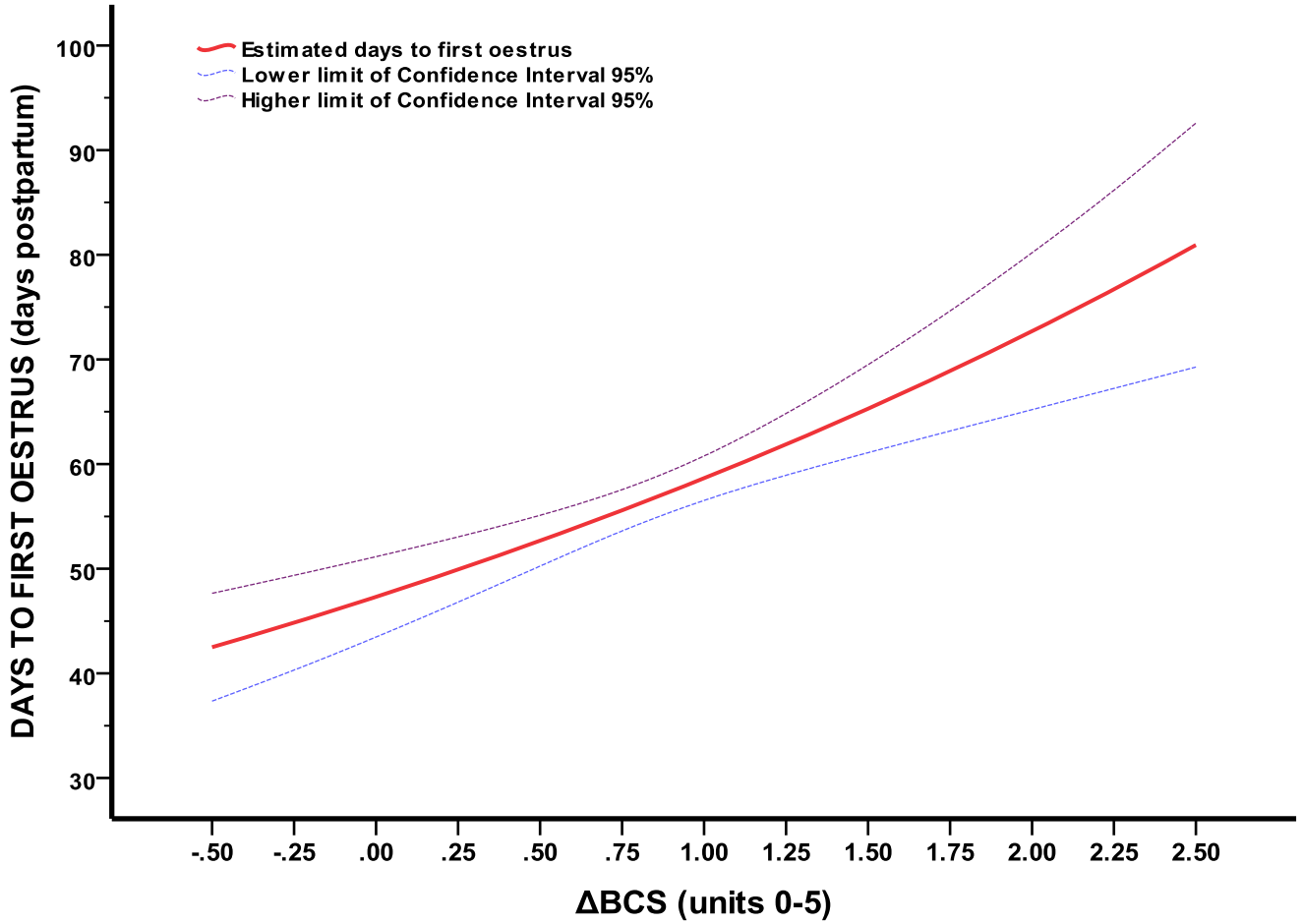


Figure 5.7: Effect of postpartum change in body condition score (ΔBCS) on days to first oestrus (n= 57, *P*=0.012). GLM with *Poisson* error distribution, *log* link function, and robust standard error estimation. In this model, dependent variable was days to first oestrus whereas ΔBCS and parity were added as continuous covariates. Red solid line represents the estimated probability whereas blue and purple dashed lines show the upper and lower 95 % Confidence Interval (CI). ΔBCS values with negative sign indicate gain of BCS whereas positive ΔBCS values denote loss of BCS.

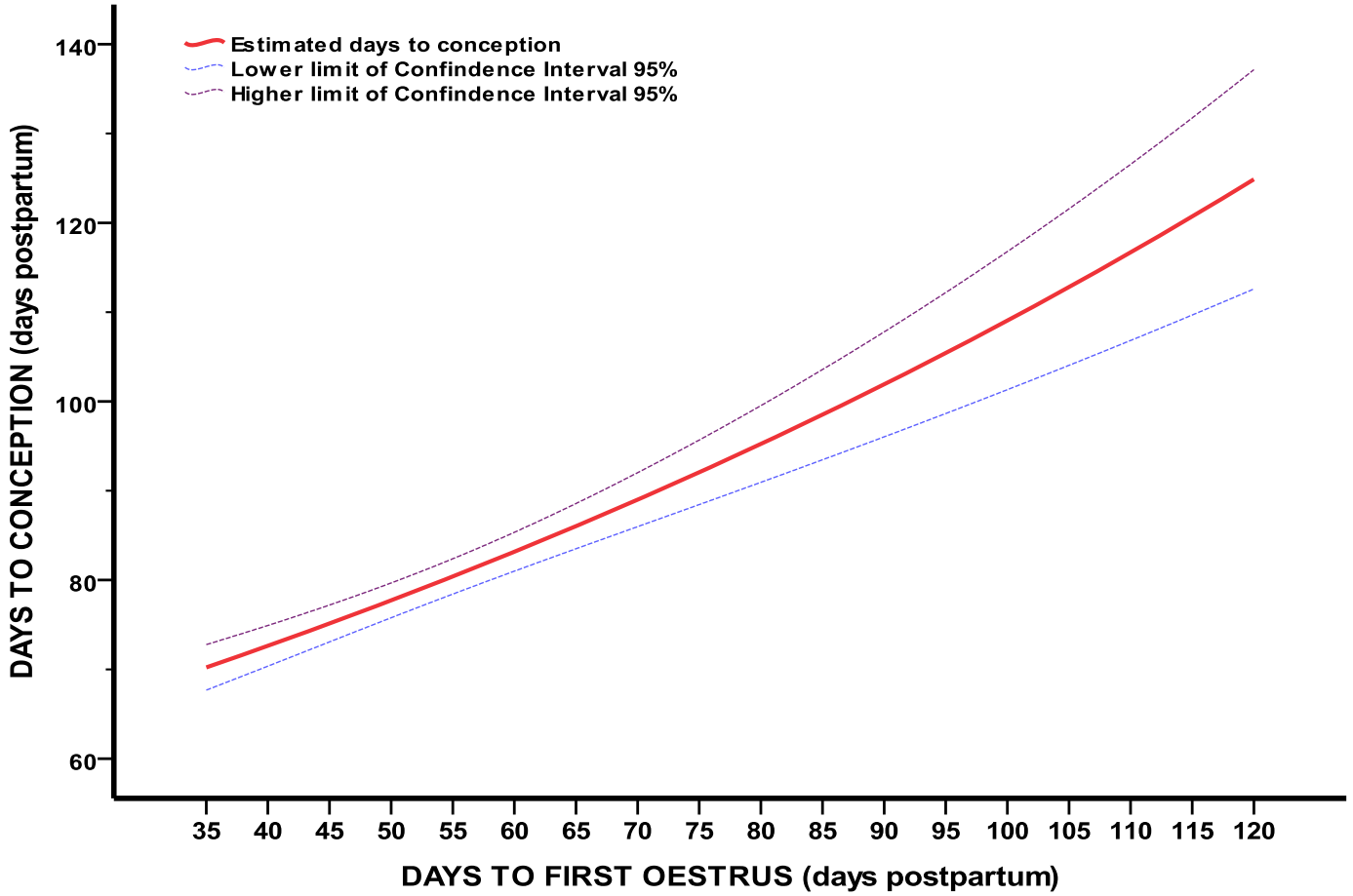


Figure 5.8: Effect of days to first oestrus on days to conception (n= 21, *P*=0.001). GLM with *Poisson* error distribution, *log* link function, and robust standard error estimation. In this model, dependent variable was days to conception whereas days to first oestrus and parity were added as continuous covariates. Red solid line represents the estimated probability whereas blue and purple dashed lines show the upper and lower 95 % Confidence Interval (CI).

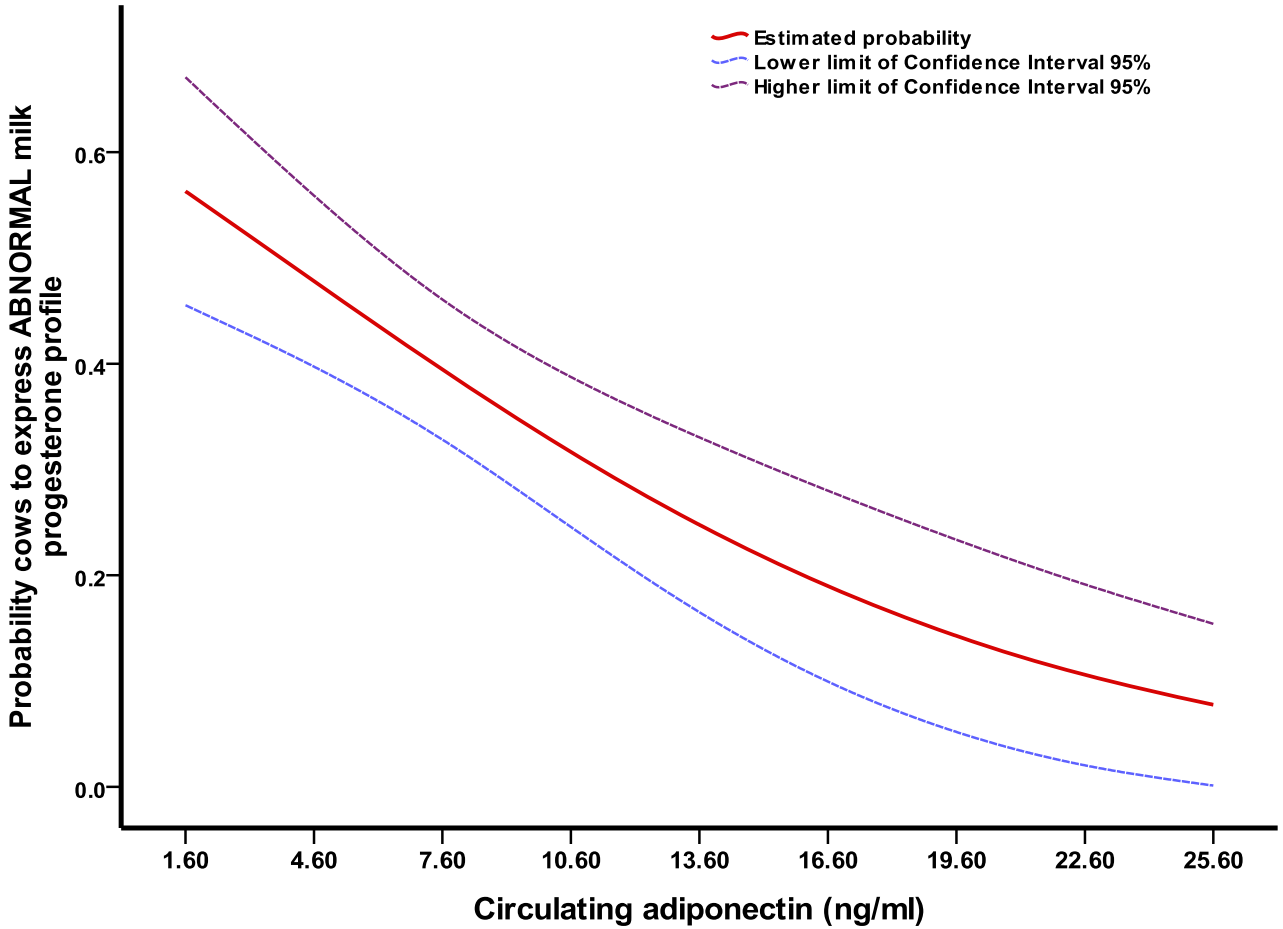


Figure 5.9: Effect of circulating adiponectin on probability for cows to express abnormal milk progesterone profiles (n= 60, *P*= 0.037, exp (*b*) =0.88, CI for exp (*b*); [0.79, 0.99], *beta* coefficient (*b*) = -0.12, AUC_{ROC}=0.71±0.07, GLM with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was progesterone profile (0= normal, 1= abnormal) whereas circulating adiponectin and parity were added as continuous covariates. Red solid line represents the estimated probability whereas blue and purple dashed lines show the upper and lower 95 % Confidence Interval (CI).

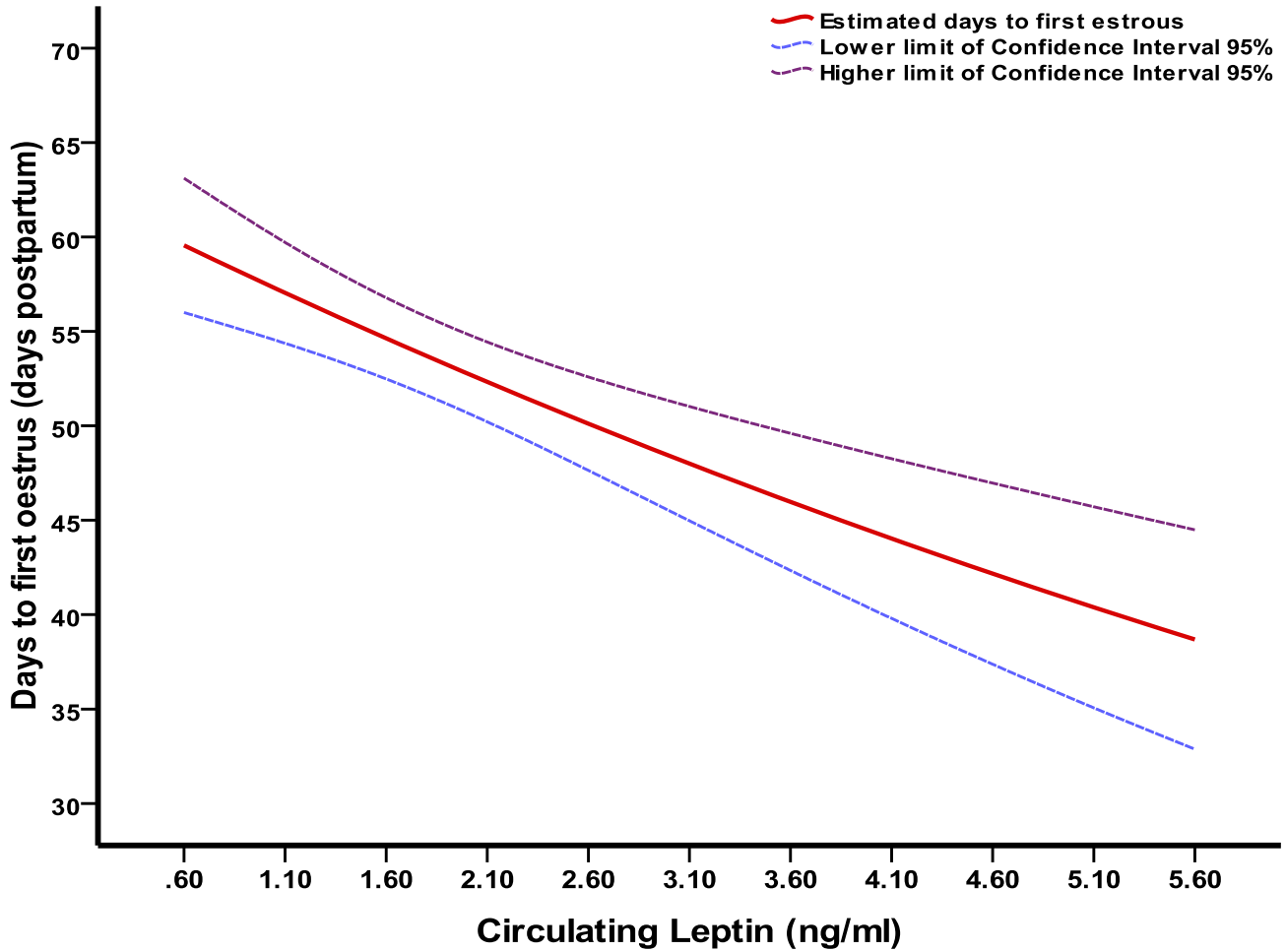


Figure 5.10: Effect of circulating leptin on days to first oestrus (n= 58, *P*=0.023), GLM with *Poisson* error distribution, *log* link function, and robust standard error estimation). In this model, dependent variable was days to first oestrus whereas circulating leptin and parity were added as continuous covariates. Red solid line represents the estimated probability whereas blue and purple dashed lines show the upper and lower 95 % Confidence Interval (CI).

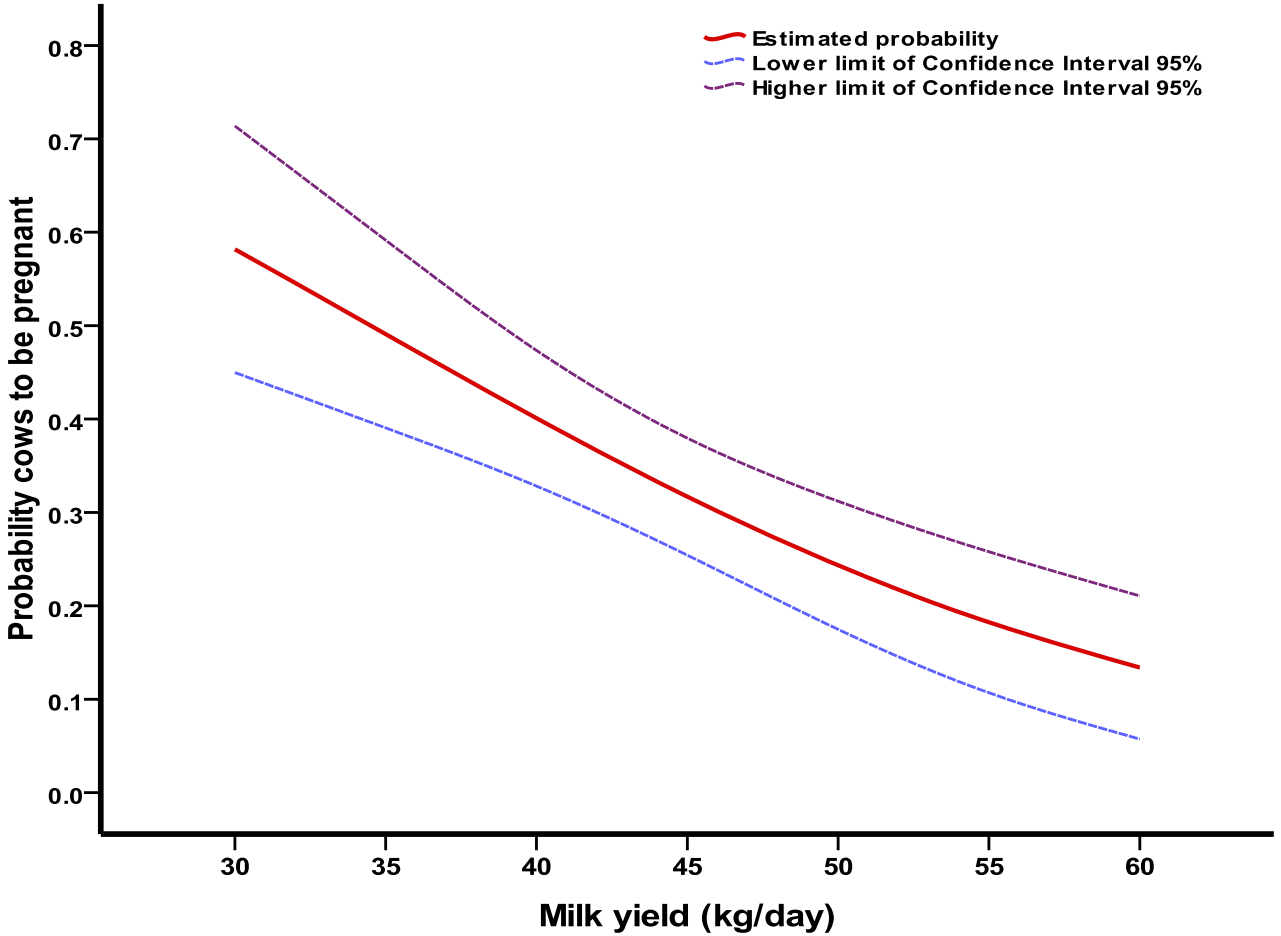


Figure 5.11: Effect of milk yield on probability of cows to be pregnant (n= 60, *P*=0.024, exp (*b*) =0.92, CI for exp (*b*); [0.86, 0.98], *beta* coefficient (*b*) = -0.08, AUC_{ROC}=0.68±0.07, GLM with *Bernoulli* error distribution, *logit* link function, and robust standard error estimation). In this model, dependent variable was pregnancy (0= normal, 1= abnormal) whereas milk yield and parity were added as continuous covariates. Red solid line represents the estimated probability whereas blue and purple dashed lines show the upper and lower 95 % Confidence Interval (CI).

Table 5.10: Effects of milk yield, DMI, GH, and parity on milk progesterone profile

Parameters in the model:	exp (*b*)†	95% CI for exp (*b*)		Beta (*b*) coefficient	*P*
		Lower	Upper		
Constant	-	-	-	-3.18	*0.251*
Milk yield	1.21	1.03	1.41	0.19	*0.02*
DMI	0.68	0.44	1.04	-0.39	*0.07*
GH	1.31	0.97	1.76	0.27	*0.07*
Parity	1.86	0.91	3.75	0.62	*0.09*

Likelihood ratio test **(χ^2=10.6, *df*=4, *P*=0.031);** ***Hosmer & Lemeshow test for fitness*** **(χ^2=12.6, *df*=8, *P* =0.128); AUC_{ROC}=0.73±0.07**
† **exp (*b*) is the odds ratio**

Table 5.11: Effects of insulin, glucose, GH, NEFA, and parity on follicular cyst formation

Parameters in the model:	exp (*b*)†	95% CI for exp (*b*)		Beta (*b*) coefficient	*P*
		Lower	Upper		
Constant	-	-	-	16.84	0.012
Insulin	~0 ($2.4\ 10^{-11}$)	0.00	0.004	-24.43	0. 004
Glucose	0.006	0.00	0.74	-5.06	0.035
GH	2.10	1.06	4.21	0.75	0.044
NEFA	201.60	1.16	34,861	5.31	0.029
Parity	0.96	0.31	2.90	-0.04	0.944

Likelihood ratio test **(χ^2=25.3, *df*=5, *P*=0.001);** ***Hosmer & Lemeshow test for fitness*** **(χ^2 =3.5, *df*=8, *P*=0.90); AUC_{ROC}=0.95±0.02**
† **exp (*b*) is the odds ratio**

Table 5.12: Effects of insulin, milk yield, and IGF-I on days to conception

Parameters in the model:	Beta (*b*) coefficient	95% CI for *b*		*P*
		Lower	Upper	
Constant	3.65	3.210	4.096	*0.001*
Insulin	0.98	.423	1.544	*0.001*
Milk yield	0.01	.006	.022	*0.001*
IGF-I	-0.002	-0.0034	-0.0007	*0.002*

***Likelihood ratio test* (χ^2=31.5, *df*=4, *P*=0.001)**

Table 5.13: Model fitted values for different insulin concentrations

Insulin *(ng/ml)*	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1.0**
Days to conception† *(days postpartum)*	57.4 [46.43, 68.34]	63.3 [54.62,72.03]	69.9 [63.78, 75.94]	77.1 [73.16, 81.01]	85.1 [79.75, 90.34]	93.8 [83.79, 103.88]	103.5 [87.06, 120.02]	114.2 [89.87, 138.60]	126.1 [92.25, 159.85]	139.1 [94.09, 184.06]

† **Row is mean with 95% CI**

Table 5.14: Effect of circulating adiponectin levels (ADP LEVELS) on metabolic hormones and metabolites

Treatment:	ADP LEVELS					
Parameters:	**NORMAL**†	n_1✧	**HIGH**†	n_2 ✧	**SED**‡	***P***
Adiponectin (*ng/ml*)	7.12	346	21.32	41	1.328	*0.001*
Insulin (*ng/ml*)	0.40	337	0.42	40	0.043	*0.730*
Glucagon *(pg/ml*)	95.2	178	96.6	38	9.38	*0.885*
GH (*ng/ml*)	4.7	173	3.1	33	0.52	*0.002*
Leptin (*ng/ml*)	1.66	338	2.30	40	0.367	*0.081*
IGF-I *(ng/ml)*	118.7	167	154.9	30	14.78	*0.015*
Glucose (*mmol/l*)	3.51	220	3.08	26	0.210	*0.041*
Urea (*mmol/l*)	2.33	223	2.55	26	0.303	*0.475*
BOHB (*mmol/l*)	0.66	223	0.53	26	0.070	*0.093*
NEFA (*mmol/l*)	0.46	226	0.36	23	0.084	*0.219*

† **Columns are means**
✧ ***n_1* and *n_2* are the repeated observations of animals with NORMAL and HIGH circulating adiponectin levels, respectively.**
‡ **SED = standard error of the difference between treatment means**

Table 5.15: Effect of circulating adiponectin levels (ADP LEVELS) on production traits

Treatment:	ADP LEVELS					
Parameters:	**NORMAL**†	n_1✧	**HIGH**†	n_2 ✧	**SED**‡	***P***
DMI (*kg/d*)	21.7	316	19.5	36	0.99	*0.028*
Milk Yield (*kg/d*)	44.1	316	38.2	36	2.17	*0.01*
LWT (*kg*)	657.1	316	659.3	36	7.74	*0.776*
BCS at calving	3.45	54	3.13	6	0.134	*0.017*
MEBAL *(MJ/d)*	-5.4	316	-6.2	36	2.18	*0.697*
ΔBCS (*units 1-5*)	0.70	53	0.25	6	0.185	*0.015*

† **Columns are means**
✧ ***n_1* and *n_2* are the repeated observations of animals with NORMAL and HIGH circulating adiponectin levels, respectively.**
‡ **SED = standard error of the difference between treatment means**

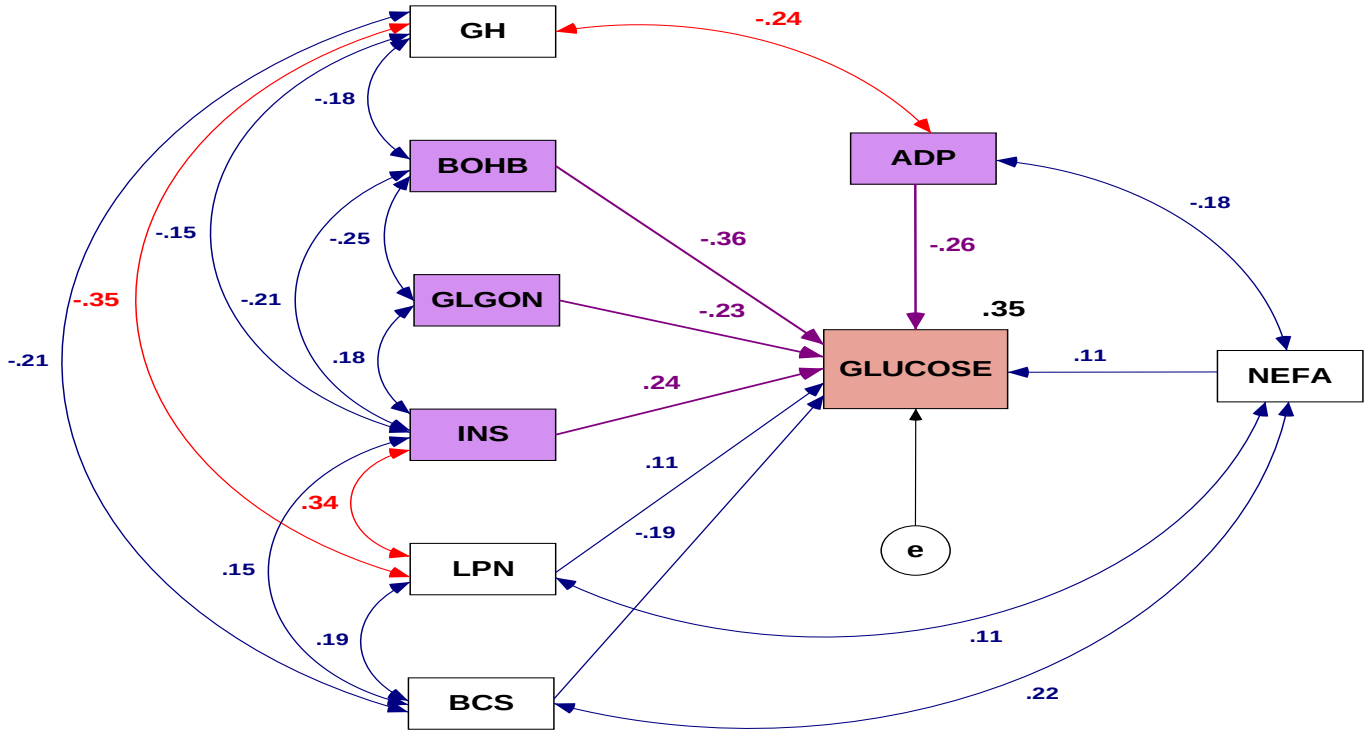


Figure 5.12: The basic model: Glucose interrelationships with adiponectin, BCS, metabolites, and metabolic hormones (n= 60; χ^2 =5.9, df =15, P=0.981; Bollen-Stine nonparametric bootstrap P=0.954, N=1,000 samples; CFI=1.00; NFI=0.918; RMSEA=0.0001; AIC=83.51; DIC=86.02, P=0.62, Effective number of parameters=33.32, convergence was achieved after (68,500)*2 burn-outs, 500 samples per each burn-in, convergence cut-off limit 1.002). In this basic model, single headed arrows (→) represent effects of BCS, metabolites, and metabolic hormones on glucose, and they are quantified as standardized regression coefficients placed in the middle of the arrows. Double headed arrows represent partial correlations and their coefficients are approximately located in the middle of arrow curvature. Purple single headed arrows (→) and red double headed arrows (↞) mark significant effects or correlations (P<0.05), whereas dark blue single headed arrows (→) and dark blue double headed arrows (↔) mark non-significant effects or correlations. Thus, the correlations INS↔LPN (0.34, P=0.012), LPN↔GH (-0.35, P=0.01), and GH↔ADP (-0.24, P=0.042) and the effects (→) of INS (0.24, P=0.036), GLGON (-0.23, P=0.036), BOHB (-0.36, P=0.001), and ADP (-0.26, P=0.014) on glucose are significant. Partial correlation coefficients and standardized regression coefficients are interpreted the same, as classical correlation coefficients. Model squared multiple correlations coefficient (which is equal to adjusted R^2 of linear regression models) is 0.35. Variable e is the residual error of the model. Model parameter estimates (mean±SE) are illustrated in Table B.1 in appendix.

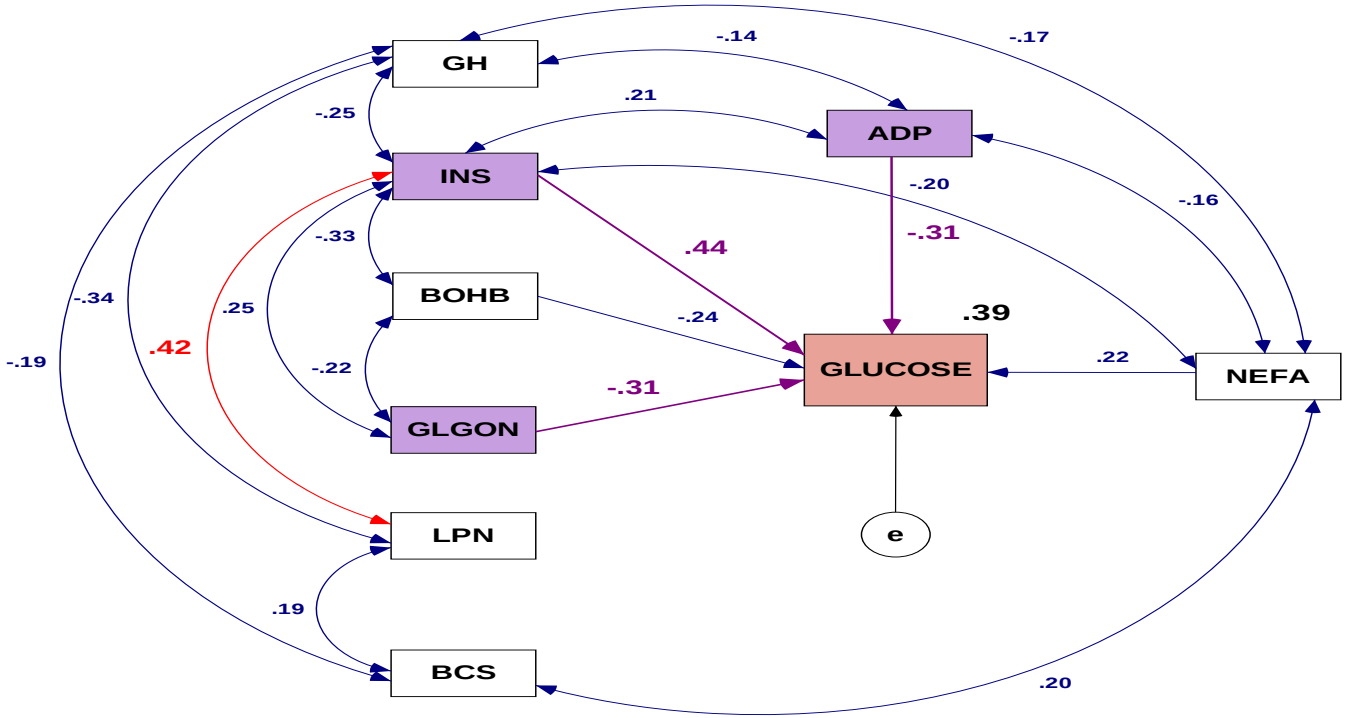


Figure 5.13: Glucose interrelationships with adiponectin, BCS, metabolites, and metabolic hormones in cows with LOW previous milk production (n= 30; χ^2=5.9, df=17, P=0.996; CFI=1.00; NFI=0.865; RMSEA=0.0001; AIC=79.86; DIC=83.17, P=0.56, Effective number of parameters=24.18, convergence was achieved after (77,500)*4 burn-outs, 500 samples per each burn-in, convergence cut-off limit 1.002). In this model, single headed arrows represent (→) effects of BCS, metabolites, and metabolic hormones on glucose and they are quantified as standardized regression coefficients placed in the middle of the arrows. Double headed arrows represent partial correlations and their coefficients are approximately located in the middle of arrow curvature. Purple single headed arrows (→) and red double headed arrows (↔) mark significant effects or correlations (P<0.05), whereas dark blue single headed arrows (→) and dark blue double headed arrows (↔) mark non-significant effects or correlations. Thus, the correlations INS↔LPN (0.42, P=0.022) and the effects (→) of INS (0.44, P=0.008), GLGON (-0.31, P=0.043), and ADP (-0.31, P=0.041) on glucose are significant. Partial correlation coefficients and standardized regression coefficients are interpreted the same, as classical correlation coefficients. Model squared multiple correlations coefficient (which is equal to adjusted R^2 of linear regression models) is 0.39. Variable e is the residual error of the model. Model parameter estimates (mean±SE) are illustrated in Table B.1 in appendix.

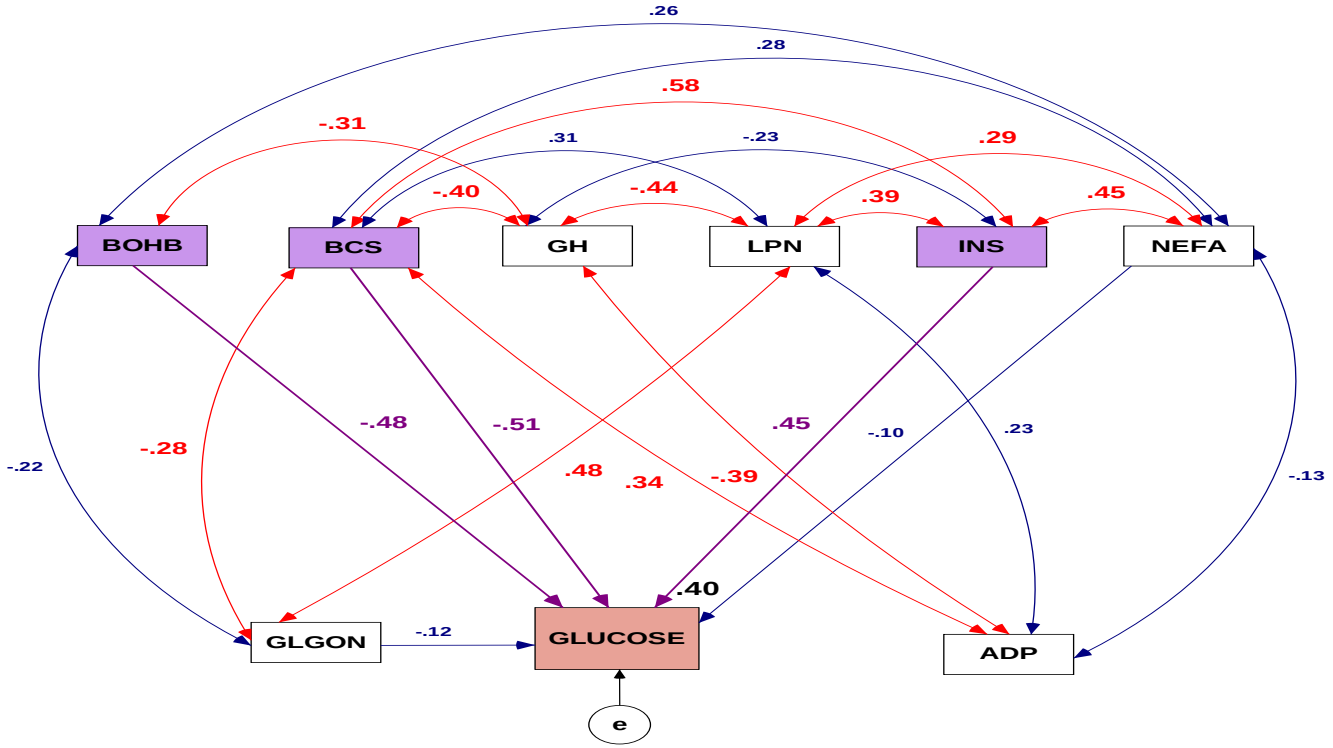


Figure 5.14: Glucose interrelationships with adiponectin, BCS, metabolites, and metabolic hormones in cows with HIGH previous milk production (*n*= 30; χ^2=3, *df*=13, *P*=0.998; CFI=0.998; NFI=0.966; RMSEA=0.001; AIC=85.01; DIC=88.29, *P*=0.43, Effective number of parameters=23.34, convergence was achieved after (89,500)*4 burn-outs, 500 samples per each burn-in, convergence cut-off limit was 1.002). In this model, single headed arrows (→) represent effects of BCS, metabolites, and metabolic hormones on glucose and they are quantified as standardized regression coefficients placed in the middle of the arrows. Double headed arrows represent partial correlations and their coefficients are approximately located in the middle of arrow curvature. Purple single headed arrows (→) and red double headed arrows (↔) mark significant effects or correlations (*P*<0.05), whereas dark blue single headed arrows (→) and dark blue double headed arrows (↔) mark non-significant effects or correlations. Thus, the correlations of INS↔LPN (0.39, *P*=0.02), INS↔BCS (0.58, *P*=0.003), INS↔NEFA (0.45, *P*=0.02), LPN↔NEFA(0.29, *P*=0.05), LPN↔GLGON(0.48, *P*=0.004), LPN↔GH(-0.44, *P*=0.01), BCS↔GH(-0.40, *P*=0.027), GH↔BOHB(-0.31, *P*=0.05), GH↔ADP(-0.39, *P*=0.036), GLUGON↔BCS(-0.28, *P*=0.049), and ADP↔BCS(0.34, *P*=0.031), and the effects (→) of INS (0.45, *P*=0.035), BCS (-0.51, *P*=0.01), and BOHB (-0.48, *P*=0.001) on glucose are significant. Partial correlation coefficients and standardized regression coefficients are interpreted the same, as classical correlation coefficients. Model squared multiple correlations coefficient (which is equal to adjusted R^2 of linear regression models) is 0.40. Variable *e* is the residual error of the model. Model parameter estimates (mean±SE) are illustrated in Table B.1 in appendix.

5.5. DISCUSSION

5.5.1 Effect of circulating adiponectin levels on metabolic hormones, metabolites, productive traits, and reproductive performance

Raddatz *et al.,* (2008) reported that individual cows had consistently high or low adiponectin levels throughout the sampling period and this result is in agreement with the present study. Moreover, the present study found that the group of the cows showed persistently HIGH adiponectin levels had 3 times higher circulating adiponectin than cows with consistently NORMAL adiponectin levels. The present study for first time, to the best of our knowledge, analyzes the effects of differences observed in circulating adiponectin levels in dairy cows. These differences were associated with differences in metabolic, hormonal, productive and reproductive traits. Unfortunately, only 10% of cows in this experiment expressed HIGH adiponectin levels. This point was a drawback in the statistical analysis because the data were unbalanced and this limits the conclusions. Nevertheless, large differences were observed in metabolic, hormonal, productive and reproductive traits, and different patterns in metabolic and reproductive regulation were demonstrated between cows expressing NORMAL and HIGH adiponectin levels.

In the present study, cows with HIGH adiponectin levels had lower GH and glucose than cows with NORMAL adiponectin levels. Moreover, cows with HIGH adiponectin levels had trends for higher circulating leptin and BOHB than cows with NORMAL adiponectin levels. Cows with HIGH adiponectin levels ate less feed and produced less milk than cows with NORMAL adiponectin profiles. Finally, cows with HIGH adiponectin levels lost less BCS postpartum, and had lower BCS at calving than cows with NORMAL adiponectin levels. Studies in humans and rodents indicate that the relation between adiponectin and GH is negative (Lam *et al.,* 2004; Nilsson *et al.,* 2005; Rodriguez-Pacheco *et al.,* 2007). In accordance with this, cows with HIGH adiponectin levels had lower GH than cows with NORMAL adiponectin levels. It is known that GH has rapid direct catabolic actions (enhances lipolysis, decreases lipogenesis and restricts glucose transport) whereas

adiponectin has insulin-sensitizing actions (decreases glucose output, increases FFA oxidation, and increases influx of NEFA) (Davidson, 1987; Lucy, 2000). Thus, low GH and high adiponectin in cows with HIGH adiponectin levels may explain why these animals had lower glucose and a trend for lower BOHB.

In humans, acromegalic and anorexia nervosa patients expressed decreased circulating leptin and increased circulating GH (Popovic *et al.,* 2001; Scacchi *et al.,* 1999) and this may indicate the relationship between GH and leptin is negative at least in extreme conditions with elevated circulating GH. Also, Leifers *et al.* (2005) suggested that leptin expression in adipose tissue is possibly regulated in early lactating cows by the negative impact of GH. Block *et al.* (2001) also demonstrated that the correlation between GH and leptin is negative. Moreover, restriction of energy intake suppresses GH secretion in rodents, but stimulates it in humans and ruminants (Block *et al.,* 2001; Nagatani *et al.,* 2000). In line with these results, cows with HIGH adiponectin levels in this experiment had low GH and DMI, and a trend for high leptin. It is known that the main central effect of leptin is suppression of food intake (Chilliard *et al.,* 2005), and this may explain why cows with HIGH adiponectin levels in this experiment had lower DMI than cows with NORMAL adiponectin levels. Furthermore, leptin stimulates free fatty acids (FFA) oxidation and increases glucose uptake in the skeletal muscles, and decreases glucose output and increases FFA oxidation in the liver (Yildiz & Haznedaroglu, 2006; Kokta *et al.,* 2004; Macajova *et al.,* 2004). This may provide further explanation why glucose was low, and BOHB had a trend to be low in cows with HIGH adiponectin levels. In the present study, cows with HIGH adiponectin levels had higher adiponectin and a tendency for higher leptin than cows with NORMAL adiponectin levels. Some studies suggest that the relationship between adiponectin and leptin is negative (Matsubara *et al.,* 2002; Huypens, 2007). However, other studies reported positive correlations between leptin and adiponectin (Pardo *et al.,* 2004; Rossi *et al.,* 2005) or no correlation (Park *et al.,* 2004). However, in acromegalic patients GH down-regulated both circulating adiponectin and leptin (Popovic *et al.,* 2001; Lam *et al.,* 2004). Thus, at least in extreme conditions, it is possible for adiponectin and leptin to be positively correlated, especially when circulating GH is very high or very low. In the present study, cows with HIGH adiponectin levels had

approximately 34% lower circulating GH compared with cows with NORMAL adiponectin levels, and maybe that is the reason why cows with HIGH adiponectin levels had higher circulating adiponectin and a trend for higher leptin than cows with NORMAL adiponectin levels. TNF-*α* is another interesting adipokine and it has been found to exert regulatory roles on adiponectin and leptin. Classically, TNF-α is increased in periparturient cows and decreased in early lactating cows (Ametaj, 2005). Also, TNF- α is a potent inhibitor of adiponectin expression and secretion by adipose tissue, and negatively correlated with insulin sensitivity (Fasshauer *et al.*, 2002; Ruan *et al.*, 2002; Ronti *et al.*, 2006). Moreover, TNF-*α* has been demonstrated to stimulate leptin (Zumbach *et al.,* 1997) in humans. Unfortunately, the present study did not measure TNF- α, but it is possible that this adipokine is the link that explains the high circulating leptin and adiponectin observed in cows with HIGH adiponectin levels.

In early lactating cows, low circulating insulin is responsible for uncoupling of the GH – IGF-I axis in the liver due to down-regulation of GH receptors (Lucy; 2000). Consequently, IGF-I production in the liver is suppressed and the negative feedback of IGF-I at the level of the hypothalamus/ pituitary is removed, resulting in increased circulating GH. This situation can be restored by improving insulin sensitivity (Wathes *et al.,* 2007; Garnsworthy *et al.,* 2008a; Leroy *et al.,* 2010). Cows with HIGH adiponectin levels had higher circulating IGF-I, but lower circulating GH than cows with NORMAL adiponectin levels in this experiment. However, circulating insulin did not differ between the two groups. In line with this experiment, animals with low IGF-I have greater circulating GH (Lucy, 2000). Elevated circulating GH can be the main reason for lower circulating IGF-I in cows with NORMAL adiponectin levels due to uncoupling of the GH–IGF-I axis in the liver of these animals. It is known that IGF-I stimulates the synthesis of adiponectin (Williams *et al.,* 2004) and that may explain why cows with HIGH adiponectin levels had elevated adiponectin and IGF-I. Moreover, the combination of high circulating adiponectin and leptin along with decreased glucose and the same levels of circulating insulin in cows with HIGH adiponectin levels may imply better insulin sensitivity of these animals. High insulin sensitivity is associated with weight gain, and accretion of condition is positively associated with circulating IGF-I (Wathes *et al.,* 2007). IGF-I concentrations

were positively correlated with insulin sensitivity in humans (Sesti *et al.,* 2005) and that might be another reason why circulating IGF-I was higher in cows with HIGH adiponectin levels.

According to Meikle *et al.* (2004) leptin and IGF-I are positively correlated in periparturient dairy cows and this is in agreement with this study. Also, the lower circulating glucose for the same levels of circulating insulin in cows with HIGH adiponectin levels might suggest better response of insulin to glucose and thus better insulin sensitivity than cows with NORMAL adiponectin levels. Low insulin sensitivity is common in high yielding cows (Chilliard *et al.,* 1998; Gutierrez *et al.,* 2006). High GH concentrations not only stimulate milk production, but also stimulate glucose production in the liver, and adipose tissue mobilization. The resulting high circulating NEFA, BOHB, and GH inhibit insulin action and create a further state of peripheral insulin resistance (Leroy *et al.,* 2010). Furthermore, milk yield and IGF-I are negatively correlated (Wathes *et al.,* 2007). In the present study, cows with NORMAL adiponectin levels had higher milk yield and greater BCS loss postpartum than cows with HIGH adiponectin levels and this also agrees with the lower circulating levels of IGF-I in cows with NORMAL adiponectin levels.

Differences in productive traits were generally matched by differences in metabolic and hormonal profile in this experiment. Cows with HIGH adiponectin levels had lower DMI and ΔBCS, and produced lower milk yield than cows with NORMAL adiponectin levels. The lower DMI in cows with HIGH adiponectin levels may be was the result of elevated circulating leptin in these animals (as increased leptin suppresses feed intake). In addition, decreased circulating GH and glucose in cows with HIGH adiponectin levels may be was the main reason for decreased milk yield and decreased ΔBCS. It is known that fatter cows at calving lose more body condition than thinner cows (Garnsworthy & Topps, 1982), and this was also true in the present study because cows with HIGH adiponectin levels were thinner at calving and they lost less condition throughout the experiment compared to cows with NORMAL adiponectin levels. Moreover, BOHB had a tendency to be elevated in

cows with NORMAL adiponectin levels and that indicates greater dependence on fat tissue mobilization for supporting increased milk yield.

Reproductive functions are closely related to nutritional status (Boland *et al.,* 2001; Garnsworthy *et al.,* 2008; Santos, 2007). Adipokines such as leptin and adiponectin are known to be implicated in reproductive functions at different levels, including central effects on the hypothalamus and pituitary, peripheral effects on the ovary and reproductive tract, and direct effects on the oocyte and embryo (Mitchell *et al.,* 2005). In the present study, cows with HIGH adiponectin levels showed higher probability to become pregnant than cows with NORMAL adiponectin levels. Moreover, cows with NORMAL milk progesterone profile were more likely to be pregnant than cows with ABNORMAL milk progesterone profile, and there was a tendency for non-random association between cows expressing HIGH circulating adiponectin levels and cows with NORMAL milk progesterone profile. This may explain in part the higher pregnancy rate in cows with HIGH adiponectin levels, but it needs to be investigated further.

The present study showed that the association of ΔBCS with the probability of a cow to be pregnant was non linear. Furthermore, cows with HIGH adiponectin levels lost less BCS (0.25 units *versus* 0.70 units) than cows with NORMAL adiponectin levels. Moreover, cows losing 0.25 units of BCS or less were more likely to become pregnant than cows losing 0.70 units or more (Figure 5.3). This is another explanation for the higher pregnancy rate in cows with HIGH adiponectin levels (Table 5.7). Also, the association of ΔBCS with days to first oestrus was negative (Figure 5.7) in the present study and this may explain why cows with HIGH adiponectin levels had fewer days to first oestrus than cows with NORMAL adiponectin levels (Table 5.7).

IGF-I was associated with the probability for a cow to become pregnant in this experiment. Cows with HIGH adiponectin levels had higher circulating IGF-I than cows with NORMAL adiponectin levels. Cows with average circulating IGF-I equal to or greater than 155 *ng/ml* throughout the experimental period were more likely to become pregnant than cows with average circulating IGF-I equal to or less than 120 *ng/ml*. Also, milk yield was

negatively associated with the probability of cows to be pregnant in the present study. These may further explain the higher pregnancy rate in cows with HIGH adiponectin levels.

Cows with HIGH adiponectin levels had fewer days to first oestrus than cows with NORMAL adiponectin levels, and days to first oestrus were positively associated with the probability of cows to express ABNORMAL milk progesterone. Also, circulating adiponectin was negatively associated with the probability of cows to express ABNORMAL milk progesterone profile. Taken together these findings may explain why in this experiment there was a tendency for non-random association between cows expressed HIGH circulating adiponectin levels and cows with NORMAL milk progesterone profile. According to Dupont *et al.* (2008) adiponectin activates AMPK and that leads to decreased steroidogenesis in the granulosa cells. Indeed, *in vitro* studies showed a possible role of adiponectin in modulating ovarian steroidogenesis (Ledoux *et al.*, 2006; Lagaly *et al.*, 2008; Chabrolle *et al.,* 2009; Gutman *et al.,* 2009; Pierre *et al.,* 2009; Maillard *et al.,* 2010), but results are controversial, which may imply species-specific differences in the regulation of steroidogenesis by adiponectin (Dupont *et al.,* 2008; Brochu-Gaudreau *et al.,* 2010).. Moreover, women treated with chorionic gonadotrophin expressed increased circulating adiponectin, which correlated with circulating progesterone and oestradiol (Liu *et al.,* 2006). Furthermore, short-term treatment of rat pituitary gonadotroph cells with recombinant murine adiponectin abolishes GnRH-induced LH secretion (Rodriguez-Pacheco *et al.,* 2007). Adiponectin has been detected in porcine and human follicular fluid (Ledoux *et al.,* 2006; Chabrolle *et al.,* 2009) and AdipoR1 and AdipoR2 are present in theca and granulosa cells, oocytes, and the corpus luteum in dairy cows (Tabandeh *et al.,* 2010). However, either AdipoR1 or AdipoR2 knock-out mice is fertile, and this may imply adiponectin is not necessary for normal ovarian function (Campos *et al.,* 2008; Brochu-Gaudreau *et al.,* 2010).The effect of adiponectin on milk progesterone profile needs to be investigated further.

Treatment with leptin reverses fasting-induced anoestrus in Syrian hamsters (Schneider *et al.,* 1998). Moreover, female mice injected with leptin reproduced up to 9 days earlier than

controls and showed earlier maturation of the reproductive tract (Chehab *et al.,* 1997). Furthermore, higher leptin concentrations were associated with shorter intervals to first observed estrus in dairy cows (Liefers *et al.*, 2003). Leptin was negatively associated with days to first oestrus in this experiment, which is in line with the afore-mentioned studies. Moreover, cows with HIGH adiponectin levels had lower days to first oestrus and a tendency for higher leptin than cows with NORMAL adiponectin levels. Thus, the tendency for higher leptin may explain the lower days to first oestrus in cows with HIGH adiponectin levels.

In lactating cows, delayed resumption of cyclicity is associated with decreased circulating leptin, whereas increased circulating leptin resulted in improved reproductive performance (Kadokawa *et al.*, 2000; Block *et al.*, 2001; Liefers *et al.*, 2003; Meikle *et al.*, 2004; Konigsson *et al.*, 2008). According to Meikle *et al.* (2004) cows with better reproductive performance had higher circulating IGF-I and Butler *et al.* (2000) found a negative relationship between postpartum circulating IGF-I and the interval to the resumption of ovarian cyclicity. Also, Huszenicza *et al.* (2001) found higher IGF-I levels in cows with ovulation occurring within 35 days postpartum. In the present study, cows with HIGH adiponectin levels had higher IGF-I, leptin (tendency), and reproductive performance than cows with NORMAL adiponectin profiles and those findings are generally in agreement with the above mentioned studies.

It is known that cows with high genetic merit show increased incidence of delayed commencement of luteal activity (CLA) **(**Windig *et al.,* 2008) and increased circulating GH and milk yield (Veerkamp *et al.,* 2003). Moreover, cows with greater milk production have lower peak progesterone concentrations **(**Windig *et al.,* 2008; Taylor *et al.,* 2003; Remppis *et al.,* 2011) and secretion of progesterone is reduced by energy deficit (Villa-Godoy *et al.,* 1988). According to Veerkamp *et al.* (2000) increasing genetic merit for feed intake improves CLA in dairy cows. Moreover, cows in higher parities are more at risk of suffering from prolonged luteal phases (Opsomer *et al.*, 2000). In accordance with these studies, multiple logistic regression analysis demonstrated that the risk for abnormal milk

progesterone profile increased with milk yield, GH (tendency), and parity (tendency), whereas it decreased with DMI (tendency) in this experiment.

It has been shown that ovarian follicular cyst formation frequently occurs in the early postpartum period when the cow changes from the acyclic state during pregnancy to the establishment of regular cyclicity (Webb *et al.,* 1998; Vanholder *et al.,* 2006). Low circulating IGF-I in early lactating cows could predispose to anovulation and development of cystic follicles (Zulu *et al.,* 2000). Moreover, decreased circulating insulin and insulin resistance in early lactating cows may affect cyst formation (Vanholder *et al.,* 2005) whereas leptin may play a role in cyst development as a minimum level of circulating leptin is required to induce the first postpartum LH surge (Vanholder *et al.,* 2006). Furthermore, cows with cysts were associated with higher milk production (Garverick, 1997; Webb *et al.,* 1998) and cows with high circulating NEFA after calving were twice as likely to develop cystic ovarian disease (Jackson *et al.,* 2011). Additionally, Borromeo *et al.* (1998) demonstrated that GH concentrations in plasma and follicular fluid were higher in dairy cows with cystic follicles and Kawashima *et al.* (2007) showed that cows did not ovulate had higher circulating GH and lower circulating glucose during the peri-partum period than cows ovulated. In aggreement with the above mentioned studies, multiple logistic regression analysis demonstrated a negative effect on cystic body formation of circulating glucose and insulin, whereas circulating GH and NEFA had a positive effect on cystic body formation. Although all the animals expressed HIGH adiponectin levels were assessed without cystic bodies, there was no association between adiponectin level and follicular cysts. However, this needs to be investigated further as it is possibly biased by the small numbers of cows observed with follicular cysts (only 8 out of 60) and the small number of cows with HIGH adiponectin levels (6 out of 60) (Table 5.9). Moreover, follicular cyst formation delayed days to first oestrus in the present study (Figure 5.6) and may be another explaination why cows with HIGH adiponectin levels had fewer days to first oestrus than cows with NORMAL adiponectin levels (Table 5.7).

Interval from calving to conception was not affected by circulating adiponectin levels, whereas days to conception positively associated with days to first oestrus, and cows with

HIGH adiponectin levels had fewer days to first oestrus (43 days *versud* 56 days) than cows with NORMAL adiponectin levels in the present study. According to Figure 5.8 animals with 43 days to first oestrus (HIGH adiponectin levels) did not have statistically different days to conception (74 days *versus* 80 days) compared with animals with 56 days to first oestrus (NORMAL adiponectin levels). Insulin was the main factor influenced days to conception, but circulating insulin was not different between cows with HIGH and NORMAL adiponectin levels. This latter may further explain why interval from calving to conception was unaffected by circulating adiponectin levels.

Why individual cows had consistently high or low adiponectin levels is difficult to explain in this experiment. In humans, circulating adiponectin has a strong genetic component with heritability estimated to be between 30% and 50% (Comuzzie *et al.*, 2001). Moreover, low circulating adiponectin is observed in several forms of diabetes, with insulin resistance, and in non-alcoholic fatty liver disease (Xu *et al.,* 2007). In dairy cows, there has been no genetic association study to examine the role of adiponectin polymorphisms on indices of insulin sensitivity, circulating GH levels, fatty liver disease, and glucose tolerance. However, it is possible that insulin resistance, genetics, and fatty liver disease in dairy cows to regulate circulating adiponectin levels similarly to man. However, this hypothesis needs to be further investigated.

To conclude, cows with HIGH adiponectin levels had fewer days to first oestrus and a higher probability to be pregnant than cows with NORMAL adiponectin levels. All, cows with HIGH adiponectin levels exhibited normal milk progesterone profiles. However, there were no significant difference between cows with HIGH adiponectin levels and cows with NORMAL adiponectin levels for days to pregnancy or follicular cyst formation. Differences in metabolic and hormonal profile and production traits may at least in part explain the differences in reproductive performance between cows with HIGH adiponectin levels and cows with NORMAL adiponectin levels. However, further work is required to clarify the reasons for existence of such “special” animals (with increased adiponectin, decreased GH, and superior fertility), and the relations of high circulating adiponectin with metabolic and hormonal profile, productive traits, and reproduction.

5. 5.2. Interrelationships of adiponectin with glucose, BCS, metabolic hormones, and metabolites in high and low yielding cows

Path diagrams and structural equation modelling (SEM) analysis for all the 60 cows in this experiment proved that glucose balance in dairy cows was regulated by the negative influences of circulating adiponectin, BOHB, and glucagon, and the positive influence of circulating insulin on circulating glucose. It is known that high circulating adiponectin levels were associated with a lower risk of development of type 2 diabetes and adiponectin was found to negatively correlate with circulating glucose (Hotta *et al.,* 2000; Weyer *et al.,* 2001; Spranger *et al.,* 2003) and this is in agreement with the current study. Also, the hypoglycaemic effect of insulin action is antagonized by glucagon, and insulin is positively correlated with glucose (Brockman, 1978, 1979). Moreover, BOHB is negatively correlated with insulin and glucose in dairy cows (Drackley, 1999; Bobe *et al.,* 2004; Veerkamp *et al.,* 2003; Leroy *et al.,* 2010). These findings are also in line with the current study. In the present study, GH, NEFA, leptin, and BCS had no direct effect on circulating glucose, but GH was negatively correlated with adiponectin and leptin, whereas leptin was positively correlated with insulin. Human obese patients showed decreased circulating GH and increased circulating leptin (Casanueva & Dieguez, 1998) whereas acromegalic and anorexia nervosa patients expressed decreased circulating leptin and increased circulating GH (Popovic *et al.,* 2001; Scacchi *et al.,* 1999). Also, studies in humans and rodents showed that the relation between adiponectin and GH is possibly negative (Lam *et al.,* 2004; Nilsson *et al.,* 2005; Rodriguez-Pacheco *et al.,* 2007). Insulin was positively correlated with leptin in periparturient and lactating dairy cows (Block *et al.,* 2001; Garnsworthy *et al.,* 2008c). These findings are also in agreement with the present study.

Previous milk yield (PMY) was used in the present study as an indicator of genetic merit for milk yield. However, it is known that other factors, such as lactation persistency, BCS at calving, DMI, calving interval, parity, health status, and diet contribute to differences observed in milk yield (Rook & Thomas, 1983). Cows were grouped according to milk yield in the previous lactation (PMY) with a cut-off point between high and low yielding cows of 10,000 kg/cow/lactation. This cut-off point is higher than the current average yield

per cow in the UK (7,406 kg/cow/lactation) (DEFRA, 2011) and it was chosen to be as high as possible. This was to ensure that the nature of the differences observed in the PMY groups was mainly due to genetic differences between cows. All instances of ill health of cows were recorded in this experiment, although subclinical diseases cannot be excluded from the factors affecting PMY. Parity was not statistically different for high PMY cows compared to low PMY cows (the majority of the cows (39 out of 60) were in their 2nd lactation) and the length of lactation used to calculate PMY was 305 days. In addition, high PMY cows had higher milk yield, DMI, and BCS at calving and a trend for higher ΔBCS than low PMY cows in the present lactation, which is in agreement with the profile of the high genetic merit cow for milk production (Veerkamp *et al.,* 2003).

Structural equation modelling analysis for cows with LOW PMY revealed a simple network of interrelationships of adiponectin with glucose and other metabolic signals in these animals. In PMY LOW cows, glucose homeostasis was regulated by the positive effect of insulin and the negative effects of adiponectin and glucagon. GH was not related to leptin or adiponectin. It is known that low yielding cows have low circulating GH (Veerkamp *et al.,* 2003), which is a possible reason for the lack of correlation between GH and adiponectin. In these PMY LOW cows, adiponectin was the main insulin sensitizing hormone due to its direct negative effect on circulating glucose, whereas leptin may have exerted insulin stimulatory effects because it was correlated with insulin.

In contrast, PMY HIGH cows showed completely different patterns in regulating glucose homeostasis. In these animals, the relationships between metabolic hormones and metabolites were very complicated. It is known that selection for milk yield resulted in decreased circulating glucose and increased circulating GH and BCS (Roche *et al.,* 2009; Coffey *et al.,* 2003; Veerkamp *et al.,* 2003). Possibly, that is the reason GH showed strong negative correlations with adiponectin and leptin. It has been demonstrated that GH is up-regulated in human diabetes type 2 (Holt, 2003); high yielding cows tend to be genetically and phenotypically thinner (Garnsworthy, 2006); and fatter cows at calving lose more body fat than thinner cows (Garnsworthy & Topps, 1982). This latter might explain the strong negative correlations of BCS with glucose, glucagon and GH and the strong positive

correlations of BCS with insulin and adiponectin. Furthermore, glucose homeostasis in PMY HIGH cows was regulated by the positive effect of insulin and the negative effects of BCS and BOHB. In these cows, adiponectin did not play any direct role in regulating glucose balance (because it was strongly down-regulated by GH) but leptin, as in PMY LOW cows, was the main insulin stimulatory hormone because it was positively correlated with insulin. However, adiponectin was positively correlated with BCS and BCS had a significant effect on glucose balance. That means that, the greater was the BCS of the high yielding cows postpartum (cows that did not lose a lot of condition), the lower was the circulating GH and glucagon, and consequently the lower was the milk yield. Moreover, the lower the circulating GH, the higher was the circulating adiponectin, leptin, and BOHB. The higher the circulating leptin, the higher was the circulating insulin, NEFA, and glucose. Finally, this aetiological chain concludes to a profile of high yielding cows with elevated circulating adiponectin, leptin, insulin, glucose, NEFA, and BOHB. According to Figure 5.14, adiponectin and leptin are not active in terms of lowering glucose, and consequently that aetiology leads to elevated circulating glucose, insulin, NEFA, and BOHB which is common in human metabolic syndrome, insulin resistance, and diabetes mellitus. Oligomerisation of adiponectin structure is considered very important for its biological function (Berg *et al.,* 2002; Barb *et al.*, 2007) and could explain at least theoretically why high circulating adiponectin was possibly inactive under such physiological conditions. Moreover, impaired oligomerization might be an important causative factor for type 2 diabetes (Xu *et al.,* 2007). The positive correlations of NEFA with insulin and leptin also imply insulin or/and leptin resistance in PMY HIGH cows (Krentz, 1996; Fam & Andrikopoulos, 2007; Myers *et al.,* 2008). Indeed, many studies have reported low insulin sensitivity in early lactation cows (Sano *et al.,* 1991, 1993; Chilliard *et al.,* 1998; Gutierrez *et al.,* 2006; Hayirli, 2006). Besides, if this aetiological chain runs for a high yielding cow which loses a lot of condition postpartum, then it would fit the profile of an animal with high GH, milk yield, and glucagon and low glucose, insulin, leptin, adiponectin, NEFA, and BOHB. Of course, this is an incorrect prediction of the model because at least NEFA and BOHB are reported to be elevated in such conditions (Jorritsma *et al.,* 2003; Veerkamp *et al.,* 2003). However, the majority of commercial cows are in-between the two main cases examined by the model, and other physiological

regulatory factors are likely to contribute to the whole complicacy of glucose homeostasis in high yielding cows.

The main conclusion of this analysis is that the relationship between adiponectin and GH was generally antagonistic in dairy cows. Also, the relation between circulating glucose and circulating adiponectin was negative and that might indicate insulin sensitizing traits of adiponectin in dairy cows. Moreover, high yielding cows might regulate glucose homeostasis differently to low yielding cows, which was mainly achieved by higher secretion of GH and lower or inactive adiponectin. GH possibly down-regulated leptin and adiponectin, but this was mainly in high yielding cows. Leptin was positively correlated with insulin in both high yieldings and low yielding cows, and it was likely to exert insulin stimulatory effects. However, further work is needed to elucidate the perplexities of glucose homeostasis and its interrelationships with hormonal and metabolic profile in dairy cows.

5.5.3 Conclusions

In summary, the results from this study confirm that cows with circulating adiponectin levels up to three times normal had a better reproductive performance than cows with normal circulating adiponectin levels. In addition, differences in metabolic and hormonal profile and production traits may at least in part explain the superior fertility of cows with circulating adiponectin levels up to three times normal. More importantly, the relation between circulating glucose and circulating adiponectin was negative and that might indicate insulin sensitizing traits of adiponectin in dairy cows. Finally, high yielding cows might regulate glucose homeostasis differently to low yielding cows due to increased GH and decreased adiponectin concentrations. However, these results need to be investigated further

6. General discussion

6.1. INTRODUCTION

The control of glucose homeostasis and homeorhesis is of outmost importance in understanding hormonal and metabolic profiles, relating to NEB in early lactation (Bell, 1995; Bauman, 2000). The postpartum cow has to deal with energy deficit and the great demand for nutrients, especially glucose, to meet the high needs of the mammary gland (Butler & Smith, 1989; Bell, 1995; Nebel & McGilliard, 1998). Decreased insulin allows cows to partition glucose preferentially to the mammary gland, and increased circulating GH and prolactin facilitate this adaptation (Bauman & Currie, 1980). Elevated circulating NEFA, BOHB, and urea are mainly produced as a result of excessive mobilization of adipose and muscle tissue to support the demand for nutrients by the mammary gland (Bell, 1995; Grummer, 1995). Decreased circulating IGF-I may be a consequence of deteriorating functionality of the liver which in early lactation prioritizes intensification of processes such as gluconeogenesis, lipolysis, and ketogenesis (Butler *et al.,* 2003; Wathes *et al.,* 2007, 2008). Adiponectin, because of its insulin sensitising actions and its negative correlation with body condition (Berg *et al.,* 2002; Matsuzawa *et al.*, 2004; Kadowaki & Yamauchi, 2005), could be another putative regulator of metabolism during the transition from pregnancy to lactation (Mazaki-Tovi *et al.,* 2005; Mitchell *et al.,* 2005). Body condition score is considered an indirect measure of nutritional status (Short *et al.,* 1990; Garnsworthy, 2006; Bewley & Schutz, 2008) and nutrition has been demonstrated to interact with reproduction (Boland *et al.,* 2001; Garnsworthy *et al.*, 2009).

The overall objective of the present study was to examine the impacts of different nutritional and body condition treatments on metabolic and hormonal profiles, circulating adipokines, and reproductive performance in dairy cows. A special focus was directed to circulating adiponectin and its association with metabolic and hormonal status and reproduction. Work in this thesis has shown some novel effects of circulating adiponectin on cow fertility. In addition, the effects of lactational stage, diet, and BCS at calving on

circulating adiponectin were assessed. Also, adiponectin was shown to be present in bovine milk for the first time. Moreover, optimum insulin concentration in terms of reproduction, and its association with dietary and condition treatments was explored. Furthermore, a subpopulation of dairy cows with consistently high circulating adiponectin levels was identified for the first time, and the hormonal, metabolic, reproductive, and productive profiles of these animals were studied. Last but not least, the association of adiponectin with glucose homeostasis and other metabolic and hormonal signals was determined in cows with different milk yield potential. To our knowledge, no other study has explored associations between circulating adiponectin and hormonal and metabolic stimulus with regard to milk yield potential in dairy cows.

6.2. SUMMARY OF MAIN POINTS

6.2.1. Effect of diet and body condition score at calving on reproductive performance in dairy cows

Results from the present study demonstrated that feeding dairy cows with a HS diet or a HF diet for the first 4 months of lactation would not result in changing reproductive performance (probability of cows to be pregnant, days to first oestrus, and days to conception, were not affected by dietary treatment). That is mainly because important metabolic hormones (e.g. leptin, IGF-I, and GH), production traits (milk yield, BCS, ΔBCS, nadir week, and nadir BCS), and metabolites (e.g. NEFA and glucose) were not influenced by diet (Chapter 3). In addition, moderately elevated insulin concentration that exceeded optimum insulin concentration and induced by feeding dairy cows the HS diet may account for the higher probability to express ABNORMAL milk progesterone profile (Chapter 3). The results of the present study are not in agreement with the study of Gong *et al* (2002b) in which cows fed the HS diet had better reproductive performance than cows fed the HF diet. An explanation for the discrepancies between the present study and the study of Gong *et al* (2002b) could be that the animals were dietary manipulated for longer (up to 2 times) in the present study than the animals of the study of Gong *et al.* (2002b). Moreover, all animals exceeded minimum insulin concentration in terms of reproduction in the present study whereas some animals were below minimum insulin concentrations in the

study of Gong *et al.* (2002b) and that might be another explanation for discrepancies between the two studies (Chapter 3).

The results of the present study clearly indicated that THIN cows at calving (BCS≤ 3.25), which lost less than 0.5 units of BCS postpartum had better reproductive performance than FAT cows at calving (BCS>3.25), which lost more than 0.8 units of BCS postpartum (Chapter 3). FAT cows at calving had higher circulating NEFA and lower circulating IGF-I than THIN cows at calving. Elevated circulating NEFA may detrimentally affect follicular growth and development (Leroy *et al.,* 2008, 2010) and reduced circulating IGF-I negatively influence postpartum reproductive performance (Butler *et al.,* 2000; Huszenicza *et al.,* 2001; Meikle *et al.,* 2004). Moreover, THIN cows at calving had higher probability of being pregnant and lower probability of expressing irregular oestrous cycles than FAT cows at calving, although the animals had the same insulin concentration, which ranged from 0.4 to 0.6 *ng/ml*. This latter result could also explain why THIN cows showed superior reproductive performance compared with FAT cows in the present study (Chapter 3).

6.2.2. Reproductive performance and optimum insulin concentration

Long term moderately elevated insulin concentration (>0.3 *ng/ml*) had a negative impact on pregnancy rate and milk progesterone profile in the present study, whereas optimum insulin concentration that maximized pregnancy rate and minimized the probability for cows to express abnormal milk progesterone profile varied from 0.2 to 0.3 *ng/ml*. Furthermore, insulin concentration greater than 0.6 *ng/ml* reduced the probability of cows being pregnant towards zero, whereas insulin greater than 0.4 to 0.6 *ng/ml* tended to maximize the probability for cows to express an abnormal milk progesterone profile (Chapter 3). Also, insulin concentration greater than 0.7 *ng/ml* resulted in calving to conception interval greater than 100 days, whereas animals with insulin concentration within the range 0.2-0.3 *ng/ml* had a calving to conception interval of less than 70 days (Chapter 5). Therefore, the results suggest that optimum insulin concentration in terms of reproduction is within the range 0.2-0.3 *ng/ml,* whereas insulin concentration greater than

0.6 to 0.7*ng/ml* is detrimental for cow fertility. Interestingly, the current study found that BCS at calving was a critical modulator of fertility in lactating dairy cows when circulating insulin was suboptimum (≥0.4 *ng/ml*). Moreover, THIN cows at calving had higher probability to be pregnant and lower probability to express abnormal milk progesterone profile than FAT cows at calving, when the animals had approximately the same insulin concentration and insulin concentration ranged from 0.4 to 0.6 *ng/ml*. Thus, achieving a BCS of less than 3.25 units at calving is essential to ensure optimum cow fertility. Furthermore, cow fertility (pregnancy rate and milk progesterone profile) was not affected by dietary treatments when insulin concentrations were about the same and insulin ranged from 0.1 to 1 *ng/ml*. However, the current work showed that HF diet must be the preferable feeding strategy to maximise reproductive performance of dairy cows when insulin concentration ranges from 0.1 to 0.3 *ng/ml* and BCS at calving is equal or lower than 3.25 units.

6.2.3. Adiponectin

6.2.3.1. Measurement of circulating adiponectin in dairy cows

The present study showed that plasma adiponectin in dairy cows varied from 6.47±0.75 to 11.15±1.06 *ng/ml*, and some cows had constantly elevated plasma adiponectin throughout the experiment. In addition, circulating adiponectin values were not normally distributed and mean circulating adiponectin was significantly greater than mean insulin, leptin, GH and glucagon. Furthermore, the high autocorrelation of circulating adiponectin values might indicate that plasma adiponectin concentration was tightly controlled genetically and other factors had only a minimal impact (Chapter 4).

6.2.3.2. Measurement of adiponectin in bovine milk

Milk adiponectin concentration was measured for the first time in bovine milk and the mean concentration was 11.5±2.6 *ng/ml* (Chapter 4). This result is in agreement with the studies of Martin *et al.* (2006), Weyermann *et al.* (2006), and Bronsky *et al.* (2006) which confirmed the presence of adiponectin in human milk. Milk and plasma adiponectin

concentrations were similar in the current study, which may imply that milk adiponectin is excreted at concentrations similar to the peripheral circulation (Chapter 4). Adiponectin concentrations in human breast milk ranged from 3.9 to 87.9 *ng/ml* (Bronsky *et al.,* 2006; Martin *et al.,* 2006). Normal adiponectin concentrations in human serum ranged from 5 to 30 *μg/ml* (Ahima, 2006) and this may imply adiponectin is present in human milk in concentration lower than in blood, which is not in agreement with the present study. Ohtani *et al.* (2011) demonstrated the existence of an autocrine-paracrine system of adiponectin action in bovine mammary gland and the presence of adiponectin receptors was shown in human breast cancer cell line MCF-7 (Dieudonne *et al*., 2006). However, there is no study that directly compared circulating and milk adiponectin or examined the action, synthesis, and excretion of adiponectin in the mammary gland. Human milk adiponectin concentrations decrease approximately 5% – 6% with each month of lactation (Newburg *et al.,* 2010; Savino & Liguori, 2008). In the present study, milk adiponectin concentrations did not show the same trend, although samples were collected only for the first two months of lactation; nevertheless, adiponectin varied with days postpartum. Another finding of this study was that milk adiponectin was not different for cows with either high or low milk yield, but this needs to be confirmed (Chapter 4).

The milk adiponectin assay, because it is non-invasive, offers the potential for more widespread studies in dairy cows under commercial conditions. It was intended to apply the technique on a larger scale in the present study, but time and financial constraints precluded this. The effects of adiponectin and adiponectin receptors in mammary gland and milk synthesis need to be elucidated by explicit experiments.

6.2.3.3. The impact of lactational stage, diet and BCS at calving on circulating adiponectin in dairy cows

This study showed that, as in humans (Asai-Sato *et al.,* 2006; Ritterath *et al.,* 2008), the transition from pregnancy to lactation in dairy cows was associated with a reduction in plasma concentration of adiponectin. Moreover, the present study found that after the 4th week postpartum circulating adiponectin was significantly increased and remained at the same level until the 12th week postpartum. Furthermore, this study suggests that after the

12th week postpartum there was a significant reduction in circulating adiponectin (Chapter 4).

In the present study, stage of lactation affected the majority of circulating metabolic hormones, metabolites and production traits, but circulating adiponectin showed correlations only with circulating GH and postpartum BCS loss (ΔBCS). However, there was a weak negative association between plasma adiponectin and glucose. The weekly changing pattern of circulating adiponectin can be partially explained by the negative relationship between GH and adiponectin. This is the first *in vivo* study to demonstrate a negative relationship between GH and adiponectin in dairy cows (Chapter 4).

There was no effect of either diet or BCS at calving on circulating adiponectin. Circulating GH and glucose were not altered by dietary and BCS treatments in this study and this was possibly the reason the diet and BCS at parturition, had no effect on circulating adiponectin concentrations (Chapter 4). However, dietary factors have modulated circulating adiponectin (Reis *et al.,* 2010) in other species. High consumption of magnesium (Qi *et al.,* 2005; Cassidy *et al.,* 2009), caffeine (Williams *et al.,* 2008), n-3 PUFA (Duda *et al.,* 2007), and dietary salt (Lely *et al.,* 2007) are associated in humans with higher circulating adiponectin. Prepartum thiazolidinediones (TZD) administration dramatically decreased ΔBCS and NEFA and increased DMI and the proportion of dairy cows ovulating by 21 days postpartum (Schoenberg *et al.,* 2008; Smith & Overton, 2008). TZD is a known PPARγ agonist, which increases adiponectin production in both humans and rodents (Xu *et al.*, 2007; Yoon *et al.,* 2009). It is likely that the therapeutic effects of TZD are mediated by induction of adiponectin (Xu *et al.*, 2007; Yoon *et al.,* 2009). TZD administration of cows requires drug approval; however, application of dietary strategies enhancing circulating adiponectin may be an inexpensive alternative. Thus, properly designed experiments need to be conducted in order to evaluate putative roles of nutrients in regulating circulating adiponectin in dairy cows

A characteristic trait of circulating adiponectin is its negative correlation with adiposity in non-pregnant human and rodent models (Ahima, 2006). However, circulating adiponectin values throughout pregnancy are not correlated with maternal BMI in humans (Mazaki-

Tovi *et al.*, 2005; Jansson *et al.*, 2008). According to Raddatz *et al.*, (2008) and Puntenney (2006) adiponectin was not correlated with BCS in periparturient cows and this finding is in accordance with the current study. Thus, it is likely that BCS is not correlated with circulating adiponectin in periparturient and early lactating dairy cows. However, further research would determine if this hypothesis is correct.

Postpartum BCS loss (ΔBCS) was negatively correlated with leptin and adiponectin in the current study and may be a useful tool to study circulating adipokines in periparturient and early lactating cows (Chapter 4). The negative correlation of leptin with BCS loss is documented in heifers (Leon *et al.*, 2004) and this is in agreement with the finding of the present study. Weight and BMI loss lead to increased circulating adiponectin in humans (Reinehr *et al.,* 2004; Bobbert *et al.,* 2005; Engl *et al.,* 2007), but this is not in line with the present study. A possible explanation for this discrepancy between the present study and human studies could be differences between human and bovine animal models in regulation of circulating adiponectin. Also, it is known that human adiponectin is down-regulated when hyperinsulinemia and insulin resistance coexist (Kadowaki & Yamauchi, 2005). The animals of the present study had moderately elevated insulin concentrations and that might be another reason why ΔBCS was negatively correlated with adiponectin. In humans, circulating adiponectin showed a strong negative relationship with visceral body fat, which is strongly related to BMI (Matsubara *et al.,* 2002; Cnop *et al.,* 2003; Lara-Castro *et al.,* 2006). It is uncertain if ΔBCS is related to visceral body fat of the cow.

6.2.3.4. Circulating adiponectin and milk yield

Evidence accumulated from *in vitro* studies suggests that human mammary epithelial cells of various cancer cell lines express receptors of adiponectin (Dieudonne *et al*., 2006; Treeck *et al.,* 2008; Jarde *et al.,* 2009). Treatment of cancer mammary epithelial cells with recombinant adiponectin deters their growth, by activating cell apoptosis pathways and by inhibiting the cell cycle (Dieudonne *et al*., 2006). Moreover, obesity causes suppression of circulating adiponectin levels (Kadowaki & Yamauchi, 2005) and genetic improvement of cows for higher milk yield results in increased postpartum BCS loss (Dechow *et al.*, 2002) and circulating GH (Veerkamp *et al.,* 2003). Furthermore, circulating GH was negatively

correlated with circulating adiponectin in dairy cows (as shown in Chapter 4) and adiponectin receptors were present in bovine mammary epithelial cells (Ohtani *et al.,* 2011). It is likely; therefore, to assume low circulating adiponectin (as a result of elevated GH concentrations) leads to increased milk production in high yielding cows. High yielding cows lost more condition postpartum, ate more feed and had higher circulating GH, milk yield and DMI but lower circulating adiponectin, leptin, and insulin than low yielding cows in the current study (Chapter 4). Moreover, cows with circulating adiponectin levels up to three times normal (21.2±1.32 *ng/ml*) had lower GH and milk yield and higher leptin (tendency) than cows with normal circulating adiponectin levels (7.1±0.23 *ng/ml*) as shown in Chapter 5. This is the first study to suggest that due to increased circulating GH and its antagonistic relationship with adiponectin and leptin, high yielding cows may have lower circulating leptin and adiponectin concentrations than low yielding cows.

6.2.3.5. Adiponectin and feed intake in dairy cows

This is the first study to show that adiponectin is likely to be related to DMI in dairy cows, but this relationship is minimal and dependent on BCS at calving (Chapter 4). Kubota *et al.,* (2007) showed that adiponectin enhances AMPK activity in the murine arcuate hypothalamus through AdipoR1 receptor, and stimulates food intake. There is a need for further study of the effect of adiponectin on food intake.

6.2.3.6. Effect of circulating adiponectin levels on reproductive performance

For the first time, a subpopulation of dairy cows with consistently high circulating adiponectin levels was identified, and the hormonal, metabolic, reproductive, and productive profiles of these animals were studied. Cows with circulating adiponectin levels up to three times normal (21.2±1.32 *ng/ml*) had a higher probability to be pregnant and fewer days to first oestrus than cows with normal circulating adiponectin levels (7.1±0.23 *ng/ml*). Also, cows with circulating adiponectin levels up to three times normal had a tendency to be associated with normal milk progesterone profile than cows with normal circulating adiponectin levels. However, there were no significant difference between cows

with circulating adiponectin levels up to three times normal and cows with normal adiponectin levels in the interval from calving to conception and formation of follicular cysts. Differences in metabolic and hormonal profiles and reproductive and production traits may at least partly explain the differences in reproductive performance between cows with high circulating adiponectin and cows with normal circulating adiponectin levels (Chapter 5).

Unfortunately, only 10% of cows in this experiment expressed circulating adiponectin level up to three times normal. In humans, circulating adiponectin has a strong genetic component with heritability to be estimated between 30% and 50% (Comuzzie *et al.*, 2001). Moreover, the high autocorrelation of circulating adiponectin values in the present study may indicate that plasma adiponectin concentration is tightly regulated genetically. Genetic association studies need to be performed in dairy cows to investigate further if there is any association between adiponectin gene polymorphisms and indices of insulin sensitivity, circulating GH levels, circulating adiponectin levels, and glucose resistance.

6.2.4. Glucose homeostasis

Glucose balance in dairy cows was regulated mainly by the negative influence of circulating adiponectin, BOHB, and glucagon on circulating glucose, and the positive influence of circulating insulin on circulating glucose (Chapter 5).

High yielding cows might regulate glucose homeostasis differently to low yielding cows, which was mainly achieved by higher secretion of GH and lower or inactive adiponectin. GH possibly down-regulated leptin and adiponectin, but this was mainly in high yielding cows. Leptin was positively correlated with insulin in both high yielding and low yielding cows, and it was likely to exert insulin stimulatory effects (Chapter 5).

6.2.5. Reproductive performance and adipokines

Reproductive indices (such as pregnancy rate, days to first oestrus, interval from calving to conception, milk progesterone profile, and follicular cyst formation) were measured and the effects of circulating adiponectin and leptin were examined. Leptin was negatively associated with days to first oestrus in the present study (Chapter 5), and this is in agreement with the studies of Liefers *et al.* (2003), Kadokawa *et al.* (2000), Block *et al.* (2001), and Meikle *et al.* (2004) in dairy cows.

Circulating adiponectin was negatively associated with the probability of cows to express abnormal milk progesterone profiles and cows with circulating adiponectin levels up to three times normal had a tendency to be associated with normal milk progesterone profiles (Chapter 5). According to Campos *et al.* (2008) there is plenty of evidence to support a beneficial role of adiponectin in reproduction, but it is not yet clear that adiponectin is required for normal reproductive performance. This is the first study to suggest that hypoadiponectineamia might be associated with increased milk production and a predominance of atypical milk progesterone profiles. This could be another mechanism that leads to infertility in the modern dairy cow; however, explicit experimentation is needed to further investigate this hypothesis.

6.3. INTEGRATION OF RESULTS AND IMPACT ON REPRODUCTIVE MANAGEMENT

Cow fertility was the final output of complicated interactions of BCS at calving, diet, metabolic and hormonal stimulus, production traits, and reproductive indices in the current project. An overview of factors influenced reproductive indices and subsequent cow fertility is presented in Figure 6.1.

Results in this thesis have demonstrated the predominant role of insulin in cow fertility. Pregnancy rate, interval from calving to conception, and milk progesterone profile were associated with insulin and analysis showed that optimum insulin concentration that benefited these indices was within the range 0.2 to 0.3 *ng/ml*, whereas insulin concentration greater than 0.6 to 0.7 *ng/ml* deteriorated them. However, analysis in Chapter

5 showed that insulin concentration greater than 0.6 to 0.7 *ng/ml* could benefit cow fertility due to decreased incidence of follicular cyst formation. Thus, insulin effects on fertility can vary significantly according to insulin concentrations and reproductive indices assessed. Follicular cyst formation positively associated with days to first oestrus and HS diet increased circulating insulin in this study. This latter may suggest that HS diets can be used to benefit fertility in dairy herds with history of low insulin concentration, increased incident of follicular cyst formation, and delayed onset of postpartum ovarian activity. However, it needs to be further elucidated.

Proportion of atypical oestrous cycles was low and pregnancy rate was high when insulin concentration was within the optimum range in the current project. Also, the pregnancy rate was higher when the proportion of atypical oestrous cycles was lower. Therefore, cow fertility was maximized when insulin concentration was within the optimum range. Moreover, optimum calving interval is 12 months in dairy cows (Ball & Peters, 2004) and maintenance of insulin concentration within the optimum range can assure on time reproduction of commercial dairy herds, which is important for their profitability. Consequently, results of this study support the concept that cow fertility will be enhanced if insulin concentration is within the range 0.2 to 0.3 *ng/ml* for a period of 4 months postpartum. This significant finding should be taken in account when a postpartum dietary strategy is designed to improve reproductive performance of dairy cows. Of the managerial tools studied in the current project, HS and HF diets showed a potential to regulate insulin concentrations and should be components of an integrated management strategy that ensures better cow fertility. However, long term use of HS diet should be avoided because it increases the proportion of irregular oestrous cycles.

Results of this study support the concept that ΔBCS and BCS at calving are critical modulators of fertility in lactating dairy cows. Optimum range of ΔBCS in terms of reproduction was 0.25-0.50 units of BCS, whereas ΔBCS greater than 0.70-0.80 units of BCS was detrimental for cow fertility. Moreover, THIN cows at calving (BCS≤ 3.25) had better reproductive performance than FAT cows at calving (BCS>3.25). Furthermore, the results demonstrated that optimum insulin concentration was dependent on BCS at calving in lactating dairy cows and THIN cows at calving (BCS≤ 3.25) had better reproductive

performance than FAT cows at calving (BCS>3.25) when insulin concentration was within the range 0.4-0.6 *ng/ml*. Also, THIN cows at calving had lower ΔBCS and circulating NEFA, and higher circulating IGF-I than FAT cows at calving. NEFA was positively associated with follicular cyst formation and IGF-I was positively associated with pregnancy rate, and negatively with interval from calving to conception in the current study. In addition, ΔBCS was positively associated with days to first oestrus and negatively with pregnancy rate. Therefore, achieving a BCS of less than 3.25 units at calving is essential to ensure optimum cow fertility. The results also suggest that HF diet must be the preferable feeding strategy to maximize reproductive performance of lactating dairy cows when insulin concentration ranges from 0.1 to 0.3 *ng/ml* and BCS at calving status is THIN.

The main effects of adipokines on reproduction were: adiponectin negatively influenced the proportion of animals expressed irregular oestrous cycles, whereas leptin negatively influenced days to first oestrus. ΔBCS was negatively correlated with leptin and adiponectin and may be a useful tool to study circulating adipokines in periparturient and early lactating dairy cows. Moreover, animals with HIGH adiponectin levels were thin at calving (ΔBCS=0.25, BCS at calving= 3.13) whereas animals with NORMAL adiponectin levels were fat at calving (ΔBCS=0.70, BCS at calving= 3.45). This latter may further explain why animals with HIGH adiponectin levels showed superior reproductive performance.

In summary, the results from this study suggest that an integrated management strategy that maximizes cow fertility should be based on the following principles:

(1) Optimum insulin range (0.2-0.3 *ng/ml*) must be maintained for a period of 4 months postpartum.
(2) BCS at calving of less or equal than 3.25 units must be achieved
(3) ΔBCS optimum range (0.25-0.50 units of BCS) must not be exceeded for a period of 4 months postpartum.
(4) Selected prepartum and postpartum feeding strategy must meet the above-mentioned goals.

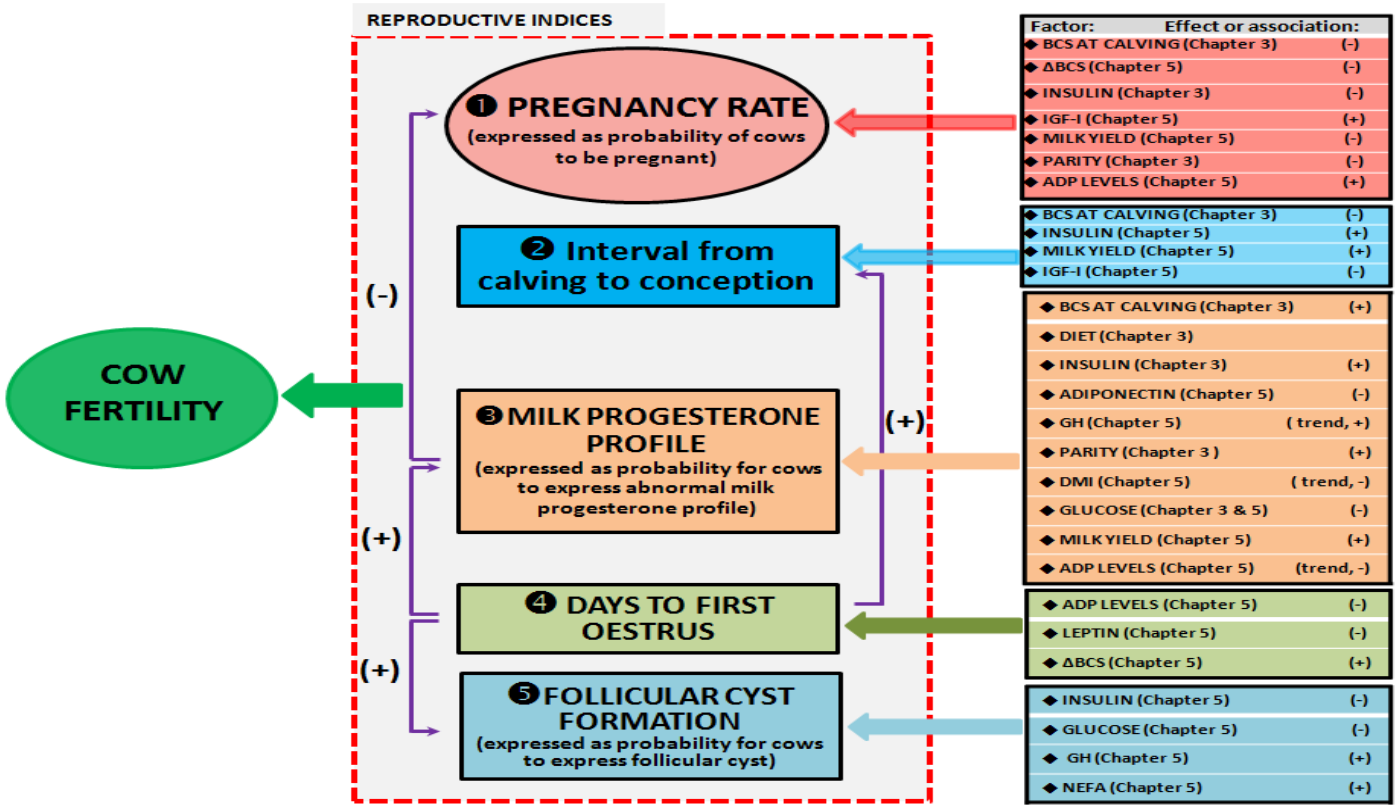


Figure 6.1: Overview of factors influenced reproductive indices and subsequent cow fertility in the present project. ❶ Pregnancy rate (expressed as probability for cows to be pregnant) was affected mainly by BCS at calving (Chapter 3), postpartum BCS loss (ΔBCS) (Chapter 5), circulating insulin (Chapter 3), circulating IGF-I (Chapter 5), milk yield (Chapter 5), parity (Chapter 3), and milk progesterone profile (Chapter 3 & 5). Also, circulating adiponectin levels had an effect on pregnancy rate (Chapter 5), whereas pregnancy rate was not influenced by dietary treatments (Chapter 3). ❷Interval from calving to conception was influenced by BCS at calving (Chapter 3), circulating insulin (Chapter 5), circulating IGF-I (Chapter 5), and milk yield (Chapter 5); it was unaffected by the dietary treatments (Chapter 3) and circulating adiponectin levels (Chapter 5). Also, days to conception was influenced by days to first oestrus (Chapter 5). ❸Milk progesterone profile (expressed as probability for cows to express abnormal milk progesterone profile) was affected mainly by the dietary and BCS at calving treatments (Chapter 3), circulating insulin (Chapter 3), days to oestrus (Chapter 5), circulating adiponectin (Chapter 5), glucose (Chapter 3), parity (Chapter 3), and milk yield (Chapter 5). Also, cows with circulating adiponectin levels up to three times normal had a tendency to be non-randomly associated with normal milk progesterone profile (Chapter 5). Moreover, milk progesterone profile showed a trend to be influenced by circulating GH (Chapter 5) and feed intake (Chapter 5). ❹ Days to first oestrus was affected mainly by ΔBCS (Chapter 5) and leptin (Chapter 5). Also, circulating adiponectin levels had an effect on days to first oestrus (Chapter 5), whereas days to first oestrus was not influenced by the dietary and BCS at calving treatments (Chapter 3). ❺Follicular cyst formation was affected mainly by circulating insulin, glucose, GH, and NEFA (Chapter 5). Also, follicular cyst formation positively associated with days to first oestrus. Positive sign (+) indicates positive effect or association, whereas negative sign (-) indicates negative effect or association.

6.4. LIMITATIONS OF THE STUDY

There are certain limitations in the present study that need to be acknowledged. The first limitation concerns the experimental design, which was repeated measurements (Davis, 2002). The data obtained from this study were panel or longitudinal data. Cows were the panels and treatments were nested within week of experiment. Repeated measurement design, was seen as the most appropriate design for the present study because it allowed definition of individual patterns of adiponectin values (Chapter 5). In contrast, cross-sectional designs would require significantly more cows at different stages of lactation to achieve the same statistical power, without allowing assessment of individual patterns (Minkle, 1997; Davis, 2002; Liu & Li, 2005).

Missing or incomplete data are inherent in studies where repeated measurements are obtained from animals (Little & Rubin, 1987; Allison, 2001; Frees, 2004). However, missing data in panel studies can be handled by *maximum likelihood* (*ML*), *restricted maximum likelihood* (REML), and *multiple imputation estimation* (*MI*) (Allison, 2001; Schafer, 1997; Frees 2004; Enders, 2010) methods. Generalized linear models (GLM), generalized estimating equations (GEE), and *log-linear Poisson* models utilize *ML* to calculate parameter estimates and were used in the present study (McCullagh & Nelder, 1989; Lindsey, 1997; Dobson, 2002). GEE and GLM do not assume normality, but allow choice of error distribution and link function, leading to more efficient parameter estimates (McCullagh & Nelder, 1989; Lindsey, 1997).

The second limitation concerns measurement of plasma and milk adiponectin. There was no species specific validated RIA kit for measuring bovine adiponectin. Serial dilution of bovine plasma and fat-free milk led to samples contained adiponectin concentrations below the sensitivity of the assay. Thus, validation of the method was not feasible. However, the Linco kit has been used for cows (Raddatz *et al.*, 2008), horses (Gordon & McKeever, 2005; Gordon *et al.*, 2006; Kearns *et. al.*, 2006; Pratt *et al.*, 2005), and dogs (Gayet *et al.*, 2007; Brunson *et al.*, 2007). Adiponectin was assessed in bovine milk by using the same kit, which is an extra reason to believe that kit can at least in part measure bovine adiponectin. Circulating adiponectin, as measured by the Linco kit, revealed some of its

basic heterologous traits (Chapter 4), but development of a species specific bovine kit to measure circulating adiponectin might offer an opportunity to estimate plasma adiponectin more precisely in cattle. Analysis of circulating adiponectin levels in Chapter 5 was retrospective and few animals expressed HIGH adiponectin levels. Retrospective analysis is susceptible to bias error sources, but when the examined trait is rare or takes a long time to develop this design is the best choice in practice (Woodward, 1999).

Parameter estimation in structural equation models uses maximum likelihood (*ML*), which is based on certain assumptions (*i.e.* large sample and multivariate normality). It is known that combining small sample sizes, non-normal data, and weak empirical relationships between variables can lead to estimation problems and unreliable results (Werner & Schermelleh-Engel, 2009). It is questionable if the findings of the present study (Chapter 5) can be generalized beyond the cases studied. However, the research findings of the present study concerning glucose homeostasis should be tested in larger numbers of animals, to refine the models suggested.

As discussed above, another limitation of the analysis in the present study was that reproductive performance (Chapter 3 & 5) was assessed in a small sample of cows. Moreover, reproductive hormones were not measured in the present study. Milk progesterone profile was transformed to a binominal variable with two levels (NORMAL and ABNORMAL). This was the best approach in practice because only a small minority of animals expressed DOV1, DOV2, PCL1, and PCL2, although the components of ABNORMAL milk progesterone patterns are not exactly the same in terms of reproduction (Lamming & Darwash, 1998; Taylor *et al.,* 2003).

Another consideration is that BCS at calving was generally high in both BCS groups (Chapter 3, 4, & 5), so the groups consisted of fat versus over-fat cows. Because some hormonal and metabolic traits (*e.g.* adiponectin and leptin) are affected by adiposity, and the differences between the BCS groups were small, they possibly did not allow significant responses to be investigated fully.

6.5. RECOMMENDATIONS FOR FURTHER RESEARCH

The research conducted in this project has led to some novel results and conclusions. The purpose of this section is therefore to identify and discuss the need for further research. The areas of further research include the following:

There is a critical need for additional comprehensive studies on the potential for reproductive performance in dairy cows to be modulated by BCS at calving and insulin. It is important to determine the best BCS at calving and optimum insulin concentration range in terms of reproduction, and what are the best management approaches for obtaining an optimum ΔBCS and insulin range. Moreover, experiments need to be conducted to give a better understanding of the importance and role of insulin and ΔBCS in fertility and productivity of dairy cows, and their association with hormonal and metabolic profiles.

It is known that genetic selection for milk yield led to higher circulating GH in dairy cows (Veerkamp *et al.*, 2003) and insulin resistance is commonly observed in early lactating cows (Sano *et al.,* 1991, 1993). This makes the early lactating dairy cow the most suitable animal model to study associations of adiponectin with GH and glucose. In the present study, the relationship between adiponectin and GH was antagonistic and the relation between circulating glucose and adiponectin was also negative. However, additional research is needed to confirm this negative association and the causes.

The current prevalence of obesity in humans in the UK is around 20%, but rates are increasing rapidly (Norman, 2010). Obesity is related to diabetes mellitus and both are associated with metabolic complications such as hypoadiponectineamia, and subfertility (Ramsay *et al.,* 2006). Increased risk of breast cancer is also associated with reduced circulating adiponectin (Mantzoros *et al.,* 2004). Bovine adiponectin protein sequence shares about 85% homology with human adiponectin protein sequences (Berg *et al.*, 2002). Interestingly, the presence of the insulin-sensitizing adipokine adiponectin in fat-free cow milk was ascertained by the present study. According to Xu *et al.* (2007) direct supplementation of recombinant adiponectin in human subjects is extremely expensive. Thus, consumption of fat-free cow milk (or dairy products) could be an alternative

inexpensive source of adiponectin for humans, but this needs to be elucidated further by interdisciplinary research. Furthermore, 10% of the animals in the present study expressed circulating adiponectin levels up to three times normal, and milk and plasma adiponectin concentrations were similar, which may imply that milk adiponectin is excreted at concentrations similar to those found in blood. If that is the case, then selection of animals with circulating adiponectin levels up to three times normal would allow production of distinctive label milk with high adiponectin. However, explicit experimentation is needed to investigate if this hypothesis is true.

6.6. OVERALL CONCLUSIONS

This thesis has confirmed previous reports indicated that BCS at calving is a critical regulator of fertility in lactating dairy cows. THIN cows at calving (BCS≤3.25), which lost less than 0.5 units of BCS (ΔBCS) during the first 4 months of lactation, had superior reproductive performance, and that was because of increased circulating IGF-I and decreased circulating NEFA. Moreover, this study clearly showed that there was an optimum insulin concentration (0.2 to 0.3 *ng/ml*) necessary for normal reproductive performance, while insulin concentration greater than 0.6 *ng/ml* impaired reproductive performance in lactating dairy cows. The results strongly suggested that optimal reproductive performance of dairy cow was dependent on insulin concentration and BCS at calving.

Adiponectin showed a potential to regulate glucose homeostasis and feed intake in the current study, and it was detectable in bovine milk. Also, genetic selection for milk production due to increased circulating GH and its antagonistic relationship with adiponectin may lead to hypoadiponectineamia in modern high yielding cows. More importantly, hypoadiponectinemia could be another mechanism that contributes to poor fertility in dairy cows. Based on the results of the present study, adiponectin is a plausible regulator of metabolism and reproduction in dairy cows. Nevertheless, the specific role of adiponectin in bovine physiology is yet to be clarified.

APPENDIX

A.1. Blood sampling and post-collection processing

Blood samples for hormonal assays, and plasma metabolites were collected from the coccygeal veins of cows into evacuated 10 *ml* vacutainer tubes (BD Vacutainer Systems®, Belliver Industrial Estate, Plymouth, UK) containing heparin anticoagulant. The blood samples were collected on ice and centrifuged within 10 minutes at 3000 *rpm* for 15 minutes at 4 ºC. Plasma samples was separated into duplicate aliquots and stored frozen in 5 ml screw cap containers at -20 ºC until subsequently assayed.

A.2. Experimental diets

The two diets were formulated to be iso-energetic (ME~12.5 *MJ/kg* DM) and iso-nitrogenous (CP~180 *g/kg* DM). The only difference between the two diets was that the High Starch diet had higher starch content than the High Fat diet (182 *g/kg* DM versus 98 g/kg DM) and the High Fat diet had higher fat content than the High Starch diet (53 *g/kg* DM versus 39 *g/kg* DM).

Table A.1: Formulation and composition of High Starch and High Fat diets (Garnsworthy *et al.,* 2009)

	DIET	
	HIGH STARCH [HS]	**HIGH FAT [HF]**
1. Ingredients (g/kg DM):		
Grass silage	404	409
Maize / whole-crop wheat silage	119	120
Sugar beet pulp	114	211
Wheat	209	77
Soyabean meal	76	85
Rapeseed meal	57	61
Fatty acid supplement[a]	11	27
Minerals and vitamins[b]	10	10
	1000	1000
2. Composition[†]:		
DM (*g/kg*)	556	553
ME (*MJ/kg* DM)	12.5	12.6
CP (*g/kg* DM)	179	181
NDF (*g/kg* DM)	285	293
Starch (*g/kg* DM)	182	98
Sugars (*g/kg* DM)	57	67
Fat (*g/kg* DM)	39	53

[†]DM, Dry Matter; ME, Metabolisable Energy; CP, Crude Protein; NDF, Neutral Detergent Fibre

[a] Megalac, calcium salts of palm fatty acids; Volac International, Royston, UK.

[b] Bibby HiPhos: ABN Ltd, Peterborough: Calcium, 18%; Phosphorus, 10%; Magnesium, 5%; Salt, 17%; Copper, 2000 mg/kg; Manganese, 5000 *mg/kg*; Cobalt, 100 *mg/kg*; Zinc, 6000 *mg/kg*; Iodine, 500 *mg/kg*; Selenium, 25 *mg/kg*; Vitamin A, 400,000 *IU/kg*; Vitamin D3 80,000 *IU/kg*; Vitamin E, 1000 *mg/kg*.

A.3. Measurement of adiponectin in blood plasma (*Linco Research kit #HADP-61HK*)

A.3.1. 10*x* Assay Buffer

Final concentration upon dilution is 10.0 mM Phosphate Buffer, pH 7.6 containing 0.08% Sodium Azide,

0.1 % RIA Grade BSA

Quantity: 50 *ml*/vial

Preparation: The content of the vial was diluted with 450 ml deionized water.

A.3.2. Antiserum

Rabbit anti-Adiponectin Antibody

Quantity: 13 *ml*/vial

Preparation: Ready to use

A.3.3. ^{125}I-Adiponectin

^{125}I-Adiponectin Label (specific activity 67.7 $\mu Ci/\mu g$) lyophilized for stability. Freshly iodinated label contains <3 μCi, (<111 *kBq*).

Quantity: 13.5 *ml*/vial upon hydration

Preparation: ^{125}I-Adiponectin was hydrated with 13.5 *ml* of 1*x* Assay Buffer and allowed to sit at room temperature for 30 minutes, with occasional gentle mixing.

A.3.4. Standards

Purified Recombinant Adiponectin, 200 *ng/ml* lyophilized for stability

Quantity: 1 *ml* upon hydration

Preparation: The Purified recombinant adiponectin was hydrated with 1 *ml* deionized water.

A.3.5. Quality controls 1& 2

Purified recombinant adiponectin lyophilized for stability

Quantity: 1 *ml*/vial upon hydration

Preparation: The Purified Recombinant Adiponectin reconstituted with 1 ml deionized water and mixed well.

A.3.6. Rabbit Carrier

30% Normal Rabbit Serum

Quantity: 2 *ml*/vial

Preparation: Ready to use

A.3.7. Precipitating Reagent (chilled at 4 °C before being used)

Goat anti-Rabbit IgG Serum, 3% PEG and 0.05% Triton X-100 in 0.05 M Phosphosaline, 0.025 M EDTA,

0.08% Sodium Azide

Quantity: 130 *ml*/vial

Preparation: Ready to use

A.3.8. Preparation of standards

Standards were prepared in polypropylene tubes on the day of use, as specified in the tables below.

Standards concentration (*ng/ ml*)	Volume of Deiodinized Water to add	Volume of Standards to add
200	1 *ml*	0

Stds	Std concentration (*ng/ml*)	Volume of Assay Buffer to add	Volume of Std to add (Serial Dilutions)
1	100	0.5 *ml*	0.5 *ml* of 200 *ng/ml*
2	50	0.5 *ml*	0.5 *ml* of 100 *ng ml*
3	25	0.5 *ml*	0.5 *ml* of 50 *ng ml*
4	12.5	0.5 *ml*	0.5 *ml* of 25 *ng/ml*
5	6.25	0.5 *ml*	0.5 *ml* of 12.5 *ng/ml*
6	3.125	0.5 *ml*	0.5 *ml* of 6.25 *ng/ml*
7	1.56	0.5 *ml*	0.5 *ml* of 3.125 *ng/ml*
8	0.78	0.5 *ml*	0.5 *ml* of 1.56 *ng/ml*

A.3.9. Assay Procedure

The Adiponectin assay was run in 2 days as follows:

1st Day:

- Polypropylene tubes were labeled.
- 300 *µl* of Assay Buffer pipetted to the Non-Specific Binding (NSB) tubes (3-4).
- 200 *µl* of Assay Buffer added into the Reference (B_o) tubes (5-6).
- 100 *µl* of Assay Buffer added to tubes seven through the end of the assay.
- 100 *µl* of Standards and Quality Controls added in duplicate into tubes.
- 100 *µl* of each sample (undiluted) in duplicate pipetted to tubes.
- 100 *µl* of ^{125}I-Adiponectin pipetted to all tubes.
- 100 *µl* of Adiponectin Antibody pipetted to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
- All tubes were vortexed, covered and incubated overnight (20-24 hours) at room temperature.

2nd Day:

- 10 *µl* of Rabbit Carrier pipetted to all tubes except Total Count tubes (1-2).
- 1.0 *ml* of cold (4°C) Precipitating Reagent was added to all tubes except Total Count tubes (1-2).
- All tubes were vortexed and incubated 20 minutes at room temperature.

- All tubes were centrifuged at 3,000 *rpm* for 30 minutes except Total Count tubes (1-2).
- The supernatant was decanted immediately from all tubes except TC tubes.
- Tubes left to drain for 60 seconds and the pellet counted for 1 minute on the gamma counter.

Results were calculated automatically by the instrument software (Multicalc, Wallac, Stockholm, Sweden).

The assays run five times and a composite standard curve (a plot of B/B_0 against adiponectin concentration) was created (Figure A.1). Sensitivity of the assay, expressed as effective dose 80% (ED_{80}), was calculated from this composite standard curve, and it was 2.33±0.08 *ng/ml*.

Serial dilution of bovine plasma (1/2, 1/3, 1/5, 1/7, and 1/10) led to samples contained adiponectin concentrations below the sensitivity of the assay. Thus, validation of the method by showing parallelism was not feasible.

A.3.10. Equipment

11277 Gammamaster, LKB, Wallac, Stockholm, Sweden.
2. Jouan KR422 Centrifuge.

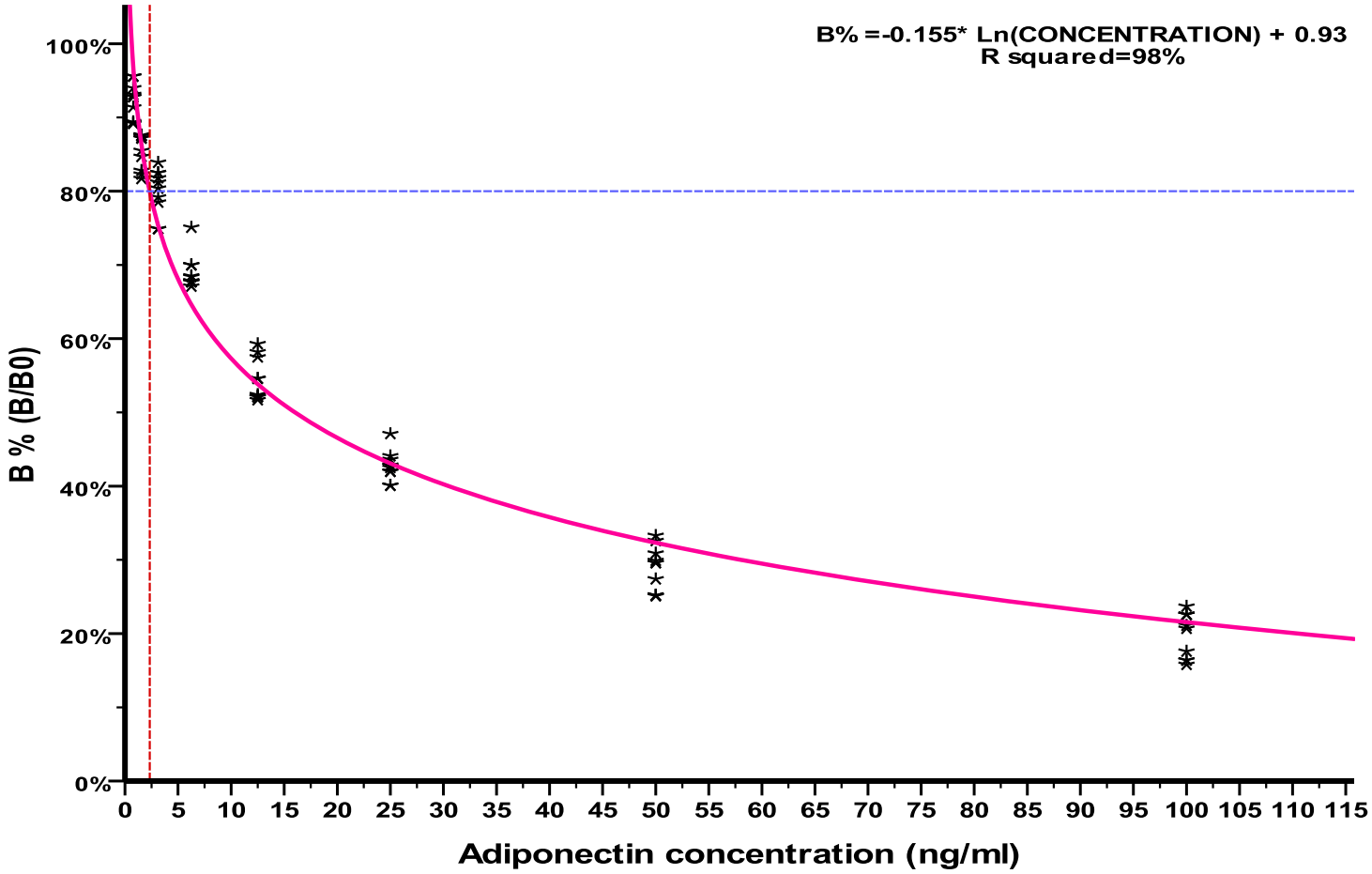


Figure A.1: Composite standard curve of plasma adiponectin assay. A plot of B/B_0 against adiponectin concentration was created for five consequtive runs of plasma adiponectin assay. Sensitivity of the assay, expressed as effective dose 80% (ED_{80}), was calculated from this standard curve, and it was 2.33 *ng/ml*.

A.4. Measurement of adiponectin in bovine milk (*Linco Research kit #HADP-61HK*)

Milk samples were collected from 6 cows in different stage of lactation. All samples were collected between 15:00 and 17:00 in the afternoon and left in the fridge (0 to 4 ^{o}C) over night. Because lipids interfere with radioimmunoassay (RIA), skim milk was used. 4 ml of whole milk of each cow was added in 6 tubes. Milk samples were vortexed and skim milk (aqueous phase) was obtained by centrifugation (1500*g*, 20 min, 4°C), after which the fat layer was removed and weighted in electronic balance (Mettler Toledo, Leicester, UK). Immunoreactive adiponectin was assessed in duplicate by using a commercial RIA kit (Linco Research, St Charles, MO) with the use of 100 μl of skim milk. The adiponectin kit assay performed as described previously and adiponectin concentration in skim milk was measured.

The assays run one time and a standard curve (a plot of B/B_0 against adiponectin concentration) was created (Figure A.2). Sensitivity of the assay, expressed as effective dose 80% (ED_{80}), was calculated from this standard curve, and it was 2.14 *ng/ml*.

Serial dilution of bovine fat free milk (1/2, 1/3, and 1/5) led to samples contained adiponectin concentrations below the sensitivity of the assay. Thus, validation of the method by showing parallelism was not possible.

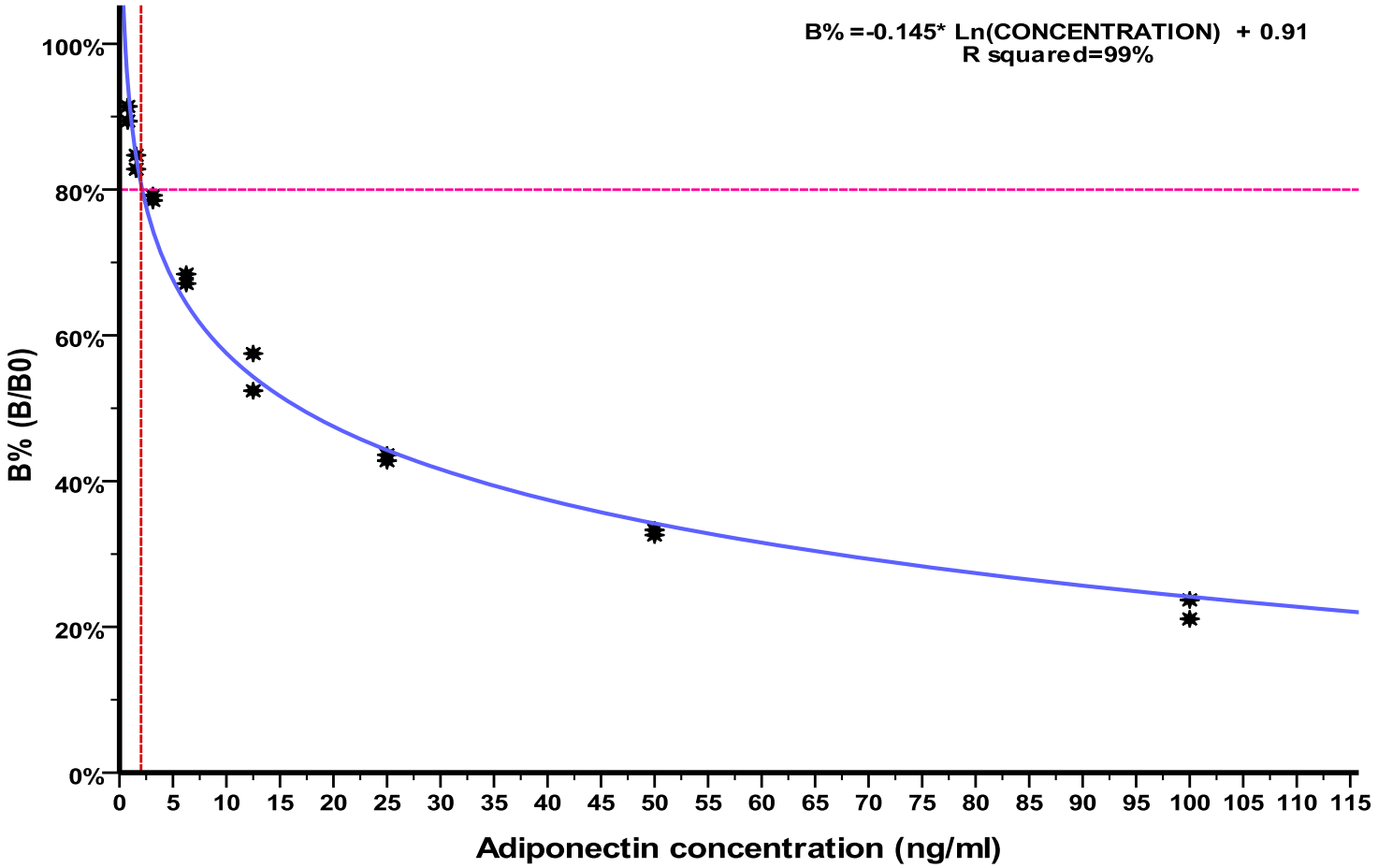


Figure A.2: Standard curve of milk adiponectin assay. A plot of B/B_0 against adiponectin concentration was created for one run of milk adiponectin assay. Sensitivity of the assay, expressed as effective dose 80% (ED_{80}), was calculated from this standard curve, and it was 2.14 *ng/ml*.

A.5. Measurement of milk progesterone (*ELISA kit, Ridgeway Scientific*) and determination of milk progesterone profile

Whole milk samples were analyzed for progesterone concentrations using a microtitre plate enzyme-linked immunosorbant assay (ELISA) kit (Ridgeway Scientific, Alvington, UK), validated by Sauer *et al.* (1986).

Samples were removed from the refrigerator (stored at 4°C after addition of Lactab MkIII tablets) at least 3 hours before analysis and warmed to 25° C. The plates are also brought at room temperature, the foil stripped from the wells and the wells emptied and tapped dry. To each well 10 *µl* of sample or standard (1 to l0 *ng/ml* progesterone in milk from an oestrus animal) was added, followed by 200 *µl* of progesterone-enzyme label (progesterone- 11 -α glucuronide-alkaline phosphatase). The plate was then vortexed and left for 1 - 1.5 hours at room temperature.

After incubation the plate was washed three times with cold water and tapped dry each time. Finally 200 *µl* of substrate in substrate buffer was added, and the plates vortexed again and left for developing the color. The samples were read on a 570 *nm* plate reader. Quality control samples could not be stored for the entire period of the trial. New quality control samples were selected from previously analyzed samples.

The reliable reading range of the ELISA was from 1.5 to 10.5 *ng/ml*. Samples reading < 1.5 *ng/ml* were taken as 1.5 *ng/ml*; samples reading >10.5 *ng/ml* were diluted to bring the reading within range. If the coefficient of variation (CV) of duplicate sample readings was > 15%, the analysis was repeated. The intra- and inter-assay coefficients of variation were < 15 and 6.6%, respectively.

Milk progesterone was measured twice in a week (either Monday and Thursday or Tuesday and Friday mornings) and a rise in progesterone was defined as above 3 *ng/ml* for two consecutive samples (Lamming & Darwash, 1998; Garnsworthy *et al.,* 2009).

Progesterone profiles were used to classify estrous cycles as normal or abnormal (DOV1, DOV2, PCL1 or PCL2), following the definitions of Lamming & Darwash (1998) (Table A.2).

Table A.2: Definition of atypical ovarian activity in dairy cattle using milk progesterone profile (Lamming & Darwash, 1998)

Atypical reproductive pattern	Definition
Delayed ovulation type I **(DOV1)**	Milk P4 concentration <3 *ng/ml* for ≥ 45 days postpartum
Prolonged inter-luteal interval, delayed ovulation type II **(DOV2)**	Milk P4 concentration <3 *ng/ml* for ≥ 12 days between two luteal phases.
Delayed luteolysis during first cycle, persistent CL type I **(PLC1)**	Milk P4 concentration >3 *ng/ml* for ≥19 days during the first postpartum oestrous cycle.
Delayed luteolysis during subsequent cycles before insemination, persistent CL type II **(PCL2)**	Milk P4 concentration >3 *ng/ml* for ≥19 days during subsequent postpartum cycles.

A.6. Measurement of metabolites in blood plasma

Blood plasma samples were analyzed for the following metabolites on a Bayer opera autoanalyzer (Bayer UK Ltd).

A.11.1. Urea

Plasma Urea concentrations were determined in a single assay, using a kit supplied by Bayer (*Bayer kit T01 182356*). The method was based on Urea hydrolyzation in the presence of water and urease. The coefficient of variation was less than 5%.

A.11.2. Glucose

Plasma glucose concentrations were determined in a single assay using a kit supplied by Bayer (*Bayer kit T01 183356*). The method was based on hexokinase catalytic phosphorylation of plasma glucose. The coefficient of variation for samples was less than 5%.

A.11.3. *β*-hydroxybutyrate (BOHB)

Plasma β-hydroxybutyrate concentrations were determined using an enzymatic kit supplied by Randox (*Ranbut RB 1008*). The method was based on the oxidation of D-3-hydroxybutyrate to acetoacetate by the enzyme 3-hydroxybutyrate dehydrogenase. The coefficient of variation for samples was less than 5 %.

A.11.4. Non- esterified fatty acids (NEFA)

Plasma NEFA concentrations were determined using an enzymatic kit supplied Waiko (*Waiko kit NEFA-C*). The method was based on the acylation of coenzyme A by the fatty acids in the presence of added acyl-CoA synthetase. The coefficient of variation for samples was less than 5 %.

B.1. Parameter estimates of models were used to explore interrelationships of adiponectin with glucose, BCS, metabolic hormones, and metabolites (Chapter 5**)**

Table B.1: Model parameter estimates

	TYPE OF MODEL		
Estimates:	**BASIC MODEL** †	**LOW PMY** †	**HIGH PMY** †
Glucose (*mmol/l*)	3.40±0.478	3.38±0.551	3.42±0.626
Insulin (INS) (*ng/ml*)	0.41±0.012	0.42±0.019	0.40±0.015
Glucagon (GLGON) *(pg/ml)*	95.1±2.47	98.9±3.51	91.2±3.3
Adiponectin (ADP) (*ng/ml*)	8.5±0.72	9.9±1.15	7.2±0.82
Leptin (LPN) (*ng/ml*)	1.73±0.115	1.9±0.203	1.57±0.106
GH (*ng/ml*)	4.55±0.266	4.16±0.37	4.95±0.394
BOHB (*mmol/l*)	0.65±0.029	0.60±0.032	0.69±0.049
NEFA (*mmol/l*)	0.43±0.033	0.45±0.057	0.40±0.036
BCS (*units 1-5*)	2.76±0.037	2.76±0.056	2.77±0.052

† **Columns are means±SE.**

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