Effect of ketosis on fertility of lactating dairy cows

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ABSTRACT

Metabolic disturbances during early lactation have long been shown to have adverse association with the reproductive performance of dairy cows. The overall objective of this doctoral research was to investigate the potential mechanisms of this negative association between postpartum metabolic disorders and fertility in lactating dairy cows. The body condition scoring (BCS) is an indicator of body fat mobilization and energy balance. Although the association between excessive BCS loss and reduced reproductive performance in early lactating cows is known, the underlying mechanisms remain unclear. In the first study, we examined the effects of severe BCS loss during early lactation on hepatic and ovarian functions in dairy cows. Cows experiencing severe BCS loss (SEV) compared to those with moderate BCS loss (MOD) had elevated concentrations of non-esterified fatty acids (NEFA), β-hydroxy butyrate (BHB) and γ–glutamyl transferase (GGT), indicative of fat mobilization and liver dysfunction. The analysis of the hepatic transcriptome in SEV and MOD cows at Week 7 after calving revealed 1,186 differentially expressed genes (DEGs), among which the pathways associated with lipid metabolism were significantly enriched. Impaired liver function in SEV cows was associated with reduced hepatic secretion of insulin-like growth factor (IGF1) and lower concentrations of IGF1 in the antral fluid of the dominant follicle. The dominant follicles of SEV cows had lower estradiol-17β along with reduced expression of the genes associated with follicular competence. These data show that severe BCS loss impairs liver function and reduces IGF1 output resulting in an incompetent ovarian dominant follicle in lactating dairy cows. In the second study, we evaluated the reproductive performance of cows with elevated milk BHB (EMB; determined using the milk recording program data), indicative of ketosis, occurring within 42 days in milk (DIM). Using the data from over 30,000 Holstein cows, we found that there was a negative association between

EMB within 42 DIM and reproductive performance after the voluntary waiting period. Of the six EMB groups, five groups had longer first service to conception, days open and calving interval compared to cows negative for EMB. Interestingly, late ketosis occurring during 15-42 DIM appeared to have more deleterious effect on fertility than other ketosis types. In the final study, we set out to uncover the mechanism underpinning the negative association between ketosis during 3-14 DIM, as determined by milk BHB concentrations and ovarian activity within 150 DIM, as assessed through the milk progesterone (P4) profile, in Holstein cows. Compared to NEG cows, SCK and CK cows had a longer median interval of calving to first luteal activity and calving to first heat within 150 DIM. Importantly, the probability of a postpartum anestrus alarm was 50% and 110% higher in cows with SCK and CK, respectively, compared to NEG cows. These data demonstrate that the negative effect of ketosis on fertility involves ovarian dysfunction. Overall, the data from this doctoral research show that severe postpartum negative energy balance impacts subsequent reproductive performance through reduced IGF1 support from the liver leading to subnormal follicular development in the ovary and the metabolic disease ketosis negative impacts subsequent fertility through sub-functional corpus luteum in dairy cows.

RÉSUMÉ

Il a été démontré depuis longtemps que les perturbations métaboliques en début de lactation ont un effet négatif sur les performances reproductives des vaches laitières. L'objectif global de cette recherche doctorale était d'étudier les mécanismes potentiels de cette association négative entre les troubles métaboliques post-partum et la fertilité chez les vaches laitières en lactation. La note d'état corporel (BCS) est un indicateur de la mobilisation des graisses corporelles et de l'équilibre énergétique. Bien que l'on connaisse l'association entre la perte excessive de BCS et la réduction des performances reproductives chez les vaches en début de lactation, les mécanismes sous-jacents ne sont pas encore clairs. Dans la première étude, nous avons examiné les effets d'une perte sévère de BCS en début de lactation sur les fonctions hépatiques et ovariennes des vaches laitières. Les vaches présentant une perte sévère de BCS (SEV) par rapport à celles présentant une perte modérée de BCS (MOD) avaient des concentrations élevées d'acides gras non estérifiés (NEFA), de β-hydroxy-butyrate (BHB) et de γ-glutamyl-transférase (GGT), ce qui indique une mobilisation des graisses et un dysfonctionnement du foie. L'analyse du transcriptome hépatique chez les vaches SEV et MOD à la semaine 7 après le vêlage a révélé 1 186 gènes différentiellement exprimés (DEG), parmi lesquels les voies associées au métabolisme des lipides étaient significativement enrichies. L'altération de la fonction hépatique chez les vaches SEV a été associée à une sécrétion hépatique réduite du facteur de croissance analogue à l'insuline (IGF1) et à des concentrations plus faibles d'IGF1 dans le liquide antral du follicule dominant. Les follicules dominants des vaches SEV présentaient un taux d'estradiol-17β plus faible ainsi qu'une expression réduite des gènes associés à la compétence folliculaire. Ces données montrent que la perte sévère de BCS altère la fonction hépatique et réduit la production d'IGF1, ce qui entraîne un follicule dominant ovarien incompétent chez les vaches laitières en lactation. Dans la seconde étude, nous

avons évalué la performance reproductive des vaches présentant un taux élevé de BHB dans le lait (EMB ; déterminé à l'aide des données du programme de contrôle laitier), indiquant une cétose, survenant dans les 42 jours dans le lait (DIM). En utilisant les données de plus de 30 000 vaches Holstein, nous avons constaté qu'il y avait une association négative entre l'EMB dans les 42 jours dans le lait et la performance reproductive après la période d'attente volontaire. Sur les six groupes EMB, cinq groupes présentaient une durée plus longue pour la première saillie jusqu'à la conception, les jours ouverts et l'intervalle entre les vêlages par rapport aux vaches négatives pour l'EMB. Il est intéressant de noter que la cétose tardive survenant entre 15 et 42 jours DIM semble avoir un effet plus délétère sur la fertilité que les autres types de cétose. Dans la dernière étude, nous avons cherché à découvrir le mécanisme qui sous-tend l'association négative entre la cétose durant la période 3-14 DIM, déterminée par les concentrations de BHB dans le lait, et l'activité ovarienne durant la période 150 DIM, évaluée par le profil de progestérone (P4) dans le lait, chez les vaches Holstein. Par rapport aux vaches NEG, les vaches SCK et CK présentaient un intervalle médian plus long entre le vêlage et la première activité lutéale et entre le vêlage et le premier chaleurs dans un délai de 150 jours DIM. Il est important de noter que la probabilité d'une alarme d'anœstrus post-partum était 50 % et 110 % plus élevée chez les vaches SCK et CK, respectivement, par rapport aux vaches NEG. Ces données démontrent que l'effet négatif de la cétose sur la fertilité implique un dysfonctionnement ovarien. Dans l'ensemble, les données de cette recherche doctorale montrent qu'un bilan énergétique négatif sévère en post-partum a un impact sur les performances reproductives ultérieures par le biais d'un soutien réduit du foie en IGF1 entraînant un développement folliculaire subnormal dans l'ovaire, et que la maladie métabolique qu'est la cétose a un impact négatif sur la fertilité ultérieure par le biais d'un corps jaune subfonctionnel chez les vaches laitières.

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CONTRIBUTIONS OF AUTHORS

This thesis follows the manuscript format of McGill University's guidelines. Three co-authored manuscripts have been included in this thesis.

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TW Alemu and Y Schuermann: conception, experimental execution, collection, analysis and interpretation of data, writing the first draft, revising and editing the manuscript. E Madogwe, A St-Yves, N Dicks, R Bohrer, V Higginson, RG Mondadori, M Priotto de Macedo, M Taibi, B Baurhoo, V Bordignon: conception, experimental assistance, data interpretation and critically revising the manuscript. R Duggavathi: conception, experimental supervision, drafting and critically revising the manuscript.

Authors of manuscript 2 (Chapter 4) entitled "Reproductive performance of lactating dairy cows with elevated milk β-hydroxybutyrate levels during first 6 weeks of lactation". Published in Journal of Dairy Science (PMID: 37225583).

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TW Alemu analyzed the data, produced tables and figures, and wrote the manuscript, DE Santschi reviewed the manuscript, RI Cue assisted with data analysis and reviewed the manuscript, and R Duggavathi conceptualized the experimental ideas and reviewed the manuscript.

Authors of manuscript 3 (Chapter 5) entitled “Adverse association between ketosis and ovarian activity in Canadian dairy cows". To be submitted to journal of Animal.

Teshome Wondie Alemu and Raj Duggavathi

TW Alemu: conception, analyzed the data, produced tables and figures, interpretation of results and wrote the manuscript and R Duggavathi: conception, interpretation of results, and critically revising the manuscript.

CONTRIBUTION TO KNOWLEDGE

Chapter 3

In the first study, we shed light on the negative impact of severe body condition loss on the hepatic output of insulin-like growth factor 1, resulting in adverse effects on the dominant follicle in the ovary of dairy cows. Cows with higher BCS at caving had lost excessive BCS after calving. Cows that experienced excessive BCS loss after calving had elevated NEFA and BHB concentrations, suggesting fat mobilization, and higher GGT levels, indicating liver dysfunction. This study is the first to evaluate IGF1 in the blood, liver and dominant follicle at the same time in early lactating cows. The results show that cows experiencing excessive BCS loss after calving had reduced hepatic, circulatory, and follicular fluid IGF1 output, leading to reduced estradiol production in dominant follicle along with reduced mRNA expression of dominant follicle competence genes in granulosa cells, including *CYP19A1, NR5A2, IGF1R,* and *LHCGR.*

Chapter 4

This is the first report that investigated the impact of early and late elevated milk BHB (EMB), indicative of ketosis, on reproductive performance of dairy cows in early lactation. More specific categorization of ketosis enabled us to provide compelling evidence that ketosis occurring at different stages of early lactation can be deleterious to reproductive performance of dairy cows. We showed that reproductive parameters: first service to conception, days open and calving interval was prolonged by all EMB categories except EARY_SUS EMB cows compared to NEG cows. It is interesting to note that late EMB during 15-42 DIM after calving has the strongest negative effects on reproductive performance of lactating dairy cows. Cows with LATE_POS EMB had the longest first service to conception, days open, and calving intervals. Overall, this

study suggests that dairy producers should pay attention to ketosis during the first six weeks of lactation to avoid its negative impact on reproductive performance of their cows.

Chapter 5

To uncover the mechanisms of deleterious effects of ketosis on the overall reproductive performance of lactating cows, we investigated the association between ketosis, diagnosed by milk BHB concentrations during 3-14 days after calving, and ovarian activity, measured by milk P4 within 150 days of lactation in Holstein cows. This is the first study examined the effect of ketosis on ovarian activity using milk BHB and milk P4 profile. We showed that cows with SCK and CK had a delayed first luteal activity and first heat after calving, and a higher probability of postpartum anestrus compared to NEG cows.

Overall, these studies highlighted the deleterious effect of metabolic disturbance in early lactation on dairy cow fertility. Therefore, monitoring body condition change and ketosis is crucial for farms to improve the reproductive performance of their cows during the transition period.

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LIST OF ABBREVIATIONS

BCS	body condition scoring
BHB	β-Hydroxybutyric acid
CCI	calving to conception interval
CDNA	complementary DNA
CFH	calving to the first heat
CFLA	calving to first luteal activity
CFSI	calving to first service interval
CK	Clinical ketosis
CYP11A1	cholesterol side chain cleavage enzyme
CYP17A1	cytochrome P450 family 17 subfamily A member 1
CYP19A1	Aromatase
DEGs	Differential expression genes
DIM	Days in milk
DNA	Deoxyribonucleic acid
E2	Estradiol
EARLY_POS	early positive
EARLY_POS_Pro	prolonged early positive
EARLY_SUSP	early suspect
EARLY_SUSP_Pro	prolonged early suspect
EMB	elevated milk BHB
FLP	first luteal phase

FSCR	first service conception rate
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
FTIR	Fourier Transform Infrared Spectroscopy
GGT	γ–glutamyl transferase
GnRH	gonadotrophin releasing hormone
GO_BP	Gene Ontology (GO) Biological Process (BP)
HDL	high-density lipoprotein
HN	Herd Navigator
HR	hazard ratio
HSD17B	17β-hydroxysteroid dehydrogenase
HSD3B	3β-hydroxysteroid dehydrogenase
HYK	Hyperketonemia
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
IGFBP	Insulin-like growth factor binding proteins
KEGG	Kyoto Encyclopedia of Genes and Genomes
LATE_POS	late positive
LATE_SUSP	late suspect
LH	luteinizing hormone
LHCGR	luteinizing hormone receptor
LP	luteal phase
MOD	Moderate

mRNA	Messenger ribonucleic acid
NEB	negative energy balance
NEFA	non-esterified fatty acid
NEG	Negative
NLA	no luteal activity
NR5A2	nuclear receptor subfamily 5 group A member 2
OR	odds ratio
P4	Progesterone
PCR	polymerase chain reaction
PGF2α	prostaglandin F2α
PPA	prolonged postpartum anestrus
RR	relative risk
SCK	subclinical ketosis
SEV	Severe
TG	Triglycerides
VLDL	very low density lipoprotein

CHAPTER 1

INTRODUCTION

The continuous selection for improved milk yield, particularly in Holstein-Friesian cows, has proven to be highly successful. North American Holstein cows have produced about 11,000 kg of milk per lactation in the past five decades (Stevenson & Britt, 2017). Despite the rise in milk yield, dairy cows' health, reproduction and longevity have been negatively affected (Dobson et al., 2007; Olds et al., 1979; Oltenacu & Algers, 2005). The primary factor behind the decline in dairy cow longevity is involuntary culling. Infertility is the leading cause of culling cows in Canadian dairy herds, resulting in a rate of about 14 - 17% in the past five years (CDIC, 2023). Reduced longevity of cows results in reduced lifetime milk yield and raising more replacement heifers that making dairy production economically and environmentally less sustainable. The increased milk production has been linked to reduced fertility performance (Albarrán-Portillo & Pollott, 2013; Dobson et al., 2007; Walsh et al., 2011). It is worth noting that dairy heifers have higher conception rates than multiparous cows (64% vs 39%, respectively) (Walsh et al., 2011). It is rational to suggest that an increase in milk production is associated with a decline in the reproductive performance of high yielding dairy cows. High yielding cows often experience metabolic disorders during a transition period that is three weeks before and three weeks after calving. This is the most critical and challenging period for diseases like metritis, displaced abomasum, milk fever and ketosis (Drackley, 1999; Melendez et al., 2009; Sundrum, 2015). Cows in early lactation required high levels of nutrients for milk production coupled with reduced feed intake, which leads to a state of negative energy balance (NEB) (Melendez et al., 2016). In response, cows attempt to balance the energy deficit through mobilization of body fat reserves, which results in increased NEFAs (Grummer, 1995) and ketone bodies concentrations in the blood, and leading to ketosis

(Ospina et al., 2010b). Ketosis is most commonly diagnosed within the first six weeks after calving, depending on the cause and the lactation stage when β-Hydroxybutyric acid (BHB) levels are elevated, ketosis has been categorized as type I (late) or type II (early) ketosis (Herdt, 2000; Holtenius & Holtenius, 1996). Type I ketosis occurs during 3-6 weeks of lactation while milk synthesis is so excessive that the requirement for glucose exceeds the capacity for glucose production. This is associated with low glucose and low insulin levels with normal or high gluconeogenesis without lipid accumulation in the liver. Whereas, type II ketosis occurs during 1-2 weeks of lactation, which is a consequence of impaired in insulin sensitivity or responsiveness, which results in continuous body fat mobilization leading to fatty liver (Grummer, 1995; Herdt, 2000; Holtenius & Holtenius, 1996).

The interval from calving to first service provides time for uterine involution and resumption of ovarian activity for next pregnancy (Stangaferro et al., 2018). Britt (1992) stated that cows' folliculogenesis and oocyte competence depend on their follicular microenvironment during the long period of follicular growth before ovulation. The follicle, encloses a single oocyte that undergoes ovulation, fertilization and subsequent formation of an embryo, is surrounded by somatic cells (granulosa and theca cells), which produce hormones and signals to ensure developmental competency of the oocyte (Kidder & Vanderhyden, 2010; Voronina et al., 2007). Early lactation metabolic and hormonal disturbance linked with alteration of the ovarian function in terms of the number of follicles, development, the size and quality of the ovulatory follicle (Hernandez-Medrano et al., 2012). Cows with high NEFAs, BHB, and lower insulin-like growth factor 1 (IGF1) resulted in decreased production of estradiol and progesterone in dominant follicle during differentiation and luteinization respectively (Walsh et al., 2012). Recent *in vitro* study that mimic NEB, has shown that high concentration of BHB hinder steroidogenesis in

cultured granulosa cells (Vanholder et al., 2006). The developmental competence of oocyte has been adversely affected by the elevated BHB (Leroy et al., 2006). Overall, high producing dairy cows have shown reduced ovarian function, lower conception rates, longer time between calving and first service, and increased days open (Rutherford et al., 2016; Walsh et al., 2007; Wathes et al., 2003). Therefore, this doctoral study aimed to investigate the potential mechanisms of negative association between postpartum metabolic disorders and fertility in lactating dairy cows.

CHAPTER 2

LITERATURE REVIEW

2.1 Follicular development

The growth, development, and maturation of ovarian follicles is a vital process in successful cattle reproduction. Ovarian folliculogenesis is a cyclic and dynamic process of follicle growth and development, that begins when primordial follicles are activated to resume their development and ends with ovulation of the dominant follicle or atresia (Monniaux et al., 2014). At birth cattle ovary is endowed with a pool of thousands of primordial follicles. The ovarian cortex contains follicles at different developmental stages (Figure 2.1), which can be classified into two distinct stages of ovarian follicle development as gonadotropin-independent and gonadotropin-dependent growth (Edson et al. 2009). According to the presence or absence of a cavity, the follicle's growth phases also are known as preantral or antral follicle stages, respectively (Williams & Erickson, 2000). The preantral/gonadotropin-independent stage is mainly regulated by locally produced growth factors acting through autocrine or paracrine mechanisms. During the antral/gonadotropin-dependent stage, most follicles undergo atresia, but a few are stimulated by gonadotropins to reach the preovulatory stage and eventually ovulate. Development of antral follicle is controlled by the pituitary gonadotropins (follicle stimulating hormone; FSH and luteinizing hormoneLH) along with growth factors (Soboleva et al., 2000).

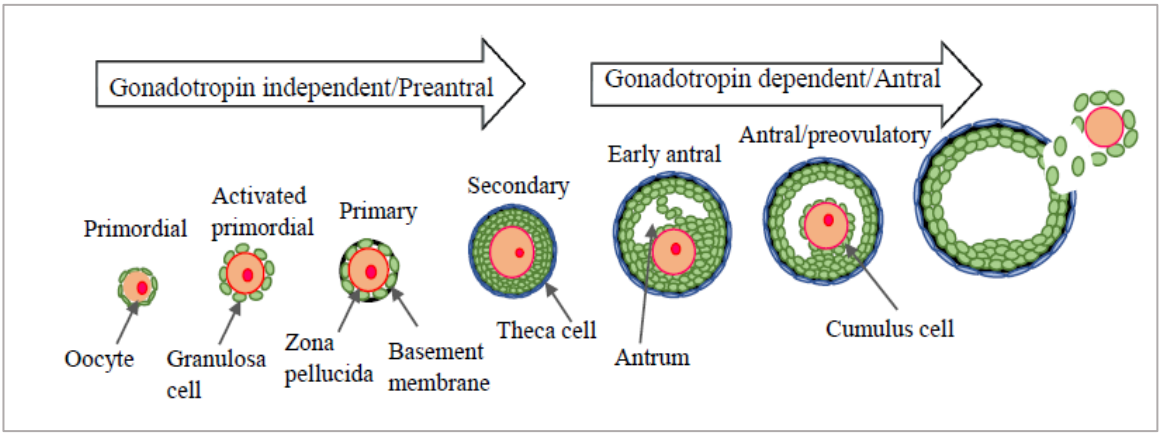

Figure 2.1 Schematic representation of the stages of follicular development in the ovary.

Preovulatory follicular steroidogenesis undergoes through "two cell two gonadotropin model" (Dorrington & Armstrong, 1979). The two cell two gonadotropin system regulates the release of gonadotropin and targets different follicular cells (Figure 2.2). LH acts on theca cells and FSH acts on granulosa cells. Cholesterol is the precursor for ovarian steroidogenesis, which can be synthesized de novo by ovarian cells or can be internalized through low-density lipoprotein (LDL) and high-density lipoprotein (HDL) receptors. Cholesterol in theca cells moves to the inner mitochondrial membrane with the help of steroidogenic acute regulatory protein (StAR) where it is converted to pregnenolone by cholesterol side-chain cleavage enzyme (CYP11A1). Pregnenolone diffuses into the smooth endoplasmic reticulum and further metabolized by either cytochrome P450 family 17 subfamily A member 1 (CYP17A1) or 3β-hydroxysteroid dehydrogenase (HSD3B) to form dehydroepiandrosterone (DHEA) or converted to progesterone, respectively. DHEA can be further converted to androstenedione by HSD3B or to androstenediol by 17β-hydroxysteroid dehydrogenase (HSD17B). Androstenedione is used to make testosterone by HSD17B or diffuse into the granulosa cells (Dorrington & Armstrong, 1979). Androstenedione in the granulosa cells used to make testosterone by HSD17B or estrone by aromatase (CYP191).

Testosterone and estrone are then transformed into 17β-estradiol by CYP19A1 and HSD17B, respectively (Sreerangaraja et al., 2020). Estradiol plays a vital role in stimulating the growth and maturation of follicular oocytes, as well as preparing uterus for potential embryo implantation.

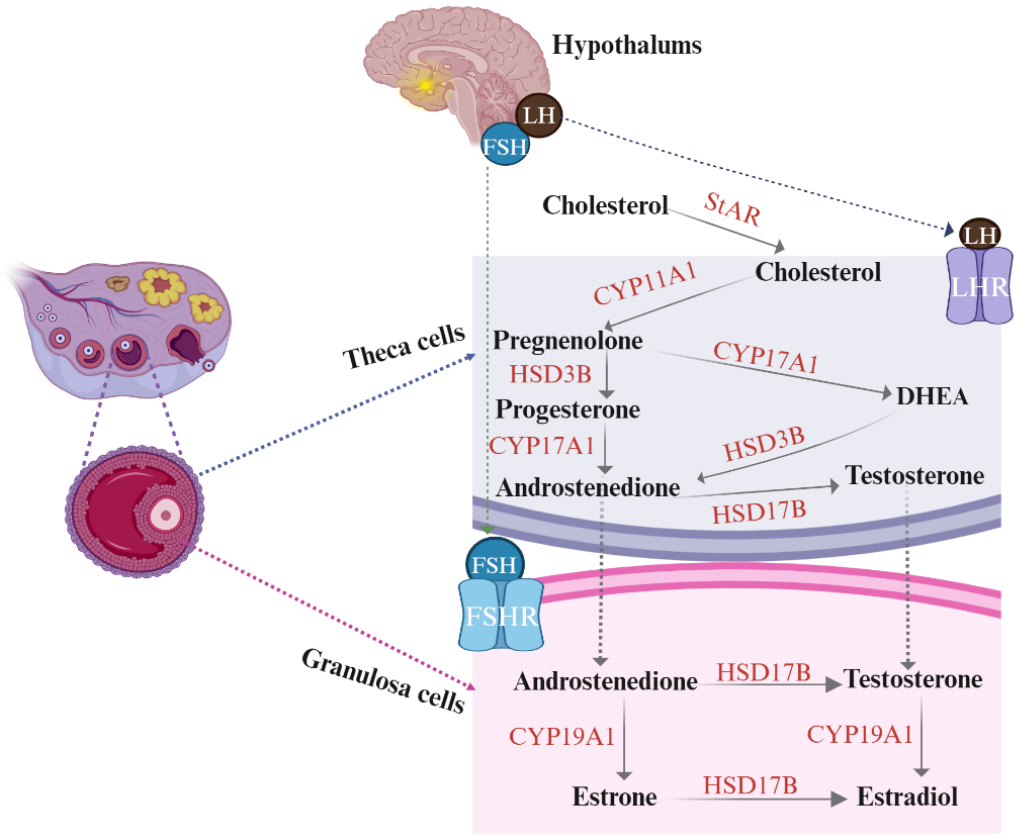

Figure 2.2 Two cell two gonadotrophin model of ovarian steroidogenesis

2.1.1 The preantral follicle development phase

Depending on the number of granulosa cells and the development of theca cell, the preantral follicle development is classified into primordial, primary, and secondary (Figure 2.1). The ovaries of mammals contain a reserve of primordial follicles, which emerge in the fetal ovary around day 90 in cattle (Fortune et al., 2010). These follicles consist of a non-growing oocyte arrested in the diplotene stage of meiosis I and are surrounded by a single layer of granulosa cells

(Hyttel et al., 1997). The activation of the primordial follicle involves a transformation of granulosa cells from flattened to cuboidal shape (Braw-Tal & Yossefi, 1997; Fair et al., 1997). Several factors and signaling pathways have been involved to activate or arrest primordial follicles including kit ligand, which activates primordial follicles. Once primordial follicles develop into primary follicles, the granulosa cells proliferate and the oocytes grow, leading to an increase in follicular size. The development of a secondary follicle begins with the formation of a second layer of granulosa cells. It involves a change in the granulosa cells from a simple cuboidal to 5 to 8 layers, and growing oocyte is surrounded by a zona pellucida. Interstitial cells surrounding the follicles become theca interna and theca externa (Williams & Erickson, 2000). This transition from primary to secondary follicles is a critical step in the process of follicular development.

2.1.2 Antral development and ovulation

The formation of a fluid-filled antrum between the granulosa cell layers marks the transformation of the secondary follicle into an early antral follicle. The cavitation (early antrum formation) is regulated by autocrine/paracrine system. It is known that pituitary gonadotrophin hormones like FSH are not essential for formation of cavitation, as demonstrated in animals that were hypophysectomized (Erickson, 1983). The induction of follicular antral-like cavity formation was observed through the *in vitro* treatment of granulosa and oocyte with activin (Li et al., 1995). In addition, the inhibition of the Kit ligand activity in the ovary hinders the development of antral follicles (Yoshida et al., 1997), demonstrating that locally produced activin and kit ligand are important for antrum formation. During antrum formation, activin and IGF1 trigger the expression of FSH receptor in granulosa cells, leading to the growth of more granulosa cells in subsequent stages of follicular development facilitated by FSH (Xiao et al., 1992).

During the transition to the antral follicle, granulosa cells differentiate into cumulus cells and mural granulosa cells. Cumulus cells surround the oocytes and help them mature, while mural granulosa cells line the follicular wall and surround the fluid-filled antrum, serving as the primary endocrine cells of the antral follicle (Williams & Erickson, 2000). Antral follicles depend on pituitary gonadotropins FSH and LH for their development and ovulation. FSH is important for antral follicle development by regulating granulosa cells proliferation, LH receptor (LHCGR) expression, and estradiol production. FSH binds to FSHR, activating the cAMP/protein kinase A pathway, which leads to the proliferation and differentiation of granulosa cells (Ulloa-Aguirre etal., 2018). The final stage of follicular development is marked by a meiotic resumption in the oocyte and the breakdown of the germinal vesicle. This crucial event is triggered by the LH surge and the disruption of cAMP inhibition on oocyte meiosis (Mehlmann, 2005). In cattle, the growth of antral follicles occurs in a wave like pattern. The first wave dominant follicles do not ovulate because they develop during the presences of corpus luteum, which inhibits the preovulatory LH surge. Even though such dominant follicles are able to ovulate if exogenous hormone treatment, such as GnRH (as in ovsynch program), they undergo atresia within a few days. With the atresia of the previous dominant follicle,a transient rise in FSH concentrations recruits a cohort of smaller ovarian follicles (3-5mm) to grow together (Mihm & Austin, 2002) as a new follicular wave. Within 48 h of the FSH peak, one follicle reaching 8 to 9 mm, selected as dominant follicle and continues to grow at a faster rate. As concentration of FSH falls below the critical threshold, the subordinate follicles become atretic (Austin et al., 2001; Mihm & Austin, 2002). Studies have shown that as the dominant follicle grows, the granulosa cells express LHCGR, HSD3B and CYP19A1 as well as the concentrations of E2 and P4 in the antral fluid (Fortune et al., 2001; Irving-Rodgers et al., 2009). The dominant follicles have higher levels of E2 and IGF1 in the

follicular fluid compared to the subordinate follicles (Fortune et al., 2001). Higher IGFl concentration in granulosa cells of dominant follicles increases E2 and LHCGR levels (Driancourt, 1991). Ovulation is triggered by an increase in LH secretion from the pituitary gland. The LH surge stimulates biochemical cascades in the preovulatory follicles, such as plasminogen activator and matrix metalloproteinases (Duffy et al., 2019). These enzymes act on the degradation of the meshwork of collagen fibers of follicular wall. After LH surge, Dow et al. (2002) have shown increased mRNA abundance and enzyme activity for plasminogen activators tissue type and urokinase type in preovulatory follicles. Elevated tissue type plasminogen activators and plasmin activity may be involved in follicular rupture and the expulsion of the oocyte. After the oocyte is released, the remaining cells (granulosa and theca interna) form the corpus luteum under the influence of LH, which is responsible for P4 secretion. (Liu, 2004).

2.2 Ovarian cyclicity in cattle

Regular estrous cycle in the dairy cattle starts at onset of puberty, between 6 to 12 months of age and estrous cycle repeats approximately every 21 days (Forde et al., 2011). Estrous cycle begins with the observable estrus behaviors, commonly referred as heat. Signs of estrus include vaginal discharge, swollen vulva, restlessness, and being mounted by another animal. The average length of standing estrus is approximately 15 hours, with a range of 6 to 24 hours. Milk yield impairs estrus behavior as high producing cows have a shorter estrus duration than low producing cows (Cutullic et al., 2012; Lopez et al., 2004). The estrous cycle consists of two distinct phases: The long luteal phase (14 -18 days) under the influence of P4 and a shorter follicular phase (4 - 6 days) under the influence of E2. The luteal phase starts after ovulation and the formation of the corpus luteum, while the follicular phase follows the regression of the corpus luteum until next ovulation. The dominant follicle, which grows during the follicular phase, eventually ovulates

(Forde et al., 2011). The estrous cycle is governed by hormones of the hypothalamus (gonadotrophin-releasing hormone; GnRH), anterior pituitary (FSH and LH), ovaries (E2, P4 and inhibins), and uterus (prostaglandin F2α; PGF2α) through positive and negative feedback mechanisms (Pring et al., 2012). Towards the end of luteal phase, P4 levels drop and E2 levels rise from dominant follicle, leading to a surge of preovulatory GnRH and a surge of both FSH and LH. Ovulation is initiated with high LH pulses frequency, leading to the LH surge (Nordéus et al., 2012).

2.3 Postpartum resumption of ovarian activity

During the postpartum period, the uterus involution and the hypothalamic-pituitary-ovarian-axis resuming cyclic secretions of gonadotropins, bring to the first postpartum ovulation and regular estrous cycles (Krause et al., 2014). After calving, P4 and E2 decline to basal levels resulting in recurrent transient increase of FSH level (Crowe, 2008; Forde et al., 2011). The first increase in postpartum FSH level triggers the growth of the first wave of follicles, leading to the development of a dominant follicle within 7 to 10 days after calving (Crowe et al., 2014; Savio, Boland et al., 1990). The fate of the first follicular wave dominant follicle depends on its ability to secrete enough E2 to induce a gonadotrophin surge. The production of E2 from the dominant follicle during the dominance phase depends on LH pulse frequency, follicle size, and IGF1 availability (Austin et al., 2001; Bao et al., 1997; Xu et al., 1995). During the early postpartum period, LH pulse frequency with one LH pulse per hour is the major driver of ovulation of a dominant follicle (Crowe, 2008). The follicle's ability to grow and ovulate depends on the levels of growth factors like IGF1 (Mazerbourg & Monget, 2018). Molecular events also occur in the dominant follicles, such as gene expression in granulosa cells, including increased mRNA abundance of the genes coding for LHCGR, HSD3B, and CYP19A1 (Silva & Price, 2002; Stocco,

2008). The changes in molecular phenotype within the dominant follicle are vital to induce steroidogenesis. Under normal condition, dairy cows resume ovarian activity and ovulation occur within 15 to 45 days after calving (Crowe et al., 2014; Tamadon et al., 2011). However, postpartum dairy cows may experience ovarian abnormalities that delay ovulation. Reduced fertility can occur because of disrupted hypothalamus-pituitary-ovary axis caused by early postpartum NEB (Butler, 2003). NEB associated with reducing blood glucose, IGF1, and LH pulse frequency. As a result, dominant follicles produce less E2 and delayed ovulation (Butler, 2003; Taylor et al., 2004).

2.4 Transition period and negative energy balance in dairy cows

In modern dairy farming, milk yield per lactation has been significantly increased through the selection of cows that are more efficient at converting energy and body fat into high milk production (Dobson et al., 2007). The transition period is a critical stage for dairy cows, happening three weeks before and three weeks after calving. During this time, cows undergo significant metabolic adjustments as they prepare to give birth and start producing milk (Drackley, 1999). Dairy cows eat less during the transition period, but their energy needs increase for fetal development and milk production (Drackley, 1999). Thus, the increased yield and reduced feed intake cause cows to enter a state of NEB, where they use body reserves for energy, leading to hyperketonemia (HYK) (Butler, 2003; Nogalski et al., 2012). The excessive mobilization of lipids from body reserves results in the release of circulatory NEFA into the blood. In the liver, NEFAs are oxidized to acetyl-CoA, which is then oxidized through Krebs Cycle for energy production (Suriyasathaporn et al., 2000) (Figure 2.3). If there is an excess of NEFAs in the liver, unused acetyl-CoA is converted into ketone bodies like acetate, acetoacetate and BHB, which are released into the blood, milk, and urine (Suriyasathaporn et al., 2000).

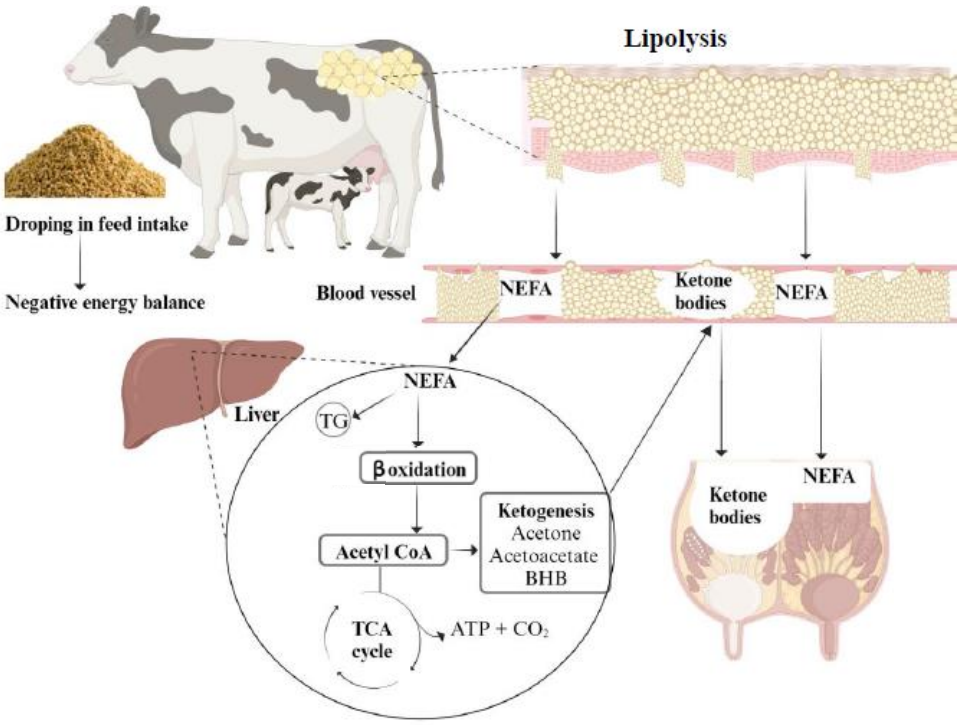

Figure 2.3 Metabolic pathways of ketogenesis.

The accumulation of excessive ketone bodies in the circulation results in postpartum ketosis. When the liver can't handle excessive NEFAs, they are re-esterified to triglycerides (TG) and can either be exported as VLDL or stored in the liver (Alves-Bezerra & Cohen, 2017; Sundaram & Yao, 2010). Fatty liver may develop when lipid uptake exceeds the liver's capacity to oxidize NEFAs or to pack and export as VLDL (Brickner et al., 2009).

2.5 Evaluation of dairy cow energy balance

Energy balance is traditionally determined by comparing energy intake and energy expenditure, but accurately calculating the difference between energy intake and energy used for maintenance and production in cows is challenging, because it is difficult to measure energy intake of individual cows within a herd (Friggens et al., 2007; Thorup et al., 2012). Thus, indirect

techniques like body condition scoring (BCS) and body fluid metabolites analysis, have been developed to evaluate a cow's energy balance and well-being.

2.5.1 Body condition scoring

Bod condition score is a noninvasive, quick, and inexpensive method to assess the level of energy balance during the transition period. BCS provides important information to help dairy farmers manage nutrition, health, production, and reproduction. BCS involves assessing fat reserves on a scale of 1 to 5 with 0.25 increments. This is done by visual and tactile appraisal of specific areas of a cow's body, like the transverse and spinous processes of the lumbar vertebrae or the tail head. The BCS scale of 1-5 is stratified as 1 being emaciated, 2 thin, 3 average, 4 fat, and 5 obese (Edmonson et al., 1989; Wildman et al., 1982). Changes in body condition levels of cows are related to the stage of lactation and gestation. Maintaining the optimal BCS at calving is crucial for dairy cow management as it can significantly impact cow health, milk production, and reproductive performance. The optimal BCS at calving should be within the range of 3.0 to 3.5. Cows that calved with higher BCS will have a reduced feed intake due to their higher energy reserves, this reduced feed intake eventually leads to NEB, and increases the risk of postpartum metabolic disorders, like ketosis. Cows calving with BCS > 3.5 have 2.5 times greater risk of ketosis than cows caving at BCS of 3.25 (Gillund et al., 2001; Goldhawk et al., 2009). Cows that loss BCS after calving had experienced higher levels of circulating NEFAs and BHB concentrations. They also had fatty liver, which leads to delayed resumption of ovarian cyclicity, longer time to first service, and lower conception rate at first service (Barletta et al., 2017; Butler & Smith, 1989).

2.5.2 Measuring metabolites in body fluids

Another way to assess dairy cow energy balance is by measuring the concentration of metabolites in body fluids. Blood NEFA concentration is an indicator of adipose tissue mobilization, and is associated with energy balance of a cow (Wankhade et al., 2017). Cows with NEFAs level ≥ 0.3 mmol/L from 35 to 3 days before calving have been associated with NEB (Cameron et al., 1998), and those with ≥ 0.6 mmol/L NEFAs from 3 to 14 DIM have also been associated with increased risk of CK (Ospina et al., 2010a). Measuring the concentration of ketone bodies in blood or milk have been used as an indicator of energy balance status in the transition dairy cows (Denis-Robichaud et al., 2014; Forde et al., 2016; van Knegsel et al., 2007). Measuring BHB concentration in blood or milk is the most commonly used ketone body since it is more stable than acetone or acetoacetate (Tyopponen & Kauppinen, 1980). The most commonly used technique measuring blood BHB are laboratory-based, which BHB concentration is determined through a colorimetric enzymatic reaction and a subsequent spectrophotometric analysis (Chandler et al., 2018), and Precision Xtra handheld ketone meter test device (Abbott Laboratories, Abbott Park, IL) makes up on-farm testing systems, which use an electrochemical reaction with a small amount of blood to measure BHB concentration with acceptable specificity and sensitivity (Iwersen et al., 2009; Voyvoda & Erdogan, 2010). Blood sampling is a laborious and time consuming process that causes stress for animals. Milk BHB concentration has been used as a potential indicator of ketosis because of its inexpensive, non-invasive, and routine use in milk recording, making it convenient to evaluate at the herd level. Some of the most used techniques to measure milk BHB are PortaBHB milk test strips to detect cow-side milk BHB (PortaCheck Inc., Moorestown NJ). Ketoscreen test measure milk BHB (MilkoScan FT600, Foss Analytical A/S, Hillerød, Denmark). Fourier Transform Infrared (FTIR) Spectroscopy is a cost-effective and high-

throughput method for milk quality analysis, including BHB testing. As the infrared beam is passing through the milk sample, the absorption and transmission of the infrared creates a unique fingerprint. It is possible to determine and quantify the compound present in the milk by observing its absorption pattern. Technology has improved on-farm ketosis detection using automatic milk BHB analysis. Herd Navigator (HN), an automated real-time analyzer (Lattec I/S. Hillerød. Denmark), in combination with DeLaval milking robot (DeLaval Inc. Tumba. Sweden), is used to measure milk BHB concentration. The milking robot collects milk samples from each cow during milking and sends them to HN for measuring milk BHB concentration.

Several Studies have used a blood BHB threshold of 1.2 to 2.9 mmol/L for diagnosing subclinical ketosis (SCK)/HYK, while concentrations ≥ 3.0 mmol/L used as clinical ketosis (CK) (McArt et al., 2012; Suthar et al., 2013). Milk BHB have been used to diagnose cows with SCK with the range of 0.1 to 0.2 mmol/L (Carrier et al., 2004), ≥0.08 mmol/L (van der Drift et al., 2012), and > 0.1 mmol/L (Berge & Vertenten, 2014). In another study, cows were classified based on milk BHB concentration as suspect HYK with the range of 0.15 to 0.19 mmol/L or positive HYK with ⩾0.2 mmol/L (Santschi et al., 2016). The HN ketosis biomodel used milk BHB concentration thresholds of 0.08-0.12 mmol/L and ≥ 0.13 mmol/L to detect SCK and CK, respectively.

2.6 Ketosis

The metabolic disturbances, during NEB, lead dairy cows to mobilize body reserves to meet the energy requirement of lactation. However, a poor adaptive mechanism of cows to NEB leads to rapid lipolysis, results in development of ketosis (Bauman & Currie, 1980; Wankhade et al., 2017). Ketosis is a metabolic disease that occurs frequently in dairy cows after calving and compromises their reproductive performance (Raboisson et al., 2014; Walsh et al., 2007). At the

start of lactation, there is a decrease in blood glucose levels and a reduction in the insulin to glucagon ratio. This leads to the mobilization of lipids from adipose tissue, which exports NEFAs into the blood. The exported NEFAs are taken up by the liver and oxidized to acetyl-CoA and enter Krebs Cycle to produce energy for the liver's metabolic activities (Herdt, 2000). Further reduced blood glucose concentration along with peripheral insulin resistance leads to the production of ketone bodies like acetoacetate, acetone and BHB as alternative source of energy for other tissues including the brain (Adewuyi et al., 2005). However, with excessive lipid mobilization and insulin resistance, the liver makes more ketones than the body can use for energy, resulting in accumulation of circulatory ketone bodies above the threshold. Ketosis is most commonly diagnosed within the first six weeks after calving. Depending on the cause and the lactation stage when BHB levels are elevated, ketosis has been categorized either type I or type II (Holtenius & Holtenius, 1996). Both types of ketosis are associated with higher BHB levels, along with lower circulatory glucose concentrations (Table 2.1).

Table 2.1 Characteristics of Type I and II ketosis (Herdt, 2000).

Parameter	Type I ketosis	Type II ketosis
BHB	High	High
Glucose	Low	Low
Period of occurrence	3 - 6 weeks of lactation	1-2 weeks lactation
Gluconeogenesis	Normal	Low
Fatty liver	No	Yes

Type I ketosis occurs in dairy cows during 3-6 weeks after calving, while milk production is very high and the body can't make enough glucose to meet the demand. This results in low glucose and insulin levels, with normal gluconeogenesis, and without fat accumulation in the liver. Whereas, type II ketosis occurs 1-2 weeks after calving with reduced gluconeogenesis as consequences of impaired insulin sensitivity and responsiveness; insulin resistance resulting in continuous body fat mobilization and fatty liver (Herdt, 2000). Ketosis is clinically classified as either SCK or CK. SCK is difficult to detect as it lacks clinical signs such as decreased appetite, reduced milk yield and drastic weight loss seen in clinical ketosis. The definitive diagnosis of SCK is determined by serum BHB concentrations within the range of 1.2 to 2.9 mmol/L in circulation (McArt et al., 2012). Cows with a blood BHB concentration of 1.2 to 2.9 mmol/L have a higher risk of developing metritis, lameness, displaced abomasum, and CK (Koeck et al., 2014; Suthar et al., 2013; Vanholder et al., 2015). Cows with blood BHB values $\geq$ 3.0 mmol/L are categorized as CK (Vanholder et al., 2015). The time of planned breeding after calving is crucial for follicular development as important events occur, such as communication between somatic cells (granulosa cells and theca cells) and the oocyte, and activation of the oocyte transcriptome (Fair, 2010). These

follicles would have grown during the postpartum period while being exposed to BHB and other metabolic changes.

2.6.1 Prevalence of ketosis

Ketosis prevalence is calculated by dividing the number of cows with ketosis by the total number of cows sampled in a specific period. The prevalence of ketosis varies depending on days in milk and frequency of sampling. The highest prevalence has been diagnosed during the first two weeks of lactation, and then declines (Koeck et al., 2014; Santschi et al., 2016; Tatone et al., 2017; van der Drift et al., 2012). Measuring blood BHB concentration shows ketosis prevalence range from 7.1% (van Knegsel et al., 2010) to 35.4% (Ribeiro et al., 2013). The range of ketosis prevalence was between 14% (Koeck et al., 2014; Renaud et al., 2019) to 39% (Berge & Vertenten, 2014) when milk BHB was considered Table 2.2.

Table 2.2 A summary of ketosis prevalence in the literatures.

DIM	Threshold	Prevalence (%)	References
Blood BHB			
≤ 7	1.4	12	(Geishauser et al., 2000)
≤ 14	1.4	16.6 - 18.6	(Duffield et al., 2009)
2 - 5	1.2	21.8	(Suthar et al., 2013)
7 - 14	1.2	7.2	(Vanholder et al., 2015)
3 - 14	1.2	30.5	(Mann et al., 2016)
7 - 21	1.2	17	(Rutherford et al., 2016)
5 - 60	1.2	11.2	(van der Drift et al., 2012)
< 63	1.2	7.1	(van Knegsel et al., 2010)
7-14	0.96	35.4	(Ribeiro et al., 2013)
Milk BHB			
≤ 14	0.1	26	(Walsh et al., 2007)
3 - 35	⩾ 0.15	22.6	(Santschi et al., 2016)
5 - 100	⩾ 0.2	14	(Koeck et al., 2014)
< 30	0.15	21	(Tatone et al., 2017)
< 25	0.14	14	(Renaud et al., 2019)
7 - 21	0.1	39	(Berge & Vertenten, 2014)

2.6.2 The effect of ketosis on ovarian activity

Maternal genes are transcribed to mRNA, translated into protein, and stored in the oocyte during follicular growth to help with embryo development before the embryonic genome is activated (Lonergan et al., 2003; Zhang & Smith, 2015). At 8-16 cell stages, the cow embryo activates its genome and using its own formed DNA to produce transcription factor (Frei et al., 1989). This is a highly critical stage in early embryo development. Consequently, any negative consequence that happens during oocyte growth and maturation can subsequently alter the viability of the embryo, even in the presence of successful fertilization. Britt (1992) hypothesized that the process of follicle development from activated primordial follicle to the ovulatory stage is lengthy and lasts approximately 100 days. The follicle and the oocyte that ovulated at the time of breeding may have been impacted by NEB during the transition period. Thus, ketosis during early lactation may prevent primary follicles from producing sufficient E2 and post ovulatory P4 (Britt, 1992). The elevated BHB concentrations are reflected in the follicular fluid of dominant follicles in early lactating dairy cows (Sanchez et al., 2014; Schuermann et al., 2019). *In vitro* study showed that the increased BHB concentration inhibits oocyte maturation rate, resulting in relatively lower rates of fertilization, cleavage, and blastocyst formation (Leroy et al., 2006). Furthermore, it has been shown that BHB reduced E2 and P4 production and induce apoptosis in cultured granulosa cells (Vanholder et al., 2006).

2.7 The effect of metabolic stress on reproductive performance

After calving in cattle, an increase in FSH concentration stimulates the first waves of follicular development around 5-7 DIM. The dominant follicle from the first wave can undergo various outcomes: (1) ovulation during the first wave (16-20 DIM); (2) development of a first-wave anovulatory dominant follicle followed by additional waves of follicular development before

first ovulation; or (3) development of a first-wave dominant follicle that becomes cystic ovulation (Beam & Butler, 1997). If ovulation occurs in the first wave, a corpus luteum is formed, while the first dominant follicle fails to ovulate, it degenerates, and more follicular waves will occur until ovulation (Beam & Butler, 1997; Sartori et al., 2004). The regression of the first wave dominant follicle or formation of a follicular cyst results in prolonged interval to first ovulation (31 and 52 DIM, respectively) compared with that of postpartum cows that ovulate their first dominant follicle (16 DIM) (Beam & Butler, 1997). LH pulse frequency is crucial for ovulation. The dominant follicle must produce high levels of E2 to inhibit FSH production and release one LH pulse per hour (Crowe et al., 2014; Sunderland et al., 1994). The impact of energy balance on the hypothalamus-pituitary-ovary axis has been studied. Insufficient energy intake after calving has been linked to the loss of pulsatile LH secretion in cows (Hill et al., 2008; Nagatani et al., 1998). Moreover, cows with NEB, the dominant follicle produces less E2 and takes longer time to increase its diameter and produce the final adequate LH pulse frequency (Lopez et al., 2004; Sartori et al., 2004). Insufficient IGF1 concentrations after calving can result in reduced E2 secretion and failure of dominant follicles to ovulate (Beam & Butler, 1997).

Reproductive performance of dairy cows can be measured using various parameters. The interval from calving to first service, first service to conception, days open (interval from calving to conception), number of services per pregnancy and the interval between consecutive calving (calving interval) are reproductive performance indicators commonly used by dairy cow farmers (LeBlanc, 2010; Norman et al., 2009). These reproductive performance indicators in high producing dairy cows are affected by a metabolic disease during the transition period. Cows under NEB have low fertility and longer unproductive period. They also take a longer time from calving to first service and reduced conception rates, resulting in longer days open (Rutherford et al., 2016;

Walsh et al., 2007). Table 2.3 provides a summary of the effects of elevated circulatory BHB concentration on reproductive performance indicators.

The traditional recommendation for dairy farming has been one calf for each cow per year. To achieve this goal, cows should conceive within 80 days after calving, since the gestation length is approximately nine months. However, postpartum cows with NEB have a delay in the resumption of ovarian cyclicity or become non-ovulatory (Crowe et al., 2014; Walsh et al., 2007). After calving ovarian follicle cells undergo multiple events to prepare a competent oocyte for fertilization. These events include proliferation, differentiation, and synthesis of mRNAs, proteins and hormones, as well as completion of meiosis. Metabolic disorders during transition period have an adverse effect on fertility in dairy cows. Therefore, understanding the occurrence of ketosis and its negative effects on ovarian function is very important to improve the reproductive performance of lactating dairy cows.

Table 2.3 Effects of elevated circulatory BHB concentration on reproductive performance of postpartum dairy cows.

Outcome	BHB (mm/L)	Weeks in milk	HR, OR or RR (95% CI)		*P value*	References
EC	≥0.96	1 to 2	OR	0.64 (0.39-1.04)	0.07	(Ribeiro et al., 2013)
FSCR	≥1.0	1 & 2	OR	0.47 (0.29-0.77)	0.003	(Walsh et al., 2007)
CFSI	≥1.2	1 to 2	RR	0.90 (0.80-1.2)	0.55	(McArt et al., 2012)
CFSI	≥1.0	1 & 2	HR	0.93 (0.76- 1.1)	0.48	(Walsh et al., 2007)
CCI	≥1.2	1 to 2	HR	0.90 (0.7- 11.1)	0.40	(McArt et al., 2012)
CCI	≥1.0	1 & 2	HR	0.029 (0.004-0.21)	<0.001	(Walsh et al., 2007)
CCI	≥1.0	1 to 2	HR	0.87	0.1	(Ospina et al., 2010a)

EC = Estrous cyclic (corpus luteum presence at ultrasound)

FSCR = first service conception rate

CFSI = calving to first service interval

CCI = calving to conception interval

HR = hazard ratio; OR = odds ratio; RR = relative risk

2.8 Hypothesis and objectives

2.8.1 Hypothesis

We hypothesize that metabolic stress associated with ketosis compromises ovarian functions and fertility in dairy cows.

Therefore, to test this hypothesis we have established the following objectives:

2.8.2 Objectives

1. To evaluate the effect of excess BCS loss during the transition period on liver functions and the status of the dominant follicle in the ovary as the cow approaches the breeding period.

2. To investigate associations between timing and amplitude of elevated milk BHB (EMB) and reproductive performance of lactating dairy cows

3. To investigate the association between ketosis, determined by milk BHB concentrations during 3-14 days in milk (DIM), and ovarian activity, measured by milk progesterone (P4) profiles within 150 DIM in Holstein cow.

CHAPTER 3

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Severe body condition loss lowers hepatic output of IGF1 with adverse effects on the dominant follicle in dairy cows

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3.1 Highlights

- Severe peripartum body condition loss impacts liver function and transcriptome.
- Hepatic dysfunction results in lower IGF1 production.
- Low circulating IGF1 concentration is reflected within the dominant follicle.
- Reduced IGF1 support results in abnormal granulosa cell gene expression.
- Severe body condition loss results in sub-functional ovarian follicles.

3.2 Abstract

The severe loss of body condition score (**BCS**) during the early lactation period has been associated with infertility in cows. However, the mechanisms are not fully understood. The aim of this study was to examine the effect of BCS loss on liver health, and ovarian functions in cows during early lactation. Retrospectively multiparous cows from two farms were categorized based on units of BCS (1–5 scale) loss as Moderate (**MOD**, <0.75 units; n = 11) or Severe (**SEV**, ≥0.75 units; n = 9) loss groups. From Weeks −3 to 7, relative to calving, MOD and SEV cows lost on average 0.4 and 1.0-unit BCS, respectively. All data except hepatic transcriptomes were analyzed with PROC MIXED procedure of SAS. The plasma concentration of non-esterified fatty acids at Week 0 and 1, ß-hydroxy butyrate at Week 1, and γ-glutamyl transferase at Weeks 1 and 7 relative to calving were higher in SEV cows. Hepatic transcriptome analysis showed that 1 186 genes were differentially expressed in SEV (n = 3) compared to MOD (n = 3) cows at Week 7 after calving. Pathway analysis revealed that significant DEGs in SEV cows enriched in lipid metabolisms including, lipid metabolic process, ether lipid metabolism, fatty acid beta-oxidation, fatty acid biosynthetic process, fatty acid metabolic process, fat digestion and absorption, linoleic acid metabolism, alpha-linolenic acid metabolism. The impaired liver function in SEV cows was associated with 1.5-fold reduction of hepatic *IGF1* gene expression and lower serum IGF1

concentrations. At the ovarian level, SEV cows had lower IGF1 concentration in the follicular fluid of the dominant follicle of the synchronized follicular wave compared to that of MOD cows at 7 weeks after calving. Further, the follicular fluid concentration of estradiol-17β was lower in SEV cows along with lower transcript abundance of genes from granulosa cells associated with dominant follicle competence, including *CYP19A1*, *NR5A2*, *IGF1R*, and *LHCGR*. These data show that SEV loss of BCS during early lactation leading up to the planned start of breeding is associated with liver dysfunction, including lower IGF1 secretion, and impaired function of the dominant follicle in the ovary.

Keywords: Follicular fluid, Granulosa cells, IGF1, Liver, Transcriptome

3.3 Implications

Excessive body condition loss during the early lactation period has been associated with reduced fertility. However, the underlying mechanisms are not well understood. In this study, cows with severe body condition loss during early lactation experienced increased lipid mobilization, and liver dysfunction as demonstrated by transcriptomic deficiencies. The hepatic production of insulin-like growth factor 1 in these cows was lower, potentially reducing the insulin-like growth factor1 support for normal ovarian functions. Consequently, the dominant follicle in their ovaries had low estradiol levels, an indicator of follicular health, due to an underlying abnormal gene expression in granulosa cells. Overall, this study provides a molecular basis for the negative relationship between excessive body condition loss and ovarian function.

3.4 Introduction

The time between three weeks before calving to three weeks after calving is known as the transition period, which is the most challenging physiological stage of dairy cows. With the onset of lactation, dairy cows experience high energy demand to support rapidly increasing milk production. The energy demand for milk synthesis cannot be met with feed intake resulting in a negative energy balance (Wankhade et al., 2017). Cows depend on lipid mobilization from body reserves to meet the energy demands not met with by feed intake. Cows undergo varying degrees of lipid mobilization, which involves the release of non-esterified fatty acids (**NEFAs**) from adipose tissue (Drackley, 1999, Trevisi and Minuti, 2018). The NEFAs are metabolized in the liver in multiple ways including beta-oxidation for hepatocytes, conversion to ketone bodies as an alternative to glucose for other tissues and repackaging into fat for systemic redistribution (Alemu et al., 2023). However, if the NEFA load exceeds the liver's metabolic capacity, the excess fatty acids are stored leading to fatty liver thereby perturbing the hepatic functions. Negative

energy balance during the transition period has a negative impact on the liver, and the consequent metabolic alterations may compromise immune and reproductive functions in dairy cows (LeBlanc, 2012). Supporting this, studies have shown significant changes in liver transcripts associated with lipid_metabolism, cholesterol_metabolism, and gluconeogenesis in dairy cows during the transition period (Schlegel et al., 2012, Kessler et al., 2014, Gross et al., 2015, Ha et al., 2017).

Monitoring the change in body condition score (**BCS**) is a noninvasive tool for assessing body fat and lipid mobilization in transition dairy cows. The change in BCS during the transition period has been shown to impact hepatic transcriptome in grazing dairy cows (Akbar et al., 2015, Vailati-Riboni et al., 2016). Previous report has shown that cows that lost more than 1 unit of BCS in the first five weeks of lactation suffer from fatty liver, longer time to commencement of luteal activity, time to first estrus, and time to first service and lower first service conception rate compared to cows losing less than 0.5 units (Butler and Smith, 1989). Cows that lost BCS during the transition period had higher concentrations of circulating NEFAs and ß-hydroxy butyrate (**BHB**), and had a lower rate of cyclic cows by 50 days in milk, longer time to commencement of luteal activity and a lower rate of pregnancy per first service compared to maintained or gained BCS (Carvalho et al., 2014, Barletta et al., 2017). These studies clearly demonstrate an adversarial relationship between BCS loss and reproductive functions of lactating cows.

Although several studies have shown that excess lipid mobilization during the transition period negatively impacts reproductive performance (Britt, 1992, Lopez-Gatius et al., 2003, Bewley and Schutz, 2008, Carvalho et al., 2014, Luttgenau et al., 2016, Barletta et al., 2017), the mechanistic basis of this negative relationship is not clearly understood. Further studies

are needed to determine the molecular mechanisms by which BCS loss relates to liver function and the competence of ovarian function. To our knowledge, no one has yet evaluated the impact of BCS loss on the hepatic and ovarian health during the transition period leading up to the time planned for breeding. Therefore, the objective of the present study was to evaluate the effect of excess BCS loss during the transition period on liver functions and the status of the dominant follicle in the ovary as the cow approaches the breeding period.

3.5 Materials and Methods

3.5.1 Animals and body condition scoring

Twenty multiparous lactating Holstein dairy cows from two farms (farm A = 14 and farm B = 6) were housed in a tie-stall setting. Cows were fed with standard diets of alfalfa silage and dry hay during the dry period, and switched to alfalfa silage, corn silage, and soybean meal beginning at the onset of lactation. All cows were milked at approximately 0600 h and 1730 h twice daily. Cows were selected based on normal dry off, no history of adverse health events in the previous lactation. None of the cows in the present study had calving difficulty. The sample/data collection period ranged from –3 weeks and ended at +7 weeks relative to calving; these times were selected to include the transition period (Grummer, 1995) to the week approaching the planned start of breeding (i.e. 7 weeks after calving) (Miller et al., 2007). BCS was evaluated using a 5-point scale in 0.25 increments, where 1 is emaciated and 5 is obese (Edmonson et al., 1989). BCS loss during early lactating cows should not be more than 0.5–1 units (Butler, 2014); therefore, cows were retrospectively allocated to one of two groups based on units of BCS lost throughout the 10-week sampling periods: cows that had moderate (**MOD**, losing < 0.75 units; n = 11) or severe (**SEV**, losing ≥ 0.75 units; n = 9) loss of BCS.

3.5.2 Blood collection and analyses

At least 30 minutes prior to morning feeding, blood samples were obtained from all cows on −3, 0, +1, and +7 week relative to calving by coccygeal vein venipuncture using 21G vacutainer needles into blood plasma collection tube Monoject 10 ml (ethylenediaminetetraacetic K3) and blood serum collection tube Monoject 10 ml. Samples were placed immediately on ice and centrifuged for 10 min at 1500*g*. The plasma and serum samples were collected in 5-mL tubes and frozen in liquid nitrogen before storing at −80 °C for downstream analyses. Briefly, plasma was

used to measure for NEFAs, BHB, glucose, γ-glutamyl transferase (**GGT**), and haptoglobin (**HP**) using a Roche Cobas 6 000 c501 automated chemistry at Animal Health Laboratory (University of Guelph, ON, Canada) (MOD; N = 9 and SEV; N = 8). Except for NEFA and BHB (Randox Laboratories (Crumlin, UK)), all test reagents were supplied by Roche diagnostics (Indianapolis, IN). Serum taken only at week 7 postpartum was used to measure total IGF1 at the Prairie Diagnostic Centre (University of Saskatchewan) using the IMMULITE/IMMULITE 1 000 IGF1 enzyme-labeled chemiluminescent immunometric assay (SIEMENS).

3.5.3 Liver biopsy

The liver biopsy was collected from all cows in the morning during week +7 relative to calving using the Tru-Cut type biopsy tool (Care Express Products, Cary, Illinois, USA). Briefly, the skin was clipped and disinfected, and the local area was anesthetized with 2 ml of 2% lidocaine solution (Lidocaine HCL 2%, Bimeda, Cambridge, CA). The skin was cut (2 cm) using a scalpel blade and 14G, six-inch-long Tru-Cut needle was inserted through the 10th intercostal space on the right side of the cow at approximately 2/3 from the top of the cow into the liver, and about 10–12 mg of liver tissue was collected. The procedure was repeated two to three times on each collection day to obtain an adequate amount of liver tissue. Samples were immediately frozen in liquid nitrogen and stored at −80 °C upon arrival at the laboratory.

3.5.4 Follicular wave synchronization and follicular fluid and granulosa cells collection

Follicular wave synchronization was applied for each cow entering the 6th week of her lactation as described in our previous study (Sanchez et al., 2014). On the first day, the ovaries were monitored by transrectal ultrasonography using a 7.5 MHz linear-array transducer, where all follicles >5 mm were aspirated through transvaginal aspiration. At the end of follicular ablation, cows were given 2 ml of a synthetic prostaglandinLutalyse® 5 mg/ml (103843, CDMV, Saint-

Hyacinthe, QC, Canada) and an intravaginal progesterone-releasing device containing progesterone CIDR 1380 Intravaginal Progesterone (108 316, CDMV, Saint-Hyacinthe, QC, Canada) were placed. This is expected to result in the emergence of a new follicular wave in 36–48 h after the first follicular aspiration. CIDR was removed on day six, and dominant follicles (>10 mm) were aspirated by ultrasound-guided intrafollicular aspiration (MOD; N = 9 and SEV; N = 7). Follicular fluids were centrifuged at 3 000*g* for 7 minutes to collect granulosa cells. Granulosa cells and follicular fluids were frozen in liquid nitrogen until arrived at the laboratory and stored at −80 °C for further analysis.

3.5.5 Follicle and follicular fluid analysis

The size and number of follicles were determined by ultrasonography just before the final follicular fluid aspiration. The hormones estradiol (**E2**) and IGF1 were analyzed in follicular fluid (MOD; N = 9 and SEV; N = 7). The concentration of E2 in the follicular fluid was determined by a multispecies E2 ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). Intra- and inter-assay coefficients for the E2 were 3.48 and 14.86%, respectively. IGF1 was measured at the Prairie Diagnostic Centre, as mentioned above.

3.5.6 RNA Library construction and Illumina sequencing from liver samples

The total RNA from liver biopsy samples from MOD and SEV cows sampled at 7 weeks after calving was isolated with TRIzol® (Thermo Fisher Scientific [Life Technologies, Inc.], Burlington, ON, Canada) using the Direct-Zol RNA MiniPrep Isolation kit (R2050, Zymo Research, Cedarlane Laboratories, Burlington, Canada) according to manufacturer's protocol. RNA was quantified using the Nanodrop 2 000 spectrophotometer (Thermo Fisher Scientific). RNA samples were sent to Génome Québec Innovation Centre at the University of McGill (Montreal, Canada) for quality analysis with Bioanalyzer (2100 Bioanalyze, Agilent) and library

preparation. Using the best quality samples from three MOD and three SEV cows, RNA libraries were constructed from the rRNA-depleted RNA, and libraries were then sequenced using the Illumina HiSeq4000 in paired-end mode with 100 base pair ends (PE100).

3.5.7 RNA- sequence data analysis

Paired-end reads were harvested, and quality control was performed by FastQC. The reads containing adaptors were trimmed with trimmomatic-0.39 (Bolger et al., 2014). The clean reads were mapped to the bovine reference genome ARS-UCD1.2 and counted reads using STAR, splice aware alignment software (Dobin et al., 2013), 92.7−94.7% of the reads were uniquely aligned. The read counts were then uploaded to the NetworkAnalyst tool (Xia et al., 2015) to perform differential gene expression (DEG) analysis using the edgeR package. The genes with $P < 0.05$ and |fold change| ≥1.5 of SEV vs MOD BCS loss cows at week 7 relative to calving were considered as DEGs. Since we used biopsy samples, we used this modest stringent cut off similar to a recent study (Shahzad et al., 2019). The DEGs were subjected to Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathways and Gene Ontology (**GO**) Biological Process (**BP**) using the NetworkAnalyst tool (Xia et al., 2015). The KEGG pathways and GO_BP terms with $P < 0.05$ were considered statistically significant.

3.5.8 Quantitative Real-Time PCR

All granulosa cell samples (MOD; N = 9 and SEV; N = 7) were used for qPCR assays, which were performed using previously described protocols from our laboratory (Schuermann et al., 2018). Briefly, total RNA was purified from granulosa cells similar to liver biopsies as mentioned above. Complementary DNA was synthesized from 250 ng of total RNA using the iScript cDNA Synthesis kit (Bio-Rad, Mississauga, Canada) with the following temperature program: 25 °C for 5 min (Priming), 46 °C for 20 min (Reverse transcription) and 95 °C for 1 min

(Reverse transcription inactivation). Sample dilution factors for granulosa cells were 1:50. Relative transcript abundance for each gene of interest was calculated by dividing their respective starting quantity (**SQ**) values by the average SQ values of reference genes in granulosa cells (*ACTB*, *PPIA*, and *RPL19*). The primer sequences of transcripts measured in this study can be found in Table 1, where primer design was performed using the NCBI Primer-BLAST. If variants of a gene were present, the primers were designed to include all variants.

3.5.9 Statistical Analysis

The PROC MIXED procedure of SAS (SAS 9.4 Inst. Inc., Cary, NC, USA) was used to analyze all data except RNAseq data, the analysis of which is described above. The statistical model used was $Y_{ijkmn} = \mu + Group_i + Farm_j + Time_k + (Group \times Time)_{ik} + Cow_m + e_{ijkmn}$, where Y_{ijkmn} is outcome variables; μ overall mean; $Group_i$ is the fixed effect of the ith BCS loss group (i = SEV and MOD); $Farm_j$ is the fixed effect of the jth farm (j = A and B); $Time_k$ is the fixed effect of the kth sampling time (k = −3, 0, 1 and +7 in relative to calving); $(Group \times Time)_{ik}$ is the fixed effect of the interaction between ith group and jth time; Cow_m is the random effect of the mth cow (m = 1–20); and e_{ijkmn} is the random error. The various sampling time points were not equally spaced, an exponential correlation covariance structure SP (POW) was used for repeated measures. Data were checked for normality using Shapiro-Wilk (PROC UNIVARIATE) and log-transformed if needed. Differences between groups were done using Scheffé's adjustment. The data are presented as LS means ± SEM. The *P*-values of <0.01 and <0.05 were declared as significantly different, while $0.05 < P < 0.1$ were declared as tendencies for differences.

3.6 Results

3.6.1 Body condition score loss

Cows in MOD group that lost 0.4 ± 0.08 units of BCS had a mean BCS of 3.2 at 3 weeks before calving and 2.8 at 7 weeks after calving. Cows in SEV group that lost 1 ± 0.10 units of BCS had a mean BCS 3.6 at 3 weeks before calving and 2.6 at 7 weeks after calving. Analysis of BCS profile from Weeks −3 to +7 relative to calving showed a significant effect of group (Fig. 1A; $P < 0.05$) as well as group and time interaction (Fig. 1A; $P < 0.01$). Although cows in SEV group had higher BCS compared to MOD cows during the week of calving (Fig. 1A; $P < 0.05$), their BCS decreased by week 7 after calving compared to 3 weeks before and the week of calving (Fig. 1A; $P < 0.01$). Normalizing the % BCS lost during the sampling period to the BCS observed at three weeks before calving revealed a dramatic decrease in BCS of 28% in SEV cows compared to 12% in MOD cows (Fig. 1B).

3.6.2 Severe loss of body condition is associated with increased plasma non-esterified fatty acids ß- hydroxybutyrate and γ-glutamyl transferase

The plasma NEFA and BHB concentration is an indicator of lipid mobilization. The analysis of NEFA concentrations in SEV and MOD cows showed a tendency for group effect ($P < 0.1$) and significant effects of time as well as interaction between group and time ($P < 0.05$). There was an overall tendency for SEV cows to have higher plasma NEFA concentrations compared to MOD cows (Fig. 2A; $P < 0.1$). Within each group of cows, there was a significant effect of time, wherein plasma NEFA levels increased from Week −3 to Weeks 0 and 1 relative to calving, thereafter, decreasing by week 7 after calving. The NEFA concentrations were higher in SEV compared to MOD cows during Weeks 0 and 1 relative to calving (Fig. 2A; $P < 0.05$). With respect to plasma BHB concentrations, the group effect was not significant (Fig. 2B; $P > 0.05$) but

there were significant effects of time as well as group and time interaction (Fig. 2B; $P < 0.05$). There was an increase in BHB concentrations from week −3 to week 1 (Fig. 2B; $P < 0.05$) and week 7 (Fig. 2B; $P = 0.06$) in SEV cows, while such a significant increase was not evident for MOD cows (Fig. 2B; $P > 0.05$). The plasma concentrations of GGT, a marker of liver injury, were higher at weeks 1 and 7 after calving in SEV than in MOD cows (Fig. 2C; $P < 0.05$). There was no significant difference between MOD and SEV cows in plasma glucose (Fig. 2D; $P > 0.05$) and haptoglobin (Fig. 2E; $P > 0.05$) levels.

3.6.3 Severe loss of body condition is associated with abnormal liver transcriptome

As SEV cows had elevated GGT along with higher NEFA and BHB concentrations indicating potential liver impairment, we explored the transcriptome differences between MOD and SEV cows at 7 weeks after calving. There were 1 186 ($P \leq 0.05$ and |fold change| ≥ 1.5) differentially expressed genes in SEV compared to MOD cows with 858 upregulated and 328 downregulated transcripts (Supplementary Table 1 deposited on the FigShare repository). The KEGG pathway enrichment analysis of data from SEV vs. MOD cows at weeks 7 after calving demonstrated that 43 significantly enriched pathways including linoleic acid metabolism, alpha-linolenic acid metabolism, ether lipid metabolism, fat digestion and absorption, citrate cycle (TCA cycle), protein digestion and absorption, insulin secretion, cAMP signaling pathway, calcium signaling pathway, ECM-receptor interaction, ovarian steroidogenesis, and tyrosine metabolism (Supplementary Table 2 deposited on the FigShare repository). Of these, linoleic acid metabolism, alpha-linolenic acid metabolism, ether lipid metabolism, fat digestion and absorption pathways along with shared genes together indicate that SEV cows had increased lipid metabolism (Fig. 3 and Table 2). The GO_BP term analysis demonstrated 36 significantly enriched GO_BP terms, including lipid metabolic process, fatty acid beta-oxidation, fatty acid biosynthetic process, fatty

acid metabolic process, carbohydrate catabolic process, oligosaccharide metabolic process, cellular homeostasis, cellular response to stress, protein targeting to membrane, and negative regulation of apoptotic process (Supplementary Table 3 deposited on the FigShare repository). Once again, these enriched biological processes along with shared genes reveal that SEV cows had increased lipid metabolism (Fig. 4 and Table 3).

3.6.4 Liver dysfunction is associated with lower IGF1 production

Increased GGT and higher expression of genes involved in fatty acid metabolism were indicative of a higher lipid burden on the liver in SEV cows. We examined if this impacted IGF1 production in those cows. Our differentially expressed gene data revealed that the hepatic expression of *IGF1* was lower in SEV cows compared to MOD cows at week 7 after calving (Fig. 5A; $P < 0.05$). Cows in SEV also had lower hepatic expression of *IGFBP1* ($P < 0.05$) and *IGFBP3* ($P < 0.001$) than MOD cows (Supplementary Table 1deposited on the FigShare repository). This lower expression of IGF-system genes in the liver was associated with lower serum IGF1 concentrations in SEV cows in comparison with MOD cows at 7 weeks after calving (Fig. 5B; $P < 0.01$).

3.6.5 Reduced IGF1 support impacts ovarian follicular functions

As IGF1 is a well-known regulator of follicular dominance in cattle (Beg et al., 2001), we examined if the reduction in IGF1 levels in the circulation impacted ovarian functions in SEV cows. Ultrasonography showed that there was no difference in the size of the dominant follicle of the synchronized follicular wave between MOD and SEV cows at 7 weeks after calving (Fig. 6A; $P > 0.05$). However, the lower hepatic expression and serum levels of IGF1 in SEV compared to MOD cows were reflected in the follicular microenvironment. The follicular fluid of the dominant follicles in SEV cows had lower total IGF1 than those in MOD cows at week 7 after

calving (Fig. 6B; $P < 0.01$). In addition, the follicular fluid of the dominant follicles from SEV cows had lower estradiol concentrations compared to those from MOD cows (Fig. 6C; $P < 0.05$).

3.6.6 Reduced IGF1 support impacts gene expression in granulosa cells of the dominant follicle

To investigate the molecular basis of reduced estradiol levels in the dominant follicles of SEV cows, we measured gene expression in granulosa cells from MOD and SEV cows at 7 weeks after calving. Transcript levels of *CYP19A1* were lower in granulosa cells of SEV than in MOD cows (Fig. 7; $P < 0.05$). Furthermore, *NR5A2* and *IGF1R* transcript abundance were also lower in granulosa cells of SEV than MOD cows (Fig. 7; $P < 0.05$). The LH receptor (*LHCGR*) mRNA abundance tended to be lower in granulosa cells of SEV cows (Fig. 7; $P < 0.1$).

3.7 Discussion

This study examined the association between BCS loss, liver health, and ovarian functions in early lactating dairy cows. In the cow's body, nutrient partitioning is a normal physiological adaptation to support metabolic health and reproductive functions along with milk production (Walsh et al., 2007, Caputo Oliveira et al., 2020, White, 2020). Imbalance in metabolic adaptation can result in liver dysfunction, metabolic disorders, and infertility. A previous retrospective study showed that cows losing BCS after calving had higher BCS at calving than cows maintaining BCS after calving (Britt, 1992). Cows that lost BCS had a lower conception rate at first service and a lower overall conception rate than cows that maintained BCS after calving (Britt, 1992). Another study found that cows losing excessive BCS (≥1 unit) after calving had extended intervals to the first ovulation (Shrestha et al., 2005).

Therefore, we investigated metabolic profiles, hepatic transcriptome, hormone concentrations, and gene expression of ovarian tissue. We found that a loss of ≥0.75 BCS had drastic implications leading to compromised postpartum dominant follicles. SEV cows fell in accordance with the results described in the previous studies as unfavorable levels of BCS loss (Kim and Suh, 2003). Our results are also in line with the reports that show cows with higher BCS at calving lose more BCS after calving and are more likely the cows with reduced appetite due to being over-conditioned (Britt, 1992, Roche et al., 2007, Bewley and Schutz, 2008). Several studies have demonstrated that excess BCS before calving leads to excessive loss of BCS after calving, resulting in declined subsequent reproductive performance of cows (Buckley et al., 2003, Roche, 2006, Roche et al., 2009, Stefańska et al., 2016). Circulatory NEFAs and BHB are used as the key indicators of lipid mobilization and hyperketonemia diagnosis in early lactating cows. We found that there was an overall tendency for SEV cows to have higher plasma NEFA concentration than

MOD cows. In accordance with this, previous studies have shown that greater loss of body condition during early lactation was associated with elevated NEFA concentration (Akbar et al., 2015, Luttgenau et al., 2016, Barletta et al., 2017). Prepartum over-conditioned cows lost higher body conditions and had higher NEFA concentration during early lactation, which was associated with delayed time to first ovulation (Rukkwamsuk et al., 1999). Cows that lost ≥1 unit of BCS during the transition period had higher BHB concentrations (Rathbun et al., 2017). In line with this, we observed that BHB concentrations increased drastically in SEV but not MOD cows.

The GGT is an enzyme that predominantly exists in the liver and its elevated levels in circulation indicate the destruction of the hepatocyte, including fatty liver (Sevinc et al., 2001). Our result showed that GGT of SEV cows increased at 1 and 7 weeks after calving, and the overall GGT level of SEV cows was higher than MOD cows. Therefore, this elevated plasma GGT level, along with higher NEFA and BHB indicative of hepatic lipid burden in SEV cows, hint at liver dysfunction. This interpretation is supported by previous studies that have shown that cows with fatty liver had higher plasma GGT (Ohtsuka et al., 2001) and elevated levels of blood NEFA and BHB (Rukkwamsuk et al., 2000, Mohamed et al., 2004).

This evidence of liver injury compelled us to explore hepatic transcriptome differences between MOD and SEV cows at 7 weeks after calving. Week 7 represents the ideal time to study ovarian health as it is the period approaching the end of the ideal voluntary waiting period practiced by most dairy producers in North America. In the present study, the hepatic transcriptome of cows experiencing severe body condition loss was found to be significantly affected as evidenced by the differentially expressed genes and the enriched pathways among those genes. This suggests that severe body condition loss through the transition period leading up to planned breeding time had a substantial impact on the hepatic gene expression patterns in lactating dairy cows. A deeper

probing of the transcriptome data revealed several lipid metabolism pathways or processes being enriched among the differentially expressed genes in SEV cows. Previous gene expression studies have found that there are significant differences in hepatic pathways including those associated with lipid metabolism in metabolically stressed cows during transition (Loor et al., 2007, McCabe et al., 2012, Ha et al., 2017, Shahzad et al., 2019). With these observations, our data demonstrate that lipid metabolic pathways in cows with severe body condition loss are dysregulated. There is evidence in the literature that hepatic stress, especially elevated GGT, can impact IGF1 production.

Cows that had higher BCS at calving, similar to SEV cows of this study, had higher expression of genes involved in fatty acid oxidation and lower expression of IGF1 (Akbar et al., 2015). In line with this, we found that SEV cows had lower transcript levels of *IGF1*and IGF-binding proteins as well as lower serum concentrations of IGF1. Likewise, cows experiencing a severe negative energy balance during early lactation had lower levels of circulating IGF1 or IGF-binding proteins (Fenwick et al., 2008). IGF-binding protein 3 is the most abundant protein that carries and increases the half-life of IGF1 in circulation (Varma Shrivastav et al., 2020). The SEV cows in the present study showed decreased expression of hepatic IGF1 and its binding proteins (IGFBP1 and IGFBP3). These observations suggest that SEV cows not only had lower hepatic IGF1 output but may also have a lower half-life of IGF1 in the circulation during their early lactation period leading to the breeding period. It is well established that IGF1 is a metabolic hormone that plays a significant role in ovarian folliculogenesis (Lucy et al., 1992, Spicer et al., 1994, Beam and Butler, 1999), it is mainly produced in the liver and also secreted from the ovary (Sjogren et al., 1999, Velazquez et al., 2008). In fact, most of the IGF1 found in the bovine follicular fluid is of hepatic origin as ruminant granulosa cells do not produce a significant amount of IGF1 (Velazquez et al., 2008). We and others have shown that cows in early lactation with a

severe negative energy balance had lower levels of circulating IGF1 (Fenwick et al., 2008; Sanchez et al., 2014). In this study, reduced hepatic IGF1 expression and lower serum IGF1 concentration in SEV were reflected in the follicular fluid of the dominant follicles. Further, we found that granulosa cells of SEV cows had lower expression of IGF1R further reducing the IGF1 support to their dominant follicle. Taken together, it is possible to draw a link from hepatic stress due to severe loss of body condition to reduced IGF1 support to the dominant follicle in the ovary. Lower plasma concentrations of IGF1 in early lactating cows are linked with decreased follicular competence, estrogen-inactive dominant follicles, anovulation, longer time to commencement of luteal activity and lower conception rates to first service (Beam and Butler, 1997, Beam and Butler, 1999, Taylor et al., 2004, Patton et al., 2007, Castro et al., 2012, Dupont et al., 2014). In a growing and differentiating dominant follicle, the increase in IGF1R expression is also linked to an increase in granulosa cell estradiol production (Spicer et al., 1994, Beam and Butler, 1999). In agreement with these observations, we found that the dominant follicles of SEV cows were less estrogenic compared to those from MOD cows. This drastic reduction in estrogen production by the dominant follicle of SEV cows was underpinned by lower expression of genes involved in the follicle health and function such as *LHCGR*, *NR5A2* and *CYP19A1*. Multiple studies support this argument. LHCGR in granulosa cells is also essential for the process of converting androgens to estradiol through aromatization (Duffy et al., 2019). Although granulosa cell-specific deletion of *NR5A2* was linked to increased expression of CYP19A1 in mice (Duggavathi et al., 2008), in rat granulosa cells, testosterone-induced CYP19A1 expression was abolished when NR5A2 was knocked down (Saxena et al., 2007, Wu et al., 2011). In luteinized bovine granulosa cells, overexpression of *NR5A2* increased the activity of the CYP19A1 promoter, indicating that NR5A2 is needed for CYP19A1 expression (Sahmi et al., 2014) and estradiol production.

3.8 Conclusion

In conclusion, the data from this study provide the mechanistic basis, at least in part, between severe BCS loss and ovarian dysfunction. Even though others have investigated more drastic changes in body condition loss during early lactation (Kim and Suh, 2003, Shrestha et al., 2005), we show that even at ≥0.75 loss of BCS from during early lactation leads to an adverse metabolic profile and hepatic dysfunction including reduced IGF1 production. The metabolic disorder and hepatic dysfunction appeared to drastically reduce IGF1 support for the development of the dominant follicle and estradiol production in cows that had severe BCS loss (summarized in Fig. 8). Therefore, preventing drastic body condition loss during early lactation will stabilize a cow's metabolic process and prevent fluctuation in blood metabolites, leading to improved liver health and production of adequate levels of IGF1, which could potentially regulate the acquisition and maintenance of the dominant follicle for eventual ovulation. The main takeaway from this study is to minimize a dairy cow's body condition loss from the transition period through to the planned time of breeding through changes in management both in the cow's environment and nutritionally. Maintaining an optimal BCS during this period is essential for the health and reproductive success of lactating cows. Implementing the best on-farm nutrition management practices during the transition period, including proper monitoring of energy components in the ration, can prevent over-conditioning in prepartum cows and ease their metabolic adaptation into early lactation (Cardoso et al., 2013, Drackley and Cardoso, 2014). Cows that avoid severe BCS loss have reduced peripartum health events and better reproductive performance (Fricke et al., 2023).

3.9 Ethics approval

This study was conducted on two Canadian dairy farms: A) the Macdonald Campus Farm from McGill University and B) a commercial dairy farm located close to McGill University. The Facility Animal Care Committee of the Faculty of Agricultural and Environmental Sciences of McGill University approved all animal procedures (Protocol #: 7550).

3.10 Data and model availability statement

Supplementary data are deposited on FigShare repository (https://figshare.com/s/255792b0a6c9bfa22f85). Additional information can be made available from the authors upon request.

3.11 Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

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3.14 Declaration of interest

None

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3.17 Tables

Table 3.17.1 Sequence of primers used in quantitative reverse transcriptase PCR. Transcript abundance analysis by qPCR was performed on granulosa cells of dominant follicles in cows with severe (SEV) and moderate (MOD) body condition loss collected at week 7 after calving.

Gene[1]		Forward Primer
CYP19A1	F	CTGAAGCAACAGGAGTCCTAAATGTACA
	R	AATGAGGGGCCCAATTCCCAGA
IGF1R	F	ATCCCAAGTCGAGGATCAGC
	R	GTCGTCTTGGCCTGAACGTA
LHCGR	F	GCACAGCAAGGAGACCAAATAA
	R	TTGGGTAAGCAGAAACCATAGTCA
NR5A2	F	CTACAGACTACGACCGCAGC
	R	TCCACGTAGGAGTAGCCCAT
ACTB	F	TCTGGATCAGCAAGCAGGAGTA
	R	TGCGCAAGTTAGGTTTTGTCA
PPIA	F	GGTCATCGGTCTCTTTGGAA
	R	TCCTTGATCACACGATGGAA

	F	GCCAACTCCCGTCAGCAGA
RPL19		
	R	TGGCTGTACCCTTCCGCTT

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[1] *CYP19A1*: cytochrome P450 19A1; *IGF1R*: insulin-like growth factor 1 receptor; *LHCGR*: luteinizing hormone receptor; *NR5A2*; nuclear receptor 5A2; *ACTB*: actin B; *PPIA*: Peptidylprolyl Isomerase A (Cyclophilin A); *RPL19*: ribosomal protein L19; F: forward; R: reverse.

Table 3.17.2 Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and significantly upregulated (with *) and downregulated (without *) genes within each pathway in cows with severe (SEV) compared to cows with moderate (MOD) body condition loss. Liver biopsies were collected from cows with SEV (N = 3) and MOD (N = 3) body condition loss at week 7 after calving.

KEEG pathway	P value	Enriched genes
alpha-Linolenic acid metabolism	0.002	*PLA2G2C*, PLA2G4E*, PLB1*, PLA2G4B*, LOC618367*
Citrate cycle (TCA cycle)	0.03	*OGDHL*, SUCLG1, MDH1, SDHC, FH*
Ether lipid metabolism	0.01	*PLA2G2C*, PLA2G4E*, PLB1*, PLA2G4B*, PLA2G, GDPD1, LOC618367*
Linoleic acid metabolism	0.01	*PLA2G2C*, PLA2G4E*, PLB1*, PLA2G4B*, LOC618367*
Fat digestion and absorption	0.03	*PLA2G2C*, LOC618076*, ABCA1, LOC518526, GOT2*
Insulin secretion	0.006	*ATP1A4*, CAMK2B*, ADCY8*, STX1A*, RYR2*, CACNA1D*, ATP1A2*, PCLO*, CACNA1C*, ATP1B3, CREB3L1*
Calcium signaling pathway	< 0.0001	*SLC8A3*, CACNA1E*, TNNC2*, TNNC1*, GRIN2A*, CAMK2B*, ADCY8*, PTGFR*, SLC8A2*, RYR3*, ATP2B3*, RYR2*,*

		CACNA1D, CACNA1B*, CACNA1C*, GRIN2C*, CACNA1A*, RYR1*, MYLK3*, PLCE1*, F2R*
cAMP signaling pathway	< 0.0001	*PLCE1*, GABBR1*, GRIN2C*, CACNA1C*, ADCY10*, ATP1A2*, CACNA1D*, RYR2*, ATP2B3*, GIPR*, ADCY8*, CAMK2B*, GRIN2A*, MAPK10*, GRIA1*, ATP1A4*, CNGA3*, PDE4C*, HCN4*, CREB3L1, NFKBIA, F2R, GNAI1, ATP1B3*
Protein digestion and absorption	< 0.0001	*SLC8A3*, COL17A1*, COL4A4*, COL11A1*, COL27A1*, ATP1A4*, COL4A3*, COL13A1*, KCNJ13*, COL9A2*, SLC8A2*, ATP1A2*, COL11A2*, PRSS2, CPB2, ATP1B3*
Tyrosine metabolism	0.03	*AOX2*, ADH6*, ALDH1A3, GOT2, MIF*

Note: cAMP – cyclic adenosine mono-phosphate

Table 3.17.3 Enriched gene ontology-biological process (GO_BP) terms and significantly upregulated (with *) and downregulated (without *) genes within each GO-BP term in cows with severe (SEV) compared to cows with moderate (MOD) body condition loss. Liver biopsies were collected from cows with SEV (N = 3) or MOD (N = 3) body condition loss at week 7 after calving.

GO_BP	P value	Enriched genes
Protein targeting to membrane	< 0.0001	*HCN4*, CNGA3*, KCNJ9*, CACNA1E*, SCN5A*, SCN10A*, CLCNKA*, KCND3*, KCNJ13*, SLC4A10*, KCNK7*, KCNH5*, SLC9A2*, SLC5A11*, GRIA1*, GRIN2A*, CHRNA1*, SLC24A2*, CNGB1*, SLCO5A1*, TRPV6*, ATP2B3*, RYR2*, CACNA1D*, ATP1A2*, TRPC4*, CACNA1B*, GABRE*, GRIN2C*, CACNB2*, SLCO3A1*, SLC26A9*, SCNN1A*, CACNA1A*, RYR1*, ATOX1, SLCO1A2, ATP5PO, ATP1B3, ATP5PB, ATP5PF, ATP6V0E1, KCNJ2, ATP6V1F, SCARA5, CHRNE*
Protein export from nucleus	< 0.0001	*ANO4*, SLC28A2*, HCN4*, CNGA3*, KCNJ9*, CACNA1E*, SCN5A*, SCN10A*, CLCNKA*, KCND3*, LOC527385*, KCNJ13*, SLC4A10*, KCNK7*, KCNH5*, SLC9A2*, SLC5A11*, GRIA1*, GRIN2A*, RAB3C*, CHRNA1*, SLC4A11*, SLC6A7*, RAB15*, SLCO5A1*, TRPV6*, ATP2B3*, RYR2*, CACNA1D*, ATP1A2*, TRPC4*, CACNA1B*, LCN12*, GABRE*,*

		SLC6A5, GRIN2C*, CACNB2*, SLCO3A1*, SLC26A9*, SLC25A34*, SLC26A7*, SCNN1A*, SLC25A30*, CPT1B*, CACNA1A*, RYR1*, OSBPL7*, SLC25A47*, SERPINA5, ATOX1, SLCO1A2, ATP5PO, ABCA1, ATP1B3, AQP11, AP1M1, GABARAP, ATP5PB, SNX3, ATP5PF, GOT2, ATP6V0E1, SNX10, KCNJ2, TOMM6, HPX, ATP6V1F, MFSD1, HIKESHI, TTR, SCARA5, SERPINA6, CHRNE, SLC7A5, AP1M2*
Fatty acid biosynthetic process	< 0.0001	*SLC8A3*, CACNA1E*, GRIN2A*, SLC24A2*, SLC8A2*, TRPV6*, ATP2B3*, RYR2*, CACNA1D*, TRPC4*, CACNA1B*, CACNB2*, CACNA1A*, RYR1**
Fatty acid metabolic process	< 0.0001	*HCN4*, ATP1A4*, SCN5A*, SCN10A*, SLC9A2*, SLC5A11*, SLC4A11*, ATP1A2*, SCNN1A*, ATP1B3*
Regulation of cell adhesion	< 0.0001	*HCN4*, CNGA3*, ATP1A4*, KCNH5*, GRIN2A*, CHRNA1*, CNGB1*, GABRE*, GRIN2C*, CACNA1A*, CHRNE*
Oligosaccharide metabolic process	0.001	*CACNA1A*, OTOA*, SPTBN4*, SOD1*
Carbohydrate catabolic process	0.003	*SLC4A10*, ATP1A4**

Lipid metabolic process	0.01	*HCN4*, KCNJ9*, ATP1A4*, KCND3*, KCNJ13*, KCNH5*, ATP1A2*, ATP1B3, KCNJ2*
Fatty acid beta_oxidation	0.01	*SLC4A10*, SLC26A9*, SLC26A7*, SLC4A11**
Transforming growth factor beta receptor signaling pathway	0.02	*CNGB1*, GUCA1A**

3.18 Figures

Figure 3.18.1 Body condition score (BCS) loss in cows during early lactation. (A) Change in BCS from 3 weeks before calving to 7 weeks after calving from retrospectively grouped cows that had moderate (MOD; N=11) or severe (SEV; N=9) loss of BCS. All data are expressed as a mean ± SEM, where significant differences over time within a group are labelled with superscripts A and B and significant differences between the two groups at a particular time-point are labelled with the superscript F, $P < 0.05$. (B) Loss of body condition in MOD and SEV cows represented as a percentage and normalized to 3 weeks before calving.

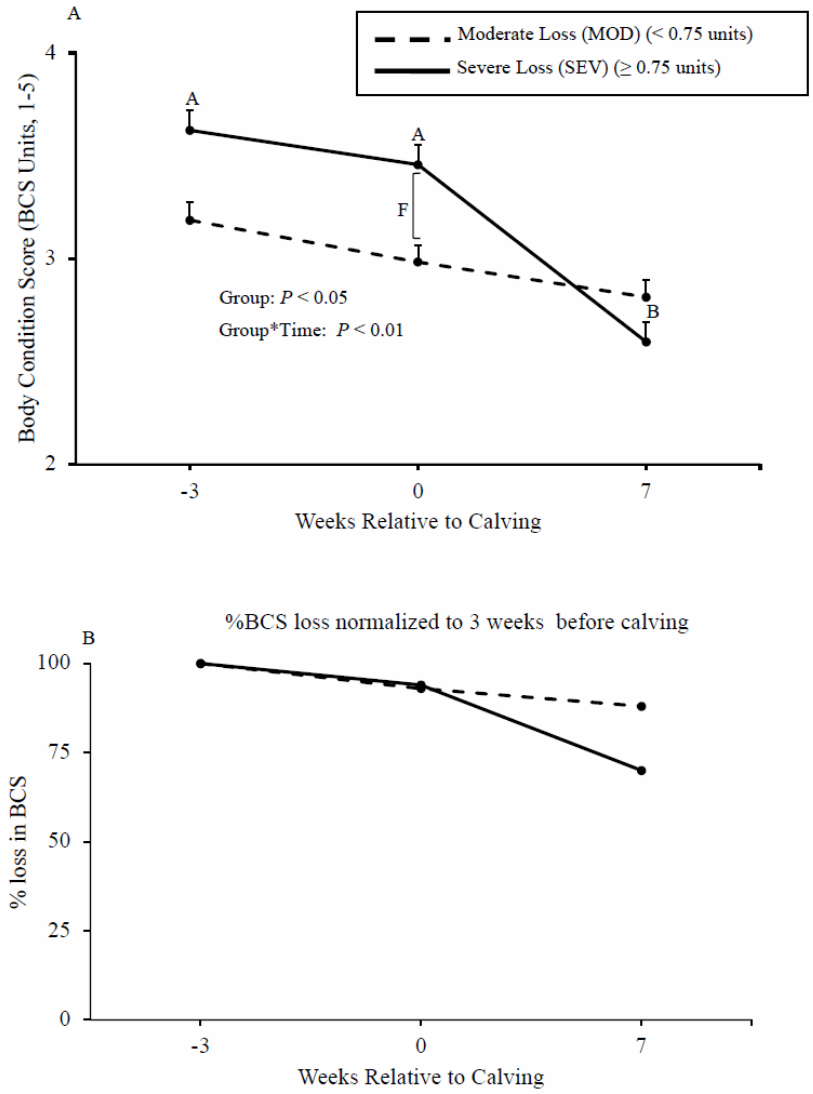


Figure 3.18.2 Severe loss of body condition is associated with increased plasma non-esterified fatty acids (NEFA), ß-hydroxybutyrate (BHB) and γ-glutamyl transferase (GGT). Plasma concentrations of NEFA (A), BHB (B), GGT (C), glucose (D), and haptoglobin (E) during weeks 3 before calving, week of calving, and weeks 1 and 7 after calving in cows that had moderate (MOD; N=9) or severe (SEV; N=8) loss of body condition score (BCS). Asterisk (*) indicates a significant difference between groups within a timepoint. Double-asterisks (**) and hashtag (#) represent significant difference and tendency for the difference in BHB levels from Week -3 within SEV cows. All data are expressed as a mean ± SEM.

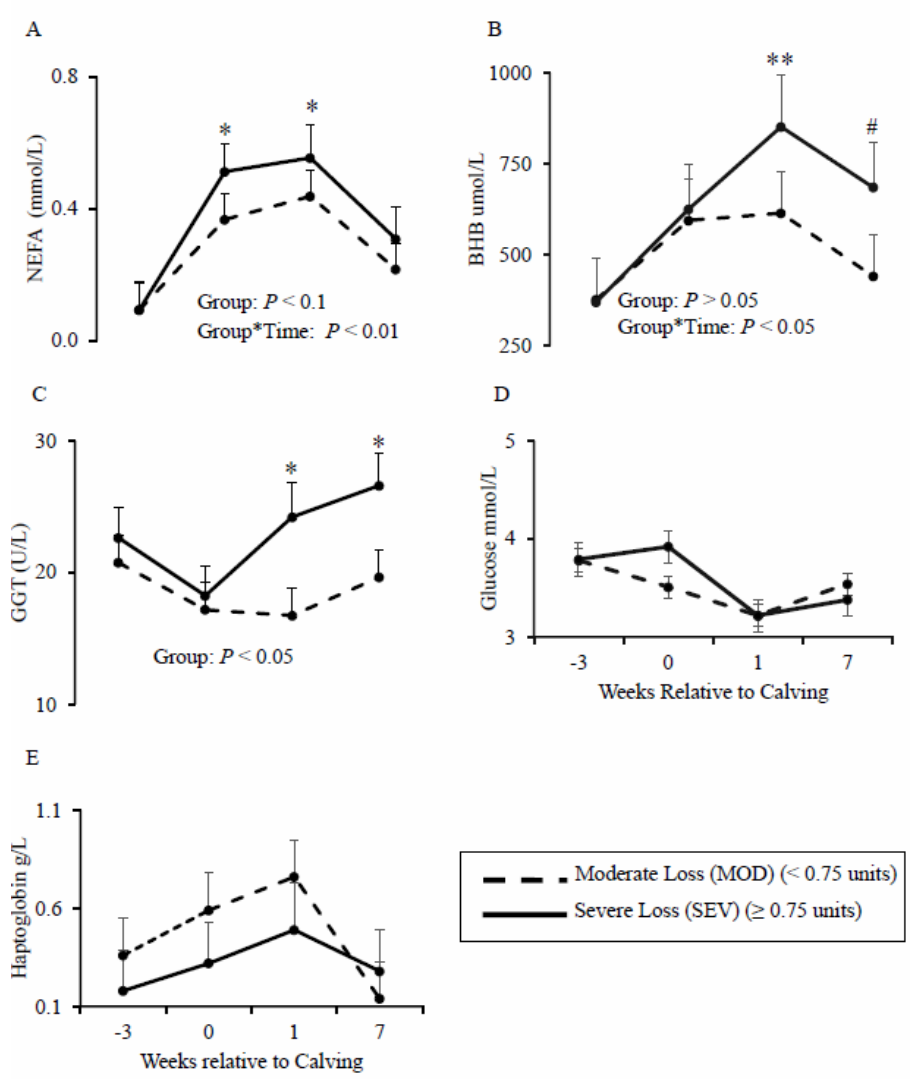


Figure 3.18.3 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched among differentially expressed genes in the liver at week 7 after calving in cows with severe (SEV; N = 3) relative to those with moderate (MOD; N = 3) body condition loss during early lactation. Important genes belonging to multiple pathways are highlighted with upregulated genes in red and downregulated genes in blue fonts. Full lists of genes involved in each pathway are presented in Table 2.

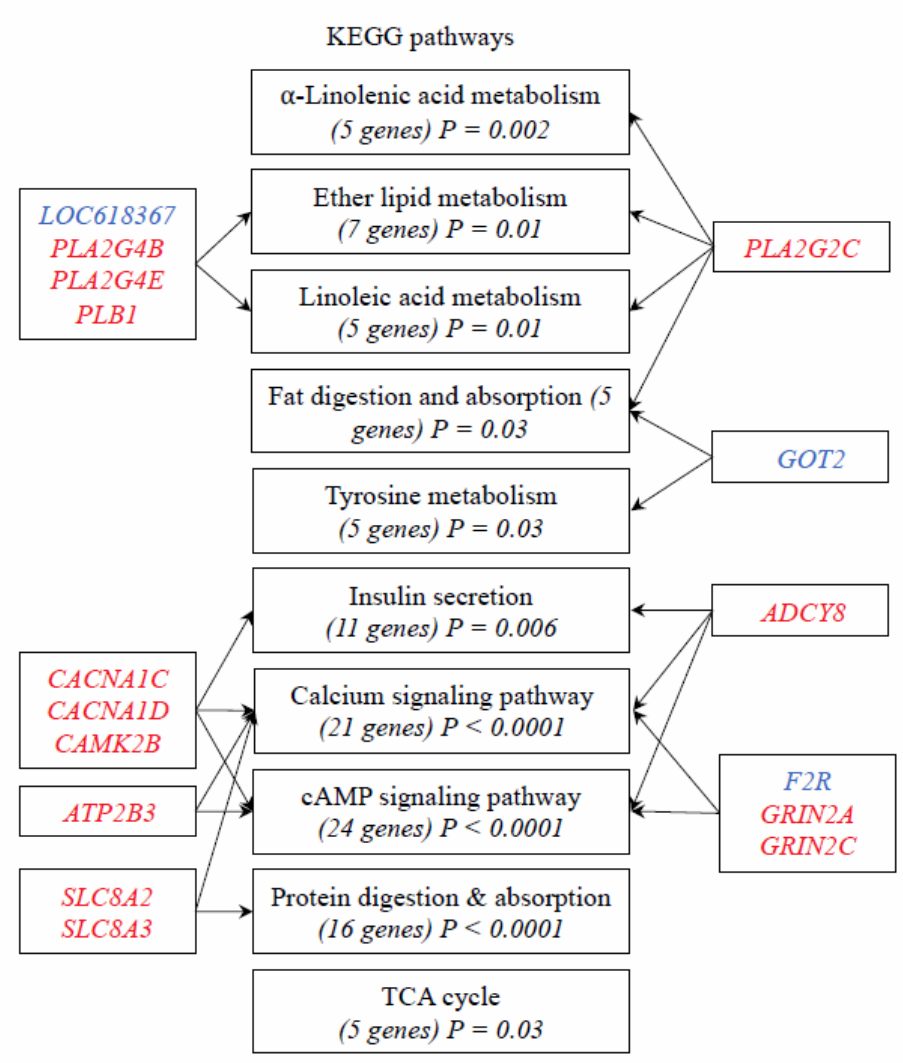


Figure 3.18.4 Gene Ontology (GO) biological process (BP) terms enriched among differentially expressed genes in the liver at week 7 after calving in cows with severe (SEV; N = 3) relative to those with moderate (MOD; N = 3) body condition loss during early lactation. Important genes belonging to multiple GO_BP terms are highlighted with upregulated genes in red and downregulated genes in blue fonts. Full lists of genes involved in each GO_BP term are presented in Table 3. Note: TGFβ-R – transforming growth factor β receptor.

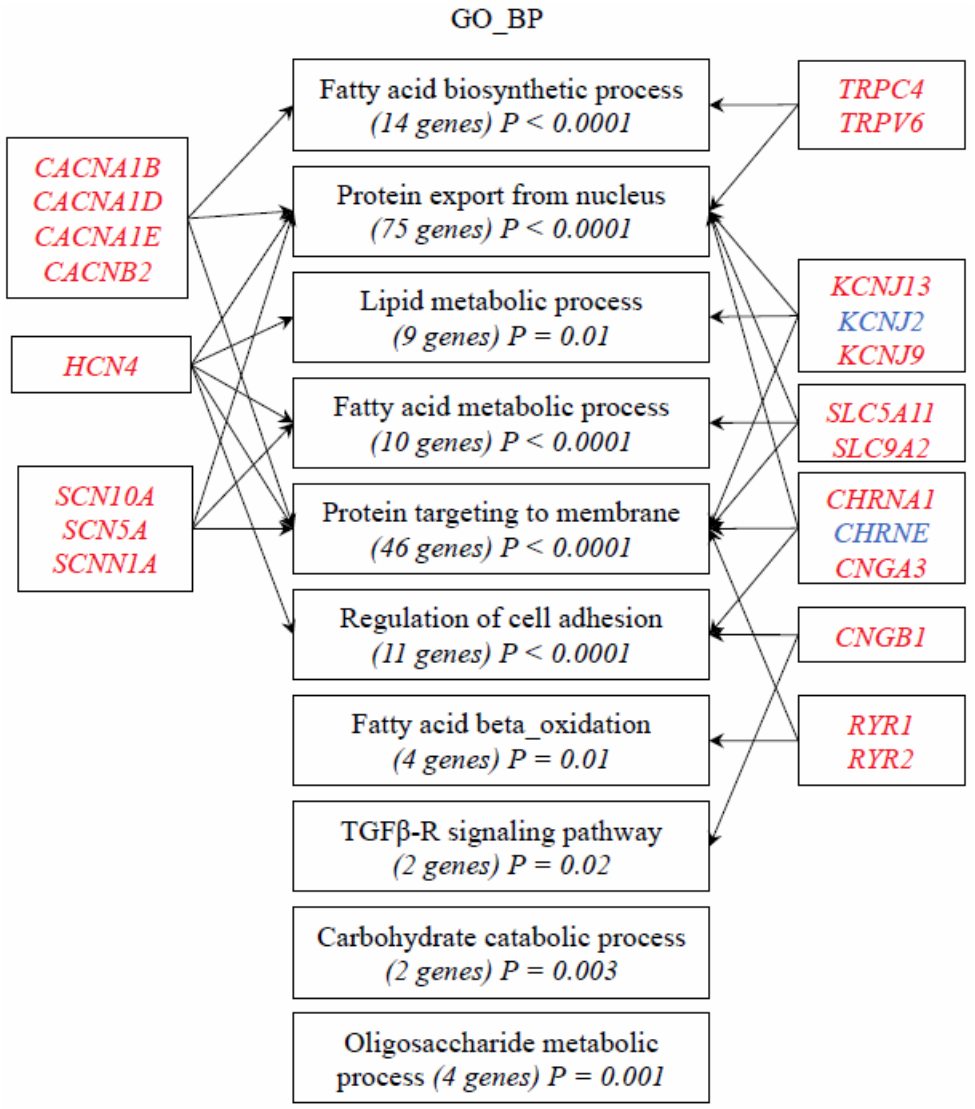


Figure 3.18.5 Hepatic differential expression and hormonal analysis of serum in week 7 after calving between cows experiencing a moderate (MOD) or severe (SEV) degree of body condition loss. Hepatic differential expression of IGF1 (MOD; N=3 and SEV; N=3) (A) and IGF1 concentrations measured in serum (MOD; N=9 and SEV; N=8) (B) at 7 weeks after calving. Significant differences between groups are marked with the superscript * ($P < 0.05$) and ** ($P < 0.01$). Data are expressed as a mean ± SEM.

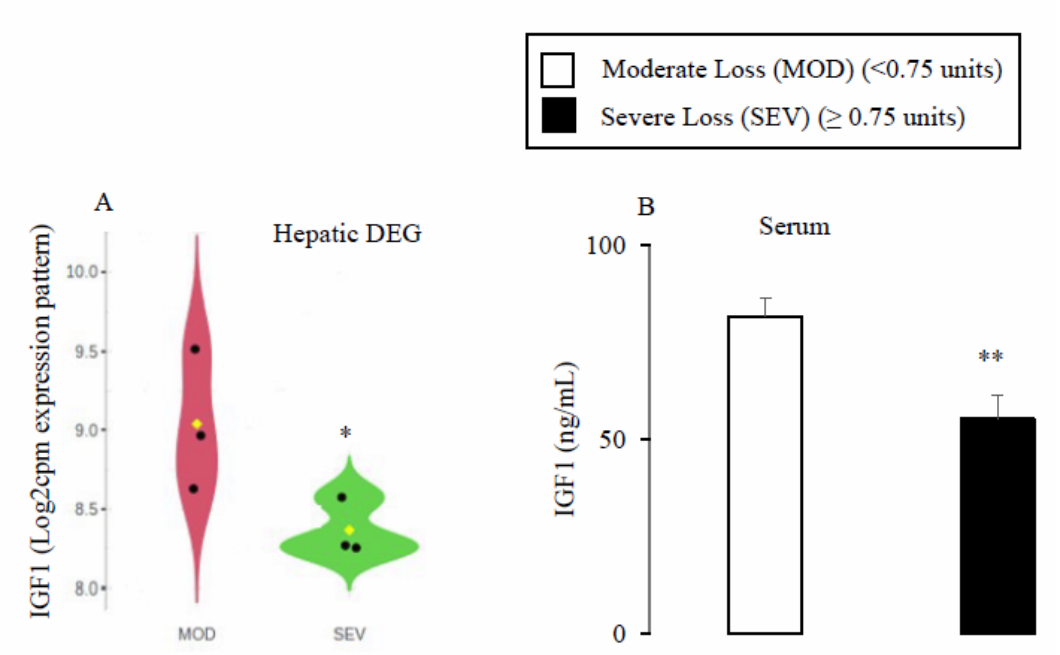


Figure 3.18.6 Follicle size and hormonal analysis of dominant follicle at week 7 after calving between cows experiencing a moderate (MOD; N=9) or severe (SEV; N=7) degree of body condition loss. Follicle size was measured for all cows at the time of follicular aspiration (A), IGF1 concentration in follicular fluid (B), and Estradiol levels measured in the follicular fluid of dominant follicles (C) at 7 weeks after calving. Significant differences between groups are marked with the superscript * ($P < 0.05$) and ** ($P < 0.01$). Data are expressed as a mean ± SEM.

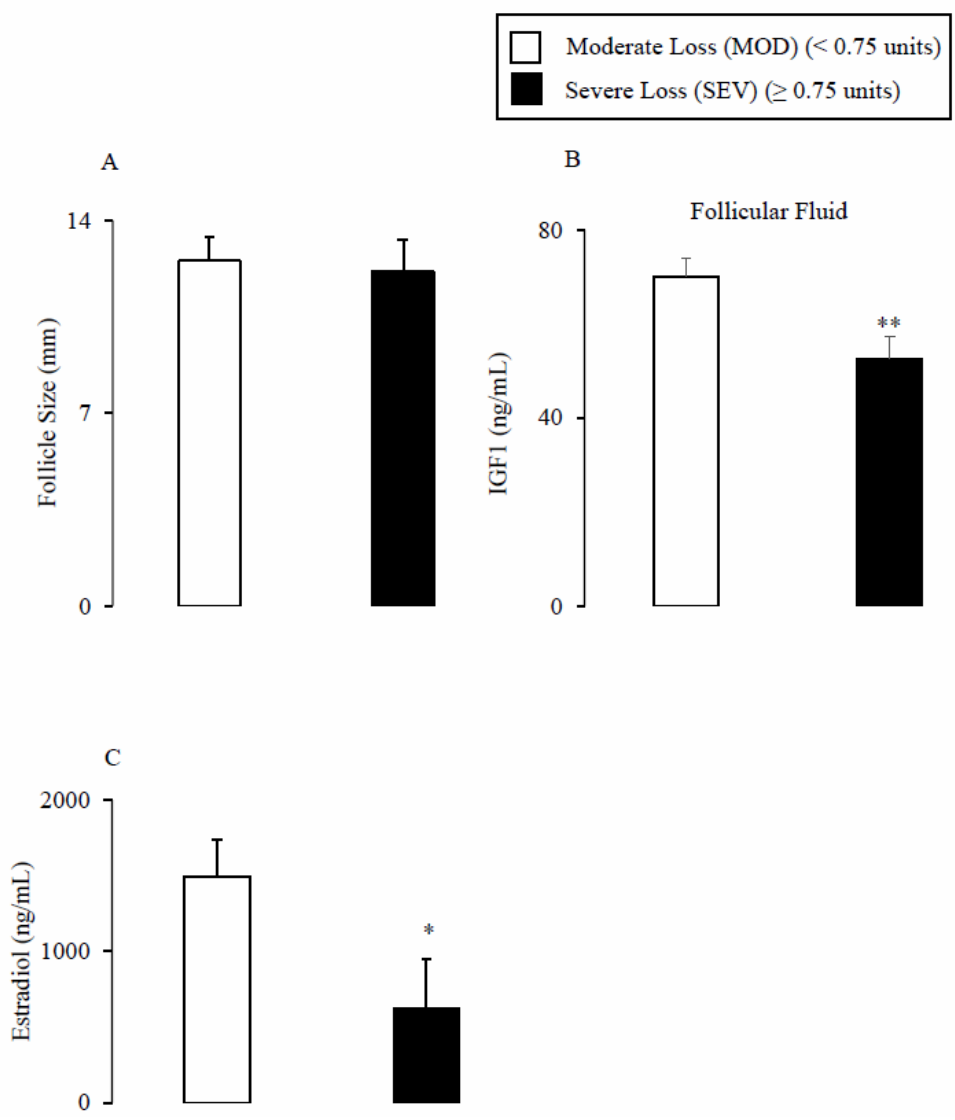


Figure 3.18.7 Transcript abundance of the genes, *IGF1R*, *CYP19A1*, *NR5A2*, and *LHCGR* in granulosa cells of cows experiencing moderate (MOD; N=9) or severe (SEV; N=7) body condition loss from 3 weeks before calving to 7 weeks after calving. Granulosa cells were collected in week 7 after calving. Transcript abundance of each gene was normalized to reference genes *ACTB*, *RPL19*, and *PPIA*. Significant differences between groups are marked with the superscript * ($P < 0.05$), while tendencies for differences are marked with the superscript # ($P < 0.1$). All data are expressed as a mean ± SEM. Note: The gene symbols are described in Table 1.

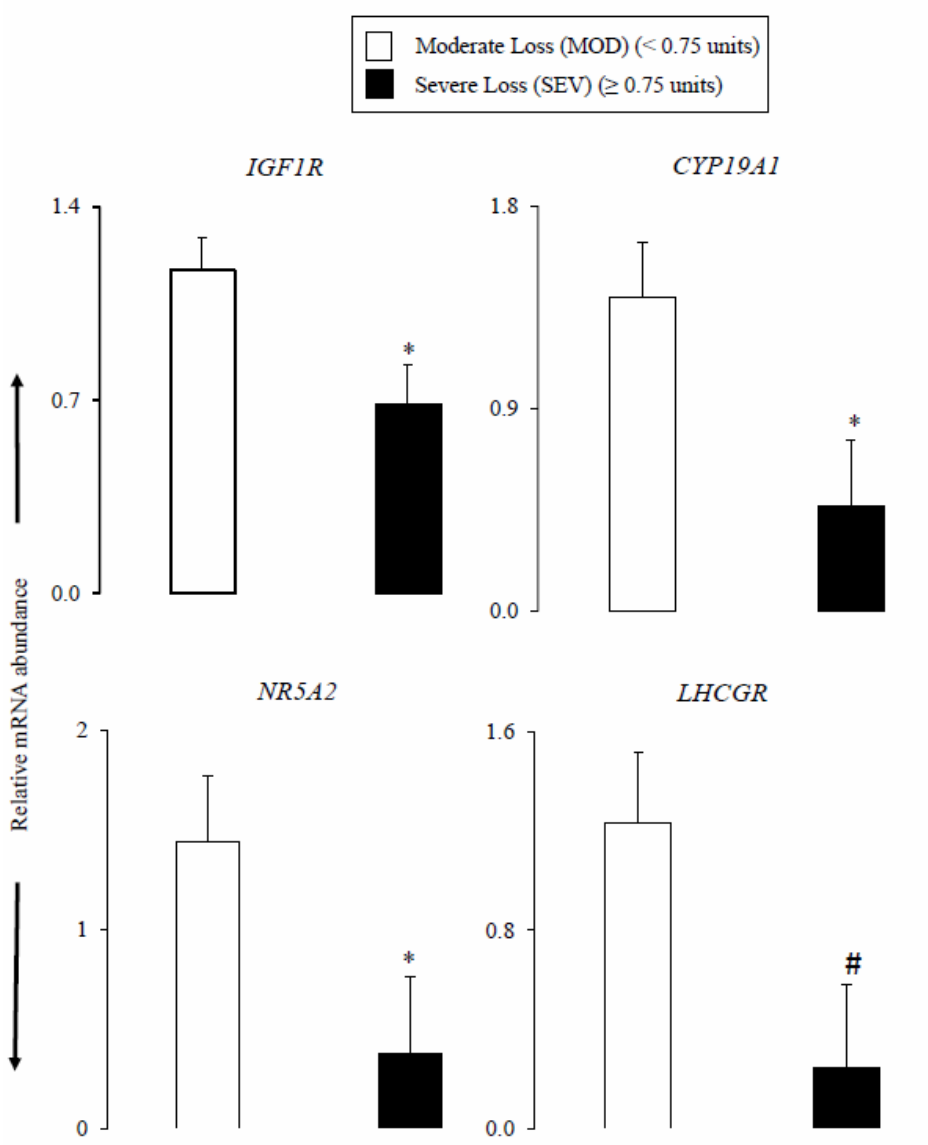
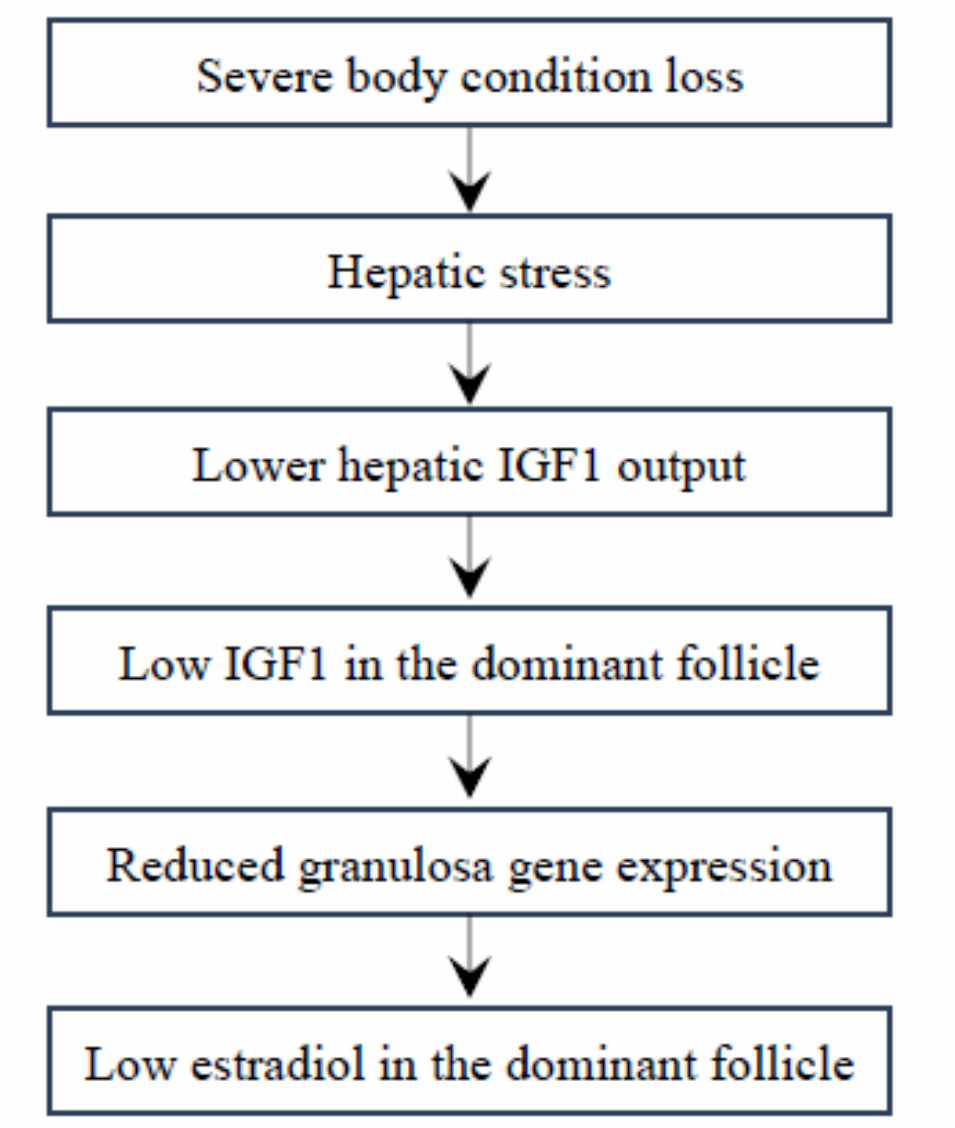


Figure 3.18.8 Proposed mechanism of impact of severe loss of body condition during early lactation on ovarian follicular function in lactating dairy cows. Severe body condition loss after calving had altered hepatic health, leading to abnormal gene expression, disrupted in metabolic pathways, lower circulatory IGF1 concentrations. The lower hepatic IGF1 support was associated with lower follicular fluid IGF1 and estradiol-17β concentration because of disrupted gene expression in granulosa cells of the dominant follicle at seven weeks after calving.



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CONNECTING STATEMENT 1

Body condition score (BCS) reflects body fat reserves in dairy cows. A decrease in BCS during the transition period can cause health problem and lead to infertility. In the first study, we investigated the effect of severe BCS loss on liver health and ovarian function in early lactation cows. Our results demonstrated that impaired metabolic and liver function due to severe BCS loss during early lactation resulted in ovarian dysfunction. This finding directed our attention towards exploring how elevated BHB concentration affects the reproductive performance of dairy cows. Therefore, in the next study, we investigated the association between time and amplitude of elevated milk BHB (EMB) occurring within 42 DIM and subsequent reproductive performance of lactating Holstein cows.

CHAPTER 4

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Reproductive performance of lactating dairy cows with elevated milk β-hydroxybutyrate levels during first 6 weeks of lactation

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4.1 Abstract

Although there is evidence that ketosis negatively affects fertility, the effect of late and early ketosis on the reproductive performance of lactating cows has not been systematically investigated. The aim of this study was to evaluate the association between time and amplitude of elevated milk BHB (EMB) occurring within 42 d in milk (DIM) and subsequent reproductive performance of lactating Holstein cows. The dairy herd information data of 30,413 cows with 2 test-day milk BHB recordings during early lactation periods 1 and 2 (5–14 and 15–42 DIM, respectively) assessed as negative (<0.15 mmol/L), suspect (0.15–0.19 mmol/L), or positive (≥0.2 mmol/L) for EMB were used in this study. Based on the time and amplitude of milk BHB, cows were grouped into 7 groups: (1) healthy cows negative in both periods 1 and 2 were classified as NEG; (2) suspect in period 1 and negative in period 2: EARLY_SUSP; (3) suspect in period 1 and suspect/positive in period 2: EARLY_SUSP_Pro; (4) positive in period 1 and negative in period 2: EARLY_POS; (5) positive in period 1 and suspect/positive in period 2: EARLY_POS_Pro; (6) negative in period 1 and suspect in period 2: LATE_SUSP; and (7) negative in period 1 and positive in period 2: LATE_POS. The overall prevalence of EMB within 42 DIM was 27.4%, with the highest prevalence being EARLY_SUSP (10.49%). Cows in EARLY_POS and EARLY_POS_Pro, but not other EMB categories, had a longer interval from calving to first service compared with NEG cows. For the reproductive parameters, first service to conception interval, days open and calving interval, cows in all EMB groups except EARLY_SUSP had longer intervals compared with NEG cows. These data indicate that there is a negative association between EMB within 42 d and reproductive performance after the voluntary waiting period. The intriguing findings of this study are the unaltered reproductive performance of EARLY_SUSP cows, and the negative association between late EMB and reproductive performance. Hence,

monitoring and prevention of ketosis during the first 6 wk of lactation is necessary to optimize reproductive performance of lactating dairy cows.

Key words: early lactation, elevated milk BHB, Holstein cow, reproductive performance

4.2 Introduction

Dairy cows experience negative energy balance during early lactation because feed intake alone cannot support the energy demand for body maintenance as well as milk production (Wathes et al., 2007). In addition, nutrient partitioning in terms of glucose utilization is favored toward the mammary gland for milk synthesis, exacerbating the negative energy balance. The high metabolic demand during lactation-induced negative energy balance is met with by mobilization of fat from adipose tissue to release nonesterified fatty acids (**NEFA**) for energy. The NEFA can either be used as fuel by other tissues or further metabolized in the liver. The metabolic consequences of NEFA in the liver include utilization as metabolic fuel for hepatocytes, conversion to ketone bodies as alternate to glucose for other tissues and repackaging into very-low density lipoproteins as fat for systemic redistribution. However, if the NEFA load exceeds the liver's metabolic capacity, the excess fatty acids are stored leading to fatty liver. In contrast, rapid and excessive ketogenesis can lead to ketosis (Duffield et al., 2009), defined as high concentration of ketone bodies in the blood.

The prevalence of ketosis is high during the first 2 wk (Duffield et al., 1998; Walsh et al., 2007; McArt et al., 2012; Santschi et al., 2016), although the risk of ketosis lasts at least until 42 d of lactation (Mahrt et al., 2015). Depending on the presence or absence of clinical signs, ketosis is classified as clinical (**CK**) and subclinical ketosis (**SCK**), respectively. Some studies have also categorized ketosis into 2 “types” depending on the etiological background and the lactational stage when hyperketonemia occurs in relation to calving (Holtenius and Holtenius, 1996; Herdt, 2000; Zhang and Ametaj, 2020). Type I ketosis usually develops between 15 to 42 DIM as a result of reduced feed intake leading to lower gluconeogenesis, hypoglycemia and hypoinsulinemia, which stimulate rapid ketogenesis and lower fat accumulation in the liver (Holtenius and Holtenius, 1996). Type II ketosis usually develops in the first 14 DIM as a result of insulin

resistance resulting in hyperinsulinemia, hyperglycemia and lower gluconeogenesis, which promote excessive body fat mobilization resulting in increased ketogenesis and fat accumulation in the liver (Grummer, 1993; Holtenius and Holtenius, 1996; Herdt, 2000).

The gold standard for diagnosing ketosis is measuring the concentration of the major ketone body, BHB, in blood (plasma or serum) samples. Various studies have used automated analyzers or hand-held cow-side meters to measure concentration of BHB and have used different thresholds to diagnose ketosis. The most commonly used thresholds of blood BHB concentrations are 1.2–2.9 mmol/L and 3.0 mmol/L to categorize subclinical and clinical ketosis, respectively (McArt et al., 2011; Borchardt and Staufenbiel, 2012; Suthar et al., 2013). Recent technological advances in milk analysis have shown that milk can be a noninvasive sample suited for monitoring BHB levels (Denis-Robichaud et al., 2014). Indeed, milk is routinely put through Fourier-transformed infrared (**FTIR**) testing for quality analyses including BHB measurement. Although several studies have used direct measurement techniques used for blood BHB assay, recent studies have used FTIR data to study milk BHB and ketosis in lactating cows (Benedet et al., 2019). Similar to blood BHB levels, different thresholds of milk BHB have been used to determine ketosis. However, recent studies have used, based on correlation between BHB measurement by direct and FTIR methods, the thresholds of 0.15–0.19 and ≥0.20 mmol/L of milk BHB as suspect and positive for hyperketonemia, respectively (Koeck et al., 2014; Santschi et al., 2016).

Several authors have indicated an association between ketosis and reproductive performance (Walsh et al., 2007; Wathes et al., 2007; Ospina et al., 2010; McArt et al., 2012; Rutherford et al., 2016). In contrast, other studies have shown that SCK and healthy cows had similar reproductive performance parameters such as first service to pregnancy (Kessel et al., 2008; Chapinal et al., 2012; McArt et al., 2012), pregnancy at first insemination (Chapinal et al., 2012),

and calving to pregnancy (Wathes et al., 2003; Ospina et al., 2010; Chapinal et al., 2012; McArt et al., 2012). Although ketosis occurs in 2 different etiological background as mentioned above, there are no studies investigating the effect of late and early ketosis on reproductive performance. Thus, the objective of our study was to use FTIR milk BHB and actual breeding data from the Lactanet database to investigate associations between timing and amplitude of elevated milk BHB (**EMB**) and reproductive performance of lactating dairy cows. Because the data on circulating concentration of BHB were not available, we chose to use the following terminologies: Negative EMB, Suspect EMB and Positive EMB to classify data based on the levels of milk BHB. Because there is no clear evidence if Type I and II ketosis are associated with abnormal insulin concentration and resistance found in human diabetes, we will refer to EMB occurring during wk 1–2 of lactation as early EMB and that occurring during wk 3–6 of lactation as late EMB. Establishing the relationship between EMB "types" and reproductive performance will enhance our understanding of the effect of ketosis subtypes, which is imperative for the development of individualized strategies to optimize reproductive performance of lactating dairy cows.

4.3 Materials and Methods

4.3.1 Data Management

Facility Animal Care Committee approval was not necessary for this study, as no animals were used for the sample collection and all data used for the study were part of the routine milk recording program.

The original data set obtained from Lactanet, collected between January 01, 2014, to November 30, 2016, contained records of 726,259 cows coming from 4,596 herds (median size of 137 cows) with the following information: cow identification number, herd code, parity, calving date, test-day milk BHB, 305-d milk yield, lactation end reasons (i.e., dry off, death, or sale), and breeding service records, and parental EBV for heifers (305-d milk yield, 305 d fat yield, and 305 d protein yield). To classify EMB into different types, we divided the 5–42 DIM interval into 2 periods, period 1 (5–14 DIM) and period 2 (15–42 DIM), representing, as mentioned above, the time of occurrence of early and late ketosis (Herdt, 2000). The details criteria and number of cows retained in the database at each inclusion-exclusion step are given in Figure 1A. First, we retained cows having 2 milk BHB records, one in each lactation period. Of the 33,821 cows retained, 27,095 cows had and 6,726 cows did not have a subsequent calving recorded. To analyze cows that had a reasonable breeding management, we restricted the interval of calving to first service to 20–200 DIM. With this restriction, there were 26,946 cows with a subsequent calving and 6,624 cows without a subsequent calving retained in the database. For cows with a recorded subsequent calving, we retained 26,517 cows with a reasonable calving interval of 300 to 600 d (Pryce et al., 2000). For these cows with a subsequent calving, the conception date was defined as the date of the last service. Gestation length was derived from the last service date and subsequent calving date and cows with a reasonable gestation length of 265 to 295 d (Olds et al., 1979) were retained,

leaving 24,110 cows with reasonable calving intervals and gestation lengths in the database. From among the combined 30,734 (6,726 + 24,110) cows, we retained cows that had "lactation end reason" as normally dried or sold or died and excluded cows with no known lactation end reason. Following these inclusion and exclusion exercises, the final data set had a total of 30,413 lactating Holstein cows from 3,172 herds, with number of cows included for analysis ranging from 1 to 274 and a median of 6 cows per herd. The frequency distribution of cows during both period 1 (5–14 DIM) and period 2 (15–42 DIM) in the final data set is presented in Figure 1B. Finally, the season of calving was classified into 4 categories (fall: September to November; winter: December to February; summer: June to August, and spring: March to May).

4.3.2 Categorization of Milk BHB Data

The threshold levels for milk BHB concentration were establish based on the criteria outlined by the previous studies (Denis-Robichaud et al., 2014; Santschi et al., 2016): <0.15 mmol/L = negative (**NEG**); 0.15 to 0.19 mmol/L = suspect (**SUSP**); and ≥0.20 mmol/L = positive for EMB. Based on these threshold levels of milk BHB during the 2 lactation periods, cows were categorized into 7 different EMB types (Table 1). To account for the effect of previous milk yield, multiparous cows were classified into production-level categories according to their previous lactation yield and primiparous cows were classified based on their parental EBV (ECM, kg/305 d). The production level categories were low (bottom 25%), medium (middle 50%), or high (top 25%) producers. Energy-corrected milk yield of 305 d was calculated as ECM (kg/305 d) = 12.55 × fat (kg/305 d) + 7.39 × protein (kg/305 d) + 0.2595 × milk yield (kg/305 d) (Tyrrell and Reid, 1965; Santschi et al., 2016).

4.3.3 Reproductive Parameters Analyzed

The association of EMB with reproductive performance of lactating cows was studied by analyzing 4 major fertility performance indicators: (1) calving to first service interval, defined as number of days between calving and first service; (2) first service to conception interval, defined as number of days between first service after calving and conception; (3) days open, defined as the number of days between calving and service that resulted in a conception; and (4) calving interval, defined as number of days between 2 consecutive calvings.

4.3.4 Statistical Analysis

Statistical analysis was performed using SAS 9.4 (SAS Institute Inc.). Descriptive statistics were generated with the FREQ procedure of SAS. A generalized logit function was used to determine the risk factors associated with the dependent variable being EMB category (nominal categorical outcomes): NEG, EARLY_SUSP, EARLY_SUSP_Pro, EARLY_POS, EARLY_POS_Pro, LATE_SUSP or LATE_POS in early-lactation cows. The risk factors in the model included parity, previous 305-d milk yield, calving season, calving year, and region.

Kaplan and Meier (1958) estimates of the survival function were used to measure the reproductive parameters using the LIFETEST procedure of SAS. Cows that had a lactation end reason of died or sold and cows ended a lactation normally, but missed a subsequent calving record were right censored. The hazard ratios of reproductive parameters were determined using the PHREG procedure of SAS with Cox proportional hazard ratio (Cox, 1972) fitted for time calving to first service, first service to conception, days open and calving interval. The model used was

$$\lambda i(t|Xi) = \lambda 0(t) \exp(xi'\beta),$$

where $\lambda i(t|Xi)$ is the hazard ratio of having the first service after calving at time t, conception after the first service at time t, conception after calving at time t (days open) or calving interval at time t; $\lambda 0(t)$ is the baseline hazard function; β is an unknown vector of regression coefficients for the covariates; x' is a vector for the fixed effect of EMB type (Table 1), parity (1, 2, 3, and ≥ 4), previous 305-d milk yield (low, medium, and high), calving season (fall, summer, winter, and spring), calving year (2014 to 2016), and region (1 to 18), EMB × parity, and EMB × previous 305-d milk yield, and herd as a random effect; and i is the combination of fixed effect levels for a specific observation. If statistically nonsignificant ($P \geq 0.05$), the interaction factors were manually removed by manual backward stepwise elimination. Cows that had a lactation end reason of died or sold and cows that ended a lactation normally, but missed a subsequent calving record were considered as right censored. The Cox proportional hazard model assumptions were found to be supported by a nonsignificant relationship between Schoenfeld residuals against the survival time ($P > 0.05$).

4.4 Results

After preliminary editing, the final data sets used for the analyses included a total of 30,413 cows (Figure 4.8.1A), of which 9,716 (32%) were parity 1, 8970 (29.5%) were parity 2, 6251 (20.5%) were parity 3, and 5,476 (18%) were parity ≥4. The distribution of cows with elevated milk BHB level during period 1 (5–14 DIM) or period 2 (15–42 DIM) is represented in Figure 4.8.1B. The prevalence of EMB within the first 6 wk of lactation is shown in Figure 4.8.2. The overall prevalence of EMB using a threshold ≥0.15 mmol/L of milk BHB was 27.4% within 42 DIM, with early EMB (20.96%) 3 times more prevalent than late EMB (6.44%). Of the 6 EMB types the highest prevalence was of EARLY_SUSP (10.49%). The prevalence of POS was lower than SUSP in both late and early EMB. The differences in least squares means of test-day milk BHB concentrations during the period of 5–80 DIM among different EMB type cows were tested using Scheffe's multiple comparison tests (Figure 4.8.3). As expected due to our grouping method, there were differences in milk BHB levels during the period of 5–42 DIM in different EMB group cows compared with NEG cows ($P < 0.05$). We included the period of 43–80 DIM to examine if the milk BHB concentrations of cows with EMB were different from those of healthy (NEG) cows during the period from the end of the ketosis-risk period (Mahrt et al., 2015) to the median day of first service after calving (Stangaferro et al., 2018). The mean milk BHB levels were well below the POS and SUSP ranges through the period from 5 to 80 DIM in NEG cows. The mean milk BHB levels during lactational period 1 (5–14 DIM) were within SUSP range in both EARLY_SUSP and EARLY_SUSP_pro groups of cows. The mean milk BHB levels during lactational period 2 (15–42 DIM) were below SUSP range in EARLY_SUSP cows, but continued to be within or above SUSP range in EARLY_SUSP_pro cows. The mean milk BHB levels during lactational period 1 were above SUSP range in both EARLY_POS and EARLY_POS_pro cows.

The mean milk BHB levels during lactational period 2 were below SUSP range in EARLY_POS cows, but continued to be within or above SUSP range for EARLY_POS_pro cows. The mean milk BHB levels during lactational period 1 were below SUSP range for both LATE_SUSP and LATE_POS groups of cows, whereas the milk BHB levels during lactational period 2 were within and above SUSP range in LATE_SUSP and LATE_POS cows, respectively. Most importantly, the levels of milk BHB in all EMB type cows were similar to NEG cows ($P > 0.05$) and below SUSP range during the period from the end of the ketosis-risk period to the median day of first service after calving (43–80 DIM).

4.4.1 Risk Factors for Developing EMB During Early Postpartum

We used a generalized logit model to relate explanatory variables with EMB during the risk period of ketosis. The explanatory factors of EMB included parity, energy-corrected previous 305-d milk yield, calving season, year of calving, and agricultural regions. Parity was a significant source of variation for risk with increasing parity being associated with an increased risk of developing EMB during the first 6 wk of lactation. However, parity one cows were 21% and 30% more likely to develop EMB types EARLY_SUSP and EARLY_POS, respectively, than parity 2 cows (Table 4.7.2). Milk yield in previous lactation was associated with EMB (Table 4.7.3). Cows with a medium level of 305-d milk yield in their previous lactation had 11% higher risk of EARLY_SUSP EMB than cows with high level of 305-d milk yield in their previous lactation. Cows with medium and high 305-d milk yield had 13% and 15% lower risk of developing EARLY_POS EMB, and 18% and 20% lower risk of LATE SUSP EMB, respectively than cows with a low level of 305-d milk yield in their previous lactation. However, previous lactation milk yield had no significant risks associated with EARLY_SUSP_Pro, EARLY_POS_Pro or LATE_POS EMB (Table 4.7.3). Season of calving was associated with risk of developing EMB,

with higher risk of EMB types observed when calving occurred in winter (Table 4.7.4). Other risk factors which were significantly associated with developing EMB in the model were year of calving ($P < 0.001$) and agricultural region ($P = 0.01$).

4.4.2 Days from Calving to First Service

The median days from calving to first service interval and the hazard ratio of first service after calving for different EMB categories and other covariate variables, obtained from the Kaplan-Meier survival and Cox proportional hazard ratio analyses, are presented in Figure 4.8.4 and Supplemental Figure S1 (https://doi.org/10.6084/m9.figshare.22718047.v1; Alemu et al., 2023). Compared with NEG cows with median interval of 73 d of calving to first service, the rate of first service was 7% lower chance ($P = 0.011$) and 2 d delay for cows classified as EARLY_POS, and with 12% of lower chance ($P = 0.005$) and 5 d delay for EARLY_POS_Pro cows for receiving their first service after calving. However, the rate of first service and the median calving to first service interval for cows in other EMB categories were not significantly different from that of NEG cows ($P > 0.05$). Considering the effect of the other determinant factors (Figure 4.8.4), the high previous 305-d milk yield cows had a lower rate of first service ($P = 0.004$), with a median calving to first service interval of 74 d, compared with low yield cows (median 73 d). Whereas there was no significant difference in the median calving to first service interval between low and medium yield cows ($P > 0.05$). Multiparous cows had lower rate of first service after calving ($P < 0.0001$) and longer calving to first service interval (73–76 d) as compared with the first parity cows (median 72 d). The median calving to first service interval was shorter with a higher rate of first service for cows calving in the summer (median of 73 d; $P = 0.0002$) and winter (median of 72 d; $P < 0.0001$) compared with cows calving in the fall (median of 74 d). Other determinant variables which were significantly associated with interval from calving to first service after calving in the

model were the year of calving (P = 0.009), agricultural region (P < 0.0001), and the random effect of herd (P < 0.0001).

4.4.3 Days from First Service to Conception

The median days of first service to conception interval and hazard ratio of conception in cows of different EMB categories and covariate variables are presented in Figure 4.8.5 and Supplemental Figure S2 (https://doi.org/10.6084/m9.figshare.22718047.v1; Alemu et al., 2023). Cows NEG for EMB had a median of 42 d interval from first service to conception, an equivalent of 2 estrous cycles. Compared with NEG cows, the rate of conception was 10% lower for EARLY_SUSP_Pro (P = 0.013) with a median of 55 d interval from first service to conception. EARLY_POS_pro cows had an 8% lower rate (P = 0.049) with a median of 48 d interval from first service to conception. LATE_POS cows had an 11% lower rate of conception (P = 0.011) with a median of 60-d interval from first service to conception. Additionally, EARLY_POS and LATE_SUSP cows had a modestly lower rate of conception (5%, P = 0.072 and 6%, P = 0.057, respectively) with median intervals of 55 and 51-d from first service to conception, respectively. Finally, there was no difference in rate of conception and the interval from first service to conception between NEG and EARLY_SUSP cows (P = 0.35). Considering the effect of the other determinant factors (Figure 4.8.5), cows with higher previous 305-d milk yield had lower rate of conception (P < 0.01) and longer median interval from first service to conception (44 d and 45 d for the medium and high milk yield, respectively) as compared with cows with low milk yield (median interval of 41 d). Parity had significant effect on rate of conception and first service to conception interval. Fourth and greater parity cows had 32% lower rate of conception (P < 0.0001) followed by third parity (24% lower; P < 0.0001) and second parity (18% lower; P < 0.0001) with longer median interval from first service to conception (73–76 d) compared with first parity cows

(72 d). The median interval from first service to conception was shorter and rate of conception was higher (P = 0.0003) for cows calving in the summer compared with cows calving in fall, with no difference between cows calving during the fall, spring and winter seasons. Other determinant variables which were significantly associated with interval from first service to conception in the model were the year of calving (P < 0.0001) and the random effect of herd (P < 0.0001), whereas agricultural region did not have a significant association (P > 0.05).

4.4.4 Days Open

The median interval of days open (calving to conception interval) and hazard ratio of conception for different EMB categories and other covariates are presented in Figure 4.8.6 and Supplemental Figure S3 (https://doi.org/10.6084/m9.figshare.22718047.v1; Alemu et al., 2023). Cows NEG for EMB had a median of 118 d days open. Rate of conception was lower for cows in all EMB categories (P < 0.01) except for EARLY_SUSP compared with NEG cows. The median interval for cows of all EMB categories, except for EARLY_SUSP, was 5 to 24 d longer than that of NEG cows. The rate of conception was lower with longer median days open for cows with medium (P = 0.004) and high (P < 0.0001) previous 305-d milk yield compared with low milk yield. Multiparous cows had longer days open and lower rate of conception than first parity cows (P < 0.0001). The rate of conception was lower for cows calving in the spring (P = 0.002) with longer median days, and higher for cows calving in the summer (P = 0.007) with shorter median days compared with cows which calved in the fall. Other determinant variables which were significantly associated with days open in the model were the year of calving (P < 0.0001), agricultural region (P = 0.016) and the random effect of herd (P < 0.0001).

4.4.5 Calving Interval

The median days of calving interval and hazard ratio of subsequent calving for different EMB categories and other covariates are presented in Figure 4.8.7 and Supplemental Figure S4 (https://doi.org/10.6084/m9.figshare.22718047.v1; Alemu et al., 2023). Cows NEG for EMB had a median of 397 d days of calving interval. Rate of subsequent calving was lower for cows in all EMB categories ($P < 0.01$) except EARLY_SUSP compared with NEG cows. The median calving interval for cows all EMB categories, except for EARLY_SUSP, was 7–24 d longer than that of NEG cows. The rate of subsequent calving was lower with longer median calving interval in cows with medium ($P = 0.005$) and high ($P < 0.0001$) level of previous 305-d milk yield compared with low level of milk yield (Figure 7). Multiparous cows had longer calving interval and lower rate of subsequent calving interval than first parity cows ($P < 0.001$; Figure 7). Rate of subsequent calving was lower for cows calving in spring ($P = 0.002$) with longer median calving interval, and higher for cows calving in the summer ($P = 0.006$) with shorter calving interval compared with cows calved in fall, whereas cows calving in the winter ($P = 0.85$) had no difference (Figure 7). Other determinant variables which were significantly associated with calving interval in the model were the year of calving ($P < 0.0001$), agricultural region ($P = 0.021$), and the random effect of herd ($P < 0.0001$).

4.5 Discussion

Although numerous studies have assessed the association between ketosis (clinical or subclinical) and various reproductive parameters (Walsh et al., 2007; Ospina et al., 2010; Raboisson et al., 2014; Rutherford et al., 2016), no study has examined the effect of ketosis subtypes – based on the timing and amplitude of hyperketonemia – on reproductive performance of lactating cows. Heterogeneity in the data analyzed by combining ketosis subtypes may be one of the causes of lack of association between hyperketonemia and reproductive parameters in some studies (Kessel et al., 2008; Chapinal et al., 2012; McArt et al., 2012). It is also possible that breeding and therapeutic strategies applied, if any, by the producer would have differed depending on whether ketosis is subclinical or clinical and lactation stage, thus masking the ketosis effect on reproductive performance. Therefore, we sought to examine the association between individual ketosis subtypes, using the milk BHB data as an indicator, and reproductive performance indicators in this study.

Although studies have posited that early and late ketosis occur during the first 2 wk and 3–6 wk of lactation, respectively (Grummer, 1993; Holtenius and Holtenius, 1996; Herdt, 2000) there are no large-scale data summarizing the BHB level differences among the ketosis subtypes. Our data provide the summary view of milk BHB profiles among different EMB groups of cows that milk BHB levels do rise to the levels attributable to subclinical and clinical ketosis during the first 6 wk of lactation. But most importantly, this allowed us to use test-day milk BHB data to examine the milk BHB profile beyond 42 d and up to 80 d of lactation. Our data demonstrate that even though there may be a small subset of cows that present higher milk BHB levels, it is safe to assume that BHB levels in milk stabilize to healthy levels (<0.15 mmol/L) beyond 6 wk of lactation

in most cows. This is supported by previous studies (Gillund et al., 2001; Mahrt et al., 2015) that showed that the occurrence of ketosis was distributed up to 42–50 d after calving.

In our study we found that the overall prevalence of EMB was 27.4% over 42 d with approximately 2-thirds of those cases categorized as early EMB occurring before 14 DIM. Among the EMB subtypes considered in this study, the highest prevalent subtype was EARLY_SUSP (10.49%). These observations are in line with other studies reporting the highest prevalence of EMB, using milk BHB data, to be in the first 2 wk of lactation and decreasing afterward (van der Drift et al., 2012; Koeck et al., 2014; Santschi et al., 2016). Our data further provide quantitative prevalence rates beyond 2 wk up to 6 wk of lactation, beyond which the prevalence of EMB is considerably low. It is important to note that at least a quarter of the cows with EMB in the first 2 wk (early) will continue to have EMB during the next few weeks.

Although, cows with increasing parity had overall higher odds of EMB, parity one cows were more likely to develop EARLY_SUSP and EARLY_POS EMB. In line with this, first parity cows were observe to have the highest prevalence of hyperketonemia in the first week of lactation (Santschi et al., 2016). This increased odds of EMB in primiparous cows can be attributed to multiple factors including energy requirement to support their own growth, late fetal development, mammary development and the stress linked to transition from heifer to cow status. Surprisingly, we found that increased previous 305-d milk yield was associated with lower odds of some, but not with other EMB types. A previous study found that cows with higher milk yield in previous lactation had increased milk BHB concentrations at the first test day (Viña et al., 2017). The apparent difference between this study (Viña et al., 2017) and our study could be due to the fact that we did not have data to include other risk factors for higher milk BHB in our analysis, such as body condition at calving and length of dry period. Also, the study by Viña et al. (2017)

considered the milk BHB on the first test-day that occurred between 5 and 35 DIM, whereas our study considered 2 test-day milk BHB values during 5–42 DIM to categorize cows into different types of EMB. In our study calving season showed an association with developing EMB types. Our observation that winter calving cows had higher risk of developing EMB compared with cows calving in fall, spring or summer, confirms the results from previous studies that the winter is a period of higher risk for ketosis (Berge and Vertenten, 2014; Vanholder et al., 2015).

The existing evidence on the effect of ketosis on reproductive parameters has been inconsistent. Some studies have shown that cows with subclinical ketosis had decreased conception rates (Wathes et al., 2003) and longer calving to first service interval and days open (Rutherford et al., 2016). Conversely, other studies have found no association between SCK and first service to pregnancy (Kessel et al., 2008; Chapinal et al., 2012; McArt et al., 2012), pregnancy rate at first insemination (Chapinal et al., 2012), and days open (Wathes et al., 2003; Ospina et al., 2010; Chapinal et al., 2012; McArt et al., 2012). The inconsistency could be explained by multiple factors such as: variation in the included covariates; different BHB concentration threshold to define ketosis; using milk or blood BHB data; and considering ketosis without subclassifications as early and late. In the present study, we used 2 test-day milk BHB data to classify cows into different EMB types and also carefully considered possible covariates, the information for which was available on the databases we used. Following systematic iterations to exclude the factors that did not have a significant effect on the reproductive parameters assessed, we included parity, previous milk yield, calving season, agriculture region of the herds and calendar year in the final model assessing the association between EMB subtypes and reproductive parameters. Inclusion of the other determinant variable allowed us to evaluate the effect of EMB types on reproductive

performance over and above the effects of other variables such as parity, previous 305-d milk yield and calving season.

The calving to first service interval was longer with lower rate of first service for cows with EARLY_POS and EARLY_POS_Pro EMB, but not other EMB types. Assuming ketosis signs and comorbidities (e.g., abomasal displacements and metritis) in these 2 categories, the delayed first service could be a reflection of problems with reproductive organs and the proactive producer's decision including delaying breeding and using assisted reproductive technologies. Unfortunately, it was not possible to specify which was the reason for the delayed first service with the data available for analysis in this study. The calving to first service interval and rate of first service were not affected in cows with EARLY_SUSP and EARLY_SUSP_Pro EMB. This is in contrast with a previous study showing 15% lower rate of first service with a median 8-d delay in cows with SCK during the first or second week of lactation (Walsh et al., 2007). However, the same study (Walsh et al., 2007) found that the cows with SCK diagnoses in both of the first 2 wk had similar odds of first insemination compared with non-SCK cows. In addition, this study (Walsh et al., 2007) included all cows ≥1.0 mmol/L during wk 1 and cows ≥1.4 mmol/L during wk 2 after calving, whereas our study EARLY_SUSP and EARLY_SUSP_Pro EMB cows had 0.15–0.19 mmol/L of milk BHB (which corresponds to 1.2–2.9 mmol/L of serum BHB). These differences between our studies may explain the different observations. Also, it is intriguing that cows in LATE_SUSP and LATE_POS EMB categories did not have delayed first service.

The interval from the first insemination to conception is an important indicator of reproductive efficiency in dairy cows. It is the interval between a producer's decision to breed and conception for each cow, and a delay in this interval can be attributed to the prolonged effects of metabolic perturbations during the early stages of lactation. Compared with NEG cows, the first

service to conception interval was 3 to 18 d longer in EMB cows, except for those in EARLY_SUSP EMB group. An increase in the first service to conception interval by 3 d would mean that 1 in 7 cows would have taken one additional insemination and an additional estrous cycle of 21 d in length. Considering early EMB events occurring in the first 2 wk, our observations are in line with a previous study that the rate of pregnancy after first service was approximately 50% lower in cows classified as SCK in the first 2 wk after calving (Walsh et al., 2007). Further, our observation of longer first service to conception interval in both prolonged EMB (EARLY_SUSP_Pro and EARLY_POS_Pro) cows was 6–13 d longer than NEG cows, it is apparent that prolonged elevation of EMB is more deleterious to reproductive performance of lactating cows. Another study found that the first service to conception interval was not different between ketosis and negative groups (Kessel et al., 2008), although this study had a lower threshold of 1 mM BHB during a much wider period of observation of 14 wk after calving. The most important finding of our study is that the LATE_POS cows had the longest first service to conception interval. Although emphasis has been on ketosis during the first 2 wk of lactation (early) because of its high prevalence, our data demonstrate that late ketosis is equally detrimental to reproductive processes in lactating cows.

Days open is another important indicator of reproductive performance as this parameter is a combination of the calving to first service and first service to conception intervals. Compared with NEG cows, the median days open was 5 to 24 d longer in all EMB cows except for those in EARLY_SUSP EMB category. Our finding that EARLY_SUSP cows were not affected is in contrast to previous studies. Cows with elevated serum BHB in the first 2 wk of lactation had decreased risk of pregnancy after 70 d (Ospina et al., 2010), although this study included all cows with BHB ≥1.0 mmol/L potentially combining both CK and SCK cows. Cows with elevated serum

BHB (≥1.2 mmol/L) in the first week of lactation had 30% reduction in pregnancy by 150 DIM, whereas cows with elevated serum BHB in the second week did not have reduction in pregnancy compared with nonhyperketonemia cows (Rodriguez et al., 2022), although this study also potentially combined CK and SCK cows. It should be pointed out that it is not possible to rule out such mixing, although minimal, of CK and SCK cows in our study given this study is based on test-day milk samples. Another study found that among cows diagnosed as SCK during the first 2 wk of lactation, the rate of pregnancy was lower until 140 d into lactation, but not thereafter in SCK compared with non-SCK cows (Walsh et al., 2007). It should be noted that we included cows with up to 600-d calving interval indicating that cows with the potential conception day up to 320 d of lactation were included in our analysis. Finally, calving interval was also significantly longer for all EMB categories except for EARLY_SUSP. As calving interval is the sum of days open and gestation length, it was also prolonged by 4 to 24 d in affected EMB categories compared with NEG cows, with LATE_POS cows having the longest median calving interval.

In line with the literature (Gröhn and Rajala-Schultz, 2000; Grimard et al., 2006), cows of second parity or greater had significantly lower hazard ratio of calving to first service, conception to first service, days open and calving interval than cows of first parity. Our finding that all reproductive parameters analyzed were negatively affected by the level of the previous 305-d milk production concurs with a previous study showing high producing cows required more services and longer time to pregnancy than low producing cows (Berger et al., 1981). The observation that cows that calved during summer had shorter first service to conception, days open and calving intervals compared with cows calving in fall was unexpected and in contrast to a previous report that summer calving cows had lower rate of pregnancy than fall calving cows (Farin et al., 1994).

The reproductive parameters analyzed in this data set were also influenced by the agriculture regions, which is attributable to changing feed quality and climate year-to-year in different regions.

The readers are cautioned that the factors that could affect reproductive performance, such as body condition score, peripartum morbidities, hormone levels, could not be included in the statistical model due to unavailability of such information. Given the expected norm of 25–30 d gap between test-day milk collection, there were fewer cows with test-day collection during wk 3 and 4. Because they represented field conditions of variable frequency of test-day collections by the producers and exclusion of such cows did not influence our statistical inferences, those cows were retained in the analyses presented in this study. More specific categorization of EMB, and meticulous inclusion and exclusion of data from more than 30-thousand cows have enabled us to provide compelling evidence that EMB occurring at different stages of early lactation can be deleterious to reproductive performance of dairy cows. Late ketosis occurring between wk 3 and 6 of lactation appears to have the strongest adverse effects on the fertility of lactating dairy cows as LATE_POS cows had the longest first service to conception interval, days open and calving interval. Separating EARY_SUS EMB from other categories revealed that it may not have a drastic negative effect on cow fertility as reproductive parameters of first service to conception interval, days open and calving interval were not significantly different between NEG and EARLY_SUSP cows. Overall, the results of this study show that dairy producers should pay attention to EMB during the transition period to avoid its negative effect on reproductive performance of their cows.

4.6 Acknowledgements

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4.7 Tables

Table 4.7.1 Classification of elevated milk BHB (EMB) types based on test-day milk BHB concentrations during period 1 (5 to 14 DIM) and period 2 (15 to 42 DIM)[1]

BHB mmol/L (5 to 14 DIM)	BHB mmol/L (15 to 42 DIM)	EMB[2]
< 0.15	< 0.15	NEG
0.15 to 0.19	< 0.15	EARLY_SUSP
0.15 to 0.19	≥ 0.15	EARLY_SUSP_Pro
≥ 0.20	< 0.15	EARLY_POS
≥ 0.20	≥ 0.15	EARLY_POS_Pro
< 0.15	0.15 to 0.19	LATE_SUSP
< 0.15	≥ 0.20	LATE_ POS

[1] Milk BHB concentrations were estimated from Fourier-transform infrared analyses of milk samples collected on test days.

[2] NEG = negative; EARLY_SUSP = early suspect; EARLY_SUSP_Pro = prolonged early suspect; EARLY_POS = early positive; EARLY_POS_Pro = prolonged early positive; LATE_SUSP = late suspect; LATE_POS = late positive.

Table 4.7.2 Odds ratios (OR) and 95% CI for association of parity with development of elevated milk BHB (EMB) during early-lactation cows within 42 DIM[1]

			95% CI		
EMB type[2]	Parity[3]	OR	Lower	Upper	*P-value*
EARLY_SUSP	2 vs. 1	0.79	0.71	0.87	< 0.0001
EARLY_SUSP	2 vs. 3	0.76	0.68	0.85	< 0.0001
EARLY SUSP	2 vs. 4	0.65	0.59	0.73	< 0.0001
EARLY_SUSP	3 vs. 1	1.04	0.93	1.15	0.51
EARLY_SUSP	3 vs. 4	0.86	0.77	0.97	0.01
EARLY_SUSP	4 vs. 1	1.20	1.08	1.34	0.0007
EARLY_SUSP_Pro	2 vs. 1	0.87	0.71	1.07	0.18
EARLY_SUSP_Pro	2 vs. 3	0.44	0.36	0.54	< 0.0001
EARLY_SUSP_Pro	2 vs. 4	0.39	0.32	0.48	< 0.0001
EARLY_SUSP_Pro	3 vs. 1	2.00	1.62	2.37	< 0.0001
EARLY_SUSP_Pro	3 vs. 4	0.89	0.74	1.07	0.22
EARLY_SUSP_Pro	4 vs. 1	2.20	1.81	2.68	< 0.0001
EARLY_POS	2 vs. 1	0.70	0.61	0.80	< 0.0001
EARLY_POS	2 vs. 3	0.60	0.52	0.70	< 0.0001

EARLY_POS	2 vs. 4	0.50	0.43	0.59	< 0.0001
EARLY_POS	3 vs. 1	1.15	1.00	1.32	0.04
EARLY_POS	3 vs. 4	0.84	0.72	0.97	0.02
EARLY_POS	4 vs. 1	1.38	1.20	1.58	< 0.0001
EARLY_POS_Pro	2 vs. 1	0.91	0.72	1.15	0.42
EARLY_POS_Pro	2 vs. 3	0.40	0.32	0.50	< 0.0001
EARLY_POS_Pro	2 vs. 4	0.39	0.30	0.49	< 0.0001
EARLY_POS_Pro	3 vs. 1	2.23	1.85	2.81	< 0.0001
EARLY_POS_Pro	3 vs. 4	0.97	0.79	1.19	0.74
EARLY_POS_Pro	4 vs. 1	2.35	1.90	2.92	< 0.0001
LATE_SUSP	2 vs. 1	1.44	1.23	1.70	< 0.0001
LATE_SUSP	2 vs. 3	0.77	0.66	0.90	0.001
LATE_SUSP	2 vs. 4	0.60	0.51	0.70	< 0.0001
LATE_SUSP	3 vs. 1	1.89	1.58	2.22	< 0.0001
LATE_SUSP	3 vs. 4	0.78	0.66	0.92	0.003
LATE_SUSP	4 vs. 1	2.40	2.03	2.84	< 0.0001
LATE_POS	2 vs. 1	1.46	1.16	1.84	0.001
LATE_POS	2 vs. 3	0.53	0.43	0.65	< 0.0001

LATE_POS	2 vs. 4	0.42	0.34	0.52	< 0.0001
LATE_POS	3 vs. 1	2.75	2.19	3.45	< 0.0001
LATE_POS	3 vs. 4	0.79	0.65	0.97	0.02
LATE_POS	4 vs. 1	3.47	2.77	4.35	< 0.0001

[1] Different EMB types were based on test-day milk BHB concentrations during period 1 (5 to 14 DIM) and period 2 (15 to 42 DIM).

[2] EARLY_SUSP = early suspect; EARLY_SUSP_Pro = prolonged early suspect; EARLY_POS = early positive; EARLY_POS_Pro = prolonged early positive; LATE_SUSP = late suspect; LATE_POS = late positive.

[3] 4 = ≥4.

Table 4.7.3 Odds ratios (OR) and 95% CI for association of previous 305-d milk yield with development of elevated milk BHB (EMB) in early-lactation cows within 42 DIM[1]

EMB type[2]	Previous 305 d milk yield[3]	OR	95% CI		*P-value*
			Lower	Upper	
EARLY_SUSP	2 vs. 1	1.01	0.92	1.11	0.83
EARLY_SUSP	2 vs. 3	1.11	1.01	1.22	0.03
EARLY_SUSP	3 vs. 1	0.91	0.81	1.02	0.11
EARLY_SUSP_Pro	2 vs. 1	1.08	0.90	1.30	0.38
EARLY_SUSP_Pro	2 vs. 3	1.08	0.91	1.29	0.35
EARLY_SUSP_Pro	3 vs. 1	1.00	0.81	1.23	0.99
EARLY_POS	2 vs. 1	0.87	0.76	0.98	0.02
EARLY_POS	2 vs. 3	1.02	0.90	1.15	0.79
EARLY_POS	3 vs. 1	0.85	0.74	0.99	0.03
EARLY_POS_Pro	2 vs. 1	0.96	0.79	1.16	0.66
EARLY_POS_Pro	2 vs. 3	1.00	0.83	1.20	0.98
EARLY_POS_Pro	3 vs. 1	0.96	0.77	1.21	0.67
LATE_SUSP	2 vs. 1	0.82	0.72	0.95	0.008
LATE_SUSP	2 vs. 3	1.03	0.89	1.19	0.68

LATE_SUSP	3 vs. 1	0.80	0.68	0.95	0.01
LATE_POS	2 vs. 1	1.06	0.88	1.29	0.53
LATE_POS	2 vs. 3	1.13	0.94	1.36	0.19
LATE_POS	3 vs. 1	0.94	0.75	1.19	0.61

[1] Different EMB types were based on test-day milk BHB concentrations during period 1 (5 to 14 DIM) and period 2 (15 to 42 DIM).

[2] EARLY_SUSP = early suspect; EARLY_SUSP_Pro = prolonged early suspect; EARLY_POS = early positive; EARLY_POS_Pro = prolonged early positive; LATE_SUSP = late suspect; LATE_POS = late positive.

[3] 1 = low previous 305-d milk yield; 2 = medium previous 305-d milk yield; 3 = high previous 305-d milk yield.

Table 4.7.4 Odds ratios (OR) and 95% CI for association of calving season with development of elevated milk BHB (EMB) in early-lactation cows within 42 DIM[1]

EMB type[2]	Calving season[3]	OR	95% CI		*P-value*
			Lower	Upper	
EARLY_SUSP	Spring vs. summer	0.95	0.85	1.07	0.41
EARLY_SUSP	Spring vs. winter	0.80	0.71	0.90	< 0.001
EARLY_SUSP	Spring vs. fall	0.99	0.89	1.11	0.90
EARLY_SUSP	Summer vs. winter	0.84	0.75	0.94	0.002
EARLY_SUSP	Summer vs. fall	1.04	0.95	1.14	0.41
EARLY_SUSP	Winter vs. fall	1.24	1.11	1.38	< 0.001
EARLY_SUSP_Pro	Spring vs. summer	1.12	0.90	1.39	0.31
EARLY_SUSP_Pro	Spring vs. winter	0.61	0.49	0.76	< 0.0001
EARLY_SUSP_Pro	Spring vs. fall	0.87	0.71	1.06	0.16
EARLY_SUSP_Pro	Summer vs. winter	0.54	0.44	0.67	< 0.0001
EARLY_SUSP_Pro	Summer vs. fall	0.75	0.63	0.90	0.007
EARLY_SUSP_Pro	Winter vs. fall	1.36	1.14	1.63	< 0.001
EARLY_POS	Spring vs. summer	1.05	0.91	1.22	0.47
EARLY_POS	Spring vs. winter	0.89	0.76	1.04	0.15
EARLY_POS	Spring vs. fall	1.13	0.98	1.30	0.09
EARLY_POS	Summer vs. winter	0.85	0.73	0.98	0.03
EARLY_POS	Summer vs. fall	1.07	0.94	1.22	0.30
EARLY_POS	Winter vs. fall	1.27	1.09	1.45	0.001

EARLY_POS_Pro	Spring vs. summer	1.06	0.85	1.33	0.60
EARLY_POS_Pro	Spring vs. winter	0.73	0.58	0.92	0.007
EARLY_POS_Pro	Spring vs. fall	1.07	0.86	1.33	0.55
EARLY_POS_Pro	Summer vs. winter	0.68	0.55	0.85	< 0.001
EARLY_POS_Pro	Summer vs. fall	1.00	0.82	1.23	0.96
EARLY_POS_Pro	Winter vs. fall	1.47	1.19	1.81	< 0.001
LATE_SUSP	Spring vs. summer	1.15	0.96	1.36	0.12
LATE_SUSP	Spring vs. winter	0.77	0.65	0.93	0.006
LATE_SUSP	Spring vs. fall	0.93	0.79	1.09	0.38
LATE_SUSP	Summer vs. winter	0.67	0.57	0.80	< 0.0001
LATE_SUSP	Summer vs. fall	0.81	0.70	0.94	0.006
LATE_SUSP	Winter vs. fall	1.20	1.02	1.41	0.03
LATE_POS	Spring vs. summer	1.67	1.32	2.10	< 0.0001
LATE_POS	Spring vs. winter	0.89	0.71	1.12	0.33
LATE_POS	Spring vs. fall	1.04	0.85	1.27	0.71
LATE_POS	Summer vs. winter	0.53	0.42	0.68	< 0.0001
LATE_POS	Summer vs. fall	0.62	0.50	0.77	< 0.0001
LATE_POS	Winter vs. fall	1.16	0.94	1.43	0.15

[1] Different EMB types were based on test-day milk BHB concentrations during period 1 (5 to 14 DIM) and period 2 (15 to 42 DIM).

[2] EARLY_SUSP = early suspect; EARLY_SUSP_Pro = prolonged early suspect; EARLY_POS = early positive; EARLY_POS_Pro = prolonged early positive; LATE_SUSP = late suspect; LATE_POS = late positive.

[3] Fall = September to November; winter = December to February; spring = March to May; summer = June to August.

4.8 Figures

Figure 4.8.1 (A) Flowchart showing data cleaning and preparation for final analysis. (B) Distribution of cows with elevated milk BHB (EMB) level in early lactation: period 1 (5–14 DIM) or period 2 (15–42 DIM).

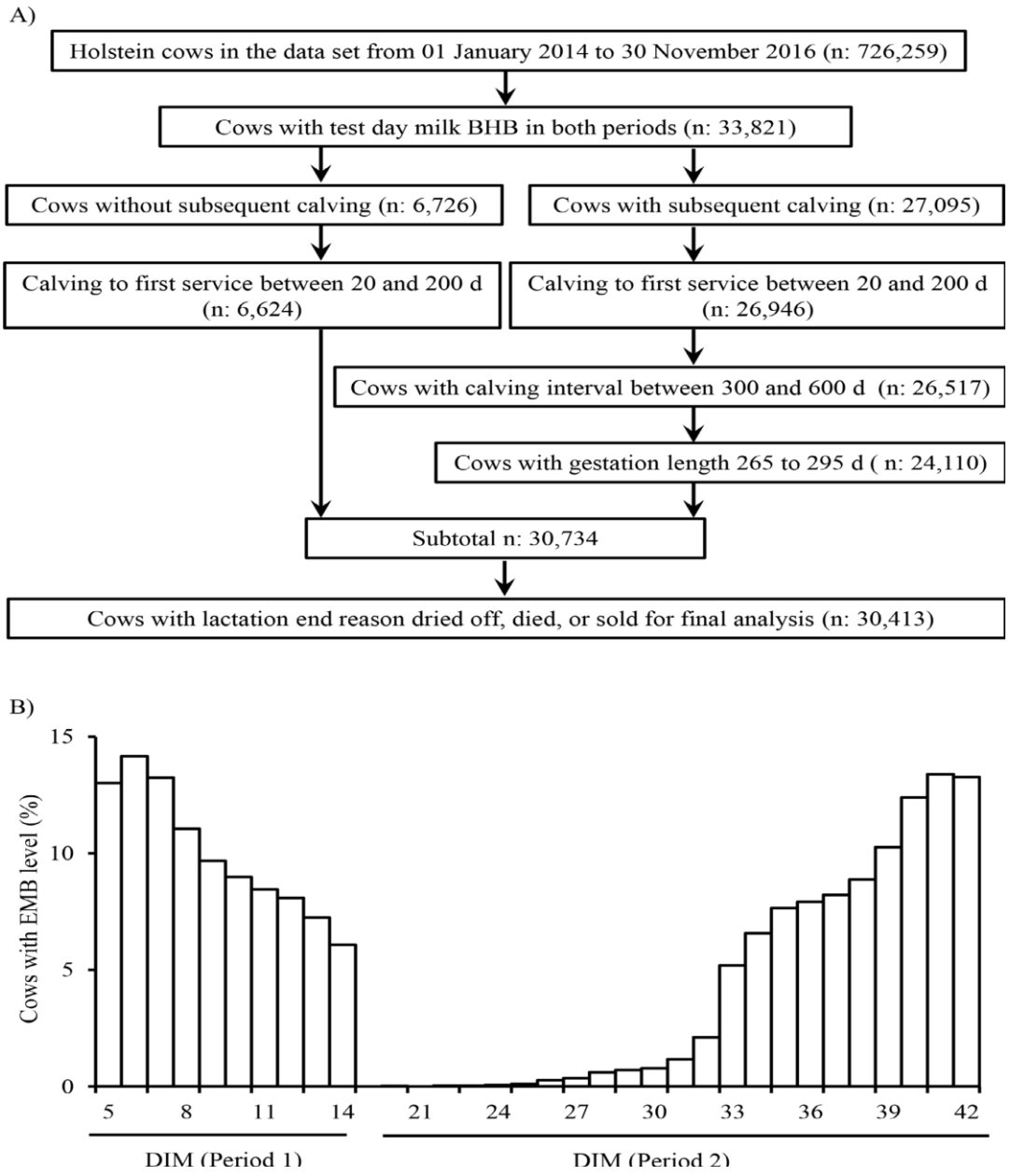


Figure 4.8.2 Prevalence (%) of elevated milk BHB (EMB) types in lactating cows. Different EMB types were based on test-day milk BHB concentrations during lactation period 1 (5 to 14 DIM) and period 2 (15 to 42 DIM). Milk BHB concentrations were estimated from Fourier-transform infrared analyses of milk samples collected on test days. EARLY_SUSP = early suspect; EARLY_SUSP_Pro = prolonged early suspect; EARLY_POS = early positive; EARLY_POS_Pro = prolonged early positive; LATE_SUSP = late suspect; LATE_POS = late positive.

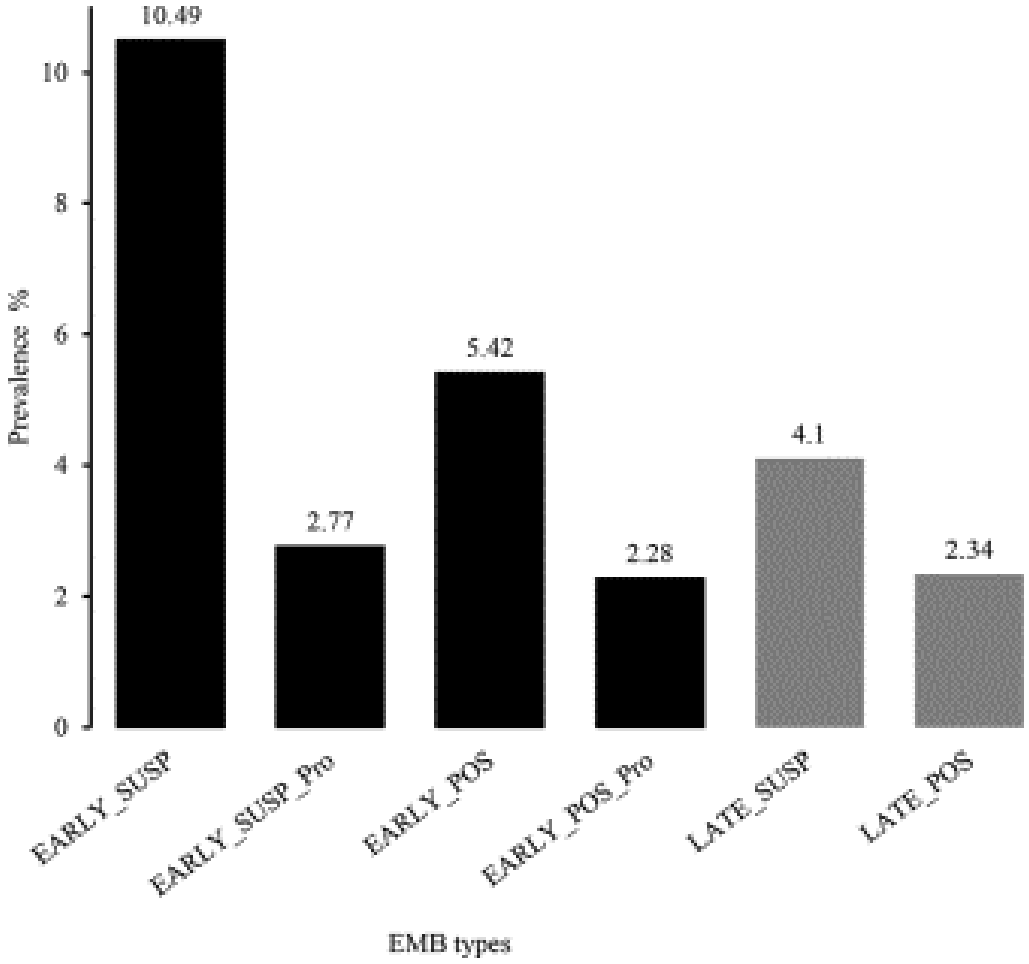


Figure 4.8.3 Daily mean milk BHB (mmol/L) of different elevated milk BHB (EMB) types in early-lactation cows. Different EMB types were based on test-day milk BHB concentrations during the period 1–80 DIM, including lactation period 1 (5 to 14 DIM) and period 2 (15 to 42 DIM). Milk BHB concentrations were estimated from Fourier-transform infrared analyses of milk samples collected on test days. EARLY_SUSP = early suspect; EARLY_SUSP_Pro = prolonged early suspect; EARLY_POS = early positive; EARLY_POS_Pro = prolonged early positive; LATE_SUSP = late suspect; LATE_POS = late positive. Data are presented as mean ± SEM.

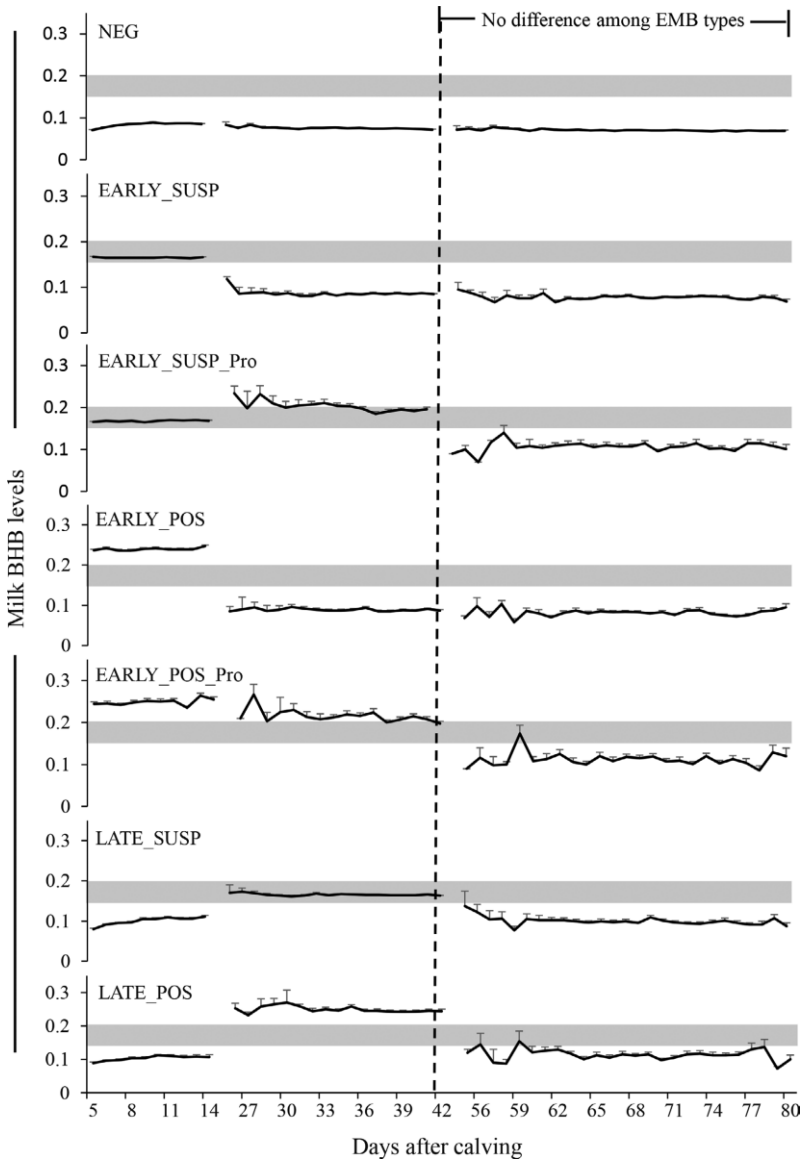


Figure 4.8.4 Estimated hazard ratio and median survival days of different elevated milk BHB (EMB) types, previous 305-d milk yield, parity, and calving season associated with days from calving to first service. Different EMB types were based on test-day milk BHB concentrations during lactation period 1 (5 to 14 DIM) and period 2 (15 to 42 DIM). Milk BHB concentrations were estimated from Fourier-transform infrared analyses of milk samples collected on test days. EARLY_SUSP = early suspect; EARLY_SUSP_Pro = prolonged early suspect; EARLY_POS = early positive; EARLY_POS_Pro = prolonged early positive; LATE_SUSP = late suspect; LATE_POS = late positive.

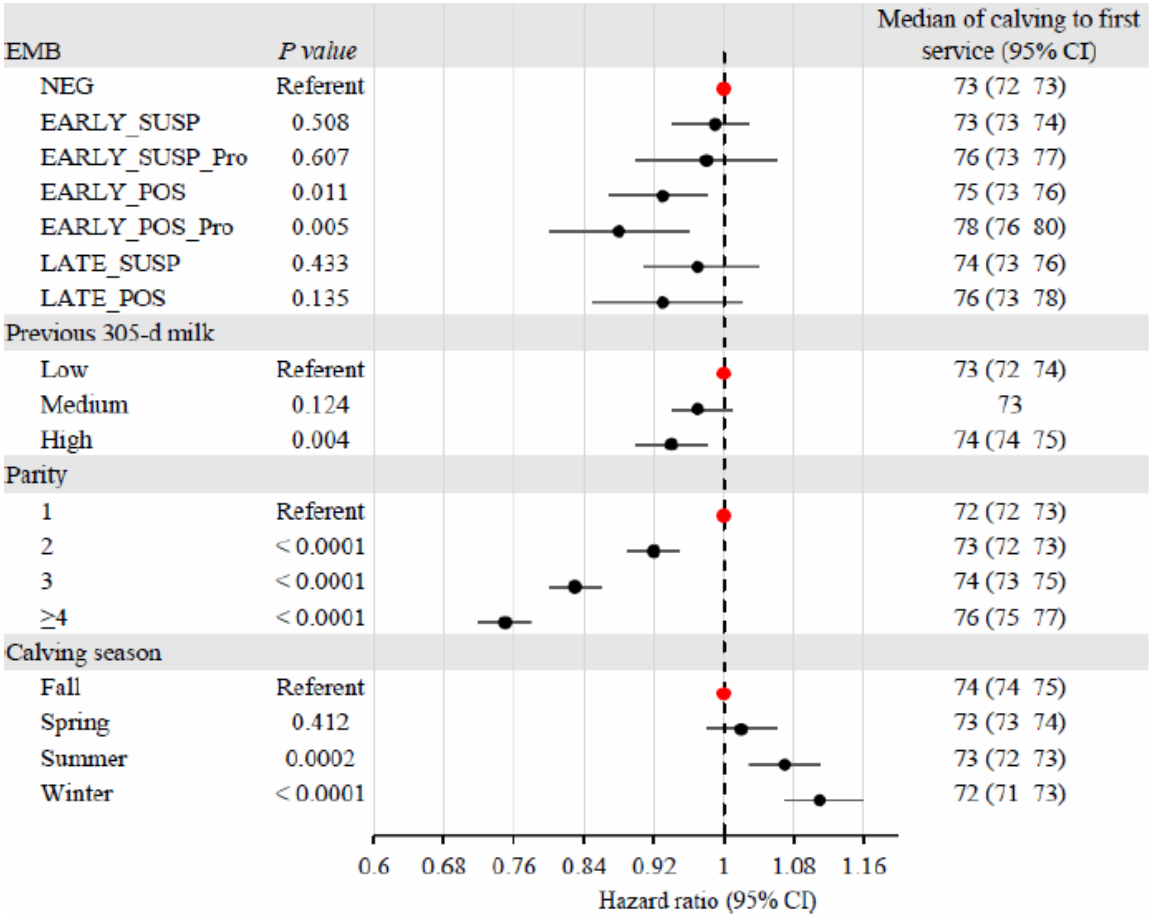


Figure 4.8.5 Estimated hazard ratio and median survival days of different elevated milk BHB (EMB) types, previous 305-d milk yield, parity, and calving season associated with days from first service to conception. Different EMB types were based on test-day milk BHB concentrations during lactation period 1 (5 to 14 DIM) and period 2 (15 to 42 DIM). Milk BHB concentrations were estimated from Fourier-transform infrared analyses of milk samples collected on test days. EARLY_SUSP = early suspect; EARLY_SUSP_Pro = prolonged early suspect; EARLY_POS = early positive; EARLY_POS_Pro = prolonged early positive; LATE_SUSP = late suspect; LATE_POS = late positive.

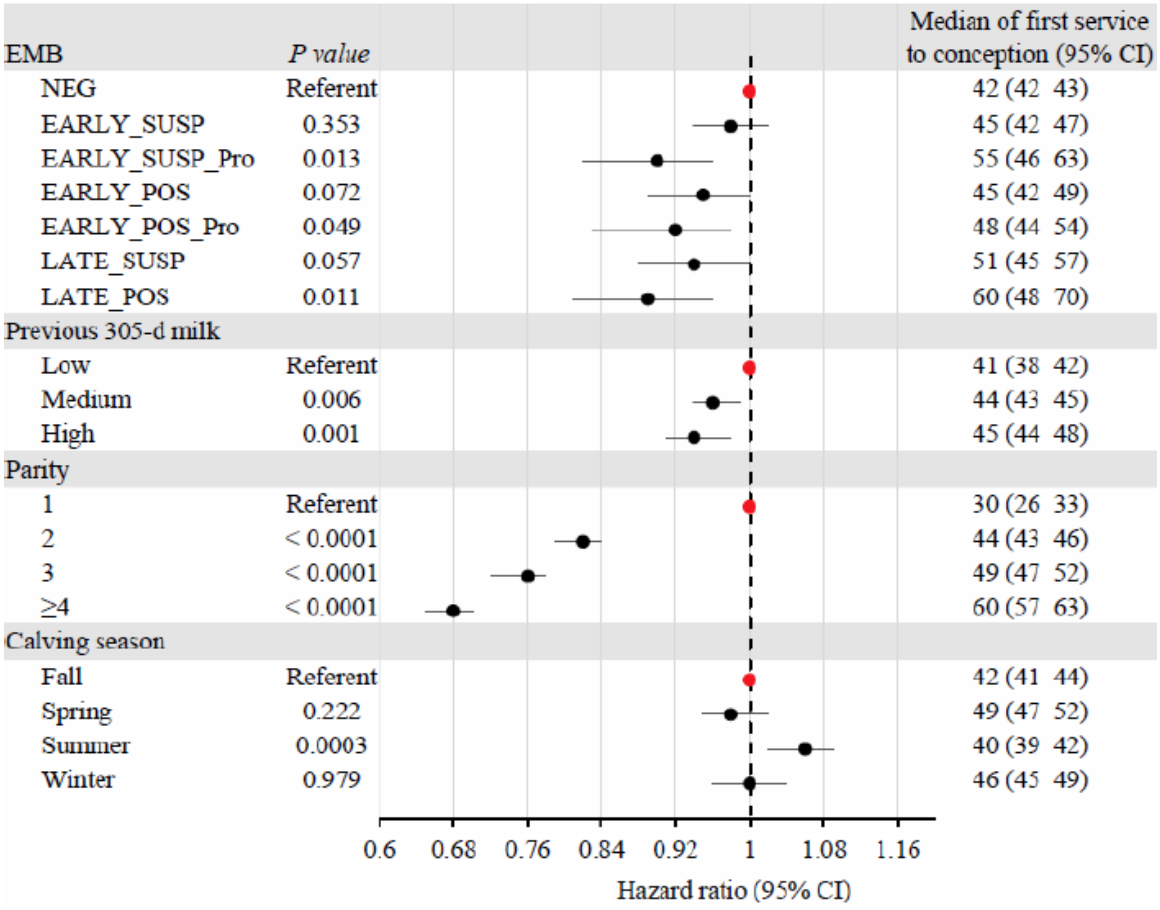


Figure 4.8.6 Estimated hazard ratio and median survival days of different elevated milk BHB (EMB) types, previous 305-d milk yield, parity, and calving season associated with days open (days from calving to conception). Different EMB types were based on test-day milk BHB concentrations during lactation period 1 (5 to 14 DIM) and period 2 (15 to 42 DIM). Milk BHB concentrations were estimated from Fourier-transform infrared analyses of milk samples collected on test days. EARLY_SUSP = early suspect; EARLY_SUSP_Pro = prolonged early suspect; EARLY_POS = early positive; EARLY_POS_Pro = prolonged early positive; LATE_SUSP = late suspect; LATE_POS = late positive.

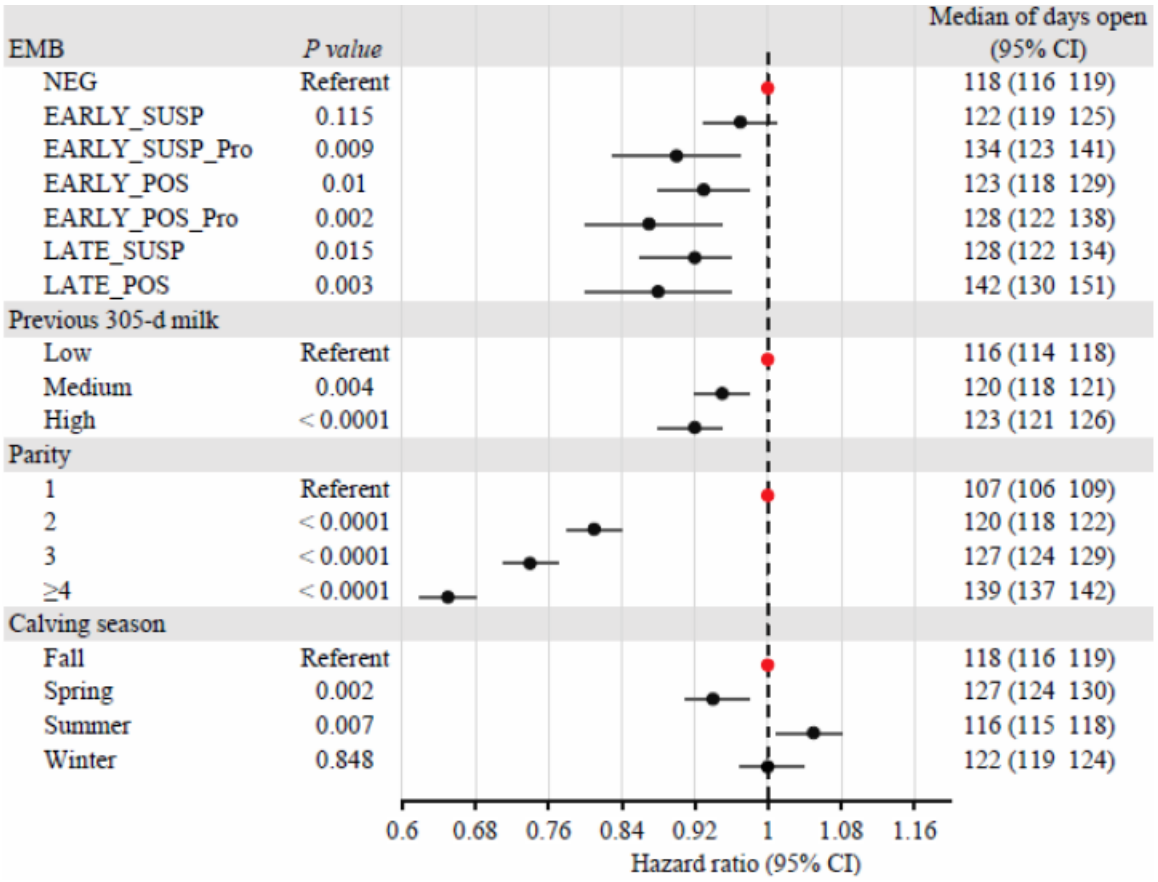
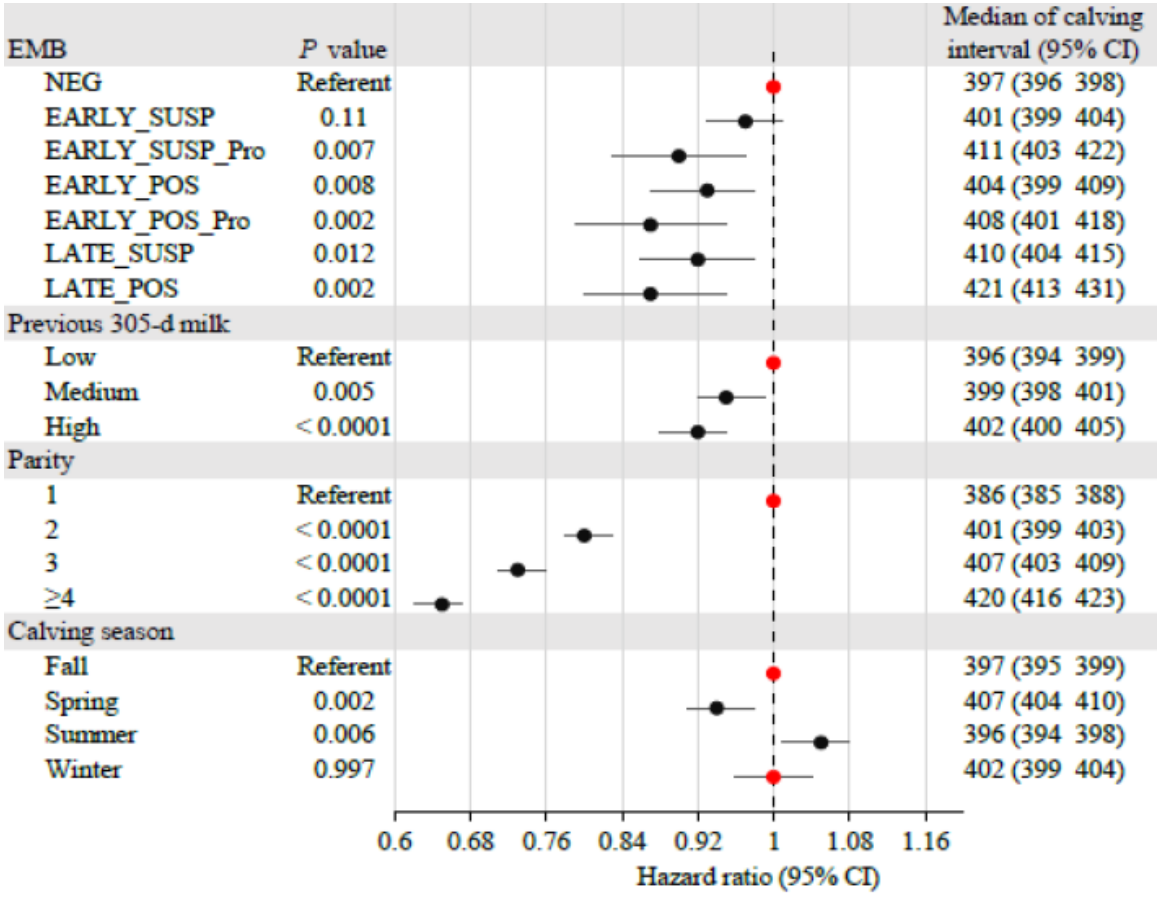


Figure 4.8.7 Estimated hazard ratio and median survival days of different elevated milk BHB (EMB) types, previous 305-d milk yield, parity, and calving season associated with calving interval. Different EMB types were based on test-day milk BHB concentrations during lactation period 1 (5 to 14 DIM) and period 2 (15 to 42 DIM). Milk BHB concentrations were estimated from Fourier-transform infrared analyses of milk samples collected on test days. EARLY_SUSP = early suspect; EARLY_SUSP_Pro = prolonged early suspect; EARLY_POS = early positive; EARLY_POS_Pro = prolonged early positive; LATE_SUSP = late suspect; LATE_POS = late positive.



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CONNECTING STATEMENT 2

In the second study, we evaluated the association between time and amplitude of elevated milk BHB occurring within 42 d DIM and subsequent reproductive performance of lactating Holstein cows. Our findings show that elevated milk BHB levels, indicative of ketosis, during the first 42 days of lactation had a negative effect on the reproductive performance of dairy cows following a voluntary waiting period. Although this and other studies have shown that ketosis has deleterious effects on the overall reproductive performance of lactating cows, the mechanisms are not clear. Therefore, in the third study, we investigated the association between ketosis, diagnosed by milk BHB concentrations during 3-14 days after calving, and ovarian activity, measured by milk P4 profile within 150 days of lactation in Holstein cows.

CHAPTER 5

Manuscript has been submitted to "Animal".

Adverse association between ketosis and ovarian activity in Canadian dairy cows

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5.1 Highlights

- Milk BHB data were used to stratify dairy cows into three ketosis status groups.
- First luteal activity after calving was delayed in ketosis cows.
- First heat after calving was delayed in ketosis cows.
- Probability of prolonged anestrus alarm was higher in ketosis cows.
- Progesterone concentration during first luteal phase was lower in ketosis cows.

5.2 Abstract

Ketosis is a major metabolic disease characterized by high circulating concentrations of ketone bodies including β-hydroxybutyrate (**BHB**) in dairy cows. Although we and others have demonstrated the negative relationship between ketosis and reproductive performance of lactating cows, the mechanisms are not yet clear. We investigated the association between ketosis, determined by milk BHB concentrations during 3-14 days in milk (**DIM**), and ovarian activity, measured by milk progesterone (**P4**) profiles within 150 DIM in Holstein cows. Milk BHB and P4 records were obtained from 7 641 lactations of 4 359 unique cows from thirteen Canadian herds. Cows were categorized into healthy (Healthy; milk BHB < 0.08 mmol/L), subclinical (**SCK**; milk BHB = 0.08–0.12 mmol/L) or clinical ketosis (**CK**; milk BHB ≥ 0.13 mmol/L) groups. The overall prevalence of ketosis during 3-14 DIM was 39.4%, with 25% SCK and 14.4% CK cows. The median intervals from calving to first luteal activity (milk P4 ≥ 5 ng/mL) and calving to first heat were longer in SCK and CK cows than Healthy cows. The probability of a postpartum anestrus alarm was 50% (P <0.0001) and 110% (P<0.0001) higher in cows with SCK and CK cows, respectively, than Healthy cows. During the first luteal phase after calving, the mean daily milk P4 concentrations were markedly lower (P < 0.05) on days 0 to 3 in SCK cows and on days 0 to 5 in CK cows, respectively, compared to Healthy cows (d0 = beginning of luteal activity). These

data demonstrate that ketosis during early lactation has adverse impact on resumption of ovarian activity and luteal function of lactating cows. Therefore, frequent monitoring of milk BHB is recommended to catch and remediate cows with ketosis to manage their reproductive performance.

Key words: BHB, corpus luteum, lactation, milk, progesterone.

5.3 Implications

The mechanisms of negative impact of ketosis on reproductive processes in dairy cows are not clear. In this study we examined the ovarian activity based on milk progesterone profiles in cows stratified by ketosis groups based on milk β-hydroxybutyrate levels. Ketosis cows had delayed first luteal activity and heat, more likely to have postpartum anestrus alarm and had lower progesterone concentrations during their first luteal phase compared to healthy cows. Overall, these data demonstrate that ketosis during early lactation has adverse impact on resumption of ovarian activity and luteal function in lactating dairy cows.

5.4 Introduction

Transitioning from a non-lactating to a lactating state is associated with dramatic metabolic adjustments with major impact on health and reproductive performance dairy cows (Staples et al., 1990; Drackley, 1999). After calving, energy demand for milk production along with decreased feed intake puts cows into a negative energy balance (**NEB**), which leads to changes in metabolic hormones and processes. The NEB is met with lipid mobilization from the adipose tissue as non-esterified fatty acids (**NEFAs**), which are used for milk fat synthesis and as energy source for body. With glucose diverted towards lactose synthesis, the liver uses NEFA to synthesize ketone bodies as alternative energy for other tissues. Hyperketonemia is a normal metabolic response to actual and apparent reduction in glucose availability. However, a rapid and excessive increase in circulating concentrations of ketone bodies such as β-hydroxybutyrate (**BHB**) leads to ketosis. Ketosis is the most common metabolic disease of dairy cows with highest prevalence during the first two weeks of lactation (Duffield et al., 1998; Walsh et al., 2007; McArt et al., 2011; Santschi et al., 2016; Alemu et al., 2023). Ketosis is categorized, depending on the presence or absence of clinical signs, as clinical (**CK**) and subclinical ketosis (**SCK**). Depending on the time of occurrence

in relation to calving, ketosis can further be classified as early (occurring during the period from 3 to 14 days in milk (**DIM**)) and late (during the period of 15-42 DIM) ketosis (Alemu et al., 2023).

The most widely used threshold of blood BHB concentrations to diagnose SCK and CK are 1.2–2.9 mmol/L and ≥3.0 mmol/L, respectively (McArt et al., 2011; Borchardt and Staufenbiel, 2012; Suthar et al., 2013). However, milk BHB measurements are also used by researchers and dairy advisors to determine ketosis in lactating cows (Denis-Robichaud et al., 2014; Koeck et al., 2014). Milk BHB measured by the Fourier-transformed infrared (**FTIR**) technology has been used to study the impact of SCK (0.15–0.19 mmol/L) and CK (≥0.20 mmol/L) on production and fertility in lactating cows (Santschi et al., 2016; Benedet et al., 2019; Alemu et al., 2023). Fluorometric analysis is another method of milk BHB measurement, which has been shown to have strong correlation with the traditional spectrophotometric method of blood BHB analysis (Larsen and Nielsen, 2005). The in-line analyzer, Herd Navigator (HN; Lattec I/S, Hillerød, Denmark) of the voluntary milking system (DeLaval Inc., Tumba, Sweden) uses this method to measure milk BHB. The HN dairy management tool uses milk BHB concentration thresholds of 0.08-0.12 mmol/L and ≥ 0.13 mmol/L to generate SCK and CK alarms, respectively (De Jong et al., 2023).

The adverse impact of ketosis occurring during the first two weeks of lactation on the subsequent reproductive performance of cows has been reported (Rutherford et al., 2016; Alemu et al., 2023). These studies were used classical fertility parameters like calving to first service, number of services per conception and first service to conception, which do not provide an insight into the underlying mechanism. Milk progesterone (**P4**) concentration profiles (Bruinjé et al., 2017), however, provide endocrine basis for the corpus luteum function directly as well as follicular development and overall ovarian activity indirectly. As NEB and adverse health events

during early lactation were associated with delayed resumption of ovarian activity (Staples et al., 1990; Opsomer et al., 2000; Mäntysaari et al., 2022), it is possible to hypothesize that delayed ovarian resumption may be the underlying mechanism of reduced fertility due to ketosis. In this study, we investigated the association of ketosis during 3-14 DIM with the ovarian activity, assessed using milk P4 concentration records, during 150 DIM in dairy cows.

5.5 Material and Methods

5.5.1 Data Management

The datasets for this study were obtained from Lattec I/S, Hillerød, Denmark. Milk sampling and recording of BHB and P4 concentrations were done by Herd Navigator (DeLaval International, Sweden). Data were collected from fifteen Canadian herds for the period from January 01, 2017 to December 31, 2021, with the following information: cow identification, herd identification, parity, calving date, milk BHB concentration, milk P4 concentration, and alarms like heat and prolonged postpartum anestrus. Two herds were excluded because milk P4 concentration recording did not start before 35 DIM. Figure 1 provides inclusion and exclusion progression of data management. All BHB datasets from 13 herds (median size of 749 lactations) containing records of 291 411 milk BHB from 9 942 lactations of 5 017 unique cows were joined. All 663 339 records of milk P4 concentration data from 9 517 lactations of 4 840 unique cows from 13 herds (median size of 708 lactations) were joined. To analyze cows that had standard breeding management, we considered the ovarian activity parameters within 150 days after calving. Considering milk P4 concentration records within 150 DIM we retained 481 277 records from 9 492 lactations of 4 830 cows. As the median days of first luteal activity in healthy cows is 35 DIM, (Mäntysaari et al., 2022) we retained cows with milk P4 records beginning before 35 DIM. The retained 476 423 records of milk P4 concentration were from 9 217 lactations of 4 679 unique cows (Figure 1). Merging of scrubbed milk BHB and P4 datasets resulted in records from 7 641 lactations of 4 359 unique cows available for further analyses. Of these, 4,220 cows (7 346 lactations) had first luteal activity (FLA) and 139 cows (295 lactations) did not have FLA within 150 DIM (Figure 1). On the other hand, 4 068 cows (7 060 lactations) had first heat (FH) and 291 cows (581 lactations) did not have FH within 150 DIM (Figure 1).

5.5.2 Stratification of cows into ketosis groups

First, we surveyed cows for BHB concentration during the period of 3-42 DIM to categorize cows into different groups (Alemu et al., 2023). The cut-off milk BHB concentrations were established, based on a previous study (Nielsen et al., 2005) and Lattec validation, as healthy (Healthy; milk BHB < 0.08 mmol/L), subclinical (SCK; milk BHB 0.08–0.12 mmol/L) or clinical ketosis (CK; milk BHB ≥ 0.13 mmol/L) (Table 1). A recent study using HN data (Mäntysaari et al., 2022) found that the median day of first luteal activity was 35 DIM. Since this falls within the period of late ketosis (15-42 DIM) (Alemu et al., 2023), we excluded all cows that had elevated milk BHB during 15-42 DIM from further analysis. We retained cows with early ketosis categorized as SCK, SCK_Pro, CK and CK_Pro groups (where "_Pro" indicates that cows in SCK and CK categories had BHB > 0.08 mmol/L beyond 15 DIM). Our initial analyses of milk P4-based parameters of ovarian activity (defined below) among the four early ketosis groups indicated no statistical differences between SCK and SCK_Pro as well as CK and CK_Pro cows. Since any impact of ketosis in cows of "Pro" categories should have begun with the first increase occurring during the period of 3-14 DIM, we joined cows of SCK_Pro group with SCK cows, and cows of CK_Pro group with CK cows for further analysis. Therefore, our subsequent analyses were based SCK and CK in comparison with Healthy cows. Upon considering early ketosis started during 3–14 DIM, we retained 243,803 records of milk BHB from 4,546 cows and 8,030 lactation (Figure 1). It is important to note that Healthy cows had milk BHB levels below the threshold of 0.08 mmol/L across 3-42 DIM. We chose to not include cows that were healthy during 3-14 DIM but had elevated milk BHB between 15-42 DIM in Healthy category as they belong to late ketosis group (Alemu et al., 2023). This allowed us to examine the knock-on effect of early ketosis

occurring during 3-14 DIM on subsequent luteal functions as compared to cows that remain healthy throughout the first six weeks of lactation.

5.5.3 Parameters indicative of ovarian activity

The following ovarian activity parameters were defined based on milk P4 concentration (Figure 2). The HN generates reproductive alarms including heat and postpartum anestrus (Friggens and Chagunda, 2005). Estrous cycle was defined as the interval between two heat alarms. Corpus luteum activity (Luteal activity) was defined as the period of at least three days when milk P4 concentration was ≥5 ng/ml. The start of luteal activity was determined on the first day, following calving or a heat alarm, when P4 concentration was ≥5 ng/ml. The end of luteal activity during an estrous cycle was defined as the last day when P4 concentration was ≥ 5 ng/ml for at least three days, before reaching <5 ng/ml. Calving to first luteal activity (**FLA**) was defined as the time interval (days) from calving to the start of luteal activity. Calving to first heat (**FH**) was defined as the time interval (days) from calving to the heat alarm for the first time since calving. The first luteal phase (**FLP**) was defined as the period (days) of first luteal activity after calving (with no preceding heat alarm), while the luteal phase (**LP**) for estrous cycles was defined as the period (days) of luteal activity during an estrous cycle excluding the first luteal phase. Peak P4 concentration was considered separately for the first luteal phase and all subsequent luteal phases (estrous cycles). Prolonged postpartum anestrus (**PPA**) alarm is notified by HN biomodel when the P4 concentration remains <5 ng/mL during the first 35 DIM.

5.5.4 Statistical Analysis

Statistical analysis was performed using SAS 9.4 (SAS Institute Inc.). Descriptive statistics were generated with the FREQ procedure of SAS. Kaplan and Meier (1958) estimates of the survival function were used to measure the time from the first elevated to the last elevated BHB

of SCK and CK, CFLA and CFH using the LIFETEST procedure of SAS. Cows that were culled or did not have CFLA and CFH within 150 DIM were right censored. The hazard ratios of FLA and FH were determined using the PHREG procedure of SAS with Cox proportional hazard ratio (Cox, 1972) fitted for time calving to FLA and calving to FH. The model used was

$$\lambda i(t|Xi) = \lambda 0(t) \exp(xi'\beta),$$

where $\lambda i(t|Xi)$ is the hazard ratio of having luteal activity after calving at time t, first heat after calving at time t; $\lambda 0(t)$ is the baseline hazard function; β is an unknown vector of regression coefficients for the covariates; x' is a vector for the fixed effect of ketosis (Healthy, SCK and CK), parity (1, 2, 3, and ≥4), calving season (fall, summer, winter, and spring), calving year (2017 to 2021), ketosis × parity, and herd as a random effect; and i is the combination of fixed effect levels. If statistically nonsignificant ($P \geq 0.05$), the interaction factor was manually removed by manual backward stepwise elimination. Cows that were culled and did not have FLA and FH within 150 DIM were right censored. The Cox proportional hazard model assumptions were found to be supported by a nonsignificant relationship between Schoenfeld residuals against survival time (*P* > 0.05).

We used mixed model to analyze the outcome variables peak P4 concentration during the first luteal activity, peak P4 concentration during estrous cycles, and number of heat alarms. The model used was:

$$Y_{ijklmn} = \mu + Ketosis_i + Parity_j + SOC_k + YOC_l + (Ketosis \times Parity)_{ij} + Herd_m + e_{ijklmn},$$

Where Y_{ijkmn} is outcome variables; μ overall mean; $Ketosis_i$ is the fixed effect of the ith ketosis (i=Healthy, SCK, and CK); $Parity_j$ is the fixed effect of the jth Parity (j=1, 2, 3, and ≥4); SOC_k is the fixed effect of the kth SOC (k=fall, summer, winter, and spring); YOC_l is the fixed effect of the lth YOC (l=2017 to 2021); $(Ketosis \times Parity)_{ij}$ is the fixed effect of the interaction between ith

Ketosis and jth Parity; $Herd_m$ is the random effect of the mth herd (m=1 to 13); and e_{ijklmn} is the random error. If the interaction factor was statistically nonsignificant ($P \geq 0.05$), we removed it manually using backward stepwise elimination.

We used multivariable mixed logistic regression models built with the GLIMMIX procedure of SAS to analyze the association between ketosis and the odd ratio of prolonged postpartum anestrus (0 = no risk alarm, 1= risk alarm). The following independent variables were fitted in the model: as fixed effect ketosis, parity, calving season, calving year, interaction of Ketosis × Parity and herd as random effect. If the interaction effect was statistically nonsignificant ($P \geq 0.05$), we removed it manually using backward stepwise elimination.

5.6 Results

5.6.1 Descriptive statistics

As shown in Figure 1, we analyzed data from 7,641 lactations of 4,359 unique cows, of which there were 2893 parity 1 (37.9%), 1937 parity 2 (25.3%), 1339 parity 3 (17.5%) and 1,472 parity ≥ 4 (19.3%) lactations. The median days of calving to the first BHB concentration record was 4 days (range: 3 -14 days) after calving. Cows retained for analysis had milk BHB records on average every 1.2 days between 3 to 14 DIM. The overall prevalence of ketosis using the Delaval cut-off ≥ 0.08 mmol/L of milk BHB was 39.4% (with 9.7% SCK, 15.3% SCK_Pro and 2.3% CK and 12.1% CK_Pro cows Figure 3). The mean daily milk BHB concentrations during the period of 3–42 DIM among different ketosis groups cows are shown in Figure 4. As expected, there were differences ($P < 0.05$) in milk BHB concentrations among cows in the four ketosis groups compared to Healthy cows during the period of 3-42 DIM (Figure 4). Kaplan-Meier survival analysis of the duration of elevated milk BHB revealed that the milk BHB levels once elevated above the cut-off remained higher for a shorter median duration of three and six days, in SCK and CK cows, respectively (Figure 5). However, the milk BHB concentrations in prolonged-ketosis category cows remained elevated for markedly longer period (median of 27 and 30 days, in SCK_Pro and CK_Pro, respectively, Figure 5).

The median days of calving to the first milk P4 concentration record was 20 days (range: 20 - 35 days) after calving. Cows retained for analysis had milk P4 records on average every 3 days between 20 and 150 DIM. Although, the milk BHB levels were different among cows in the four ketosis categories as well as Healthy cows, there were no differences in milk P4-based parameters between SCK and SCK_Pro ($P > 0.05$) as well as between CK and CK_Pro ($P > 0.05$) groups (data not shown). Therefore, as described in Methods section, we joined cows in SCK_Pro

and CK_Pro groups with SCK and CK groups, respectively, as any impact of ketosis in cows of "Pro" categories should have begun with the first increase occurring during the period of 3-14 DIM. All further analyses examining the relationship between ketosis and ovarian activity were performed with three groups, namely Healthy, SCK and CK.

5.6.2 Ketosis is associated with delayed first luteal activity

The median interval from calving to FLA and the hazard ratio of FLA during 150 DIM in cows of ketosis and covariate variable groups are shown in Figure 6 and Supplementary Figure 1. The rate of FLA during 150 DIM for SCK and CK cows was 19% and 31% lower, respectively, than Healthy cows ($P < 0.0001$; Figure 6). The median interval of calving to FLA was 5 and 10 days longer in SCK and CK than Healthy cows (Figure 6). Cows in 3rd and ≥4th parity, but not those in the 2nd parity, had a lower rate ($P < 0.007$; Figure 6) of FLA during 150 DIM with longer median interval of calving to FLA compared to first parity cows (Figure 6 and Supplementary Figure 1). Cows calving in spring and winter had a lower rate of FLA during 150 DIM ($P < 0.05$) and longer median interval of calving to FLA compared with fall-calving cows. Other determinant factor that was significantly associated with calving to FLA in the model was the random effect of herd ($P < 0.0001$), while the year of calving was not significantly associated ($P > 0.05$).

5.6.3 Ketosis is associated with delayed first heat

The median days of interval from calving to FH and the hazard ratio of FH during the 150 DIM in cows of ketosis and covariate variable groups are shown in Figure 7 and Supplementary Figure 2. The rate of FH during 150 DIM was 15% and 29% lower ($P < 0.0001$) for SCK and CK cows, respectively, with the median interval of calving to FH 7 and 12 days longer than Healthy cows (Figure 7). Cows in higher parity had lower rate of having FH during 150 DIM ($P < 0.01$), with longer median of calving to FH interval (52-59 d) compared with first parity cows (47d;

Figure 7 and Supplementary Figure 2). The rate of FH during 150 DIM was lower for cows calving in the spring ($P < 0.0001$), summer ($P = 0.02$), and winter ($P = 0.005$), with longer median calving to FH intervals compared to cows that calved in the fall. Other determinant variable that was significantly associated with calving to FH in the model was the random effect of herd ($P < 0.0001$), while the year of calving was not significantly associated ($P > 0.05$).

5.6.4 Ketosis is associated with prolonged postpartum anestrus

The odds ratio of PPA during 150 DIM in cows of ketosis and covariate variable groups are shown in Figure 8. The probability of PPA alarm was 50% and 110% higher ($P < 0.0001$) in cows with SCK and CK, respectively, compared to Healthy cows (Figure 8). Cows in the 3rd and ≥4th parity, but not those in the 2nd parity, had 30% and 70% higher probability of PPA ($P < 0.001$) compared to first parity cows. The probability of PPA was higher for cows calving in the spring and summer ($P < 0.05$), but not winter, compared with cows calved in the fall. The year of calving was significantly associated with a probability of PPA ($P < 0.0001$) in the model.

5.6.5 Associations between ketosis and corpus luteum activity and estrous cycles

The association between ketosis and other determinant covariates with indicators of corpus luteum activity and estrous cycles are presented in Table 2 and Figure 9. There was no difference among SCK, CK and Healthy cows with respect to the peak P4 concentration during the FLP ($P > 0.05$; Table 2). However, the peak P4 concentration during estrous cycles (LPs excluding the FLP) was lower ($P < 0.05$) in cows with SCK and CK than Healthy cows (Table 2). There were no differences ($P > 0.05$) in the interval from the beginning of luteal activity to peak P4 concentration (in case of FLP – 8.5, 8.5 and 8.3 days in Healthy, SCK and CK cows, respectively) and in the interval from heat to peak P4 concentration (in case of inter-heat intervals) between ketosis and Healthy cows (data not shown). However, the mean daily milk P4 concentrations from

commencement to the peak of FLP were markedly lower ($P < 0.05$) on days 0 to 3 in SCK cows and on days 0 to 5 in CK cows, respectively compared to Healthy cows (d0 = beginning of FLP; Figure 9). Further, the number of heat alarms was 3% and 10% lower ($P < 0.05$) in cows with SCK and CK, respectively, compared to Healthy cows (Table 2). Considering the parity effect, cows in the 3rd and ≥4th parity, but not those in the 2nd parity, had a lower peak P4 concentration during the FLP compared to first parity cows ($P < 0.001$; Table 2). Also, cows in higher parities had lower peak P4 concentration during subsequent estrous cycles, compared to first parity cows ($P < 0.0001$; Table 2). Cows in ≥4th parity had lower number of heat alarms compared to first parity cows ($P < 0.001$; Table 2). Considering the calving season effect, cows calving in summer had higher peak P4 during estrous cycles than cows calving in other seasons ($P < 0.01$; Table 2). On the other hand, cows calving in all other seasons had higher number of heat alarms compared to cows calving in fall ($P < 0.001$; Table 2). The variation due to random effect herd accounted for 0.4% and 2.5% of the total variation in peak P4 concentration and number of heat alarms, respectively. The year of calving was significantly associated with peak P4 concentration ($P < 0.0001$) and number of heat alarms ($P < 0.0001$) in the models.

5.7 Discussion

Although, several studies have shown that ketosis has a negative effect on reproductive performance measured by breeding and calving data (Walsh et al., 2007; Chapinal et al., 2012; Alemu et al., 2023), the mechanisms underlying this adverse relationship are not well understood. Reproduction is a result of direct interaction of the hypothalamus, pituitary, ovary and uterus (Thatcher, 2017) along with indirect regulation from non-reproductive tissues like the liver (Velazquez et al., 2008; Alemu et al., 2024). In the present study we sought to study the status of ovarian functions in cows with elevated milk BHB as ovary is the primary reproductive organ supporting development of oocytes and production of steroid hormones that regulate all reproductive processes. Endocrine parameters provide mechanistic insight into ovarian functions, which underpin reproductive performance parameters. Therefore, we examined the association between SCK and CK diagnosed by milk BHB concentrations during 3-14 DIM, and parameters indicative of ovarian activity, measured by milkP4 concentrations during 150 DIM.

The prevalence of ketosis measured in dairy herds is influenced by lactational stage and frequency of BHB measurement. Several studies have reported that the ketosis risk lasts for 50 DIM and the highest number of ketosis cases occur during the first two weeks of lactation, which decline over the next four weeks (van der Drift et al., 2012; Koeck et al., 2014; Santschi et al., 2016; Alemu et al., 2023). These studies depended on weekly or monthly measurement of BHB, which leads to underestimation of ketosis prevalence. In the dataset used in this study, milk BHB was measured in over four thousand unique cows at a frequency of almost once a day during 3–14 DIM. This frequent sampling allows for accurate estimation of ketosis prevalence, at least in the herds and time period evaluated, which was about 40%. This is greater than our recent observation, using monthly milk-recording data of over 30,000 cows, that the prevalence of elevated milk BHB

during 42 DIM was 27.4% with two-thirds of elevations occurring during 14 DIM (Alemu et al., 2023). In addition, frequent sampling allowed us to estimate the duration of SCK and CK to be three and seven days, respectively. Our data are comparable to the observation that the duration of SCK, based on twice-weekly measurement of blood BHB, was five days (McArtet al., 2012) and further provide an estimation of the duration of CK in lactating cows.

Studies have shown that SCK or CK reduced conception rates, longer calving to first service interval, first service to conception, days open and calving interval (Rathbun et al., 2017; Alemu et al., 2023). Postpartum resumption of ovarian activity, heat detection and breeding at a reasonable time are important for successful reproductive performance of dairy cows. Disrupted ovarian functions during early lactation have been associated with NEB, severe body condition loss and postpartum diseases (Butler and Smith, 1989; Beam and Butler, 1999; Reist et al., 2000; Wathes et al., 2003; Patton et al., 2007; Cheong et al., 2016). In line with this, our data demonstrate that ketosis during the first two weeks of lactation was significantly associated with delayed resumption of ovarian activity, longer intervals from calving to first heat, higher odds of postpartum anestrus, and lower number of estrous cycles, and lower progesterone production. Longer calving to FLA interval and lower probability of FLA in SCK and CK cows shows that ketosis led to delay in first ovulation after calving. In line with this, elevated milk NEFA during first three weeks of lactation had delayed FLA (Mäntysaari et al., 2022). These observations along with lower probability of FH and longer calving to FH interval in SCK and CK cows demonstrate that ketosis is associated with delayed resumption of ovarian activity after calving. Supporting this, another study found that cows with ketosis had a 37% lower probability of ovarian resumption during the first eight weeks of lactation (Shin et al., 2015), albeit based on bi-weekly measurement

of P4 concentration. The delay in the resumption of ovarian activity in SCK and CK cows resulted in lower number of heat alarms during 150 DIM than Healthy cows in this study.

In cows, estradiol from dominant follicle in the ovary is responsible for exhibition of behavioral estrus (heat) such as mucus discharge from vulva and standing to be mounted. Lower circulatory estradiol concentration was associated with shorter duration or absence of estrus in lactating cows (Lopez et al., 2004). Delayed ovarian resumption and low P4 output observed in SCK and CK cows of this study hint toward subnormal follicular growth and estradiol production as follicles producing lower estradiol result in corpus luteum after ovulation that produces lower levels of P4 (Vasconcelos et al., 2001; Perry et al., 2005). Supporting this logic, we have recently shown that cows that experienced severe body condition loss along with higher levels of NEFA and BHB in early lactation had sub-functional dominant follicle with lower estradiol production (Alemu et al., 2024). The study shows that cows with SCK during the first three weeks of lactation had delayed exhibition of heat after calving by 9 days compared to non-ketosis cows (Rutherford et al., 2016).

The HN generates prolonged anestrus alarm when milk P4 levels remain below the threshold (< 5ng/ml) until 35 DIM. We found that SCK and CK cows had dramatically higher probability of PPA alarm relative to Healthy cows indicating that there was delayed or absence of ovulation in ketosis cows. This is supported by the observation that cows experiencing severe NEB had lower LH pulse frequency and estradiol production leading to inability to ovulate dominant follicles (Cheong et al., 2016), which would result in persistent low levels of P4 in the circulation. Further, cows with postpartum diseases, including ketosis, had 30% lower rate activity monitor-based estrus (Bruinjé et al., 2023), in other words prolonged anestrus (Bretzinger et al., 2023).

Our result shows that parity, calving season and year of calving had significant association with CFLA, CFH, PPA, number of heat alarm, and peak P4 in luteal phases of estrous cycles. These observations are in agreement with other previous reports. Multiparous cows had a higher probability of anestrus (Bretzinger et al., 2023). Cows experiencing summer heat stress had reduced reproductive performance (Wolfenson et al., 2000) and lower estradiol levels in their follicular fluid (Wolfenson et al., 1997; Roth et al., 2001). Also, cows calving in summer have extended time to first ovulation after calving (Rhodes et al., 2003).

5.8 Conclusion

Overall, our results demonstrate that ketosis during early lactation has negative impact on resumption of ovarian activity and luteal function of lactating cows. Because of this adverse relationship, the cows with ketosis are at a higher risk of postpartum anestrus compared to healthy cows. It has been shown that oral administration of propylene glycol in SCK cows resolves hyperketonemia and prevents CK (McArt et al., 2011). Therefore, frequent monitoring of milk BHB is recommended as a practical strategy to catch the cows with ketosis and treat them to improve their reproductive performance.

5.9 Ethics approval

Animal Care Committee approval did not apply to this study, as all data used were part of the routine milk recording in the DeLaval Herd Navigator system.

5.10 Data and model availability statement

Supplementary data are deposited on the FigShare repository (https://figshare.com/s/c38202fecc7d42ce5f79). Additional information can be made available from the authors upon request.

5.11 Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any Generative AI and AI-assisted technologies in the writing process.

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5.13 Author contributions

TW Alemu: conception, analyzed the data, produced tables and figures, interpretation of results and wrote the manuscript and R Duggavathi: conception, interpretation of results, and critically revising the manuscript.

5.14 Declaration of interest

Authors have nothing to declare.

5.15 Acknowledgements

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5.17 Tables

Table 5.17.1 Classification of ketosis types based on milk β-hydroxy butyrate (BHB) concentrations during 3–14 DIM**.** Milk BHB concentrations were measured by the automated real-time analyzer Herd Navigator (Lattec I/S) of the voluntary milking system (DeLaval Inc.).

BHB mmol/L (3 to 14 DIM)	Ketosis
< 0.08	Healthy
0.08 to 0.12	SCK
≥ 0.13	CK

SCK = Subclinical ketosis

CK = Clinical ketosis

Table 5.17.2 Peak progesterone (P4) concentrations (N = 4,220) and number of heat alarms (N = 4,068) within 150 DIM in Holstein cows stratified by ketosis, parity and calving season. Milk β-hydroxybutyrate (BHB) and P4 concentrations were measured by the automated real-time analyzer Herd Navigator (Lattec I/S) of the voluntary milking system (DeLaval Inc.). Cows were assigned to different ketosis groups based on milk BHB elevation beginning during 3–14 DIM. Data are presented as mean ± SEM.

Effect	Peak P4–FLP	Peak P4–estrous cycles	Number of heat alarms
Ketosis group			
Healthy	19.28 ± 0.2	21.01 ± 0.1	2.9 ± 0.07
SCK	19.12 ± 0.2	20.65 ± 0.1*	2.8 ± 0.06*****
CK	18.81 ± 0.2	20.64 ± 0.1*****	2.6 ± 0.06*****
Parity			
1	19.12 ± 0.2	22.15 ± 0.1	2.9 ± 0.06
2	19.57 ± 0.2	20.95 ± 0.1*****	2.9 ± 0.06
3	18.85 ± 0.2*	20.35 ± 0.1*****	2.8 ± 0.06
≥4	18.46 ± 0.2*	19.61 ± 0.1*****	2.6 ± 0.06*****
Calving season			
FALL	19.11 ± 0.2	20.69 ± 0.1	2.7 ± 0.06
SPRING	18.77 ± 0.2	20.71 ± 0.1	2.8 ± 0.06*****
SUMMER	19.28 ± 0.2	21.13 ± 0.1*	2.9 ± 0.06*****
WINTER	19.13 ± 0.2	20.54 ± 0.1	2.8 ± 0.06*****

* Significantly different ($P \leq 0.05$) within a parameter from the reference groups, Healthy, 1 and FALL in ketosis group, parity and calving season, respectively; SCK –subclinical ketosis; CK – clinical ketosis; Peak P4–FLP – highest P4 concentration during first luteal phase; peak P4 – estrous cycles – highest P4 concentration during luteal phase of estrous cycles.

5.18 Figures

Figure 5.18.1 Flow diagram of data inclusion and data-set merging for the analysis of the association of ovarian activity with ketosis, parity and calving season in Holstein cows. Each box shows the number of milk BHB and/or P4 records from cows and lactations included for the final analysis. BHB – β-hydroxybutyrate; P4 – progesterone; DIM – days in milk; FLA – first luteal activity; FH – first heat.

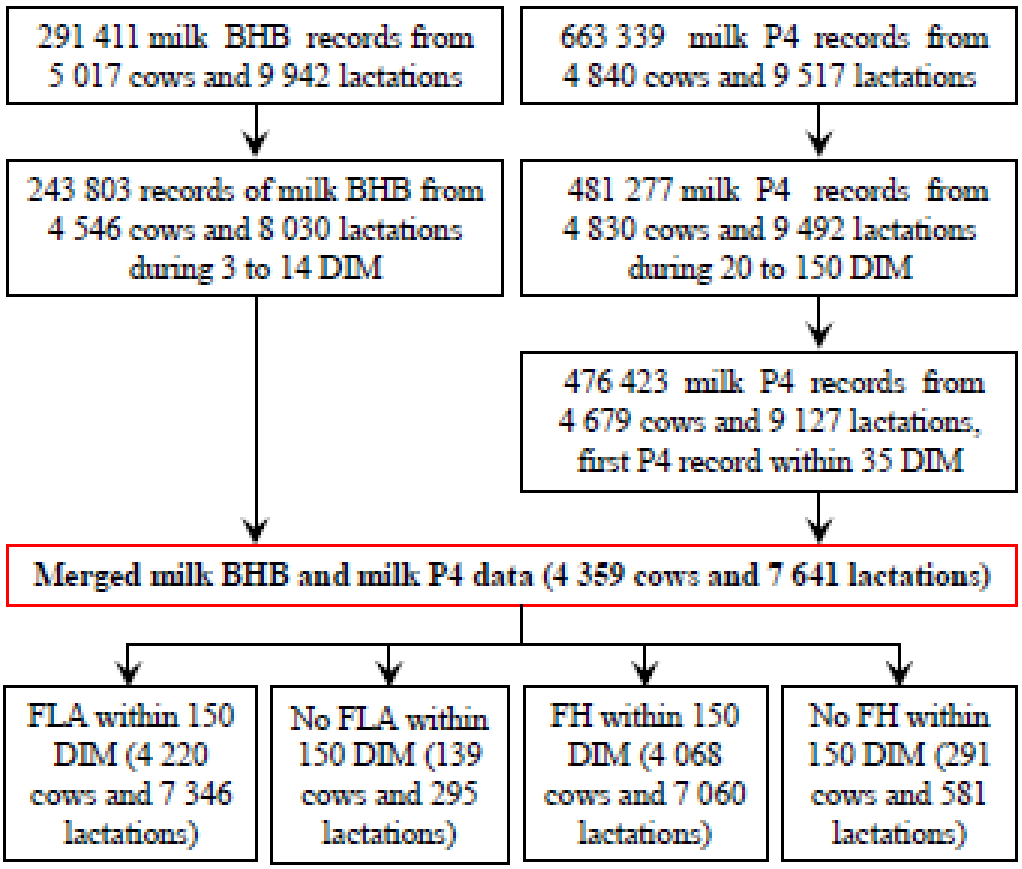


Figure 5.18.2 Hypothetical milk progesterone (P4) profile curve during 20–150 DIM showing ovarian-activity parameters analyzed to investigate the association of ovarian activity with ketosis, parity and calving season in Holstein cows. Milk P4 concentrations are measured by the automated real-time analyzer Herd Navigator (Lattec I/S) of the voluntary milking system (DeLaval Inc.). The P4 concentration ≥ 5ng/ml (red line) represents luteal activity. Ovarian-activity parameters analyzed: CFLA – calving to first luteal activity; CFH – calving to first heat; FLP – first luteal phase; LP – Luteal phase, excluding FLP; * – peak P4 concentration during luteal phase of estrous cycles; H1 and H2 – heat alarms.

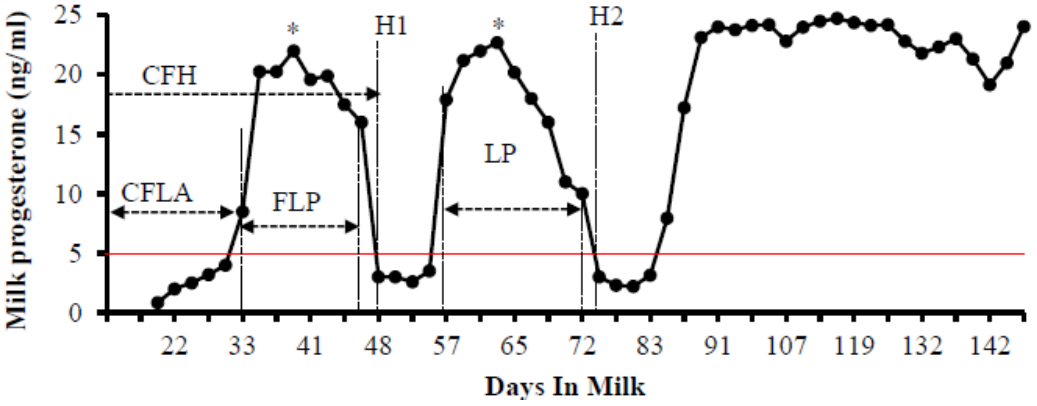


Figure 5.18.3 Frequency (%) of ketosis in lactating Holstein cows included in the analysis of the association of ovarian activity with ketosis, parity and calving season. Milk β-hydroxybutyrate (BHB) concentrations were measured by the automated real-time analyzer Herd Navigator (Lattec I/S) of the voluntary milking system (DeLaval Inc.). Cows were assigned to different ketosis groups based on milk BHB elevation beginning during 3–14 DIM. The cut-off milk BHB concentrations were established as healthy (milk BHB < 0.08 mmol/L), subclinical (milk BHB 0.08–0.12 mmol/L) or clinical ketosis (milk BHB ≥ 0.13 mmol/L). Cows in "Pro" groups had prolonged elevation of milk BHB concentration during the period of 15-42 DIM. Healthy cows did not have elevated milk BHB during 3–42 DIM. SCK – subclinical ketosis; SCK_Pro – prolonged subclinical ketosis; CK – clinical ketosis; CK_Pro – prolonged clinical ketosis.

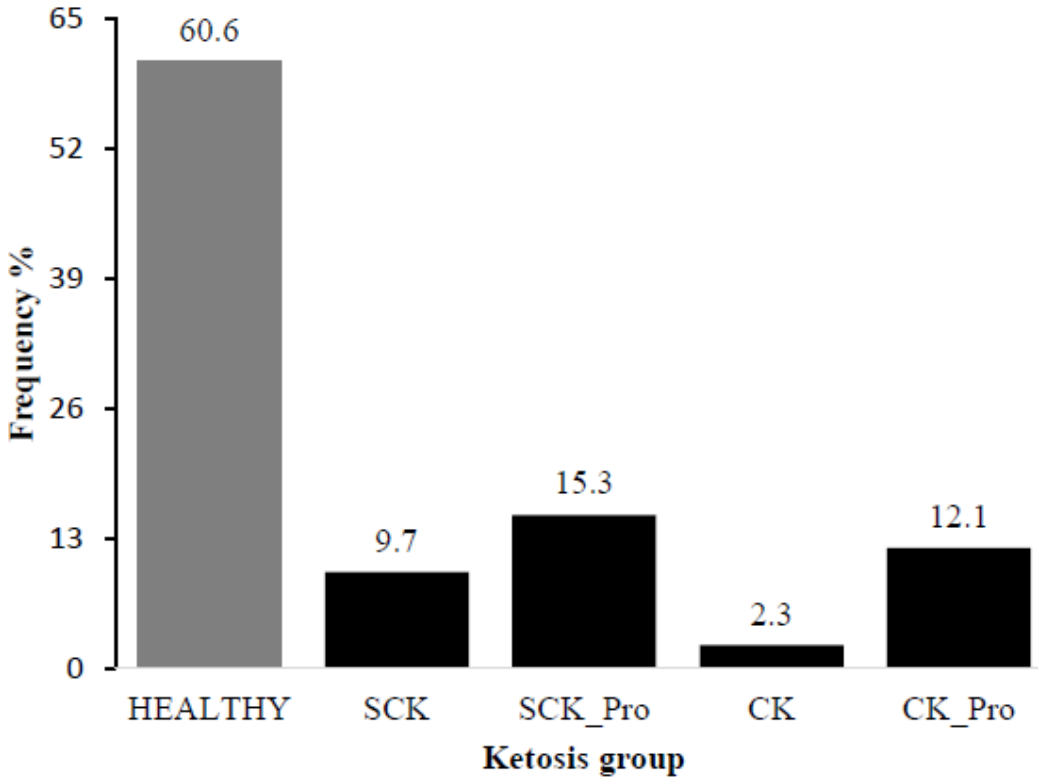


Figure 5.18.4 Daily mean milk BHB during 3-42 DIM in Holstein cows of different ketosis groups. Milk β-hydroxybutyrate (BHB) concentrations were measured by the automated real-time analyzer Herd Navigator (Lattec I/S) of the voluntary milking system (DeLaval Inc.). Cows were assigned to different ketosis groups based on milk BHB elevation beginning during 3–14 DIM. The cut-off milk BHB concentrations were established as healthy (milk BHB < 0.08 mmol/L), subclinical (milk BHB 0.08–0.12 mmol/L) or clinical ketosis (milk BHB ≥ 0.13 mmol/L). Cows in "Pro" groups had prolonged elevation of milk BHB concentration during the period of 15–42 DIM. Healthy cows did not have elevated milk BHB during 3–42 DIM. SCK – subclinical ketosis; SCK_Pro – prolonged subclinical ketosis; CK – clinical ketosis; CK_Pro – prolonged clinical ketosis. Data are presented as mean ± SEM.

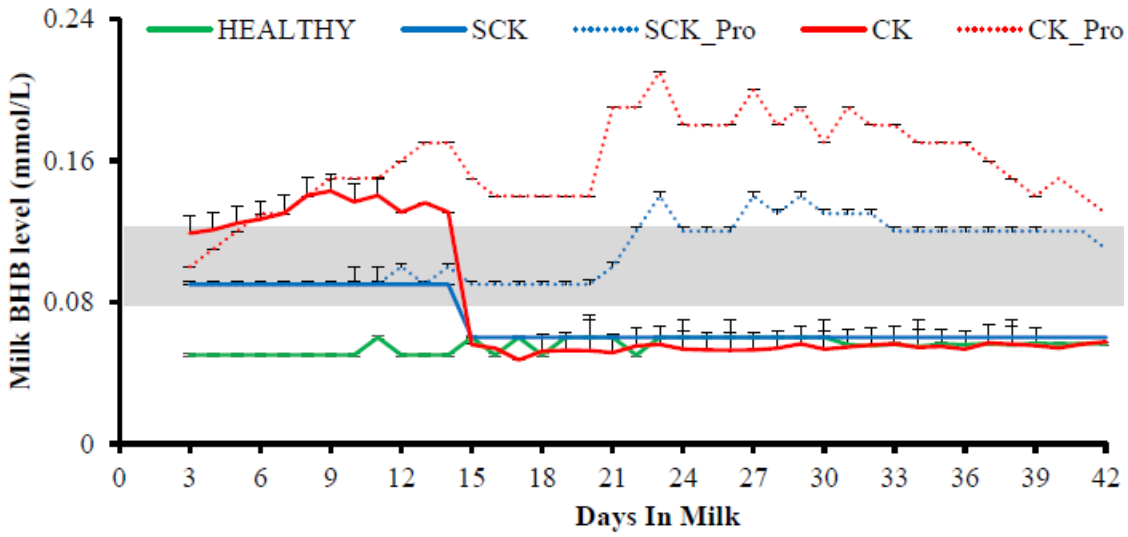


Figure 5.18.5 Duration of elevated milk β-hydroxybutyrate (BHB) in Holstein cows with ketosis. Kaplan-Meier survival curves of the duration (days) when milk BHB was higher than the threshold of ≥ 0.08 mmol/L before falling down to healthy levels (< 0.08 mmol/L) in SCK, SCK_Pro, CK, and CK_Pro cows (N = 4,359) during 3–42 DIM. SCK – subclinical ketosis; SCK_Pro – prolonged subclinical ketosis; CK – clinical ketosis; CK_Pro – prolonged clinical ketosis.

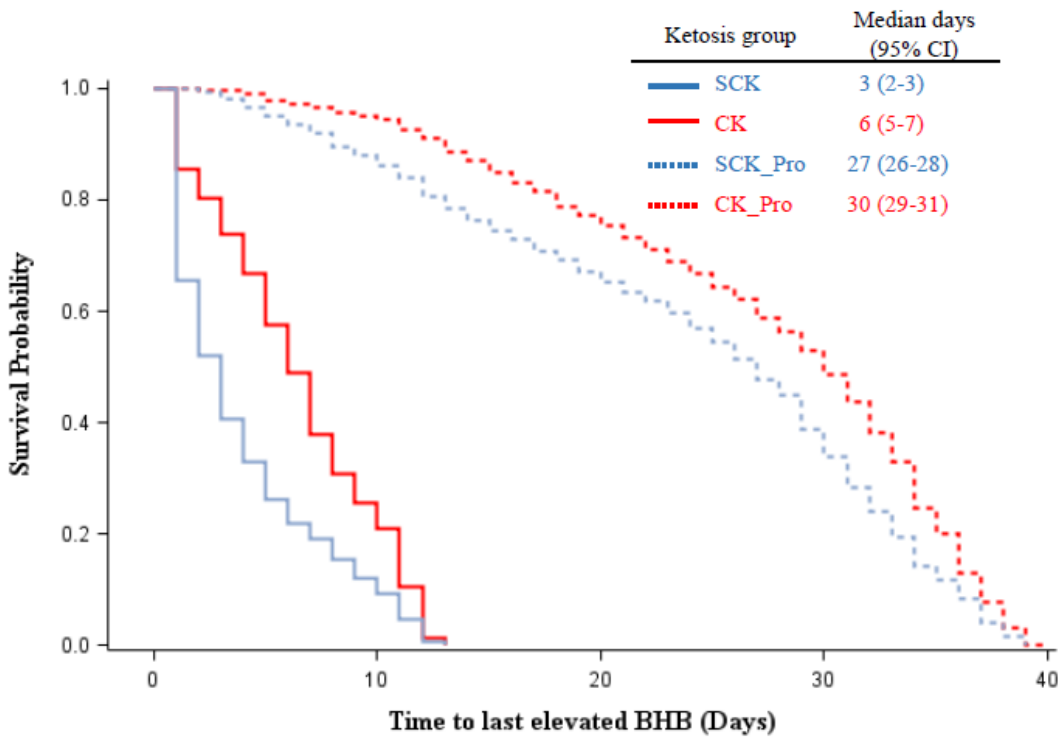
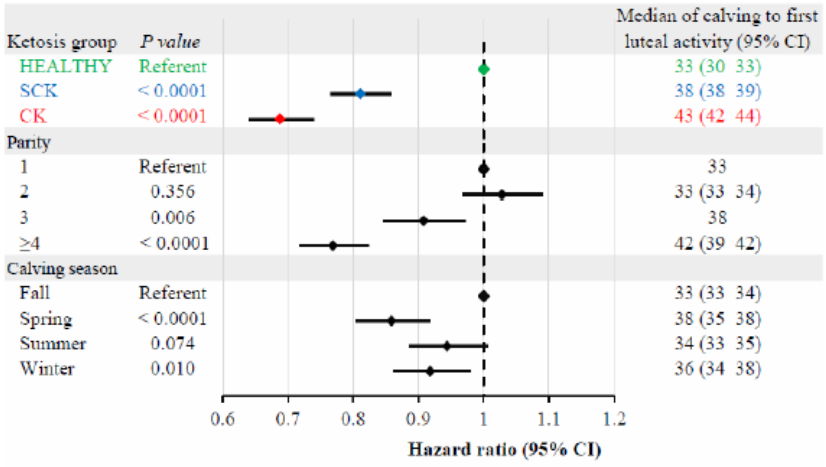


Figure 5.18.6 Association of calving to first luteal activity (FLA) interval with ketosis, parity and calving season. **A**) Estimated hazard ratio of FLA within 150 DIM and median survival days for calving to FLA interval in Holstein cows (N = 4,359) stratified by ketosis, parity and calving season. **B)** Kaplan-Meier survival curves and median days of calving to FLA interval in Holstein cows stratified by ketosis. Ketosis groups were based on milk BHB concentrations during 3–14 DIM. Survival curves and median days of calving to FLA interval of cows stratified by parity and calving season are presented in supplementary Figure 1. SCK – subclinical ketosis; CK – clinical ketosis.

A



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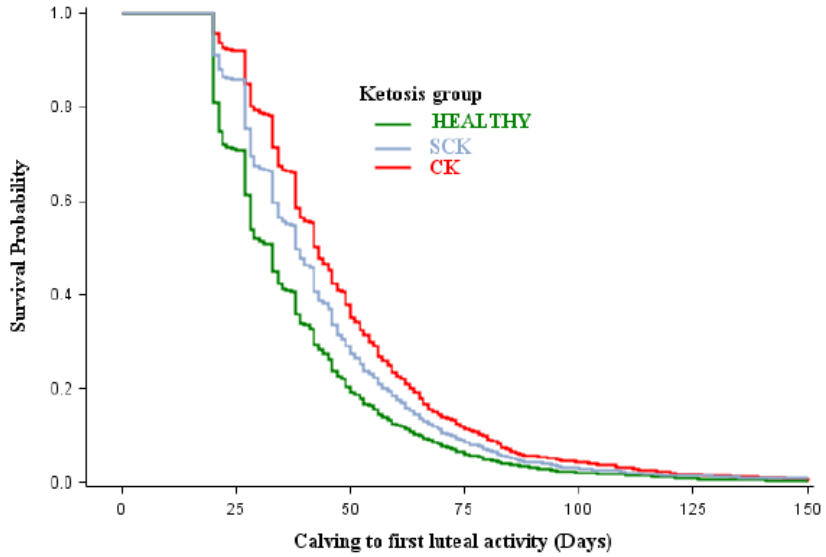


Figure 5.18.7 Association of calving to first heat (FH) interval with ketosis, parity and calving season. **A)** Estimated hazard ratio of FH within 150 DIM and median survival days for calving to FH interval in Holstein cows (N = 4,359) stratified by ketosis, parity, and calving season. **B)** Kaplan-Meier survival curves and median days of calving to FH interval in Holstein cows stratified by ketosis. Ketosis groups were based on milk BHB concentrations during 3-14 DIM. Survival curves and median days of calving to FLH interval of cows stratified by parity and calving season are presented in supplementary Figure 2. SCK – subclinical ketosis; CK – clinical ketosis.

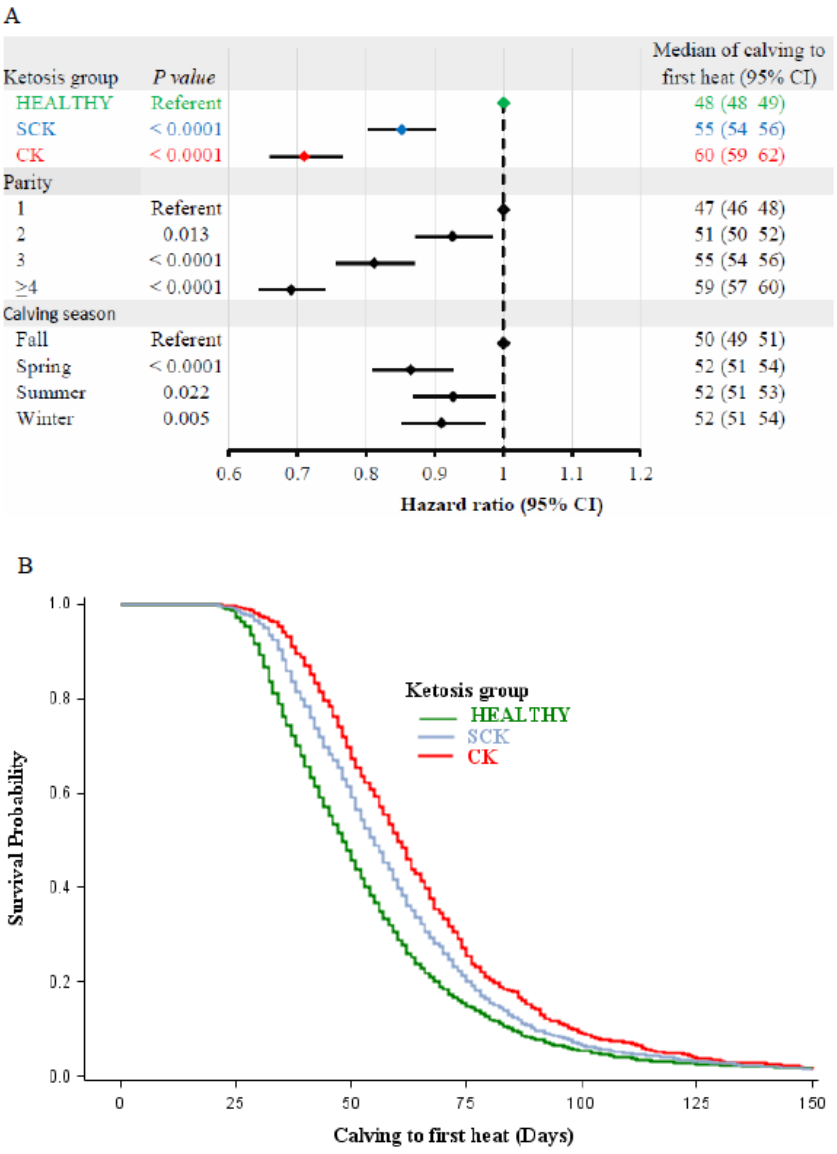


Figure 5.18.8 Probability of prolonged anestrus (PPA) alarm in cows with ketosis**.** Estimated odd ratio of prolonged anestrus (PPA) alarm in Holstein cows (N = 4,359) stratified by ketosis, parity and calving season during 150 DIM. Milk β-hydroxybutyrate (BHB) and P4 concentrations were measured by the automated real-time analyzer Herd Navigator (Lattec I/S) of the voluntary milking system (DeLaval Inc.). Cows were assigned to different ketosis groups based on milk BHB elevation beginning during 3–14 DIM. Herd Navigator uses milk P4 concentration profiles to generate PPA alarms. SCK – subclinical ketosis; CK – clinical ketosis.

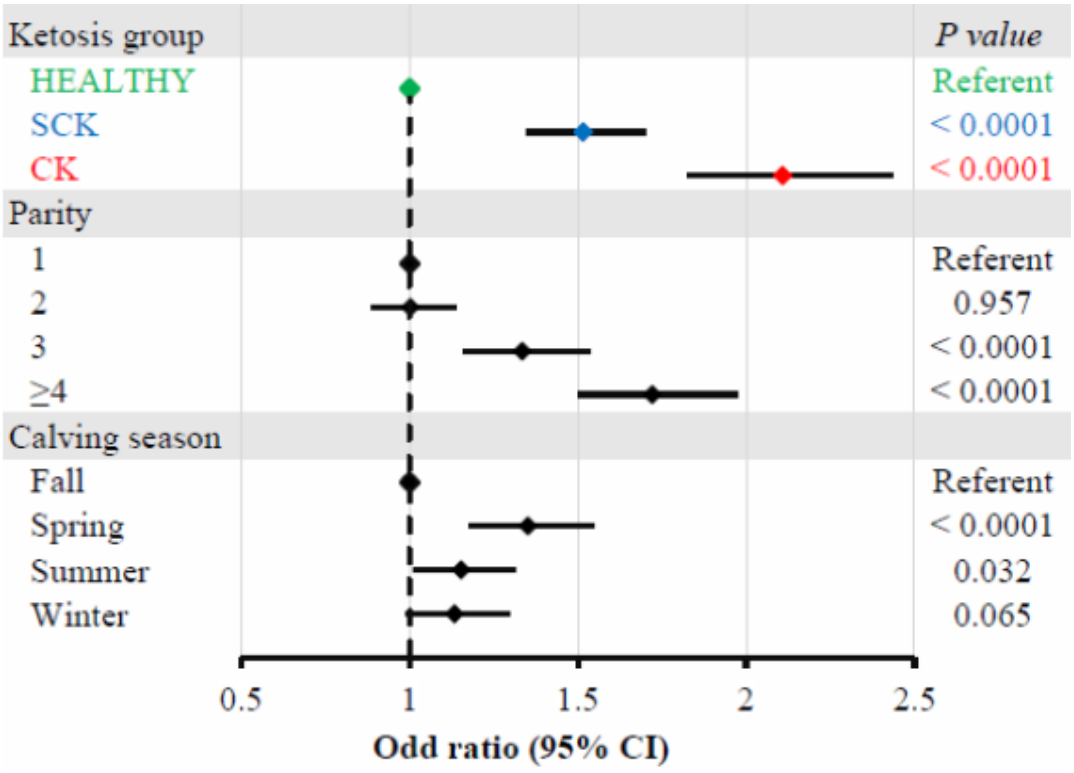
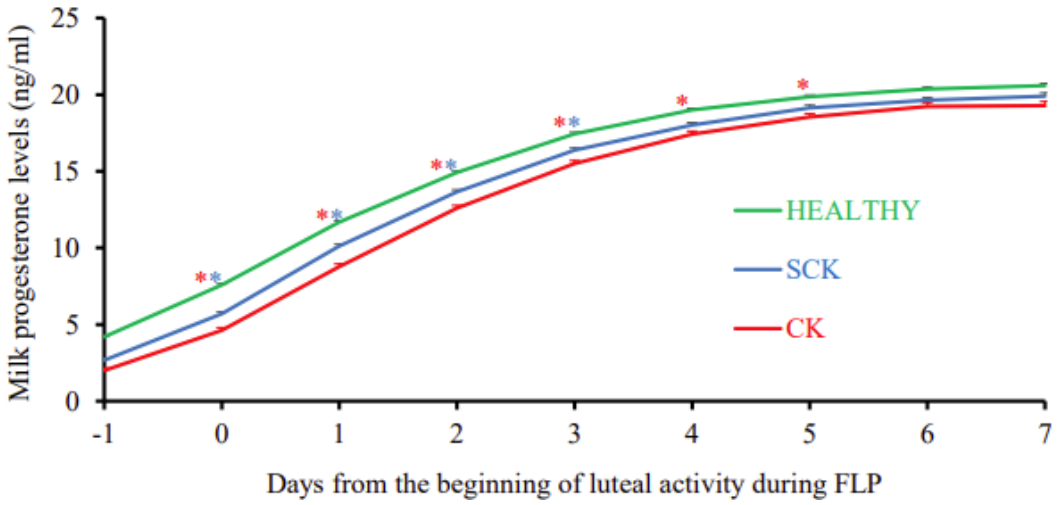


Figure 5.18.9 Corpus luteum function in cows with ketosis. Mean daily milk progesterone (P4) concentrations (ng/ml) during the first luteal phase in Holstein cows (N = 4,220) stratified by ketosis. Milk β-hydroxybutyrate (BHB) and P4 concentrations were measured by the automated real-time analyzer Herd Navigator (Lattec I/S) of the voluntary milking system (DeLaval Inc.). Cows were assigned to different ketosis groups based on milk BHB elevation beginning during 3–14 DIM. Milk P4 concentrations from the beginning of luteal activity (> 5 ng/mL) to the median day of peak P4 concentration are presented as mean ± SEM. ** $P < 0.05$ = both SCK and CK compared to Healthy; * $P < 0.05$ = CK compared to Healthy.SCK – subclinical ketosis; CK – clinical ketosis.



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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

In recent decades, there has been a substantial increase in milk production per lactation in modern dairy cows. Increased milk yield is mainly obtained through intensive genetic selection along with improved nutrition and management. After calving, cows go through NEB when they expend more energy to produce milk than they consume. This causes lower blood glucose levels and mobilization of lipids from adipose tissue as NEFAs to meet their energy needs (Drackley, 1999). Severe NEB results in excessive lipid mobilization and elevated NEFAs in the circulation. NEFAs enter the liver and are metabolized into ketone bodies, including BHB. The presence of high NEFAs and BHB levels in body fluids indicates a state of NEB and potential metabolic disorder in dairy cows (Churakov et al., 2021; Pires et al, 2022). This metabolic disorder increases the risk of fatty liver, ketosis, and impaired fertility (Ingvartsen, 2006; Rutherford et al., 2016; Shin et al., 2015). Besides NEFAs and BHB measurements, BCS is a noninvasive and inexpensive indicator of the energy status of the cow (Chebel et al., 2018; Hoedemaker et al., 2009; Thorup et al., 2012). The optimum BCS at calving is 3 to 3.5 for dairy cows (Drackley & Cardoso, 2014). Studies have shown that loss of BCS during the transition period impacted reproductive performance. In the first study, we investigated the effect of BCS loss on liver health and ovarian function during early lactation. To our knowledge, this is the first study to look at the association between changes in BCS, hepatic transcriptome, and ovarian follicular function during the transition period leading up to breeding. This experiment was performed from 3 weeks before calving to 7 weeks after calving. The cows were divided into two groups based on BCS loss; MOD cows lost 0.4 BCS units and SEV cows lost 1.0 units indicating that SEV cows mobilized more body fat reserves to provide energy for milk production. Consistent with a previous study (Roche

et al., 2007), cows that lost severe BCS after calving had higher BCS at calving. Studies have shown that excessive BCS loss after calving had a negative effect on the reproductive performance of lactating cows (Britt, 1992; Buckley et al., 2003; Pinedo et al., 2022; Roche et al., 2009). Cows diagnosed with ketosis displayed increased body condition prior to diagnosis and experienced higher body condition loss thereafter (Gillund et al., 2001). Blood NEFAs and BHB concentrations are used as an indicator of lipid mobilization during early lactation. Severe BCS loss was associated with elevated NEFAs and BHB concentrations, which agrees with previous studies (Barletta et al., 2017; Rathbun et al., 2017). Furthermore, severe BCS loss was associated with higher concentrations of plasma GGT, which is a marker of liver damage. The combination of elevated GGT, NEFAs, and BHB concentrations in SEV cows suggested that excessive lipid mobilization was the underlying cause of liver disorders. This is in line with previous findings indicating that cows with fatty liver had elevated circulatory concentrations of NEFAs, BHB and GGT (Kawashima et al., 2016; Mohamed et al., 2004; Ohtsuka et al., 2001; Rukkwamsuk et al., 2000). Liver disorders in dairy cows can negatively affect their overall health and reproductive performance. Given that elevated NEFAs and BHB concentrations, along with increased GGT an indicator of liver disorder, we investigated hepatic transcriptome differences between MOD and SEV cows at 7 weeks after calving. SEV loss cows showed significant changes in their hepatic transcriptome. This was evidenced by a higher number of significant DEGs in SEV vs. MOD cows. A total of 1186 DEGs were identified, with 858 upregulated and 328 downregulated between SEV vs. MOD loss cows at 7 weeks after calving. This suggests that severe body condition score loss during the transition period leading up to the planned breeding time had a substantial impact on hepatic gene expression patterns in lactating dairy cows.

Existing reports have highlighted the impact of BCS changes on hepatic transcriptome in early lactating cows (Akbar et al., 2015; Pascottini et al., 2021; Vailati-Riboni et al., 2016), yet there is limited research on the effect of BCS changes on hepatic transcriptome and ovarian function during transition periods till the time planned for breeding. Akbar et al. (2015) observed that cows with higher BCS at calving showed increased hepatic mRNA abundance of genes involved in fatty acid oxidation (CPT1A, ACOX1), ketogenesis (HMGCS2), and hepatokines (FGF21, ANGPTL4). These cows also had lower expression of GHR1A, IGF1, and IGFBP3, which are in line with SEV cows that had lower hepatic IGF1, IGFBP1, and IGFBP3. We performed KEEG pathways and GO_BP analyses to determine the functional roles of significant DEGs in SEV cows. Our findings revealed that several lipid metabolism pathways or biological processes were enriched among DEGs in SEV cows. Out of 43 significantly enriched KEEG pathways, several of them are involved in lipid metabolism, including linoleic acid metabolism, alpha-linolenic acid metabolism, ether lipid metabolism, and fat digestion and absorption. Similarly, GO_BP demonstrated 36 significantly enriched terms, including lipid metabolic process, fatty acid beta-oxidation, fatty acid biosynthetic process, and fatty acid metabolic process. Putting KEEG pathways and GO_BP together revealed that lipid metabolism was the most significantly enriched pathway in SEV cows. In line with our results, cows experiencing metabolic stress during the transition period had significantly enriched hepatic pathways, including lipid mobilization (Ha et al., 2017; McCabe et al., 2012; Shahzad et al., 2019). Studies have shown that NEB induced lipid metabolism can negatively impact the ovarian function of dairy cows. Elevated NEFAs concentration can disrupt follicular growth by inhibiting granulosa cell survival, steroidogenesis and oocyte development (Leroy et al., 2005; Vanholder et al., 2005). *In vitro* experiments showed that alpha-linolenic acid, linoleic acid, and oleate acid reduced the expression

of steroidogenesis-associated genes and the levels of E2 and P4 in granulosa cells (Baddela et al., 2022; Sharma et al., 2020). In our study, SEV cows showed significant enrichment in alpha-linolenic acid and linoleic acid metabolism pathways. The presence of these pathways in SEV cows might be partly associated with lower E2 levels in follicular fluid. It also leads to reduced expression of granulosa genes CYP19A1, NR5A2, LHCGR, and IGF1R which play a role in steroidogenesis and dominant follicle competence.

Severe negative energy balance in dairy cows during early lactation significantly affects the production of circulatory IGF family members (Fenwick et al., 2008). IGF1, a metabolic hormone, plays a vital role in ovarian steroidogenesis, cells survival and proliferation. It is predominantly synthesized in the liver and transported to other tissues, and it is also secreted by the ovaries (Ortega et al., 2008; Sjogren et al., 1999; Spicer & Echternkamp, 1995; Velazquez et al., 2008; Yakar et al., 1999). IGF binding protein 3 (IGFBP3) is the most abundant protein that transports and increases the half-life of IGF1 in circulation (Varma Shrivastav et al., 2020). Hepatic IGF1 and its binding proteins (IGFBP1 and IGFBP3) were found to be decreased in SEV cows at 7 weeks after calving, this reduction in IGF1 was seen in both serum and follicular fluid. In line with our findings, early lactating cows with severe negative energy balance exhibited reduced hepatic expression of IGF1 and IGFBP3 (Fenwick et al., 2008). Early lactating cows with lower plasma concentrations of IGF1 had lower E2 level in dominant follicles, delayed luteal activity, and longer calving to first service interval (Beam & Butler, 1999; Patton et al., 2007; Wathes et al., 2007). An increase in IGF1 and IGF1R expression leads to an increased E2 production and proliferation in granulosa cells (Baumgarten et al., 2017; Beam & Butler, 1999; Spicer et al., 1994). Another study showed that CYP19A1 expression and E2 production were lower in the granulosa cells of IGF1R knockout mice compared to the control group (Baumgarten

et al., 2017). In accordance with the aforementioned study, SEV loss cows showed a decreased IGF1R expression in granulosa cells, which might contributed to the lower E2 production in dominant follicles. It is well established that IGF1 plays a crucial role in the increased expression of LHCGR in granulosa cells, which is vital for follicle dominance and attainment of the ovulatory capacity (Beam & Butler, 1999; Han et al., 2021; Rawan et al., 2015; Sekar et al., 2000; Spicer & Echternkamp, 1995). In accordance with these data, the reduced in IGF1 expression in granulosa cells of SEV cows may be attributed to decreased LHCGR expression. The expression of LHCGR in granulosa cells is crucial for preovulatory follicles to respond to the LH surge, resulting in successful maturation of oocytes, ovulation, and formation of the corpus luteum (Zhang et al., 2001). LHCGR is essential for converting androgens to E2 through aromatization in granulosa cells (Duffy et al., 2019). This reduced LHCGR expression in SEV loss cows granulosa cells, might play a role for reduced E2 production in dominant follicles. The presence of NR5A2 is crucial for the survival of granulosa cells. The knockout of NR5A2 led to a reduction in granulosa cell proliferation in comparison to the control group (Meinsohn et al., 2018). The reduced NR5A2 expression in SEV cow granulosa cells might be linked to lower E2 production in dominant follicles. CYP19A1 is a crucial enzyme that has an important function in catalyzing the conversion of androgens to E2. The decreased production of E2 in dominant follicles might be partially due to reduced CYP19A1 expression in SEV cow granulosa cells. Putting all together, impaired metabolic and liver function in SEV cows leads to a decrease in dominant follicle competence.

The first study findings lead us to examine the association between elevated milk BHB and reproductive performance in early lactating cows. Studies have shown that cows with severe negative energy balance in early lactation had experienced ketosis and decreased reproductive performance (Ospina et al., 2010b; Raboisson et al., 2014; Rutherford et al., 2016; Suthar et al.,

2013; Walsh et al., 2007). Ketosis can occur in cows as either early or late ketosis, during the first 2 weeks or 3-6 weeks of lactation, respectively (Herdt, 2000). However, no study has been done to classify early lactating cows with different ketosis subtypes and examine their effect on reproductive performance. Therefore, in the second study, we classified cows with different ketosis subtypes using milk BHB concentrations and examined the association with reproductive performance parameters after a voluntary waiting period.

A previous study by (Gillund et al., 2001) using data from over 700 cows showed that the first occurrence of ketosis was distributed mainly up to 50 days after calving. The data presented in this study indicate that BHB concentrations in milk reaches to levels of SCK and CK categories during the first six weeks of lactation. It is unsurprising that, after six weeks of lactation, most cows reach healthy BHB levels (< 0.15 mmol/L). In 42 DIM, the prevalence of EMB was 27.4%, most of this prevalence was early EMB (ketosis) occurring within 14 DIM. Out of all six EMB subtypes, the highest prevalent was EARLY_SUSP (10.49%), which can be considered as Type II SCK. These results align with recent studies that found 20% ketosis based on serum BHB ≥ 1.2 mmol/L during the second week in lactation (Chapinal et al., 2012) and 22.6% HYK tested based on milk BHB ≥ 0.15 mmol/L during five weeks of lactation (Santschi et al., 2016). Cows that experienced EMB within the first 42 DIM had shown a negative association with reproductive performance indicators. These impacts of EMB types on reproductive performance parameters are discussed below.

The calving to first service interval provides insight into cow reproductive performance and herd management strategy. While resumption of estrous cyclicity and uterine health influence the calving to first service interval, the producers may also consider other disorders like mastitis and lameness to decide when to breed cows. In this study, the hazard ratio of having first service

was lower with longer median interval from calving to first service for cows with EARLY_POS and EARLY_POS_Pro, while other EMB types did not show significant association compared to NEG cows. Given that cows with EMB >0.2 mmol/L milk BHB in the first two weeks of lactation may have shown signs of ketosis and other health issues, which producers might noticed. This may have led them to delayed the first service after calving. It is also possible that severe EMB may have impacted ovarian and uterine health of these cows and thus extending the calving to first service interval. There was no significant difference in the calving to first service interval for cows with EARLY_SUSP and EARLY_SUSP_Pro EMB compared to NEG cows. This is in contrast to a previous study by Walsh et al. (2007), which showed longer calving to first service interval in cows with SCK compared to non-SCK cows in the first two weeks of lactation. In another study, there was no significant difference in the calving to first service interval between cows with ketosis and without ketosis (Gillund et al., 2001).

The interval from the first service to conception is an important indicator of reproductive performance in dairy cows as it is mainly influenced by oocyte quality and steroid hormone production along with uterine health. The voluntary waiting period provides time for metabolic recovery and complete involution of the uterus, any problems with reproduction during this time could be caused by the lingering effects of metabolic changes in dairy cows. Cows in the EARLY_SUSP EMB group were unaffected, but other EMB types had longer intervals between first service to conception compared to NEG cows. In line with our results, cows with SCK during first two weeks after calving had a decreased pregnancy rate (Walsh et al., 2007). Another study found that cows with transition disorders, including ketosis, had a reduction in first service conception rate, which is consistent with our finding (Kim & Jeong, 2019). Cows experiencing CK also had a 2-3 days delay in calving to first service and a 4-10% decline in pregnancy rate at

first service (Guliński, 2021). Our findings show that cows with prolonged EMB (EARLY_SUSP_Pro and EARLY_POS_Pro) had lower rate of conception and extended first service to conception interval compared to NEG cows, by 6-12 days. This shows that prolonged duration and severity of ketosis has a detrimental effect on the first service conception rate of lactating cows.

Days open, which is the time from calving to conception interval, is another crucial parameter in assessing reproductive performance and making economic decisions in dairy herds. In this study, the median days open for cows with EMB types were delayed by 4 to 24 d as compared to NEG cows. Except EARLY_SUSP EMB category all EMB classified cows had a significantly lower hazard of conceiving after calving. Cows displaying higher concentrations of serum NEFAs and BHB in the first two weeks after calving had a decreased pregnancy rate after a voluntary waiting period (Ospina et al., 2010a). Also, the days open for SCK cows was extended by 16 d relative to healthy cows (Walsh et al., 2007). Although our findings showed that EARLY_SUSP EMB cows were not affected, it's important to note that Walsh et al categorized all cows with serum BHB levels above 1 or 1.4 mmol/L as SCK, which may have included CK cows. Hence, the days open for EMB cows was prolonged, ultimately affecting the calving interval. It is advised for dairy cows to have a calving interval of around 12 to 13 months (Lucy et al., 1986). The median calving interval for NEG cows was found to be within the recommended range at 397 days or 13 months. The calving interval for all EMB categories was significantly delayed except for EARLY_SUSP. It was delayed up to 24 days in EMB categories compared to NEG cows. Thus, producers and veterinarians must remain vigilant regarding EMB during early lactation to enhance the reproductive performance of lactating cows.

Finally, understanding the impact of elevated milk BHB (ketosis) on reproductive performance in dairy cows led us to uncover the underlying mechanism. Therefore, in the final study, we examined the association between ketosis during 14 DIM and ovarian activity during 150 DIM. The overall prevalence of ketosis, diagnosed by milk BHB concentrations during 3-14 days after calving, was 39.4%, with 25% SCK and 14.4% CK cows. In our previous study, the overall prevalence of elevated milk BHB during 42 DIM was 27.4% with two-thirds of EMB occurring during 14 DIM. The difference in ketosis prevalence between these two studies is due to the frequency of milk BHB testing - the previous study used tested it once a month, while this study used tested it almost once a day.

The early resumption of luteal activity after calving in dairy cows is crucial for successful subsequent reproductive performance (Galvão et al., 2010). Cows that ovulated early within a month after calving had better reproductive performance than those that ovulated later (Galvão et al., 2010). In early lactation, NEB leads the body to use stored fat and produce ketone bodies, including BHB, which can affect hormone balance. Cows with NEB had delayed first ovulation after calving, leading to longer time between calving and conception (Butler, 2003). Pulsatile LH secretion is important for follicle growth, estradiol production, and ovulation (Crowe, 2008). NEB in early lactation is associated with increased NEFA concentrations and decreased LH pulse, IGF1, and estradiol production. This causes a decline in follicle competence and delays the first ovulation after calving (Bossaert et al., 2008; Butler, 2003; Cheong et al., 2016). In our first study, we found that cows losing severe BCS in early lactation had higher circulatory NEFA concentration and lower IGF1 levels in the liver, which resulted in decreased IGF1 and E2 production in dominant follicles. In the present study, cows with ketosis resumed luteal activity later than the NEG cows. Compared with NEG cows, cows in SCK and CK had a lower rate of having first luteal activity

after calving with longer calving to first luteal activity (CFLA) intervals. This is in line with studies have previously been reported that cows with elevated milk NEFA in the first three weeks of lactation had delayed CFLA interval (Mäntysaari et al., 2022), and cows with ketosis had a lower rate of resumption of luteal activity than healthy cows (Shin et al., 2015). Another study found that SCK had no effect on the resumption of ovarian cyclicity within 7 weeks after calving (Ribeiro et al., 2013), but this study had a lower threshold of 0.96 mM BHB concentration.

Timely heat detection and breeding are crucial for improving reproductive efficiency in dairy cows. It is suggested that NEB reduces LH pulses and E2 production, decreasing the exhibition of estrus activity and prolonged first heat after calving. In the present study, ketosis occurring during the first two weeks of lactation had detrimental effects on CFH within 150 DIM. CFH intervals in cows with SCK and CK were prolonged by 7 days and 12 days, respectively, when compared to NEG cows. The hazard ratio estimates showed that cows in SCK and CK had a 15% and 29% reduction in rates of first heat after calving, respectively, compared to NEG cows. In line with our results, cows in severe NEB (Mäntysaari et al., 2022) and SCK (Rutherford et al., 2016) had delayed calving to first heat (CFH) by 7 and 9 days, respectively. These cows with SCK also showed lower physical estrus activity (Rutherford et al., 2016). Moreover, a 30% decline in heat detection is noted in cows with postpartum diseases, including ketosis when compared to healthy cows (Bruinjé et al., 2023). This decrease in heat detection shows a prolonged anestrus, potentially resulting in decreased fertility. There was a greater probability of anestrus in cows diagnosed with ketosis within the first two weeks after calving, compared to NEG cows. Compared to NEG cows, SCK and CK cows had a 1.5 and 2.1 times higher probability of anestrus, respectively. Similar to our results, (Bretzinger et al., 2023) showed that cows SCK had a 1.51 higher probability of anestrus than healthy cows. SCK and CK cows showed reduced luteal

activity, as evidenced by significantly lower milk P4 concentration during the luteal phases, in line with this, Ropstad et al. (1989) reported that cows with ketosis had lower plasma progesterone levels. These studies suggest that dairy producers should focus on feeding and nutrition to maintain optimum body condition and reduce ketosis to improve the reproductive performance of early lactating cows.

The studies in this thesis have significantly contributed to our understanding of the association between metabolic disturbances and infertility in early lactating dairy cows. Moreover, this thesis provides a foundation for further research to advance our understanding on the effect of ketosis on fertility. Further studies are required to explore the direct effects of BHB on the ovarian follicular environment. A study on BHB dose response effect should be done to uncover the underlying mechanisms occurring at the cellular and molecular levels of oocytes or the surrounding cells (granulosa or theca cells). The direct effect of ketosis on ovarian follicles can be achieved by culturing oocytes or the surrounding cells (granulosa or theca cells) with various doses of BHB to mimic SCK and CK.

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