# Epidemiological assessment of hyperketonemia in dairy herds: implications for disease diagnosis and control

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# CHAPTER 1 – Literature Review

## 1.1 Chapter summary

The transition period from late gestation to early lactation is an enormously challenging time for all dairy cows (Bell, 1996). During this period, there is an energy deficit in which the nutrient intake does not cover the increased demand for nutrients to sustain fetal growth and lactogenesis (Bell, 1995). This period of negative nutrient balance, triggers various metabolic adaptations to provide alternative sources of energy to periparturient dairy cows (Herdt, 2000). These adaptations are characterized by the mobilization of body reserves, specifically fatty acids from adipose tissue that will be transformed into ketone bodies in the liver (i.e., β-hydroxybutyrate [BHB], acetate, and acetone) and used as an alternative source of energy by various body tissues. The rise of ketone bodies can be considered a fundamental strategy of the physiological adaptation to the new productive state and indicates that the cow is utilizing body reserves to fulfill the energy requirement (Herdt, 2000). However, an excessive increase of ketone bodies may jeopardize both health and productivity. Hyperketonemia is typically defined when BHB reaches a concentration of 1.2 mmol/L in blood and includes subclinical and clinical cases of ketosis (Andersson, 1988; Duffield, 2000). Hyperketonemia (HYK) is frequently observed in dairy herds affecting 14% to 30.5% of the dairy cattle in the first three weeks post-calving (Mann et al., 2016; Chandler et al., 2018). Hyperketonemia has been associated with other metabolic and infectious diseases during early lactation such as retained placenta, milk fever, mastitis, metritis, displaced abomasum, and lameness. (Duffield et al., 2009; McArt et al., 2012a; Berge and Vertenten, 2014). Additionally, HYK has been

associated with an increased likelihood of herd removal (McArt et al., 2012a; Roberts et al., 2012) and impaired reproductive performance (Walsh et al., 2007). There are a variety of methods used to diagnose HYK by measuring ketone bodies in different bodily fluids: urine, milk, and blood. The measurement of BHB in blood is the most appropriate method to diagnose HYK (Iwersen et al., 2009; Gordon et al., 2013; Rodriguez et al., 2020), because the best accuracy, stability of BHB in blood (Tyopponen and Kauppinen, 1980), and quick result. Among the treatments available for HYK (e.g., dextrose, glucocorticoids, vitamin B12), the oral administration of propylene glycol, a precursor of propionic acid that stimulates the gluconeogenesis process, is the most efficient and preferred option (Gordon et al., 2013).

The purpose of this review is to provide a brief background on hyperketonemia in terms of frequency, pathophysiology, diagnostic technics, risk factors, effect of the disease in health and productivity, and available treatments, while identifying the main gaps on these areas.

## 1.2 Hyperketonemia during the transition period

### *1.2.1 Case definition*

In research studies BHB is the most commonly measured ketone body in blood because of its stability in blood (Tyopponen and Kauppinen, 1980). The most frequently blood BHB concentration thresholds used in the definition of hyperketonemia is 1.2 mmol/L although ranging between 1.0 and 1.4 mmol/L (Tatone et al., 2016). These BHB thresholds were determined using receiver operating characteristic curves to maximize the diagnostic ability to predict diseases and impaired performance postpartum. When BHB was measured in the first week

postpartum, Walsh et al., (2007) reported reduced pregnancy rate in cows with circulating BHB concentrations of 1.0 mmol/L. Duffield et al., (2009) reported a BHB level of 1.2 mmol/L to be associated with increased risk of DA, metritis, and less milk yield including changes in milk composition, and a BHB level of 1.4 mmol/L to be associated with clinical ketosis and with a greater drop in milk yield. Others have reported a BHB level of at least 1.2mmol/L to be associated with an increased risk of DA, impairment reproductive performance, and increased culling rate (LeBlanc et al., 2005; McArt et al., 2012a). During the second week, postpartum BHB levels associated with undesirable subsequent outcomes were higher, such as 1.8 mmol/L for DA or 1.4 mmol/L for impaired reproductive performance (Walsh et al., 2007; Duffield et al., 2009).

### *1.2.2 Occurrence of hyperketonemia*

Hyperketonemia occurrence can be described by prevalence and incidence (Dohoo, 2009). The prevalence (proportion of a population that has a specific disease in a given time period), using a threshold of 1.2mmol/L of BHB in blood, ranged from 14% to 30.5% (Mann et al., 2016; Chandler et al., 2018) during the first three weeks post-calving. To measure cumulative incidence (i.e., occurrence of new cases of the disease during a specific time interval), all cows at risk must be tested twice a week in order to identify individuals with the disease because HYK typically lasts between 3 to 5 days (McArt et al., 2012a). The cumulative incidence, has ranged from 11.7% to 43.2% (McArt et al., 2012a; Rathbun et al., 2017). The peak of incidence has been detected between days 5 and 7 postpartum (McArt et al., 2012a; Tatone et al., 2017). After the first two weeks postpartum the occurrence of HYK reduces (Koeck et al.,

2014; Santschi et al., 2016). For instance, van der Drift et al., (2012) reported a reduction of 60% in the prevalence of HYK after the first two weeks postpartum. From the numerous thresholds (i.e., 1.0 to 1.4 mmol/L) and timeframes (i.e. from 7 to 60 days post-calving) reported in research studies, it appears to exist a lack of uniformity on the definition of HYK at the moment of sampling that leads to a difficult interpretation and comparison of the results (Benedet et al., 2019).

## 1.3 Pathophysiology

### *1.3.1 Energy usage in ruminants*

The peripartum period of dairy cows is characterized by a high demand of energy and a decrease in nutrient intake. During this time, feed intake gradually declines 20 to 40% because of multiple factors related to the animal, nutritional quality, management, and climate (Allen, 2000; Hayirli et al., 2002). Therefore, feed intake cannot match the energy required to meet the physiological demands during this period. This shortage of energy is known as negative energy balance (Bell, 1995). During periods of positive energy balance, carbohydrates are the primary source of energy in ruminants (Bartley and Black, 1966). From the fermentation of carbohydrates during rumen digestion it is produced propionic acid which is the only volatile fatty acid capable to act as a precursor of glucose in the gluconeogenesis process (Yost et al., 1977; Lemosquet et al., 2009). During energy shortage, carbohydrates are scarce while the modest liver and muscle glycogen depots are rapidly consumed (Orskov, 2012; Gardner et al., 2014). An alternative source of energy to produce glucose comes from proteins. Amino acids can be transformed into glucose in the gluconeogenesis process as well (Bell et al., 2000). This process is

relatively efficient making it a good alternative glucose source in the short term. However, if used for a longer period it can pose a potential risk for structural and enzyme depletion. The last and only long-term available source of energy are triglycerides (TAG) within the adiposities in the adipose tissue (Herdt, 1988). Therefore, the purpose of the homeorhetic mechanism set in place is to increase the lipolysis process to make TAG available as an alternative source of energy. The overarching goal of the adaptive process is to shift the use of energy from glucose to triglycerides. Cows that fail to make this shift accordingly face over accumulation of TAG in the liver and metabolic dysfunction.

### *1.3.2 Non-esterified fatty acid metabolism*

Triglycerides are formed by a molecule of glycerol esterified to three long-chain fatty acids. For this reason, when adipose tissue is mobilized, a biological process known as lipolysis, glycerol and three non-esterified fatty acids (NEFA) molecules are released into the bloodstream. Many tissues such as skeletal muscle, liver or mammary gland capture the NEFA released in the bloodstream to be used as an alternative source of energy during this period when the availability of glucose or the activity of insulin is decreased. Even though muscle proteins serve for the gluconeogenesis process, a key feature of the adaptive response to NEB is to stimulate the use of NEFAs over glucose by the muscle tissue. The liver is the organ that captures most of the circulating NEFA (Bergman, 1971), serving as a buffer of energy supply keeping it constant based on uptake, transformation, storage, and release. Within the hepatocyte, NEFA can be transported to the mitochondria, where it can be fully oxidized into the tricarboxylic acid cycle (TCA) to produce energy as adenosine

triphosphate (ATP), or it will be partially oxidized into ketone bodies (i.e., ketogenesis process). Otherwise, NEFA can be directed to the cytosol, in which case it will be re-esterified into TAG and stored in the liver (Herdt, 1988). Subsequently, TAG can be released in the bloodstream as very-low-density lipoproteins. Because this is a difficult process in ruminants marked by a slow rate of release, its easily saturated contributing to the over accumulation of TAG in the liver (Grummer, 1993). The pathway followed by NEFA will depend on the availability of glucose and the energy stage. As described by McGarry and Foster, (1980), under anabolic periods when the level of glucose is high, the TCA cycle will be overflow by glucose slowing down the cycle and generating a surplus of intermediate metabolites including citrate that is the first intermediate of this cycle. Citrate will be transported to the cytosol as malonyl CoA which is a potent inhibitor of the activity of the enzyme carnitine palmityl transferase I (CPT I) responsible for the translocation of NEFA into the mitochondria (McGarry and Brown, 1997). The accumulated NEFA will be re-esterified into triglycerides. On the contrary, during NEB when glucose is not available, citrate is not produced enough to be transported out of the mitochondria as malonyl CoA and CPT I are not inhibited. The NEFA transported into the mitochondria will be converted into acetone, acetoacetate and BHB. The process of ketogenesis occurs as follows (Aiello et al., 1984). Once in the mitochondria, NEFA is beta-oxidized to Acetyl CoA. By the action of the enzyme Thiolase, two molecules of Acetyl CoA are combined yielding Acetoacetyl CoA. HMG-CoA Synthase combines Acetoacetyl CoA with another molecule of Acetyl CoA resulting in 3-hydroxy-3-methyl-glutaryl-CoA. In the mitochondria the metabolite is transformed into acetoacetate, the first ketone body produced. Acetoacetate can be spontaneously

degraded into Acetone in an irreversible process or converted into beta-hydroxybutyrate by the action of beta-hydroxybutyrate dehydrogenase in a reversible process. Once ketone bodies are released in the bloodstream and reach peripheral tissues, acetoacetate and BHB are transformed back until ending in two molecules of Acetyl CoA which will enter into the TCA cycle for ATP synthesis.

### *1.3.3 Non-esterified fatty acid regulation*

Different hormones and molecules regulates the adaptive response to NEB such as insulin, glucagon, catecholamines, growth hormone, and leptin among others. These regulate the adaptive response at three levels: at the adipose tissue (by regulation of fatty acid release), at the liver (by fatty acid oxidation and re-esterification), and at the periphery (by influencing the rate of extrahepatic utilization of ketone bodies). Insulin is the principal hormone that regulates the NEB acting at the three regulatory sites. In an anabolic state, insulin induces lipogenesis and inhibit lipolysis in the adipose tissue (Metz and Bergh, 1977). When levels of glucose and glucose precursors are low, the concentration of glucagon increases stimulating the gluconeogenic process, and the concentration of insulin decreases stimulating the lipolysis process. To obtain glycerol, a precursor of glucose, TAG in the adipose tissue is broken down releasing glycerol and NEFAs into the bloodstream. In the liver, insulin inhibits the action of CPT I. Thus, during the NEB in the periparturient dairy cows, low glucose induces low insulin and CPT I is not inhibited stimulating ketone bodies synthesis. Ketone bodies will finally serve as a negative feedback regulator on NEFA release by stimulating insulin and thus suppressing adipose lipolysis (Metz and Bergh, 1977; Ikeda et al., 1987).

Catecholamines, epinephrine, and norepinephrine are also strong regulators of the adaptive response to NEB at the adipose level (Bahnsen et al., 1984; Krentz et al., 1996). These catecholamines are secreted under stress situations stimulating the lipolysis process resulting in the release of NEFAs. The growth hormone, which is naturally high in early lactation cows, is another regulator of the adaptive response to NEB, secreted under hypoglycemia stimulus (Peel et al., 1983; Bradford and Allen, 2008). Leptin, a peptide hormone, is a regulator of appetite and energy expenditure also playing a role in the adaptive response to NEB. In overconditioned ruminants, it has been observed that central administration of leptin reduced dry matter intake (Delavaud et al., 2002). Leptin acts inhibiting insulin secretion and has been positively correlated with insulin resistance (Paz-Filho et al., 2008).

## 1.4 Test to diagnose hyperketonemia

### *1.4.1 HYK diagnosed in blood*

There is a variety of cow-side devices with relatively good accuracy available to use on farm. The Precision Xtra Meter (Abbott Laboratories), a whole blood test for BHB, has reported a sensitivity of 0.94 (95%CI: 0.92, 0.97) and specificity of 0.97 (0.96, 0.98) when using a threshold of 1.2mmol/L (Tatone et al., 2016). With a threshold of 1.4 mmol/L the sensitivity was 0.89 (0.83, 0.94) and specificity of 0.98 (95%CI: 0.97, 0.99) (Iwersen et al., 2009). Pineda & Cardoso (2015) (Pineda and Cardoso, 2015) evaluated the sensitivity and specificity of Precision Xtra in serum and plasma. Using serum and a threshold of 1.2 mmol/L the sensitivity and specificity were 1.00 and 0.51 respectively. Using plasma the sensitivity and specificity were 1.00 and 0.55 respectively. For the Nova Vet (Nova Biomedical) in whole blood and using a

threshold of 1.2 mmol/L, the sensitivity and specificity were 0.95 and 0.98 (Bach et al., 2016). A caveat about this method is the increase of BHB in blood after feeding. In order to obtain consistent and comparable results, sampling needs to occur at the same time after feeding, ideally, about 4 hours after cows start feeding due to the peak of BHB concentration (Eicher, 1998; Seely et al., 2021). In addition, blood collection from the mammary vein should be avoided because of the extraction of ketone bodies in the udder besides the risk of phlebitis (Kronfeld et al., 1968).

As described the measurement of BHB in blood to diagnose HYK is a highly accurate method that provides quick result, making it an appropriate choice for herd monitoring and individual level diagnosis (Iwersen et al., 2009; Gordon et al., 2013; Rodriguez et al., 2020). The previously mentioned cow-side device Nova Vet used to measure BHB concentration is recommended for its use in whole blood and includes a calibration feature (i.e., slope calibration factor) intended to adjust for the variation of hematocrit among species. The test performance on whole blood under different slope calibrations as well as its performance on thawed serum samples, as usually are available in laboratory settings, and plasma samples that may have been collected for other purposes, remains unclear. This information would be advantageous for researchers or consultants.

### *1.4.2 HYK diagnosed in urine*

Hyperketonemia can be diagnosed in urine. The methods usually used in urine include two semi-quantitative tests, which utilizes as reagent sodium nitroprusside. Both, Ketostix (Bayer, Germany) urine strips and Acetest tablets (Bayer, Germany) detecting acetoacetate (AcAc) and acetone (Ac) respectively. In a recent meta-

analysis (Tatone et al., 2016) including multiple studies, it was reported for the Ketostix a sensitivity of 0.70 (95%CI: 0.61, 0.79) and specificity 0.96 (95%CI: 0.95, 0.97) when compared to a concentration of 1.5 mmol/L BHBA by laboratory analysis. For the Acetest the sensitivity was 0.09 (0.04, 0.19) and specificity 1.00 (95%CI: 1.00, 1.00) (Carrier et al., 2004). Besides the imperfect accuracy that tests in urine yield, this method has practical limitations given that some cows inevitably fail to urinate during the testing period.

### *1.4.3 HYK diagnosed in milk*

Measuring ketone bodies concentration in milk is simpler than in urine because of sample collection. The test characteristics vary among the entire semi-quantitative commercial milk test available. The KetoTest (Elanco) has a sensitivity of 0.83 (95%CI: 0.74, 0.93) and specificity of 0.81 (95%CI: 0.71, 0.90) when using a threshold of 0.1mmol/L and compared to a concentration of 1.4 mmol/L BHBA by laboratory analysis. When the threshold increase to 0.2 mmol/L the sensitivity was 0.39 (95%CI: 0.29, 0.52) and specificity 0.95 (95%CI: 0.92, 0.97) (Tatone et al., 2016). Another commonly used on-farm test in milk is Nitroprusside powder. The sensitivity and specificity using a threshold of 0.05 mmol/L was 0.41 (95%CI: 0.29, 0.54) and 0.99 (95%CI: 0.98, 1.00) respectively (Carrier et al., 2004). As described, this method has lower accuracy than the tests in urine and blood.

## 1.5 Risk factors associated with hyperketonemia

### *1.5.1 Body Condition Score (BCS)*

Many variables have been identified as risk factors for the occurrence of HYK. Some of them are body condition score, parity, season of calving, breed, and herd size. Body condition score (BCS) is an important risk factor associated with HYK because of the role of adipose tissue in the pathogenesis of HYK. The BCS and changes in BCS are used as indirect measures of fat reserves and its mobilization over time (Bewley et al., 2010; Morin et al., 2017). Multiple studies have found a positive association between both overconditioned cows and loss of BCS during the dry period, and elevated concentrations of BHB and incidence of HYK postpartum (Rathbun et al., 2017; Sheehy et al., 2017). According to Cummings and Foster (2003), deviations from the physiological, tightly regulated body fat composition trigger compensatory responses in terms of satiety regulators and energy usage that persist until the level of body fat storage is restored.

Most studies evaluating variations in BCS focus on the entire dry period or on early lactation (Loeffler et al., 1999; Pryce et al., 2001; López-Gatius et al., 2003). Although this is reasonable from a management perspective, considering that most diseases occur during early lactation, variations in body condition specifically during the late dry period (i.e., 21 days from calving) play a fundamental role in the control of the diseases observed later in the lactation (LeBlanc et al., 2006; Roche et al., 2009; Ribeiro et al., 2013; Sheehy et al., 2017). Therefore, a better comprehension of the influence of BCS changes during the late dry period on HYK during early

lactation can be useful in the development of strategies to monitor and mitigate high incidences of hyperketonemia in dairy herds.

### *1.5.2 Physiological Relation Between BCS And Adipose Sensitivity*

Excessive mobilization of adipose tissue, described as large loss of BCS, is the result of an inadequate adaptive response to the new physiological state characteristic of the transition period. An excessive mobilization of lipids seems driven by an increase in the sensitivity to catecholamine-stimulated lipolysis (McNamara and Hillers, 1986). This process is concomitant to a reduction in sensitivity to the antilipolytic effect of insulin. These two process are considered among the main reasons for the unsuitable response by the adipose tissue to the adaptation to NEB (McNamara and Hillers, 1986). De Koster et al., (2016) demonstrated that larger fat cells have a higher sensitivity toward lipolytic signals (De Koster et al., 2016). Hence, overconditioned cows which have the larger adipocyte volume and the greater amount of fat available to mobilize are at the greater risk of maladaptation (Jensen et al., 1989; Rukkwamsuk et al., 1999; Kokkonen et al., 2005). Obese cows have a larger amount of adipose tissue, which is associated with adipose sensitivity (Smith and McNamara, 1990). At the hepatic level, poor adaptation is related to an excess uptake of NEFA which increases partial beta-oxidation and release of ketone bodies. In the situation where the capacity of lipid oxidation in the liver is exceeded, the result is an intensification of TAG accumulated in the liver with an increased risk of metabolic dysfunction. This situation is often observed in dairy cows with maladaptation during the peripartum period.

### *1.5.3 Parity, Calving Season, Breed, and Herd Size*

Following there is a brief review of other important risk factors associated with HYK. The prevalence of HYK increases with parity. Multiparous cows are more likely to be diagnosed with HYK (Berge and Vertenten, 2014; Rathbun et al., 2017). A prevalence of 4.0% and 19.9% have been found for primiparous and multiparous Holstein cows respectively (Chandler et al., 2018). Vanholder et al., (2015) reported that the odds of cows in the second lactation of developing HYK was 2.1 times higher than for primiparous cows. In addition cows in the third lactation or greater had 2.8 times higher odds of HYK development than primiparous cows.

The season of calving has also been associated with HYK. Although there are substantially different climatic conditions across countries, this association remains relatively consistent. In the Netherlands, cows that calved during late winter and spring had 1.8 and 1.4 times higher odds of developing HYK than cows that calved in the fall (Vanholder et al., 2015). Similarly, in Canada, cows calving in winter and spring also had a higher prevalence of HYK (26%, and 25% respectively) compared to summer and fall (20%, and 18% respectively) (Tatone et al., 2017). Meanwhile, in a study including multiple countries in Europe, spring was reported as the season of the higher prevalence of HYK (Suthar et al., 2013). However, there are no clear biological reasons to explain the increased frequency of the disease in the spring. Besides temperature, factors of seasonality related to HYK could include reduced feed quality and changes in management practices such as reduced labor spent on fresh cows.

Breed is another factor associated with HYK. Jerseys have been reported to be more susceptible to HYK than Holstein cattle, having over 1.46 times higher odds of

developing HYK than the latter (Santschi et al., 2016; Tatone et al., 2017; Chandler et al., 2018). Multiple studies have found differences between Holstein and Jerseys in terms of body fat accumulation, the energy required for maintenance and lactogenesis, and milk composition (Solis et al., 1988; Bitman et al., 1996; Enevoldsen and Kristensen, 1997). These may contribute to the different risk of HYK according to the breed. However, further investigation is required to confirm and determine the reasons for different BHB concentrations across breeds.

Herd size has been identified as a potential risk factor for HYK. A European study enrolling 131 herds and sampling at least 12 cows per herd showed that a 100-cow increase in the herd size reduced the prevalence of HYK by 10% (Berge and Vertenten, 2014). A reason for this finding is a potential higher assistance of nutritional services in larger herds leading to a feed quality more suitable for the different lactation stages. Additionally, other factors such as management routine and veterinary assistance, habitual in larger herds may mediate the association between the size of the herd and occurrence of HYK.

## 1.6 Consequences of elevated BHB on productive and health outcomes

### *1.6.1 Milk yield*

Previous studies evaluating the association between HYK and milk production in early lactation have obtained conflicting results. Some studies observed milk losses among hyperketonemia cows ranging from 1.2 to 4.4 kg/day at the first test-day (Chapinal et al., 2012b; McArt et al., 2012a; Santschi et al., 2016), while others observed a milk yield increase ranging from 1.2 to 2.4 kg during the same period (Vanholder et al., 2015; Rathbun et al., 2017; Ruoff et al., 2017). The week in which

HYK is diagnosed seems to play a role in these contradictory results. Some of the studies that found a milk yield reduction, reported the largest reduction among hyperketonemic cows diagnosed in the first week of lactation (Duffield et al., 2009; McArt et al., 2012a). Cows with HYK in week 1 have been reported to have a higher level of insulin resistance, elevated fat accumulation in the liver which worse its hepatic gluconeogenic capacity, and oxidative stress compared with hyperketonemic cows diagnosed in week 2 of lactation (Emery et al., 1992; Xu et al., 2015; Youssef and El-Ashker, 2017). Therefore, a different adaptation to the new lactating physiological state by cows that developed HYK in the first or the second week of lactation may be the cause of these differences in milk yield.

The concentration of BHB is another variable that may confound studies. There is a dose-response where higher levels of BHB have a worse effect on health and productivity (Walsh et al., 2007; Duffield et al., 2009). Elevations of BHB concentration mean that the animal is mobilizing energy resources from fat storages and adapting to the NEB. However, an excess in BHB concentration will lead to a drop in milk yield. Studies that dichotomize the exposure (i.e., disease positive or negative) loss granularity especially among positive individuals.

Reports indicate that the association between HYK and milk yield differs by parity (Ospina et al., 2010). Santschi et al., (2016) observed that primiparous cows produced 2.1 kg less milk when hyperketonemic, meanwhile the reduction was 2.3 and 3.0 kg for second and third or greater lactation cows respectively. An explanation could be that cows in first lactation lack of multiple risk factors of HYK (e.g., previous lactation milk yield and metabolic diseases) compared to multiparous cows. In addition, according to Bauman and Currie (1980), first lactation cows are still

partitioning energy for growth besides maintenance and lactogenesis, and the milk yield is less compared with multiparous cows. Thus, first lactation cows mobilize energy resources more readily than the latter.

### *1.6.2 Milk composition*

The occurrence of HYK has been associated with an increase in milk fat, and reduced milk protein and lactose. Although energy corrected milk has not been extensively studied, it is reported to increase in hyperketonemic cows. First, second, and third and greater lactation hyperketonemic cows produced 0.6, 1.4, and 1.2 kg of ECM per day than their non-HYK counterparts (Santschi et al., 2016), indicating a differential increase of ECM by parity being higher in multiparous. Additionally, a greater increase in ECM has been observed in cows diagnosed in the first week rather than the second week (Rathbun et al., 2017). Milk fat increase has been reported in the range between 0.10% to 0.66% (van der Drift et al., 2012; Vanholder et al., 2015) according to different factors. Among HYK+, first, second, and third and greater lactation showed a similar increase in milk fat percentage (Santschi et al., 2016). Despite this, an increase in milk fat of 0.36% and 0.18% was observed in the first milk test-day when HYK was diagnosed in the first and second weeks respectively (Santschi et al., 2016). These results suggest that parity may not have a strong effect on milk fat variability as have the week of diagnosis of HYK. The effect of HYK is more intense when HYK occurred in the first week postpartum rather than in subsequent weeks. The composition of fat in milk also has been reported to vary in hyperketonemic cows by decreasing the de novo and medium-chain fatty acids (Mann et al., 2016). A reason for these decreases can be due to the less metabolically active

mammary gland as long-chain fatty acids captured from the bloodstream (i.e., NEFA) inhibit de novo fatty acids (Benedet et al., 2019). Cows with HYK have shown a reduction of protein percentage in the milk of 0.10% to 0.16% (Vanholder et al., 2015; Santschi et al., 2016; Rathbun et al., 2017). Parity has shown no to be associated with the percentage of protein change in milk (Santschi et al., 2016). However, since other components such as milk yield and milk fat percentage have been shown to vary across parties more studies are needed to elucidate how parity is related to protein percentage in milk. Lastly, lactose has been reported to be 0.17% and 0.12% lower in hyperketonemic primiparous and multiparous cows respectively (Santschi et al., 2016). Since glucose is a precursor of lactose, and there is an inverse relationship between BHB concentration and glucose availability this finding is expected.

As described, there is a lack of evaluation of the effect that HYK has on the entire lactation neither in milk volume nor composition which is necessary to understand the impact of the disease in the long term as most studies have follow the lactation for 1 to 3 months. In addition, these evaluations need to be done considering the moment of detection of HYK as it has been described to influence the effect of the disease in both milk production and reproduction performance.

### *1.6.3 Reproductive performance*

An association between NEB and impaired reproductive performance has been identified in multiple studies. Walsh et al., (2007) found that hyperketonemic cows had a reduction of 20% in pregnancy rate and were 50% less likely to be pregnant at the first insemination. A decrease in the hazard ratio of pregnancy success within 70

days post-voluntary waiting period (HR = 0.87, *P* = 0.10) was also reported by (Ospina et al., 2010). In the same direction, Rutherford et al., (2016) found a greater number of inseminations per pregnancy (2.8 v. 2.0), shorter estrus activity, and longer interval from calving to first observed estrus in HYK cows compared to healthy cows. However, is important to mention that not all studies have linked high NEFA and BHBA concentrations around calving with impaired reproductive performance (Chapinal et al., 2012a; McArt et al., 2012a). The detrimental reproductive performance previously mentioned may be attributed to an increased risk of delayed commencement of ovulation (Butler, 2003). The partitioning of energy to colostrum and milk yield during the peripartum period occurs at the expense of reproductive performance (Herdt, 2000). The lack of energy available during NEB affects hypothalamic responsiveness to circulating estradiol leading to a reduction in GnRH pulse frequency and thus a reduction in LH necessary for follicular ovulation (Dawuda et al., 2002). Because the ovulation of a dominant follicle is dependent on the re-establishment of pulsatile LH secretion, a failure on the re-establishment of pulsatile LH secretion can result in the development of a non-ovulatory dominant or even cystic follicle which prolongs the interval for first ovulation 40 to 50 days postpartum (Butler, 2003).

Reducing the time to first ovulation allows enough time to completion of ovarian cycles before insemination improving the conception rate (Butler and Smith, 1989). Leroy et al., (2006) observed a direct toxic effect of NEFA and BHBA on the maturation of oocytes. However, further studies are necessary to better understand the direct role of BHB on reproductive impairment.

## 1.7 Treatments for hyperketonemia

### *1.7.1 Propylene glycol*

Propylene glycol (PG) is the most efficient and preferred option (Gordon et al., 2013). Propylene glycol is a glycogenic product. In the rumen, propylene glycol is either absorbed directly or converted to propionate which will be used to stimulate the gluconeogenesis process (Herdt, 2000). After 30 minutes of PG administration, there is a spike in insulin concentration which remains increased for about 2 hours post-administration (Studer et al., 1993). This increase in insulin together with an increase in glucose concentration induces a reduction of lipolysis and consequently NEFA and ketone bodies (Nielsen and Ingvartsen, 2004). A minimum oral dosage of 300 mL of PG is necessary to decrease BHB concentration in fresh cows, while the longest lasting effect appears at 500 mL (Maurer et al., 2017).

In the trial of McArt et al., (2011), cows with subclinical ketosis (i.e., BHB ≥1.2 and ≤3.0 mmol/L) receive 300 mL of PG. Treated cows were 50% less likely to develop clinical ketosis, and were 1.5 times more likely to resolve hyperketonemia. In addition, they had a 50% reduction on early removal from the herd in the first 30 DIM, were 30% more likely to conceive at the first service, and produced 1.6 kg more milk per cow per day in certain herds, than control cows (McArt et al., 2012b). However, other researchers using variations in the protocols have reported lack of benefits of PG in terms of milk yield in early lactation (Juchem et al., 2004; Østergaard et al., 2020).

Multiple combinations between PG and other therapies such as dextrose, glucocorticoids, dexamethasone, and vitamin B12, have been investigated resulting in

various and generally poor benefits (van der Drift et al., 2015; Weerathilake et al., 2019; Capel et al., 2021).

## 1.8 Summary

The increase of BHB during the transition period of dairy cows as a response to the negative energy balance is expected and physiological. It occurs because feed intake cannot match the energy required to meet the high demand of energy necessary to carry the pregnancy and onset of milk production. Failure to rapidly shift the use of energy from glucose to TAG increases the BHB and indicates a poor adaptive response to the NEB. The increase of BHB leads to negative effects on the health, productivity and well-being of dairy cows.

As mentioned during the review, there are still multiple knowledge gaps that need to be addressed to improve management strategies to prevent, diagnose and control hyperketonemia.

Although there is available a variety of cow-side ketometer devices to diagnose hyperketonemia using blood that generates rapid results, it is unclear the performance of these devices under certain field and laboratory conditions including variations in hematocrit concentration, use of thawed samples, and/or samples containing anticoagulants. Furthermore, to implement successful preventive and control strategies of the disease, a better understanding is needed of the influence of the energy balance during the late dry period in the patters of BHB concentration and HYK in early lactation. And the role that milk yield in early lactation plays in hyperketonemic cows due to the importance that the lactogenesis process has in the energy balance needs to be explored. In terms of diagnostics, it has been reported a

differential effect of HYK in multiple health outcomes and production according to the moment of disease diagnosis. However, these effects have been evaluated only in the short term. Understanding the effect of HYK in the entire lactation according to the moment of disease diagnosis is needed to determine the optimum timeframe for diagnosis.

## 1.9 Research Objectives

The aim of this thesis was to better understand the epidemiology of HYK to guide implementation of optimal decisions to diagnose and mitigate the occurrence of the disease. The following specific objectives will address this goal:

**Objective 1.** Evaluate the test characteristics of a specific device to measure β-Hydroxybutyrate concentration in blood in reference to the laboratory assay, and the best practices to maintain an appropriate diagnostic performance in farm settings.

**Objective 2.** Describe the temporal patterns of β-Hydroxybutyrate concentration, development of hyperketonemia in early lactation, and changes in milk yield and composition based on body condition score, as both a single measurement and its change over the late dry period.

**Objective 3.** Recognize the differential impact of hyperketonemia according to the moment of diagnosis during early lactation on milk yield and composition, reproductive performance, and herd removal.

**Objective 4.** Explore the role of milk yield during the first week after parturition, on the relationship between hyperketonemia diagnosed in early lactation and reproductive performance.

# CHAPTER 2 – Diagnostic accuracy of a bovine specific electronic beta-hydroxybutyrate handheld meter in fresh blood and stored serum samples

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Z. Rodriguez; L.S. Caixeta; G. Cramer (2020). Diagnostic accuracy of a bovine specific electronic beta-hydroxybutyrate handheld meter in fresh blood and stored serum samples. Veterinary and Animal Science 11, 100159.

## 2.1 Chapter Summary

This study aims to evaluate the diagnostic accuracy of the bovine specific beta-hydroxybutyrate (**BHB**) meter Nova Vet (**NVET**). We evaluated the accuracy and agreement of the NVET in fresh whole blood and thawed serum with the reference laboratory assay; and the repeatability, the interference by anticoagulants, and the optimum slope calibration factor. Individual blood samples were collected from 200 Holstein and crossbred cows between 3-14 days post-calving from 13 dairy herds in Minnesota. Using a laboratory assay with a cut point of 1.2 BHB mmol/L hyperketonemia prevalence was 10.6% (95% CI: 6.7, 15.8). The sensitivity of NVET in whole blood and serum was 100.0% while the specificity was 98.3 and 97.7% respectively. The agreement between NVET and the laboratory assay was the highest using blood samples (concordance correlation coefficient –CCC = 96.2, 95% CI: 95.0, 97.1. The coefficient of variation including within day (intra-meter), between- days, and -batches was 13.4% when testing blood samples. Minimal interference was observed with the use of anticoagulants (K-EDTA and Li-Heparin, CCC 0.90 and

0.93 respectively) in reference to whole blood without anticoagulant. The best calibration slope factor in serum was 1.0 (Youden's index: 0.98). Results suggest that the NVET device maintained a high accuracy and precision to quantified BHB concentration when applied in fresh blood and thawed serum under field conditions using the default calibration slope (1.0), and with minimal anticoagulant interference when used in whole blood samples.

## 2.2 Introduction

Dairy cows experience a state of negative energy balance as part of a normal peripartum period. To meet the increased energy demand of milk production, dairy cows mobilize fatty acids from the adipose tissue (Bell, 1995). These fatty acids are partially oxidized into ketone bodies, including beta-hydroxybutyrate (**BHB**), to be used as an energy source by extrahepatic tissues (Drackley, 1999; Grummer, 1993). Elevated levels of BHB in blood have been associated with impaired reproductive performance, reduction in milk yield, and increased metabolic disorders (Duffield et al., 2009; LeBlanc et al., 2005; McArt et al., 2012), hence, early detection of high BHB levels is crucial.

Reference laboratory tests typically use a spectrophotometric quantitative assay following an enzymatic reaction to determine BHB concentration in blood. Concentrations between 1.2 and 1.4 mmol/L are often used as a cut point for screening programs to diagnose hyperketonemia (**HYK**; Duffield et al., 2009; Oetzel, 2004; Ospina et al., 2010). However, reference techniques tend to be laborious and time-consuming therefore, multiple electronic handheld methods have been developed to test BHB in the field with variable diagnostic accuracies (Carrier et al., 2004;

Iwersen et al., 2009; Krogh et al., 2011). In order to implement adequate screening programs to detect HYK at the herd and individual level, accurate, precise, and practical diagnostics techniques are essential (Gordon et al., 2013; Tatone et al., 2016). The Nova Vet (**NVET** - Nova Vet, Nova Biomedical Co., Waltham, MA), is a specific device to measure BHB concentration recommended for its use in whole blood, that includes a calibration feature (i.e., slope calibration factor) intended to adjust for the variation of hematocrit among species. This device showed good diagnostic accuracy in blood BHB at the recommended slope setting in 2015 (Bach et al., 2016). However, the optimum field sampling protocols, which can be advantageous for researchers or consultants (i.e., test performance on whole blood or serum samples, anticoagulant used, and unadjusted slope) remains unclear.

This project aimed to evaluate the test characteristics of the NVET in reference to the laboratory assay, and the best practices to maintain an appropriate diagnostic performance in farm settings. To achieve this aim we performed four specific objectives in two different observational studies. In the first study, (a) we evaluated the diagnostic accuracy of the device using whole blood and thawed serum, and (b) determined the best slope calibration factor for serum samples. In the second study, (c) we evaluated the precision intra-meter and between days and batches, and (d) assessed the effect of anticoagulants on test results.

## 2.3 Materials and Methods

### *2.3.1 Study Population and Data Collection – Study 1*

All animal use procedures in this research were approved by The University of Minnesota Institutional Animal Care and Use Committee (#1406-31607A).

For the first study, a convenience sample of 13 dairies in southeastern Minnesota with a history of HYK in the previous year were selected from a cohort of herds participating in the Minnesota Dairy Herd Improvement Association. Selected farms were visited at least once between June and September 2014. Blood samples (n=200) were collected from a random sample of cows between 3 to 14 days postpartum in each farm via the coccygeal vessels using 20-G x 2.54-cm blood collection needles and evacuated tubes without anticoagulant (Becton Dickinson; Franklin Lakes, NJ). The sample size was estimated to identify a desired sensitivity (**Se**) and specificity (**Sp**) of at least 90% and 85% respectively, with 80% power and an α error level of 0.05 and an expected HYK-prevalence of 30% (Dohoo, 2009). Immediately after sampling, a droplet of blood was taken directly from the tip of the needle to measure BHB concentration using the NVET handheld device at the out of the box slope setting of 1.0. Unlike the study by Bach et al, 2016 the 2013 manual for our meter did not include a recommendation to adjust the slope. Blood samples were allowed to clot in a cooler and centrifuged in the laboratory at 2,000-x *g* for 15 min within 3 hours of collection. Two aliquots of serum from each sample were stored in 2.0 mL vials (Sarstedt, Newtonm NC) and frozen at -80° C for later analysis. One aliquot was shipped on ice to the Marshfield Veterinary Laboratory (Marshfield, WI) for BHB determination using a spectrophotometric quantitative assay, LiquiColor test (EKF

Diagnostics-Stanbio, Boerne, TX) on a Beckman Coulter AU5800 (Beckman Coulter, Brea, CA, USA). The inter- and intra-assay coefficient of variation of the reference laboratory test was <1.7 and <5.2%, respectively. After obtaining the laboratory results, the second serum aliquot samples were thawed for one hour at room temperature and tested with NVET device with five different slope calibration factors (0.9, 1.0, 1.1, 1.25, and 1.5 respectively).

### *2.3.2 Study Population and Data Collection – Study 2*

For the second study, blood samples were collected from three Holstein dairy cows between 3-14 days in milk on one day. Cows were housed at the University of Minnesota Dairy Cattle Teaching and Research Facility. Based on the farm's weekly BHB monitoring routine, we randomly selected one cow that had BHB ≥1.2 mmol/L and two control cows with BHB <1.2 mmol/L. On day one, three blood samples were collected from each cow using 10 mL evacuated tubes containing either potassium EDTA (**K-EDTA** - Covidien, Mansfield, MA), Lithium heparin (**LI-HEP** - Becton Dickinson, Franklin Lakes, NJ) or no-anticoagulant (Becton Dickinson; Franklin Lakes, NJ). After collection, blood samples with anticoagulants were gently mixed inverting the tubes 20 times. To assess NVET device repeatability across time and test strip batches we selected samples containing LI-HEP anticoagulant and tested them 20 times using two different testing strips batch dates. After testing, the samples were stored on a blood tube roller (Diagnostic Products Corporation, CA) to prevent clotting, and retested following a similar procedure on day two (n=80). To assess the potential interference among the anticoagulants, individual blood samples containing

either K-EDTA, LI-HEP, or no anticoagulant were tested four times for BHB immediately after collection (n=24).

### *2.3.3 Statistical Analyses*

All statistical analyses were performed using R 3.4.4 software (R. RStudio, Inc., Boston, MA)[1]. The test characteristics in study 1 were calculated using the "epiR" package (Stevenson, 2018). The strength of the association and the level of agreement between devices and the reference method was evaluated with Pearson's correlation coefficient and the concordance correlation coefficients (**CCC**), respectively, using the "DescTools" package (Signorell, 2018). Limits of agreement plot (**LOA)** was performed to visualize the agreement, using the "BlandAltmanLeh" package (Lehnert, 2015). Receiver operating characteristic curves (**ROC**) and the area under the ROC curve (**AUC**) were calculated to determine the most appropriate slope calibration settings for the NVET meter using "pROC" package (Robin et al., 2011). From the ROC curve, the Youden's Index was calculated to set the optimal thresholds based on the highest Se and Sp combined (Dohoo, 2009). For study 2, the coefficient of variation (**CV**) was determined within and between days, and between batch variations, and LOA plot, and CCC test was used to evaluate the interference of different anticoagulants on BHB measurement.

[1] Data set and R scripts available at https://conservancy.umn.edu/handle/placeholder-to-be-determined-upon-approval-of-manuscript.

## 2.4 Results and Discussion

Of the blood samples collected, 99.9% (199/200) were successfully analyzed. 29.1% (58/199) of the animals were in the first, 27.6% (55/199) in the second, and 42.7% (86/199) in their third or greater lactation. The true HYK prevalence was 10.6% (95% CI: 6.7, 15.8) based on the reference test. It is worth noting that the prevalence observed was lower than expected when the sample size was calculated resulting in an increase of the standard errors of the estimates. The Se and Sp were similar in blood and serum samples with better Predictive Values and correlation estimates for blood samples (Table 2.1).

### *2.4.1 Results and Discussion Study 1*

High diagnostic accuracy of the BHB device is crucial for the detection of HYK. The Se and Sp for the NVET in both blood and serum were the same, 100% and 98% respectively, with overlapping confidence intervals (Table 2.1). This contrasts to previously reported results for the NVET using an unadjusted slope calibration for whole blood in which a lower Se (64%) but similar Sp (100%) were observed (Bach et al., 2016). Correlation and concordance between the NVET and the laboratory assay were slightly higher in blood (0.96 both) than in serum (0.92 and 0.89, respectively). Similarities in the results indicate a lack of evidence of either a systematic or a proportional bias. Moreover, LOA plots (Figure 2.1) show that most of the observations for both blood and serum, in reference to the laboratory assay, laid within the limits of agreements. These results indicate that serum samples collected for other purposes can be tested in batches after separation, improving efficiency, and reducing the lock-up time.

The test characteristics of each calibrated slope setting in serum are presented in Table 2.2. All the slopes had an AUC of 0.99, and 95% confidence intervals ranging from 0.97 to 1.00. Based on Youden's index, the best calibration slope factor with a BHB threshold of 1.2mmol/L was the default slope calibration factor of 1.0. Therefore, using the default slope of 1.0 is appropriate when using the NVET in serum to measure BHB concentrations. The agreement between serum and whole blood observed in our study suggests that this slope is likely also appropriate for whole blood.

### *2.4.2 Results and Discussion Study 2*

In study 2, the coefficient of variation including different testing strip batches using samples of whole blood with anticoagulant containing LI-HEP was 13.4% on day one and 12.0% on day two. The precision of the NVET meter was lower than previously reported with the use of samples containing heparin (Bach et al., 2016). However, in our study, we assessed repeatability including different batches of strips and testing days mimicking what can be expected in field conditions, which may have played a role in the lower precision estimates observed. A limitation of our precision results is that we tested a limited number of concentrations; however, these concentrations cover the clinically important range. Anticoagulants can interfere with the determination of certain analytes (Sevastos et al., 2006), which can occur through a variety of mechanisms including the chelation of minerals and the influencing of reagent enzymes (Bowen & Remaley, 2014; Tate & Ward, 2004). In our study, the enzymatic reaction between the BHB and the electrodes that take place in the chemistry layer of the testing strip did not appear to be affected by the presence of LI-

HEP or K-EDTA. The CCC when using K-EDTA was 0.90 (95% CI: 0.85, 0.94) and a LOA mean difference in BHB concentration was - 0.01 (95% LOA -0.16 and 0.14). Blood samples containing LI-HEP had a CCC of 0.93 (95% CI: 0.86, 0.96) and LOA mean difference of 0.07 (95% LOA -0.06 and 0.19.) Thus, the effect caused by K-EDTA or LI-HEP in the measurement of BHB was minimal compared to results from blood samples without anticoagulants. This suggests that in scenarios where a large number of blood samples need to be collected in a short period to obtain plasma, the use of these samples collected with an anticoagulant can be considered an alternative to immediate testing.

## 2.5 Conclusion

In summary, based on our results the NVET is an acceptable alternative to the laboratory assay to measure BHB in whole blood with or without anticoagulant or serum samples using the default calibration slope setting with acceptable accuracy and precision for screening purposes.

Table 2.1: Diagnostic test characteristics of NVET BHB meter on whole blood and thawed serum samples with a BHB threshold of ≥1.2 mmol/L.

| Characteristics of the tests | NVET on blood (95% CI) [1] | NVET on serum (95% CI) [1] |
|---|---|---|
| Apparent prevalence (%)[2] | 12.1 (7.9, 17.4) | 12.6 (8.3, 18.0) |
| Sensitivity | 1.00 (0.83, 1.00) | 1.00 (0.84, 1.00) |
| Specificity | 0.98 (0.98, 1.00) | 0.98 (0.94, 99.3) |
| Positive predictive value[3] | 0.87 (0.75, 0.99) | 0.84 (0.64, 0.95) |
| Negative predictive value[3] | 1.00 (0.98, 1.00) | 1.00 (0.98, 1.00) |
| CCC[4] | 0.96 (0.95, 0.97) | 0.89 (0.86, 0.91) |
| Pearson's correlation | 0.96 (0.95, 0.97) | 0.92 (0.89, 0.94) |

[1] Compared to the reference test (EKF Diagnostics-Stanbio, Boerne, TX). Estimates include the 95% confidence intervals of the estimate.
[2] The estimated true prevalence by the laboratory assay (reference test) was 10.6%.
[3] Predictive values were calculated based on the estimated prevalence.
[4] Concordance Correlation Coefficient between the devices and the reference test.

Table 2.2: Diagnostic test characteristics of Nova Vet (NVET) meter measuring beta-hydroxybutyrate on blood using ≥1.2 mmol/L threshold under different slopes calibration factor.

| Slope Factor[1] | Sensitivity (95% CI) | Specificity (95% CI) | Positive Predictive Value (95% CI) | Negative Predictive Value (95% CI) | Youden's Index[2] |
|---|---|---|---|---|---|
| 0.9 | 0.90 (0.70, 0.99) | 0.99 (0.97, 100) | 0.95 (0.75, 100) | 0.99 (0.96, 1.00) | 0.89 |
| 1.0[3] | 1.00 (0.84, 1.00) | 0.98 (0.94, 0.99) | 0.84 (0.64, 0.95) | 1.00 (0.98, 1.00) | 0.98 |
| 1.1 | 1.00 (0.84, 1.00) | 0.93 (0.89, 0.96) | 0.64 (0.45, 0.80) | 1.00 (0.98, 1.00) | 0.93 |
| 1.2 | 1.00 (0.84, 1.00) | 0.80 (0.74, 0.86) | 0.38 (0.25, 0.51) | 1.00 (0.97, 1.00) | 0.80 |
| 1.5 | 1.00 (0.84, 1.00) | 0.63 (0.55, 0.70) | 0.24 (0.16, 0.35) | 1.00 (0.97, 1.00) | 0.63 |

[1] Slope calibration factor.
[2] Youden's Index represents the optimal threshold of a diagnostic test that maximizes overall classification (i.e., higher sensitivity and specificity combined). It is calculated as the sum of sensitivity and specificity minus one).
[3] Default slope calibration factor.

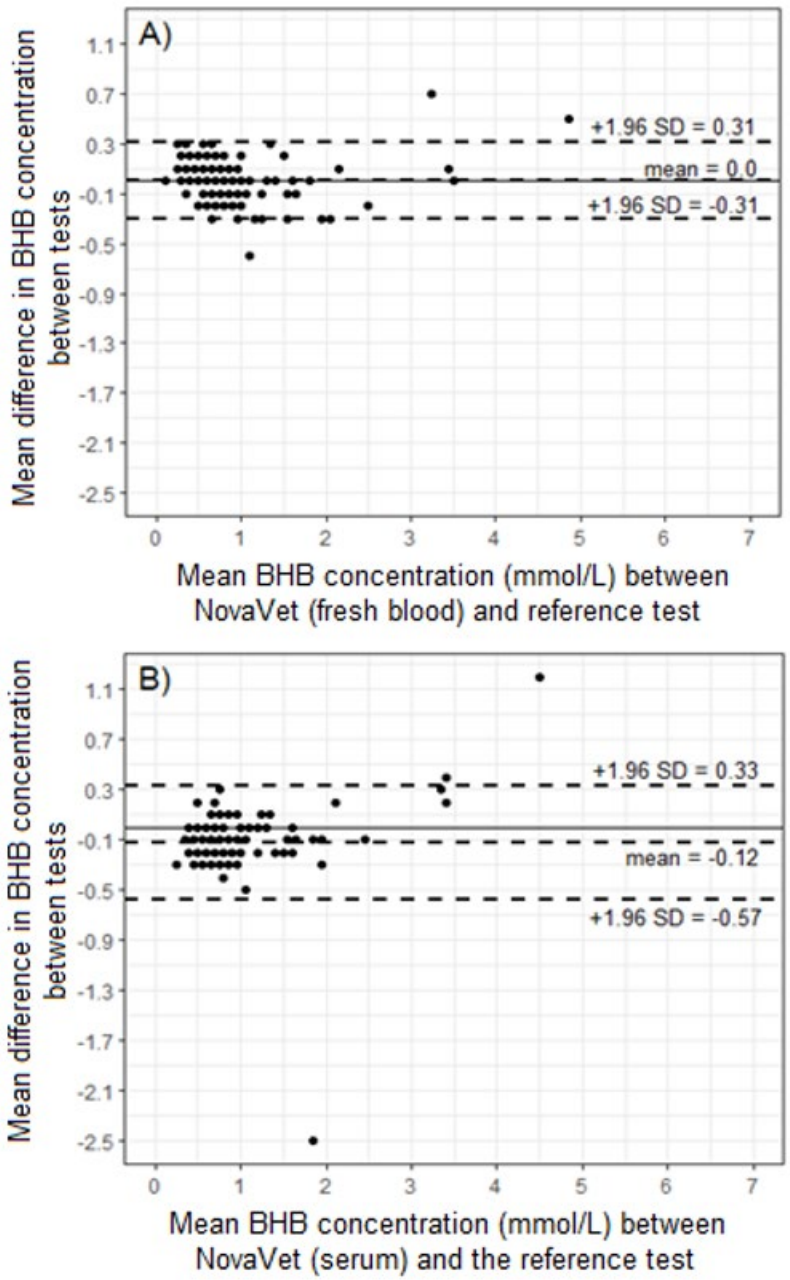


Figure 2.1: Bland Altman plot (limits of agreement) representing the differences in measurements of beta-hydroxybutyrate (BHB) concentrations between the reference test and Nova Vet on (A) fresh blood, and (B) thawed serum. The solid line represents no mean difference of BHB concentration between tests. The dashed middle line represents the mean difference. The upper and lower dashed lines represent the 95% limit of agreement. Analysis performed using n = 199 samples from Holstein cows between 3 and 14 days in milk.

# CHAPTER 3 – Association of Body Condition Score and Score Change during the Late Dry Period on Temporal Patterns of Beta-Hydroxybutyrate Concentration and Milk Yield and Composition in Early Lactation of Dairy Cows

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## 3.1 Chapter Summary

Monitoring the body condition score (BCS) of dairy cows is a management strategy that can assist dairy producers in decision-making. The BCS and its variations reflect the level of body fat reserves and fat mobilization throughout the different stages of lactation. Cows that mobilize excessive amounts of fat reserves in response to the increased energy requirements of the transition period are more likely to have higher beta-hydroxybutyrate (BHB) concentration in blood, leading to a higher incidence of hyperketonemia postpartum. In this study, our main objective was to evaluate how both BCS (at 21 d prior to the expected calving date, −21BCS) and change in BCS during the late dry period (−21 d to calving, ΔBCS) are associated with temporal patterns of blood BHB concentrations during the first two weeks of lactation. Our secondary objective was to characterize the relationship between the change in BCS in the late dry period, and milk yield and milk composition in the first milk test postpartum. In this retrospective cohort study, we assessed BCS at 21 (±3) days

before the expected calving date and within three days after calving. Blood BHB concentration was measured at days 3 (±1), 7 (±1), and 14 (±1) postpartum. Hyperketonemia (HYK) was defined as blood BHB ≥ 1.2 mmol/L. To evaluate how −21BCS and ΔBCS during the late dry period were associated with BHB in early lactation, linear mixed-effects regression models with an unstructured covariate matrix were performed. The association between ΔBCS and incidence of postpartum HYK were determined using a multivariable log-binomial model. A linear regression model was used to evaluate the association between ΔBCS and milk yield and milk composition in the first monthly test-day. Covariates used for model adjustment include parity, season, and baseline BCS. We observed that cows with BCS ≥ 4.0 at 21d before their expected calving date had the highest BHB concentration postpartum, but no evidence that BCS ≥ 4.0 at 21d was associated with fluctuations of BHB over time. Cows that experienced a large BCS loss (larger than 0.5 units) during the late dry period had a 61% (95% CI: 1.04, 2.50) higher risk of developing HYK in early lactation and had higher BHB concentrations during early lactation compared with cows with no ΔBCS prepartum. These associations were observed independently of the BCS at −21d prepartum (base-line). In addition, cows that lost more than 0.5 BCS unit in the late dry period produced 3.3 kg less milk (95% CI: −7.06, 0.45) at the first milk test compared to cows that had no ΔBCS during the late dry period. No evidence of an association between −21BCS and ΔBCS in the late dry period and milk composition was observed in our study. These results suggest that dynamic measures of BCS during the late dry period, such as ΔBCS, are better at predicting blood BHB patterns in early lactation than BCS measured at a single time point. Cows with larger BCS loss during the late dry period and with greater parity are more likely to have

higher concentrations of blood BHB post-partum, with the highest concentrations reported at 7 d post-calving.

## 3.2 Introduction

Body condition score (**BCS**) is a subjective, visual scoring system used to estimate body fat storage in cattle (Ferguson et al., 1994). The change in BCS (Δ**BCS**), rather than BCS measured at a single time point, is often used during the transition period from late pregnancy to lactation as a method of evaluating mobilization of fat reserves in dairy cattle (Bewley et al., 2010; Morin et al., 2017). The demand for energy in dairy cows during the peripartum period is elevated because of fetus growth and the concomitant onset of milk production. However, feed intake rarely matches energy requirements, leading to a period of energy deficit. Thus, the peripartum period is characterized by increased mobilization of fat and other body reserves in an attempt to overcome the shortage of energy (Herdt, 2000). When fat mobilization occurs in excess, it can overwhelm the capacity of the liver to completely oxidize fatty acids. In this situation, the breakdown of fatty acids leads to an increase in the synthesis and release of ketone bodies into the bloodstream (i.e., ketogenesis). When β-hydroxybutyrate (BHB), the most stable blood ketone body, reaches concentrations ≥ 1.2mmol/L, cows are considered to have hyperketonemia (HYK) (Herdt, 2000; Duffield et al., 2009). The importance of HYK in the dairy industry stems from its association with reduced immune function, impaired reproductive performance, decline in milk yield, and concomitant metabolic diseases (Suriyasathaporn et al., 2000; Walsh et al., 2007; Ospina et al., 2013).

A positive association between BCS and elevated concentrations of blood BHB in early lactation has been previously described by others (Rathbun et al., 2017; Sheehy et al., 2017). However, the relationship between both BCS 21d from expected calving date (**-21BCS**) and ΔBCS during the late dry period (i.e., 3 weeks before the calving date; (Dann et al., 2006)) with the fluctuations of BHB concentrations through early lactation have not yet been characterized. Furthermore, most studies evaluating ΔBCS focus on the entire dry period or on early lactation (Loeffler et al., 1999; Pryce et al., 2001; López-Gatius et al., 2003). Although this is reasonable from a management perspective, considering that most diseases occur during early lactation, variations in body condition specifically during the late dry period play a fundamental role in the control of the diseases observed later in the lactation (LeBlanc et al., 2006; Roche et al., 2009; Ribeiro et al., 2013; Sheehy et al., 2017). Therefore, a better comprehension of the influence of both -21BCS and ΔBCS on the fluctuations of blood BHB concentrations during early lactation can be useful in the development of strategies to monitor and mitigate high incidences of hyperketonemia in dairy herds.

We aimed to assess the contribution of prepartum BCS to temporal patterns of BHB concentrations during the early lactation period. Our specific objectives were to describe the temporal patterns of BHB concentration and development of HYK in early lactation on the basis of BCS as both a single measurement and its change over the late dry period. We also aimed to characterize the relationship between change in BCS and milk yield and composition in the first monthly milk test. We hypothesized that the change in BCS during the late dry period is a better variable to evaluate the temporal patterns of blood BHB concentration during early lactation than a single BCS measurement. Additionally, a substantial loss in BCS during the dry period is

expected to be associated with higher BHB concentration in early lactation and variations in milk yield and composition.

## 3.3 Materials and methods

### *3.3.1 Study Population and Data Collection*

The study was conducted according to the guidelines of the University of Minnesota Institutional Animal Care and Use Committee, protocol number: 1806-36016A. This retrospective cohort study was performed as part of another field study evaluating an algorithm for the early prediction of HYK. Therefore, the sample size of this study can be considered as a convenience sample. The study was conducted on a commercial dairy farm located in Minnesota between February and November of 2019. The herd had 1,200 lactating Holstein dairy cows, used headlocks in both dry and fresh cow pens, fed a total mixed ration formulated to meet animals' nutrient requirements, maintained reliable data records, and used a computerized data recording system (DairyPlan C21, GEA Farm Technologies). Dry cows were housed in free-stall barns with recycled manure solid bedding and were moved to a maternity stall, bedded with straw, as soon as calving signals were identified. Lactating cows were milked three times a day and the farm participated in the Dairy Herd Improvement (DHI) program.

Our research team visited the herd three times a week for sample collection. Cows were body condition scored at two time points: −21 (±3) days (**-21BCS**) and 2 (±1) days relative to calving using a 5-point scale with quarter-point increments (Ferguson et al., 1994). In this scoring system, cows scored as 1 were considered emaciated while cows scored as 5 were considered obese. The anatomical regions considered in

the measurement of BCS included the thoracic and vertebral region of the spinal column (chine, loin, and rump), the ribs, the spinous processes (loin), the tuber sacral (hip or hook bones), the Ischial tuberosity (pin bones), the anterior coccygeal vertebrae (tail head), and the thigh region (Roche et al., 2004). If cows were lying in the stalls, the research team made the cows stand for a better measurement. Scoring at calving was performed at least 1 day after calving to minimize a potential risk of over or underestimation of the BCS measured near calving due to the relaxation of anatomic regions (i.e., tail head ligaments). At the beginning of each visit, the research team rated the first 10 cows together to confirm scoring similarities between observers. The research team did not have access to the previous BCS when scoring BCS at calving. Parity and season were recorded at calving.

After calving, blood samples were collected immediately after morning milking at 3 (±2), 7 (±2), and 14 (±2) days in milk (DIM) to measure BHB concentration. All samples were collected from the coccygeal vessels using a 20-gauge, 2.54-cm blood collection needle and a vacuum tube with lithium-heparin (Becton Dickinson, Franklin Lakes, NJ). Blood samples were immediately placed in a cooler after collection and transported on ice to the laboratory. Within 3 hours of collection, samples were centrifuged at 2,000x *g* for 15 min for plasma separation and kept frozen at -20°C for later analysis. Once all samples were collected, BHB was measured in serum samples using a commercial kit colorimetric assays and an automated small-scale spectrophotometric chemistry analyzer (CataChemWell-T; Catachem Inc., Oxford, CT) as described by others (Abuelo et al., 2020). Intra- and inter-assay coefficient of variation of 3.9 and 13.3% respectively.

### *3.3.2. Statistical Analyses and Model-Building*

Statistical analyses were completed in Stata version 16.0 (StataCorp, College Station, TX). The study unit was the individual cow.

The -21BCS measurement was dichotomized as moderate (BCS ≤ 3.75 - reference) or overweight (BCS ≥ 4.0). The ΔBCS during the late dry period was calculated by subtracting the BCS at -21 days prepartum from the BCS measured around calving, and was categorized as moderate gain (ΔBCS range: > 0 to 0.5), no change (ΔBCS = 0 - reference), moderate loss (ΔBCS range: -0.5 to < 0), or large loss (ΔBCS < -0.5). The relationship between -21BCS and ΔBCS during the late dry period and differences in BHB concentration over the postpartum period was estimated by linear mixed-effects regression using an unstructured covariance pattern modeling (Hedeker & Gibbons, 2006). Thus, the interaction between BCS and time was evaluated. The unstructured error covariance matrix was deemed as the best fit after examining a number of possible covariance matrices – including independence, exchangeable, autoregressive, and Toeplitz – based on comparison of the Akaike's Information Criterion (AIC) score for each covariance matrix.

Due to the fact that the sample size was originally calculated for another field study, we performed a post hoc power calculation for the association between ΔBCS and BHB concentration change over time (main objective of the current study). This calculation was performed using Hotelling-Lawley Trace power calculation test, 0.05 significance level, three ordinal measurements, group size information from Table 3.1, the BHB concentrations observed (reported in figure 3.3), 0.8 based correlation, and 0.1 decay between repeated measurements.

To determine the association between -21BCS and ΔBCS prepartum and risk of hyperketonemia postpartum, we performed log-binomial regression models. Cows were considered to be hyperketonemia positive (HYK+) if blood BHB concentrations were ≥ 1.2 mmol/L at any time point during the first 2 weeks postpartum, otherwise they were considered hyperketonemia negative (HYK-).

The association between ΔBCS and milk yield (kg), energy corrected milk (kg), and fat and protein percentage at the first monthly milk test was evaluated using multivariable linear regression models and reported as mean differences. Energy corrected milk (ECM) was calculated using the following formula: ECM (kg) (3.5% fat, 3.2% protein) = (0.3246 ∗ kg milk) + (12.86 ∗ kg fat) + (7.04 ∗ kg protein) (Bernard, 1997).

Missing data of BCS measurements (exposures) and the BHB concentrations (outcomes) were imputed performing a multiple imputations approach. Briefly, multiple imputations allow for the uncertainty about the missing data by creating several different plausible imputed data sets and combining the results from the analysis performed on each of them. We created 10 data sets using the multiple imputations under the assumption of missing data at random (MAR) (Newgard and Lewis, 2015). We tested this assumption by evaluating significant differences in BHB concentrations between available and missing data groups at each time point, concluding that the MAR assumption holds.

To account for potential confounding effects, main and effect modification (EM) models were subjected to testing for potential confounding variables. The following covariates were offered to the models: parity (lactation =1; lactation = 2; and lactation ≥ 3) and season (spring = February – April; summer = May – July; and fall = August

– October). Baseline BCS was offered as a potential confounding variable to ΔBCS model. The confounding effects were assessed based on a 10% change in the main exposure estimate criterion (Greenland and Pearce, 2015) using a manual backward stepwise elimination procedure. Once confounding effects were assessed, biologically plausible 2-way interactions were investigated. Interactions with a $P < 0.05$ were retained in the final models.

## 3.4 Results

### *3.4.1 Descriptive Statistics*

Measurements of BCS (d -21) and ΔBCS (d -21 to d 0) were obtained in 587 and 559 cows, respectively. Measurements of BHB concentration were obtained in 591, 607, and 602 cows at 3, 7, and 14 DIM, respectively. After multiple imputations, we culminated with complete data of the 637 cows originally enrolled in the study, in each of the 10 imputed datasets. As each of the 10 imputed datasets were analyzed separately – thus resulted in different, albeit similar, estimates – the reported results are presented as the average estimate of the 10 analyses. Given that only six cows gained more than 0.5 points of BCS, we decided not creating a large gain group, and remove these cows from the analyses.

The average BCS according to the categorical level of the exposure variables are presented in Table 3.1. In general, moderate BCS cows gained or maintained BCS while overweight cows lost BCS during the last dry period. The descriptive statistics of each predictor by hyperketonemia group (i.e., HYK+ or HYK-) at each sampling point postpartum are presented in Table 3.2. The prevalence of HYK was 10.6%, 15.8%, and 11.9% on days 3, 7, and 14, respectively. The cumulative incidence of

hyperketonemia during the two weeks postpartum was diagnosed in 24.0% (n = 153) of the 637 Holstein dairy cows in the study. The incidence of HYK in the first 14 days postpartum was higher among cows with some degree of BCS loss during the late dry period than among cows with no change or gain in BCS in the same period (Figure 3.1). Primiparous cows had the lowest incidence of HYK at 4.1% followed by second parity at 25.6% and third or greater parity at 44.2%. The incidence of HYK in early lactation was 18.6% in spring, 25.0% in summer, and 30.9% in fall. The average BHB concentration across the three time points postpartum was 0.52 mmol/L for first lactation cows, 0.79 mmol/L for second lactation cows, and 0.99 mmol/L for third or greater lactation cows. Correlation between time points within-cow ranged from 0.35 (95% CI: 0.27, 0.42) to 0.46 (95% CI: 0.38, 0.53).

### *3.4.2 Body Condition Score 21 Days Before Calving Date*

The incidence of HYK in the first 14 days postpartum was 14.8% (n = 32) for moderate (BCS ≤ 3.75) and 29.6% (n = 110) for overweight cows (BCS ≥ 4.0). Cows considered overweight 21d before their expected calving date had a 63% (95% CI: 1.16, 2.28) higher risk of developing HYK than cows with moderate -21BCS. Blood BHB concentration was, on average, 0.08 mmol/L (95% CI: 0.02, 0.14) higher in overweight cows than cows with moderate -21BCS. However, when the interaction term of BCS and time was included (variation in BHB measurements across the sampling period), there was no evidence that -21BCS was associated with changes in BHB concentrations over the first two weeks postpartum ($P = 0.41$). Further analyses showed evidence that the BHB concentrations across early lactation varied according

to the parity of the cow (Figure 3.2), with BHB concentrations remaining lower in primiparous cows across all three samplings time points.

### *3.4.3 Change in Body Condition Score*

After model adjustment, cows that experienced a large loss in BCS (ΔBCS < -0.5) during the late dry period had a 61% (95% CI: 1.04, 2.50) higher risk of developing HYK in early lactation than cows with no change in BCS (ΔBCS = 0) prepartum (Table 3.3). Cows with a moderate loss in BCS (ΔBCS = -0.5 to < 0) prepartum also had an increased risk of developing HYK in early lactation but to a lower extent (RR = 1.41, 95% CI: 0.95, 2.10) than cows with large losses in BCS. Cows with a moderate gain in BCS (ΔBCS = 0 to 0.5) during the late dry period had a similar risk of developing HYK as cows that had no change in BCS. Parity was a confounder in the association between ΔBCS and HYK, unlike season, which was removed from the model (Table 3.3). We observed that the risk of HYK increased with parity. Thus, after controlling for ΔBCS during the late dry period, the risk of developing HYK was 88% (95% CI: 96, 82) and 75% (95%CI: 57, 17) lower for first and second parity cows, respectively, compared to third or greater parity cows. Unlike BCS measured at a single time point, ΔBCS was associated with variations of BHB concentration over early lactation. Figure 3.3 shows the pattern of BHB concentrations in early lactation according to ΔBCS during the late dry period. Cows that experienced a large loss in BCS during the late dry period had higher BHB concentrations during the first two weeks postpartum, with the peak BHB concentration at 7 DIM (1.07 mmol/L, 95% CI: 0.96, 1.18) and a more pronounced decline towards the end of the second week postpartum. Unlike cows with large losses in BCS, cows that experienced only a

moderate loss in BCS during the prepartum period had similar BHB concentrations over time than cows with no ΔBCS and cows with a moderate gain in BCS. The post hoc power calculation for this association was 89.5%.

Table 3.4 shows the marginal mean and mean differences in milk during the first monthly milk test by level of BCS change during the late dry period. Results suggest that cows that experienced any degree of BCS change in any direction during the late dry period experienced a decline in milk yield compared to cows that remain the same BCS, independent of -21BCS (baseline BCS). After adjusting milk yield by fat and protein percentage (i.e., energy corrected milk), the decline in milk yield remained only among the group of cows with a large loss in BCS (Table 3.5). There was no evidence that protein percentage varied with variations in BCS (Table 3.5).

## 3.5 Discussion

In the current study, we aimed to evaluate the dynamics of BHB concentrations in the first 2 weeks postpartum according to BCS at a single time point during the late dry period and the change in BCS from -21d prepartum to calving. As a secondary objective, we aimed to evaluate whether ΔBCS during the late dry period was associated with milk yield and milk composition at the first monthly milk day test.

### *3.5.1 Single Measurement of BCS Prepartum*

Our results suggest that there is no evidence that BCS measured at -21 d prepartum is associated with the changes in the BHB concentrations in early lactation. In addition, we observed that, on average, overweight cows (BCS $\geq$ 4) were more likely to have higher BHB concentration postpartum and higher risk of HYK than cows with BCS $\leq$ 3.75 during the dry period. This result is in agreement with other studies using a

similar dichotomization of BCS that reported a higher BHB concentration in overweight cows, with consequently increased risk of HYK (Vanholder et al., 2015; Rathbun et al., 2017). The positive association between prepartum BCS and postpartum BHB concentrations is likely triggered by two connected factors: a decrease in the feed intake and an increase in fat mobilization (Hayirli et al., 2002). Overweight cows have been shown to have a greater reduction in prepartum feed intake than cows with moderate body condition (Roche et al., 2015; Schuh et al., 2019), which can lead to decreases in exogenous supplies of glucose and non-carbohydrate precursors utilized in the gluconeogenesis process (Woerle et al., 2003). This situation is exacerbated by the high energy demands of the fetus and the onset of colostrum and milk production (Herdt, 2000). In terms of fat mobilization, previous studies have suggested that animals with excessive BCS generate higher BHB concentration as a consequence of exacerbated lipolysis (Herdt, 1988, 2000). A process that occurs more frequently in overweight cows as response to the increased stimulation of hormone-sensitive lipase and adipose triglyceride lipase or through the modification of the sensitivity of adipocytes to catecholamines (Vernon and Pond, 1997; Roche et al., 2009; Koster et al., 2018). Also in overweight cows, central resistance to leptin, a hormone produced by the adipocytes that induces satiety signals, can likewise lead to increases in lipolysis (Adam and Mercer, 2004; Roche et al., 2008).

### *3.5.2 Change in Body Condition Score*

Most of the cows in our study lost BCS during the late dry period. While 55.8% of the cows lost BCS, only 23.2% gained it. It is worth noting that changes in BCS during

the late dry period may differ substantially with BCS changes during the whole dry period in which body condition gains is usually observed (Nutrition During the Dry Period and Hepatic Lipid Metabolism of Periparturient Dairy Cows, 1996). The BCS changes differently through the dry period because of different physiological requirements during the early and late stages of the dry period for each individual cow (Hayirli et al., 2002). For example, cows that have the same BCS at dry-off and at calving are considered to have experienced no change in BCS throughout the dry period. However, although the majority of the cows might maintain the same or have small variations in BCS throughout the dry period, others could possibly gain BCS during the first half and lose BCS during the second half of the dry period. Then, these cows might be metabolically not equal. The latter might already be mobilizing adipose tissue by the time of calving in response to the metabolic challenge of the transition period, hence the loss of BCS during the late dry period, increasing her risk of developing HYK in accordance with results described in previous studies (Grummer, 2008; Ji et al., 2012).

Contrary to BCS assessed on a single time prepartum, ΔBCS during the late dry period was an adequate variable to describe the variability in BHB concentration during early lactation as they were associated. According to Cummings and Foster (2003) (Cummings and Foster, 2003), deviations from the physiological, tightly regulated body fat composition trigger compensatory responses in terms satiety regulators and energy usage that persist until the level of body fat storage is restored. It has been observed that, in general, cows tend to converge on their genetically programmed BCS target in early lactation (BCS = 2.5) at 12 to 15 weeks postpartum(Garnsworthy and Topps, 1982). Hence, the awareness on the relationship

between BCS change and fluctuations in BHB concentration could be beneficial for on-farm hyperketonemia management purposes and other potentially energy-related issues. For instance by controlling BCS during the late dry period to avoid large losses, or by monitoring cows especially at the end of the second week postpartum. We observed an increase in BHB concentration in the first two weeks postpartum in cows with a large loss in BCS during the late dry period. This finding is in agreement with other studies that reported a similar BHB increase in cows that lost body condition (Rathbun et al., 2017; Sheehy et al., 2017). Rathbun et al. (2017) (Rathbun et al., 2017) reported an increase of BHB concentration postpartum after the loss of 1.0 units of body condition. However, in our study, the increase in BHB was observed even at losses smaller than 0.5 units of BCS. A loss in BCS is an indication that the cow is mobilizing body reserves, including fatty acids from adipose tissue, in an attempt to overcome the energy deficit (Berry et al., 2011). Cows with higher levels of circulating fatty acids are at a higher risk of developing fatty liver as well as to have increased blood BHB concentration because the capacity of the liver to oxidize those fatty acids is overwhelmed (Herdt, 2000). Although fatty liver might influence the effect of BCS change in BHB fluctuations postpartum, other metabolic and infectious diseases around calving might also act as mediators of this association. Throughout the literature, the association between BCS change before calving and risk of dystocia or clinical mastitis are inconsistent (Gearhart et al., 1990; Heuer et al., 1999; Berry et al., 2007), but there is clear evidence of its association with uterine infections, milk fiver or displaced abomasum (Kim and Suh, 2003; Roche and Berry, 2006).

In our study, the blood BHB concentrations of cows with larger decreases in BCS tended to increase toward the end of the first week and decrease by the end of the second week postpartum. This pattern of blood BHB concentration can be the result of the low dry matter intake and the large amount of milk produced by cows immediately after calving. During the second week, milk yield continues to gradually increase but voluntary dry matter intake tend to also increase at a faster rate than in the first week postpartum reducing the energy deficit (Bell, 1995). Similarly, Barletta et al. (2017) (Barletta et al., 2017) reported an increase in BHB concentration by the day 7 of lactation in cows that lost body condition. This corresponds with the peak of HYK cases around the 5$^{th}$ day postpartum previously reported in the literature (McArt et al., 2012a). Conversely, cows that had a moderate gain in BCS had similar BHB concentration and risk of HYK than cows with no change in BCS during the late dry period, independent of the initial BCS. This suggests that attempting to gain body condition to a moderate degree in the late dry period might not be necessary and that keeping the condition steady would suffice. Although we did not have a large gain BCS group in our statistical analysis (only six cows in the study gained BCS > 0.5), body condition gain over 0.5 points in the late dry period might be negative as well. Excessive BCS gains in the dry period have been associated with a higher risk of increased fat mobilization and cows that are over-conditioned at calving are more likely to suffer the negative consequences of decreased feed intake and impaired metabolic health in the peripartum as previous discussed (Gillund et al., 2001; Ingvartsen, 2006; Roche et al., 2007).

From our study, we can infer that cows without BCS change, or those that had moderate variations, during the late dry period had a better adaptation during the

periparturient period. Nevertheless, the application of measures to correct a loss of BCS during the late dry period can be challenging in practice. Ingvartsen (2006) (Ingvartsen, 2006) suggested that the margin to pursue changes in BCS is limited because of the short period between dry-off and calving and the down-regulation of appetite observed during the late dry period. According to Heuer et al. (2000) (Heuer et al., 2000), this limited variation on BCS explains the inconsistent results reported by multiple studies on the association between BCS and production, reproductive performance, and health events (Ruegg and Milton, 1995; Walsh et al., 2007; Overton et al., 2017). Furthermore, Chapinal et al. (2011) (Chapinal et al., 2011) reported that serum metabolites (NEFA and BHB) provide a better insight into metabolic health status in the short-term than BCS.

### *3.5.3 Milk Yield and Composition*

Cows that experienced variation of BCS in any direction during the late dry period produced less milk volume that cows that maintained the same BCS. The milk volume was especially lower among the cows with a larger loss in BCS. This decrease in milk yield could be the result of an insufficient energy intake during the late dry period (Nutrition During the Dry Period and Hepatic Lipid Metabolism of Periparturient Dairy Cows, 1996). Interestingly, cows that lost BCS did not show a significant increase in milk fat percentage at the first milk test. Generally, cows with large body fat mobilization and elevated BHB concentration are expected to have increased milk fat content than their counterparts (Vanholder et al., 2015; Santschi et al., 2016). Perhaps this group of cows did not lose enough BCS to generate substantial level of fat mobilization to modify feed intake and milk fat content or the fat mobilization was

not large enough at the time that the first milk test was performed to prompt a change in milk fat percentage. Ingvartsen (2006), considers a mobilization of at least 40 kg of body weight (0.5 to 1.0 points of BCS) in Holstein dairy cows as necessary to depress feed intake and milk yield and to increase milk fat content during the mobilization period. Conversely, cows that moderately gained BCS had a larger increase in fat percentage at the first milk test day when compared with unchanged BCS cows. Considering that milk fat can originate from two different sources – the breakdown of blood lipids (e.g., NEFA), which accounts for 40 to 60% of milk fat, and de novo synthesis within the mammary epithelial cells (Bell, 1995) – the observed increase of milk fat content might have been the result of a surplus of energy intake. Ohgi et al., (2005) (Ohgi et al., 2005) suggested that cows maintaining or gaining BCS throughout the dry period increased milk fat content as a consequence of direct utilization of NEFA by the mammary gland.

In terms of milk yield, it has been reported by previous studies measuring BCS using a 5-point scale with either 0.25 and 0.50 points of increment, that cows with low BCS at calving (≤ 3.0) have increased milk yield compared to high BCS cows (Garnsworthy and Topps, 1982; Barletta et al., 2017). Conversely, in our study, cows with the lower BCS at calving, which are also those with the highest BCS at -21d (Table 3.1) and largest body condition loss, produced less milk than cows that had no change in BCS. This was similar when adjusting by fat percentage as ECM was also lower for cows with the lower BCS at calving compared to cows with moderate BCS. A lack of adjustment by variations in BCS in the aforementioned studies (Garnsworthy and Topps, 1982; Barletta et al., 2017), and by dry matter intake by our

study might be the source of disagreement as both factors are related to both BCS at calving and milk yield (Ohgi et al., 2005; Ingvartsen, 2006).

### *3.5.4 Study Limitations*

In our study, only Holstein-Friesian dairy cows were included in the analyses. Due to genetic differences between breeds in terms of fat mobilization and BHB concentration postpartum (Benedet et al., 2020), extrapolation of results to other breeds needs to be done with caution.

The short follow-up period after parturition represents a limitation of this study, impeding a conclusion regarding at which point the change in BCS would not have an effect in BHB concentration anymore. Nevertheless, it is worth noting that, even though the pattern of BHB over time was similar in all cows, those with a larger loss in BCS had higher BHB concentrations at all the time points. Another limitation of this study is the lack of information necessary to analyze and report the intra- and inter-observer agreement when assessing BCS. The inter- and intra-observer measurement of BCS have a relatively high agreement after training in BCS assessment (Kristensen et al., 2006). Despite this, a certain degree of information bias on the exposure is expected. Moreover, previous studies have suggested that the use of more than one observer would have little effect on the measurement accuracy of BCS (Ferguson et al., 1994; Kristensen et al., 2006; Morin et al., 2017). A recent study, however, reported that the ΔBCS overtime calculated from time points scored by multiple observers has moderate agreement, suggesting that a more accurate result would be obtained from the same observer doing all the measurements.

## 3.6 Conclusions

In the current research, we examine the association between both BCS at the onset of the dry period and change in BCS during the late dry period and the temporal patterns of BHB concentration during early lactation. We observed that ΔBCS is associated with variations of BHB in the first two weeks postpartum, unlike BCS measured only at -21 days relative to calving. This result suggests that, to determine how the energy deficit during the transition period affects the incidence of hyperketonemia during early lactation, changes in BCS during the dry period should be primarily considered over BCS measured at a single time point. We also observed that cows with a larger loss in BCS during the late dry period as well as older cows are more likely to have a higher concentration of BHB and a greater incidence of hyperketonemia in early lactation. Moreover, these cows also experienced a reduction in milk at the first monthly milk test day. Our results indicate that loss of BCS greater than 0.5 points during the late dry period impacts on the level of BHB concentration in early lactation and milk performance of dairy cows.

Table 3.1: Descriptive statistics for body condition score at two different points of the late dry period (-21d and at calving), presented for the four categories of change in BCS and the overall average.

| | Observations, N (%) | BCS change[1] mean (s.d.) | BCS at -21d, N (%) | | BCS at calving, N (%) | |
|---|---|---|---|---|---|---|
| BCS change[3] | | | Moderate[2] | Overweight[3] | Moderate | Overweight |
| Moderate gain | 130 (23.2) | 0.28 (0.16) | 89 (43.4) | 41 (11.6) | 50 (13.8) | 130 (23.3) |
| No change | 117 (21.0) | 0 (0) | 62 (30.2) | 55 (15.5) | 62 (17.1) | 117 (20.9) |
| Moderate loss | 247 (44.2) | -0.3 (0.13) | 53 (25.9) | 194 (54.8) | 197 (54.3) | 247 (44.2) |
| Large loss | 65 (11.6) | -0.82 (0.17) | 1(0.5) | 64 (18.1) | 54 (14.9) | 65 (11.6) |
| Total | 587 (100) | -0.16 (0.35) | 205 (100) | 354 (100) | 363 (100) | 196 (100) |

[1] ΔBCS between -21 to 0 days relative to the calving date.
[2] Moderate: BCS ≥ 4.
[3] Overweight: BCS ≤ 3.75.

Table 3.2: Descriptive statistics, sample size, and chi-square test at each sampling time point postpartum.

| | 3 DIM [1] | | | 7 DIM | | | 14 DIM | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| Variable | HYK + [2] [no. (%)] | HYK - [2] [no. (%)] | *P*-value [3] | HYK + [no. (%)] | HYK - [no. (%)] | *P*-value | HYK + [no. (%)] | HYK - [no. (%)] | *P*-value | observations (%) [5] |
| Parity | | | | | | | | | | |
| 1 | 3 (1.3) | 225 (98.7) | <0.001 | 6 (2.6) | 223 (97.4) | <0.001 | 2 (0.83) | 238 (99.2) | <0.001 | 246 (38.6) |
| 2 | 12 (8.0) | 137 (92.0) | | 22 (14.3) | 132 (85.7) | | 23 (15.1) | 129 (84.9) | | 160 (25.1) |
| ≥ 3 | 48 (22.4) | 166 (77.6) | | 68 (30.4) | 156 (69.7) | | 47 (22.4) | 163 (77.6) | | 231 (36.3) |
| Season | | | | | | | | | | |
| Spring | 19 (10.3) | 165 (89.7) | 0.311 | 20 (11.0) | 162 (89.0) | 0.013 | 19 (10.1) | 169 (89.9) | 0.640 | 199 (31.2) |
| Summer | 29 (9.5) | 277 (90.5) | | 51 (15.8) | 271 (84.2) | | 40 (12.8) | 272 (87.2) | | 328 (51.5) |
| Fall | 15 (14.8) | 86 (85.2) | | 25 (24.3) | 78 (75.7) | | 13 (12.8) | 89 (87.3) | | 110 (17.3) |
| Change in BCS | | | | | | | | | | |
| Moderate gain | 9 (7.32) | 114 (92.7) | 0.027 | 11 (9.3) | 107 (90.7) | <0.001 | 9 (7.6) | 109 (92.3) | 0.112 | 130 (23.2) |
| No change | 8 (7.1) | 105 (92.9) | | 14 (12.2) | 101 (87.8) | | 13 (11.5) | 100 (88.5) | | 117 (21.0) |
| Moderate loss | 22 (9.9) | 201 (90.1) | | 41 (17.0) | 200 (83.0) | | 32 (13.6) | 203 (86.4) | | 247 (44.2) |
| Large loss | 12 (20.3) | 47 (79.7) | | 21 (32.8) | 43 (67.2) | | 12 (20.0) | 48 (80.0) | | 65 (11.6) |
| BCS -21 [4] | | | | | | | | | | |
| Moderate | 9 (4.3) | 200 (95.7) | <0.001 | 18 (9.0) | 183 (91.0) | <0.001 | 13 (6.4) | 191 (93.6) | <0.001 | 216 (36.7) |
| Overweight | 47 (14.0) | 290 (86.0) | | 71 (19.8) | 288 (80.2) | | 55 (15.7) | 295 (84.3) | | 371 (63.3) |

[1] DIM = Days in milk.

[2] Cows with BHB concentration ≥ 1.2mmol/L were deemed positives (HYK+) otherwise they were considered negatives (HYK-).

[3] P-value reported for $X^2$ test.

[4] BCS at -21 days from expected calving. Cows with BCS 3-3.75 were deemed moderate; cows BCS 4-5 were considered overweight.

[5] Observations before multiple imputations.

Table 3.3: Association between change in BCS and HYK in early lactation, adjusted by parity.

| | Risk | Risk Ratio | 95%CI | P-value |
|---|---|---|---|---|
| ΔBCS [1] | | | | |
| Moderate gain | 0.17 | 0.89 | 0.53, 1.49 | 0.669 |
| No change | 0.19 | Ref. | — | — |
| Moderate loss | 0.27 | 1.41 | 0.95, 2.10 | 0.092 |
| Large loss | 0.32 | 1.61 | 1.04, 2.50 | 0.034 |
| Parity | | | | |
| ≥ 3 | 0.43 | Ref. | — | — |
| 2 | 0.25 | 0.59 | 0.43, 0.82 | 0.002 |
| 1 | 0.03 | 0.08 | 0.04, 0.17 | 0.000 |

[1] ΔBCS between -21 to 0 days relative to the calving date. Categorized as moderate gain (ΔBCS range: > 0 to 0.5), no change (ΔBCS = 0 - reference), moderate loss (ΔBCS range: -0.5 to < 0), or large loss (ΔBCS < -0.5).

Table 3.4: Variations in milk yield and energy corrected milk (marginal means and mean difference) at the first monthly milk test day according to BCS-change during the close-up period (d -21 to d 0).

| Change in BCS [1] | Milk yield (kg) | | | Energy Corrected Milk (kg) | | |
|---|---|---|---|---|---|---|
| | Mean | Difference in mean (95% CI) | *P*-value | Mean | Difference in mean (95% CI) | *P*-value |
| Moderate gain | 37.1 | -2.28 (-4.84, 0.29) | 0.082 | 42.8 | -0.58 (-3.61, 2.43) | 0.940 |
| No change | 39.4 | Ref. | — | 43.3 | Ref. | — |
| Moderate loss | 37.4 | -2.04 (-4.34, 0.25) | 0.081 | 42.4 | -0.89 (-3.60, 1.80) | 0.774 |
| Large loss | 36.1 | -3.30 (-7.06, 0.45) | 0.084 | 40.4 | -2.92 (-7.34, 1.49) | 0.278 |

Models adjusted by baseline BCS and parity.

[1] BCS measured at the calving date, and categorized as gain (ΔBCS 0 to 0.5), no change (ΔBCS = 0 - reference), moderate loss (ΔBCS range: -0.5 to < 0), or large loss (ΔBCS > -0.5).

Table 3.5: Variations in milk fat and protein content (marginal means and mean differences) at the first monthly milk test day according to BCS-change during the close-up period (d -21 to d 0).

| Change in BCS [1] | Fat (%) | | | Protein (%) | | |
|---|---|---|---|---|---|---|
| | Mean | Difference in mean (95% CI) | *P*-value | Mean | Mean | *P*-value |
| Moderate gain | 4.61 | 0.29 (0.00, 0.59) | 0.049 | 3.18 | 0.10 (0.00, 0.21) | 0.054 |
| No change | 4.31 | Ref. | — | 3.08 | Ref. | — |
| Moderate loss | 4.52 | 0.21 (-0.05, 0.47) | 0.118 | 3.11 | 0.03 (-0.05,0.13) | 0.444 |
| Large loss | 4.45 | 0.13 (-0.29,0.56) | 0.535 | 3.15 | 0.07 (-0.08,0.23) | 0.350 |

Models adjusted by baseline BCS and parity.

[1] BCS measured at the calving date, and categorized as gain (ΔBCS 0 to 0.5), no change (ΔBCS = 0 - reference), moderate loss (ΔBCS range: -0.5 to < 0), or large loss (ΔBCS > -0.5).

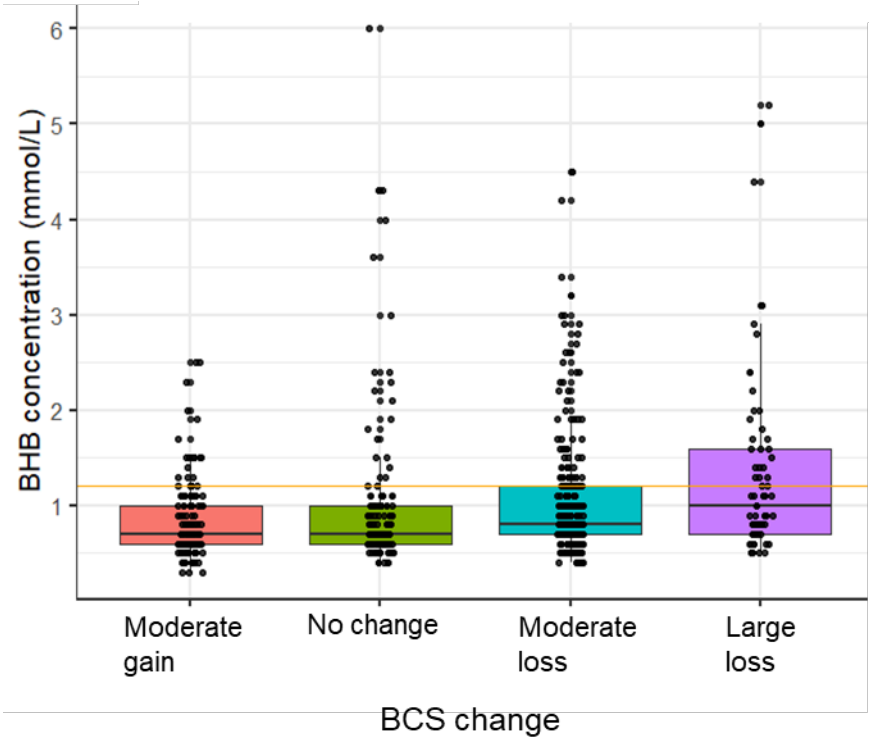


Figure 3.1: Distribution of BHB concentration during the first two weeks postpartum according to BCS change in the late dry period. Each point represents the highest BHB concentration measured during the first two weeks postpartum for each individual cow. BCS change was calculated subtracting the BCS at -21 days prepartum from the BCS measured at calving, and categorized as gain (ΔBCS 0 to 0.5), no change (ΔBCS = 0 - reference), moderate loss (ΔBCS range: -0.5 to < 0), or large loss (ΔBCS > -0.5). Number of cows with moderate gain = 130; no-change= 117, moderate loss= 247, and large loss= 65 The HYK incidence was for moderate gain 16.0%, no-change= 17.9%, moderate loss= 26.7%, and large loss= 17.9%.

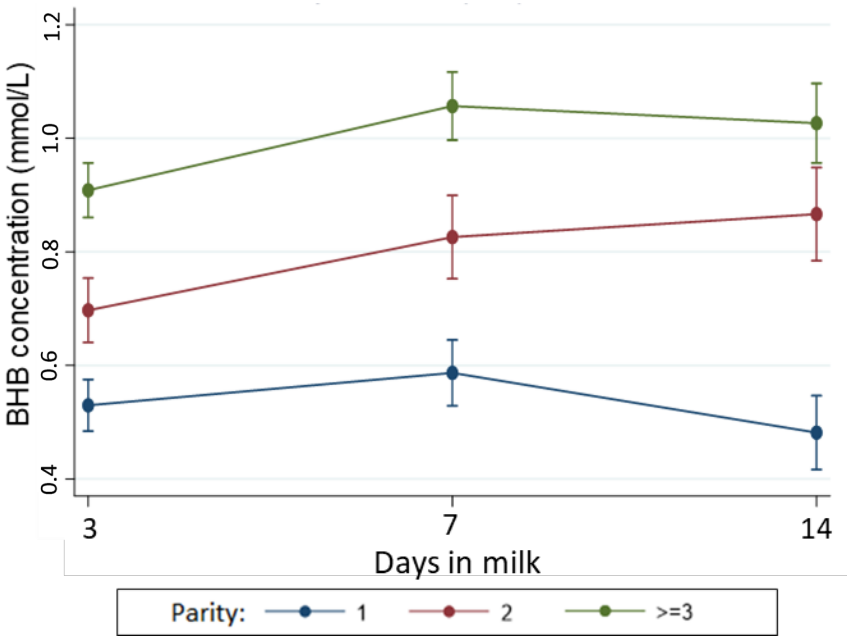


Figure 3.2: Temporal pattern of BHB concentration (with 95% confidence interval) during early lactation according to parity. The association between BHB concentrations and parity was adjusted in the model by time, BCS at 21 days prepartum, and interaction between parity and time. Number of cows with moderate gain = 130; no-change= 117, moderate loss= 247, and large loss= 65.

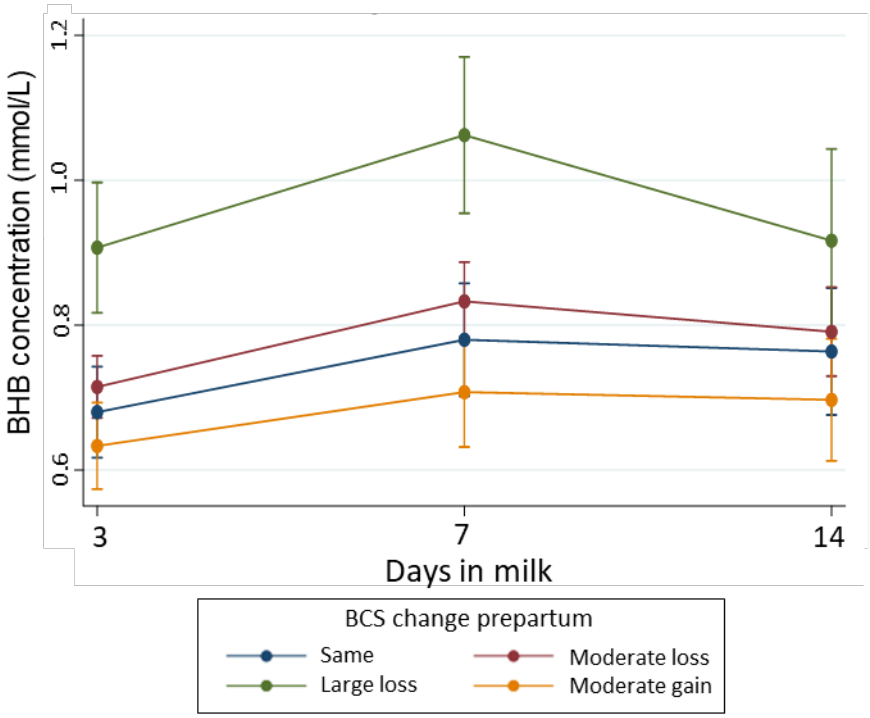


Figure 3.3: Temporal pattern of BHB concentration (with 95% confidence interval) during early lactation according to BCS change as the explanatory variable. Adjusted for BCS at baseline (-21 days), parity, time, the interaction between BCS change and time, and interaction between parity and time. BCS change was calculated subtracting the BCS at -21 days prepartum from the BCS measured around calving, and categorized as gain (ΔBCS 0 to 0.5), no change (ΔBCS = 0 - reference), moderate loss (ΔBCS range: -0.5 to < 0), or large loss (ΔBCS > -0.5). Number of cows with moderate gain = 130; no-change= 117, moderate loss= 247, and large loss= 65.

# CHAPTER 4 – Exploring the role of milk yield in the first week of lactation on the association between hyperketonemia and reproductive performance in dairy cattle

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## 4.1 Chapter Summary

The objective of this retrospective cohort study was to investigate whether the association of hyperketonemia (**HYK**) with reproductive performance differs based on milk production during the first week of lactation (**wk1**). Data between calving and 150 days in milk (**DIM**) from 2,091 Holstein dairy cows from 5 commercial dairy herds in Minnesota were collected. The concentration of beta-hydroxybutyrate (**BHB**) was measured twice between 3 and 10 DIM to diagnose HYK (defined as blood BHB ≥ 1.2 mmol/L). The average wk1 milk yield was classified into three levels (lower 25$^{th}$ percentile, 25$^{th}$ to 75$^{th}$ percentile, upper 75$^{th}$ percentile) according to parity and herd. Log-binomial and Cox-proportional hazard regression models were performed to investigate the association of HYK with pregnancy at first service and pregnancy by 150 DIM. To describe the differences in these estimates between each wk1 milk yield level, an interaction term of HYK and stratified levels of wk1 milk yield was added. The incidence of HYK between 3 to 10 DIM was 14.2% (4.6% in primiparous cows and 19.4% in multiparous cows). Among cows within the lower wk1 milk yield

level, HYK was associated with lower risk of pregnancy at first service (RR = 0.68; 95% CI: 0.43-1.09), and lower risk of pregnancy up to 150 DIM (HR = 0.73; 95% CI: 0.53-0.99). In contrast, among cows with mid-and high-wk1 milk yield levels, hyperketonemic (**HYK+**) cows had a similar reproductive performance than non-HYK cows (**HYK-**). Our results suggest that early lactation milk yield plays a role in the association of hyperketonemia with reproductive performance, and merits further investigation.

## 4.2 Introduction

The elevation of circulating ketone body concentration during early lactation is an indication that the cow is utilizing body reserves to fulfill the energy requirement of her new physiological state (Herdt, 2000). However, an excessive presence of ketone bodies jeopardizes health and productivity. Some studies have shown that the elevated concentrations of circulating BHB, also known as hyperketonemia (**HYK**), are associated with an impaired reproductive performance (Walsh et al., 2007; Ospina et al., 2010; Rutherford et al., 2016).

The evaluation of milk production during early lactation is a readily available, objective, and standardized source of information that can be used as a screening tool to monitor transition cow performance and to detect individual cows that are more likely to have experienced problems during the transition period (Nordlund and Cook, 2004). In addition, monitoring of the transition period based on early lactation performance (e.g., daily milk yield, average milk yield in the first 7 days in milk, and the difference between actual and expected milk yield) has been proposed as a useful

predictor of the probability of cows remaining healthy in early lactation and of the probability of culling (Lukas et al., 2015).

This study was designed to explore the role of milk yield during the first week after parturition, on the relationship between HYK and reproductive performance. The rationale for this study is that not all cows with HYK suffer negative outcomes and that wk1 milk yield might allow for the identification of cows at a higher risk of negative health and productive outcomes that allow for targeted interventions. The study was designed to be exploratory in nature to generate hypotheses to be tested in future confirmatory studies.

## 4.3 Materials and methods

### *4.3.1 Study Population and Data Collection*

The study was conducted according to the guidelines of the University of Minnesota Institutional Animal Care and Use Committee, protocol number: 1603-33595A. This study used a subset of data collected to evaluate the relationship between hoof lesions and HYK (Wynands and Cramer, 2018). The data was collected from five commercial dairy herds in Minnesota from January to December of 2017. All cows that calved during the collection period (N = 2,091) were enrolled independent of parity. Blood BHB concentration was measured twice between 3 and 10 DIM. Blood samples were collected from the coccygeal vessels when cows returned to their pen after morning milking. Immediately after collection, blood BHB concentrations were measured using an electronic handheld device (NovaVet™; Nova Biomedical Co., Waltham, MA), which has been previously validated for use on dairy cows (Bach et al., 2016;

Rodriguez et al., 2020). A calibration slope of 1.0 (default setting) was utilized to adjust for differences in hematocrit and to maximize the sensitivity and specificity of the test (Rodriguez et al., 2020). Calving information, health events, culling, monthly DHI records, and reproductive events to 150 DIM were entered in the farm management software (**DC305**, Valley Ag Software, Tulare, CA) by farm personnel. The average wk1 milk yield was estimated using the DC305 software prediction including peak milk production and preceding DHIA milk test results in two herds and using daily measurement of milk yield in the other three herds.

### *4.3.2 Statistical Analysis*

All statistical analyses were performed using R 3.4.4 software (R. RStudio, Inc., Boston, MA), and the individual cow was the unit of interest. The exposure variable of interest was HYK diagnosed between 3 and 10 DIM. Cows with a BHB measurement of ≥ 1.2mmol/L in at least one of the two samples taken were deemed hyperketonemia-positive (HYK+); otherwise, hyperketonemia-negative (HYK-). Average wk1 milk yield was categorized in three levels based on percentiles. The bottom 25$^{th}$ percentile was considered low milk yielding cows, cows between the 25$^{th}$ to 75$^{th}$ percentile were grouped in a medium milk yielding cows group, and cows in the 75$^{th}$ percentile were considered high milk yielding cows. Percentiles were calculated separately for different parity groups (primiparous and multiparous) and herds (Table 4.1).

The association between HYK and pregnancy by 150 DIM, was calculated using multivariable Cox proportional hazard model (package “survival”; Therneau, 2020). Individual-time at risk for pregnancy was calculated as the number of days from the

end of the waiting period until the date of pregnancy or right-censoring (i.e., death, loss to follow-up, or administrative censoring at 150 DIM). To determine the association between HYK with pregnancy at the first AI service, we performed a multivariable log-binomial regression model (package “lme4”; Bates et al., 2020). The associations between HYK and reproductive efficiency across each level of average wk1 milk yield were evaluated separately by simultaneously introducing HYK, wk1 milk yield, and their interaction (multiplicative) term (HYK x wk1 milk yield) into the regression models described previously. To account for confounding effects, overall models (ignoring interaction) and stratified models (including an interaction term) were subjected to testing for potential confounding variables. The following covariates were offered to the models: herd, parity (primiparous and multiparous), calving difficulty (1 = no assistance, 2 = mild assistance, 3 = assisted by 2 or more people, 4 = mechanical traction, 5 = surgical procedure), and disease detected by farm personnel during the first 7 days of lactation (i.e., clinical mastitis, retained placenta, metritis, and displaced abomasum). In the overall models, wk1 milk yield was also offered as a potential confounding variable. The confounding effects were assessed based on a 10% change in the main exposure estimate (i.e., HYK) (Greenland and Pearce, 2015) using manual backward stepwise elimination procedure. Results are discussed in the context of precision of the estimate using the confidence interval rather than statistical significance (Poole, 2001).

## 4.4 Results

Our analyses included a total of 2,041 Holstein dairy cows. A total of 50 cows were excluded from the initial dataset (n=2,091) because of incomplete observations of the

exposure (i.e., measurement outside the sampling range or only one measurement). We performed a sensitivity analysis to assess the effect of selecting only the three farms with daily milk measurements over including all five farms. The analyses showed similar estimates for pregnancy by 150 DIM when using only three farms (HR = 0.80, 95%CI: 0.65, 1.00), indicating that the trends remained constant regardless of wk1 milk yield determination method.

Table 4.1 contains demographic information, wk1 milk yield levels, and variables utilized in the analyses, sorted by herd. The incidence of HYK varied across herds, ranging from 4.4% to 21.5%. The overall incidence of HYK was 14.2% (289/2041), with 4.6% (33/723) of primiparous and 19.4% (256/1318) of multiparous cows reported as HYK+. The average time to 1$^{st}$ service ranged between 60 and 71 days from calving and the median time to pregnancy was 104 days across herds.
For low-yielding HYK+ cows the median time to pregnancy was between 36 and 45 days later than for all the other groups (Table 4.2). The proportion of pregnancy by 150 DIM was 58.5% for low-yielding HYK+ cows, 68.0% for HYK- low-yielding, 73.2% HYK+ high-yielding, 74.3% for HYK+ mid-yielding, and 77.8% for HYK- mid- and high-yielding cows. Models in which milk yield stratification was not included, indicated a lack of clear evidence that HYK+ cows had a lower risk of pregnancy at the first insemination (RR = 0.90, 95% CI: 0.73, 1.11) or to became pregnant by 150 DIM (HR = 0.91, 95% CI: 0.78, 1.08). However, across wk1 milk yield strata, a negative association between HYK and reproductive performance was observed only among low-yielding cows (Table 4.2). Within the group of low-yielding cows, the HYK+ had 27% lower risk of pregnancy within 150 days (HR =

0.73, 95%CI: 0.53, 0.99) compared to HYK- cows. Furthermore, when compared with HYK- high-yielding cows (which can be considered healthy), HYK+ low-yielding cows were 43% (HR = 0.57, 95% CI: 0.38, 0.86) less likely to become pregnant by 150 DIM (Table 4.3). In contrast, among cows with mid-and high-milk yield, no evidence of association was observed between HYK and pregnancy to 150 DIM due to the lack of precision (Table 4.3).

## 4.5 Discussion

Multiple studies have evaluated milk production as an outcome of HYK (Dohoo and Martin, 1984; Duffield et al., 2009). However, in our study, we explored the role of milk yield on the association between HYK and reproductive performance. It is important to make clear that this study was observational and designed to be hypothesis-generating. Consequently, our intention with this study is to determine if the interaction between milk production and HYK+ merits further study.

The incidence of HYK observed in this study was similar to some reports (Rathbun et al., 2017; Chandler et al., 2018), but was, in general, lower than the incidence levels reported by most other studies (Kaufman et al., 2016; Weigel et al., 2017). This difference can be attributed to a lower incidence on the particular herd under study, in addition to different sampling schemes between studies. A greater number of HYK+ cows are identified in studies with more frequent sampling because of an increased chance of detecting true positive cows. Recently, Tatone et al. (2016) highlighted that the abundance of protocols, sampling periods, diagnostic devices, and case definitions used in studies related to HYK led to the differences in the prevalence of HYK reported in the literature. In this study, we diagnosed HYK by measuring BHB

concentrations in blood twice between 3 to 10 DIM. This sampling scheme was selected considering the peak of HYK cases occurring at 5 DIM and HYK has a disease length of 5 days (McArt et al., 2012a). Although this strategy aimed to avoid false-negative results, cases with a shorter duration or which occurred after 10 days postpartum could have been missed, thus, not included in the analyses, resulting in a lower incidence.

Models without wk1 milk yield stratification did not show clear evidence that HYK is negatively associated with the risk of pregnancy at their first insemination or by 150 DIM. These results agree with previous reports (Chapinal 2012, McArt 2012) but conflict with other studies that have found evidence of an association (Ospina 2010, Walsh 2007). Based on our results and considering that cows need to be in a positive energy balance to fully express estrus behavior and become pregnant (Rutherford et al., 2016), we suspect that this discrepancy can be related to the energy balance during the transition period. When we stratified our dataset by early milk yield, which stands as a potential proxy for energy balance during the transition, we observed two notable findings that lend credence to the importance of energy balance in the relationship between HYK and reproductive success. First, independent of their HYK status, cows with high and mid wk1 milk yield had better reproductive performance than low-yielding cows. Second, HYK seemed to worsen the reproductive performance of low-yielding cows. These results suggest a trend of an effect modification caused by wk1 milk yield that warrants further confirmatory evaluation. In addition, results contradict the general perception that cows with high milk yield have low reproductive efficiency, a belief that has been previously challenged (Leblanc, 2010; Bello et al.,

2012). Instead, low reproductive efficiency may be attributable to greater severity of postpartum negative nutrient balance consequence caused by deficient transition management reflected in low DMI and increased incidence of disease rather than to milk yield (Gröhn and Rajala-Schultz, 2000; Lucy, 2001; Drackley and Cardoso, 2014). Consequently, we presume that HYK + cows with an wk1 milk yield falling in the lower 25$^{th}$ percentile are those that had the more severe or prolonged energy deficit. Meanwhile, HYK + cows that maintained a milk yield level in early lactation over the 25$^{th}$ percentile adapted better to the transition process by sustaining adequate feed intake, avoiding metabolic imbalance and disease, and are more likely to have a successful reproductive performance.

A strength of this study was the enrollment of cows in multiple herds varying in sizes and management strategies representing a variety of the dairy herds in the upper Midwest region of the United States. As a limitation, milk yield recording methods varied among farms. Although we estimated this limitation to have low impact in the results, bias could remain. In addition, due to our observational sampling strategy, HYK and wk1 milk yield happen at the same time after calving, and thus the temporality of these variables it is not clear. We argue that both variables started during the transition with increased fat mobilization. However, caution should be taken when interpreting the results of this exploratory study.

## 4.5 Conclusions

In conclusion, our results suggest that early lactation milk yield plays a role in the association of HYK with reproductive performance. Among low wk1 yielding cows, HYK+ cows were less likely to become pregnant. Conversely, reproductive

performance was similar among mid- and high-yielding cows, independent of HYK status between 3 and 10 DIM. The results suggest that milk yield could be considered in addition to the measurement of BHB concentration when monitoring dairy cows in early lactation. These results warrant further investigation in a larger confirmatory study, evaluating early lactation milk yield as a potential effect modifier of the association between HYK and reproduction.

Table 4.1: Descriptive characteristics of the Holstein dairy cows included in the analyses according to herds.

| Descriptive characteristics | | Herd A (n[1] = 111) | Herd B (n = 579) | Herd C (n = 548) | Herd D (n = 275) | Herd E (n = 511) |
|---|---|---|---|---|---|---|
| Herd size[2] | | 700 | 1,300 | 2,050 | 1,300 | 650 |
| Parity at enrollment (n, %) | | | | | | |
| Primiparous | | 43 (38.7) | 182 (31.4) | 182 (33.2) | 122 (44.4) | 192 (37.6) |
| Multiparous | | 68 (61.3) | 397 (68.6) | 366 (66.8) | 153 (55.6) | 319 (62.4) |
| Milk yield (kg, SD) | | | | | | |
| Primiparous ME[3] 305d | | 15,000 (2,110) | 13,000 (2,560) | 13,100 (2,010) | 13,100 (1,760) | 13,400 (2,410) |
| Multiparous ME[3] 305d | | 14,600 (3,490) | 13,700 (2,620) | 13,900 (1,900) | 12,800 (2,140) | 13,900 (2,200) |
| Percentiles 1st week milk yield (Kg) | | | | | | |
| Primiparous | 25th % | 26.1 | 23.1 | 24.5 | 23.1 | 26.3 |
| | 75th % | 33.1 | 34.8 | 32.2 | 29.3 | 33.6 |
| Multiparous | 25th % | 35.2 | 36.3 | 37.6 | 34.9 | 39.5 |
| | 75th % | 49.4 | 51.7 | 46.7 | 44.9 | 50.8 |
| Health and reproduction parameters (n, %) | | | | | | |
| HYK incidence | | 5 (4.4) | 126 (21.5) | 37 (6.7) | 42 (15.2) | 79 (15.3) |
| Pregnant at 1st service | | 45 (39.5) | 271 (46.2) | 182 (33.2) | 132 (47.8) | 180 (35.0) |
| Pregnant within 150 DIM | | 75 (65.8) | 433 (73.8) | 408 (74.3) | 210 (76.1) | 330 (64.1) |

[1] Number of animals enrolled in each herd.

[2] Average herd size during the study period.

[3] Mature equivalent 305 days in milk.

[4] Percentiles utilized for the categorization of average first-week milk yield.

Table 4.2: Model estimates for Holstein cows with at least one positive test for hyperketonemia between 3 and 10 DIM stratified by the first week of lactation milk yield.

| Events | Hazard Ratio (95% CI) | | | |
|---|---|---|---|---|
| | Overall[1] | Low milk yield[2] | Mid milk yield[2] | High milk yield[2] |
| Pregnancy at 1st service[3] | 0.90 (0.73, 1.11) | 0.68 (0.43, 1.09) | 1.00 (0.76, 1.32) | 0.93 (0.64, 1.34) |
| Pregnancy by 150d[4] | 0.91 (0.78, 1.08) | 0.73 (0.53, 0.99) | 0.93 (0.74, 1.17) | 0.94 (0.70, 1.27) |

[1] Comparison between hyperketonemic and non HYK cows without stratification by first-week postpartum milk yield average.

[2] Categories were calculated separately for primiparous and multiparous cows within each herd. Cows were considered to be low-yielding when their first-week postpartum milk yield average was under the 25$^{th}$ percentile. Similarly, cows between the 25$^{th}$ and 75$^{th}$ percentiles were considered mid-yielding. Cows over the 75$^{th}$ percentile were considered high-yielding cows.

[3] Risk ratios adjusted by parity, disease in the first week, and herd.

[4] Hazard ratios adjusted by parity, calving ease, and herd.

Table 4.3: Comparison of the estimated association of hyperketonemia (HYK) status with pregnancy at first service and pregnancy by 150 days in milk between each level of milk yield in the first week of lactation in Holstein dairy cows.

| | HYK[1] = positive | | | HYK = negative | | |
|---|---|---|---|---|---|---|
| | N w/wo outcome[2] | Median days to pregnancy | Estimate (95% CI) | N w/wo outcome | Median days to pregnancy | Estimates (95% CI) |
| Pregnancy at 1st AI[3] | | | | | | |
| Milk = high[4] | 22/50 | -- | 0.93 (0.57, 1.50) | 137/280 | -- | Ref. |
| Milk = mid | 37/80 | -- | 1.00 (0.68, 1.47) | 305/628 | -- | 1.00 (0.81, 1.23) |
| Milk = low | 17/76 | -- | 0.64 (0.35, 1.17) | 114/276 | -- | 0.93 (0.71, 1.21) |
| Pregnancy by 150d[5] | | | | | | |
| Milk = high | 52/19 | 98 | 0.94 (0.64, 1.39) | 320/92 | 90 | Ref. |
| Milk = mid | 84/29 | 92 | 0.95 (0.69, 1.30) | 708/202 | 89 | 1.01 (0.85, 1.20) |
| Milk = low | 48/34 | 134 | 0.57 (0.38, 0.86) | 238/112 | 98 | 0.79 (0.63, 0.98) |

[1] HYK = Hyperketonemia (blood BHB ≥ 1.2mmol/L).

[2] Number of cows with and without the reproductive outcome among each milk level.

[3] Estimate in risk ratio adjusted by parity, disease in the first week, and herd.

[4] Categories were calculated separately for primiparous and multiparous cows within each herd. Cows were considered to be low-yielding when their first-week postpartum milk yield average was under the 25$^{th}$ percentile. Similarly, cows between the 25$^{th}$ and 75$^{th}$ percentiles were considered mid-yielding. Cows over the 75$^{th}$ percentile were considered high-yielding cows.

[5] Estimate in hazard ratio adjusted by parity, calving ease, and herd.

# CHAPTER 5 – Assessment of milk yield and composition, reproductive performance and herd removal in multiparous dairy cattle based on the week of diagnosis of hyperketonemia in early lactation.

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## 5.1 Chapter Summary

The purpose of this retrospective cohort study was to evaluate the differential impact of hyperketonemia (HYK) according to the moment of diagnosis during early lactation on milk yield and composition, reproductive performance, and herd removal. The β-hydroxybutyrate (BHB) concentration of Holstein multiparous cows (n = 362 cows) was measured twice a week for the first two weeks of lactation for the diagnosis of HYK. Cows were considered HYK positive (HYK+) if the blood BHB concentrations were ≥1.2 mmol/L, otherwise, HYK negative (HYK−). Milk-related outcomes of interest included: monthly milk volume, milk fat and protein content, milk urea nitrogen (MUN), and linear score of somatic cell count (LSCC). Other performance outcomes of interest included: pregnancy rate within 150 days in milk (DIM) and herd removal (i.e., culling or death) within 300 DIM. Statistical models were performed separately for cows diagnosed with HYK in the 1st week (wk1) and the 2nd week (wk2). All models for the wk2 were adjusted by HYK diagnosed in the

week before along with other confounder variables. To determine the association between HYK in each wk and milk-related outcomes, we performed generalized estimated equation (GEE) models. Time to pregnancy and time to herd removal were analyzed with multivariable survival analysis using Cox's proportional hazards regression models. Seventy-eight cows (22.8%) tested positive to HYK during wk1, 60 cows (18.6%) in wk2, and 29 cows (9.3%) in both weeks. HYK during wk1 was associated with a milk volume reduction of 3.7 kg (95% CI: -6.67 to -0.76) per cow/day throughout the lactation. Meanwhile, we did not observe clear evidence of reduced milk volume in cows diagnosed with HYK during wk2. During the first two monthly milk tests, cows diagnosed as HYK+ in wk1 produced 0.42% more fat (95% CI: 0.16 to 0.67) and 0.75 mg/dl more MUN (95% CI: 0.26 to 1.24) than HYK- cows. Whereas HYK diagnosed in wk2 did not show evidence of association with these outcomes. The HYK+ cows in wk1 had a 30% (HR = 0.70, 95% CI: 0.48 to 1.01) lower pregnancy rate within 150 DIM and 2.48 times (95% CI: 1.63 to 2.89) higher risk of herd removal within 300 DIM than HYK- cows. Meanwhile, no clear evidence of association was observed between HYK in wk2 and risk of pregnancy (HR = 0.98, 95% CI: 0.64 to 1.51) or removal from the herd (HR = 0.91, 95% CI: 0.52 to 1.60). Our findings indicate that HYK diagnosed during wk1 of lactation is related with negative performance in terms of milk yield, reproduction, and herd removal. Meanwhile, cows seem less affected by HYK when diagnosed during wk2.

## 5.2 Introduction

During the transition period from late gestation to early lactation, cows endure an important metabolic adaptation (Bauman and Currie, 1980). The capacity of dairy

cows to adapt to their new physiological state will determine their health and productivity after calving (Herdt, 2000). As part of this metabolic adaptation, dairy cows mobilize body reserves, specifically fatty acids from adipose tissue which ultimately become ketone bodies, to be used as alternative sources of energy (Herdt, 2000). An excessive elevation of circulating ketone bodies, also known as hyperketonemia (HYK), may lead to compromised health and productivity. Thus, HYK can be considered one of the earlier indicators of the quality of the transition period because of its negative association with multiple outcomes after calving, including reproductive efficiency, milk production, and health-related events (Ospina et al., 2010; Suthar et al., 2013; Raboisson et al., 2014). The etiology, severity, and metabolic adaptations that cause the energy deficit that lead to the mobilization of fatty acids may differ greatly (Herdt, 2000). Following this consideration, HYK can be classified into two types according to the gluconeogenic capacity of the liver (Holtenius and Holtenius, 1996). Chronologically, type II happens early after calving (i.e., 1 to 2 weeks postpartum) and is related to strong homeorhetic processes set in place because of the energy deficit in the transition period. Conversely, type I has been described to happen closer to the peak of lactation (i.e., 3 to 6 weeks postpartum), and in high-producing cows when the demands of glucose for milk production surpass the capacity of the liver for gluconeogenesis. These cows have the gluconeogenic pathways fully stimulated and glucose production is only limited by substrate supply (Herdt, 2000).

Screening protocols to diagnose HYK typically involve the testing of cows during the first 2 weeks of lactation based on the elevated prevalence of HYK reported during this period (van der Drift et al., 2012; Suthar et al., 2013; Santschi et al., 2016).

However, previous studies have suggested that an increase of disease events and decrease in milk yield and reproductive efficiency is observed with lower BHB concentration thresholds in wk1 compared to wk2 (Walsh et al., 2007; Duffield et al., 2009). Therefore, the impact on health and production might differ according to the time in which HYK is diagnosed.

To our knowledge, there is a lack of evaluation of the long term impact of HYK based on the time of diagnosis under a unique and frequently used threshold (1.2 mmol/L). This information may serve to determine specific approaches to manage cows with HYK. The practical implications of a better comprehension of the dynamics of HYK include the enhancement of management strategies developed to reduce the impact of the disease in those animals at a higher risk of negative outcomes. Therefore, our objective was to evaluate the association of HYK in the 1st and 2nd week of lactation on milk yield and composition, reproductive performance, and herd removal. We hypothesized that HYK occurring in the 1st week postpartum negatively affects milk yield and composition, reproductive performance, and herd removal. In contrast, we expected that these differences would be less evident when HYK is diagnosed during the 2nd week of lactation.

## 5.3 Materials and Methods

### *5.3.1 Study population and data collection*

All study activities were approved by the University of Minnesota Institutional Animal Care and Use Committee, protocol number 1806-36016A.

The current study was conducted on a commercial dairy farm in Minnesota. The herd was selected by convenience due to herd size (1,200 milking cows), lack of BHB

testing protocol, reliable data records, and use of computerized data recording and management software (DairyPlan C21, GEA Farm Technologies, Bönen, Germany). Cows were housed in free stalls with recycled manure solid bedding. Lactating cows were milked 3 times a day and the farm participated in the Dairy Herd Improvement (DHI) program.

The participating herd agreed to the intended testing protocol. The data utilized in the analyses were originally collected for another study evaluating the accuracy of an algorithm for the prediction of HYK postpartum using prepartum blood metabolites. The research team visited the herd for sample collection from February to October 2019.

Enrollment of pre-fresh cows in the study occurred during the dry period at 260 ± 3 days of gestation (i.e., 3 weeks from the expected calving date). Hence, only cows that had at least 1 previous lactation were included. Blood samples were collected twice a week, 3 or 4 days apart between 1 and 14 days postpartum (days 3± 2, 6± 2, 9± 2 and 12± 2). All samples were collected from the coccygeal vessels using a 20-gauge, 2.54-cm blood collection needle, and vacuum tubes containing Li-Hep (Vacutainer; Becton Dickinson, Franklin Lakes, NJ). All collection took place in the fresh pen immediately after morning milking. Blood samples were immediately placed in a cooler after collection and transported on ice to the laboratory. Within 3 hours of collection, all samples were centrifuged at 2,000x g for 15 min for plasma separation and kept frozen at -80°C for later analysis. Samples were thawed at the end of the study for the measurement of BHB concentration using an automated small-scale spectrophotometric chemistry analyzer (CataChemWell-T; Catachem Inc., Oxford, CT). The intra- and inter- coefficient of variation for the BHB assay was

3.5% and 8.0% respectively. Hyperketonemia was defined as plasma BHB >1.2 mmol/L (Duffield, 2000). Information on milk yield and composition was obtained from 10 monthly DHI test records for each individual cow. Farm personnel recorded calving information, culling and death, and disease events (i.e., mastitis, metritis, retained placenta, left-side displaced abomasum, and milk fever). Pregnancy was diagnosed by the herd veterinarian. All information was extracted from the farm computer records.

### *5.3.2 Statistical Analyses and Model-Building Strategies*

All statistical analyses were performed using R 3.4.4 software (R. RStudio, Inc., Boston, MA), and the individual cow was the unit of interest.

The exposure variable of interest was HYK. Those individuals with BHB measurements ≥1.2mmol/L at any testing point during wk1 and wk2 were considered as hyperketonemia positives (HYK+) in their respective weeks, otherwise as negatives (HYK-). Diagnostic results were not shared with farm personnel.

The milk-related dependent variables of interest included monthly milk yield, milk fat and protein content, MUN, and LSCC. In addition, we included pregnancy rate and herd removal (i.e., culling or death).

The sample size was originally developed for a purpose other than the current study. Nevertheless, in order to provide a precise estimate of the association between HYK in both weeks and pregnancy rate, and considering the following assumptions: $\alpha = 0.05$, power = 80%, an expected HYK prevalence of 20%, and a difference in pregnancy of 10% by the end of the 150 days of follow up; a total of 360 cows would need to be enrolled.

All models were performed for wk1 and wk2 separately. To determine the behavior of milk-related outcomes, we performed generalized estimated equation (GEE) models. We selected this model because subsequent monthly measurements throughout the lactation are highly correlated. The GEE model incorporates the dependence among the variables from the same cow and provides robust variance estimates of the contrast coefficients. After evaluating several covariance structures included in the models, the autoregressive covariance structure was selected based on Akaike's information criterion. Because all models included linear and quadratic interactions terms with time, monthly pattern of milk-related outcomes are reported using graphs and as average throughout the lactation.

Time to pregnancy and time to herd removal were analyzed with multivariable survival analysis using Cox's proportional hazards regression models. Individual-time at risk for the pregnancy model was calculated as the number of days from the end of voluntary waiting period to the date of pregnancy diagnosis or censoring (i.e., death, loss of follow-up or administrative censoring at 150 days in milk). For the herd removal model, individual-time at risk was calculated as the number of days from calving date to date of culling, death, or censoring (i.e., loss to follow-up or administrative censoring at 300 days in milk). To test the proportional hazards assumption, the scaled Schoenfeld residuals were plotted against the survival time for the final models (Therneau, 2015). Although survival analysis models the time to an event, in Cox regression model the output is expressed as a hazard ratio (HR) or instantaneous relative risk of the outcome (with 95% confidence intervals) between groups in a time interval. Because these instantaneous risks are constantly proportional over time, the result can be interpreted as the relative risk or probability

of conception or removal at any time during the study period or relative pregnancy/removal rate (speed at which cows develop the outcome) (LeBlanc et al., 2002; Sedgwick and Joekes, 2015).

Independent variables offered into the multivariable models to test its confounding effect included the following: BCS measured on a 5-point scale (Ferguson et al., 1994) at the moment of enrollment; cow calving ease (assistance provided at birth) recorded using a 4-point scale (1 = no assistance provided; 2 = light assistance by one person without the use of mechanical traction; 3 = mechanical extraction of the calf with an obstetric calf-puller; and 4 = severe dystocia: surgery or fetotomy needed) (Schuenemann et al., 2011); and season of calving (spring = February through April; summer = May through July; fall = August through October). Information about the previous lactation included variables such as 305-d mature equivalent (305ME), total lactation days, gestation days, and days in the dry period. The confounding effects were assessed based on a 10% change in the estimate criterion (Greenland and Pearce, 2015) under a manual backward stepwise elimination procedure. Once confounding was assessed, biologically plausible 2-way interactions on the multiplicative scale were investigated for the remaining covariates. All wk2 models included an adjustment for HYK status in the week before independently of its statistical significance. Results are discussed in the context of precision of the estimate using the confidence interval rather than statistical significance whenever possible (Poole, 2001).

## 5.4 Results

### *5.4.1 Descriptive Statistics*

In total, 362 multiparous Holstein dairy cows were enrolled during the study period. Eleven cows were excluded from the milk-related statistical analyses, and 20 cows were excluded from the reproductive and herd removal analyses due to missing data. Characteristics of the cows enrolled in the study by HYK status and week of testing are found in Table 1. Of the 362 multiparous cows, 78 (22.8%) were diagnosed with HYK in wk1, and 60 (18.6%) were diagnosed with HYK in wk2. In addition, 29 (9.3%) cows were diagnosed with HYK in both weeks.

### *5.4.2 Milk yield and composition*

Cows diagnosed with HYK in wk1 had reduced milk volume throughout lactation (Figure 1A). When compared to HYK- cows, HYK+ cows produced on average 3.7 kg (95% CI: -6.67 to -0.76) less milk daily (Table 2). This is equivalent to 1,128.5 kg per cow during a standard 305 days lactation, representing approximately 7.9% decline in milk production. Meanwhile only a numerical difference (mean difference = -1.0 kg per cow/day, 95% CI: -4.04 to 1.05) was observed in cows diagnosed with HYK in wk2 (Figure 1B). The milk fat content throughout lactation differed in cows diagnosed with HYK in wk1 and wk2 (Figure 1C and 1D). Cows diagnosed as HYK+ in wk1 had a higher milk fat content (0.42%, 95% CI: 0.16 to 0.67) than HYK- during the first 2 monthly milk tests (Table 2). This is a difference in milk fat content of 8.5 kg per cow in 60 d of lactation. Nevertheless, the difference in fat content among cows diagnosed with and without HYK in wk1 disappeared as the lactation progressed resulting in no evidence of a difference throughout the entire lactation. In

contrast, cows classified as HYK+ in wk2 had greater milk fat content on the entire lactation than HYK- cows (mean difference = 0.29%, 95% CI: 0.08 to 0.51), which is equivalent to 114 g more milk fat/cow per day during the entire lactation period or, on average, 34.8 kg of milk fat/cow in 305d of lactation.

Milk protein content followed a similar pattern at monthly and average values for both HYK+ and HYK- cows in wk1 and wk2 (Figure 1E and 1F, Table 2). The concentration of milk urea nitrogen was higher among cows diagnosed as HYK+ in wk1 compared to HYK- during the first two monthly milk tests (mean difference = 0.75 mg/dL, 95% CI: 0.26 to 1.24; Figure 1G). No evidence of difference was observed for the average whole lactation value for HYK status or week of diagnosis (Table 2). Cows diagnosed as HYK+ in wk2 had higher LSCC than HYK- cows throughout the lactation (mean difference = 0.82, 95% CI: 0.25 to 1.39). A smaller numerical difference was observed in cows diagnosed in wk1 (mean difference = 0.49, 95% CI: -0.06 to 1.04; Table 2). LSCC was elevated for the entire lactation in cows HYK+ in wk2 (mean difference = 0.82, 95% CI: 0.25 to 1.39), but no difference was observed in wk1 (Table 2).

### *5.4.3 Reproductive performance*

The Cox Proportional-Hazard model for pregnancy risk shows that HYK+ cows diagnosed during wk1 had 30% lower pregnancy rate than HYK- cows (HR = 0.70, 95% CI: 0.48 to 1.01). During wk2 and after adjusting by HYK in the previous week, HYK+ cows had a similar pregnancy rate as HYK- cows (HR = 0.98, 95% CI: 0.64 to 1.51). The median days to pregnancy was 19 days later in HYK+ than HYK- in wk1 but only 4 days longer in wk2 (Figure 2). In addition, at 150 DIM, the proportion of

pregnancy was 69.5% and 80.0% for HYK+ and HYK- cows in wk1, and 77.6% and 76.7% for HYK+ and HYK- cows in wk2.

### *5.4.4 Herd removal*

Cows diagnosed with HYK in wk1 had 2.48 times higher risk of herd removal within 300 DIM than HYK- cows (95% CI: 1.63 to 2.89; Figure 3A). When HYK was diagnosed in wk2, HYK+ cows had a similar risk of herd removal as that of HYK- cows (HR = 0.91, 95% CI: 0.52 to 1.60; Figure 3B). At 300 DIM, the proportion of animals removed from the herd as sold or dead was 55.1% and 29.5% for wk1 HYK+ and HYK- cows, and 50.0% and 31.2% for wk2 HYK+ and HYK- cows, respectively.

## 5.5 Discussion

The present study was conducted to explore the productive and health-related consequences of HYK according to time of disease diagnosis in the first 2 weeks of lactation. We observed that the prevalence of HYK was similar in the first 2 weeks postpartum. Despite the fact that cases of HYK can occur until the 6th week postpartum, multiple studies have reported that the highest prevalence of HYK occurs during the first 2 weeks of lactation (van der Drift et al., 2012; Suthar et al., 2013; Santschi et al., 2016).

Our milk yield results showed that cows with HYK diagnosed in wk1 produced less milk volume throughout lactation than HYK- cows, whereas HYK diagnosed in wk2 did not show evidence of an association with changes in milk yield throughout the lactation. Previous studies evaluating the association between HYK during the first 2 weeks of lactation, independently of the time of diagnosis, and milk production in early lactation have shown conflicting results. Some studies reported milk losses

among HYK+ cows ranging from 1.2 to 2.4 kg/cow per day at the first test-day compared to HYK- cows (Chapinal et al., 2012; McArt et al., 2012; Santschi et al., 2016), while other studies showed an increase in milk yield from 1.28 to 2.40 kg during the same period (Vanholder et al., 2015; Rathbun et al., 2017; Ruoff et al., 2017). An explanation for these contradictory results may be related to the time of HYK diagnosis. For instance, among the aforementioned studies, those that observed a negative association reported a higher HYK prevalence in wk1, while studies that observed a positive association reported a higher HYK prevalence in wk2. This suggests that cows with HYK diagnosed in wk1 are the cows with a higher risk of milk yield reduction as observed in our study. Duffield et al. (2009) reported that cows diagnosed with HYK in the first week postpartum produced 1.22 kg/cow less milk volume at the first DHI test than HYK- cows. A smaller reduction was observed when HYK was diagnosed in wk2 (i.e., 1.03kg). Similarly, McArt et al. (2012) observed that cows that developed HYK within the first week postpartum were more likely to produce less milk than cows developing HYK after the first week of lactation. In our study, HYK diagnosed in wk2 did not show evidence of a positive association with milk production.

A reasonable explanation for the disparity between cows diagnosed with HYK in wk1 and wk2 can be related to a different adaptation to the new lactating physiological state by those groups of cows. The HYK+ cows in wk1 could have had higher level of insulin resistance, elevated fat accumulation in the liver which worse its hepatic gluconeogenic capacity, and oxidative stress (Emery et al., 1992; Xu et al., 2015; Youssef and El-Ashker, 2017) than HYK+ cows in wk2 . Therefore, despite the new physiological stage being a challenging period for all cows, those diagnosed with

HYK in wk1 could be those that are especially affected by a poor metabolic adaptation. Meanwhile, cows that were diagnosed with HYK in wk2 of lactation may have had a better adaptation to the new lactation stage. Their increased milk production would increase their glucose demand surpassing the liver capacity for gluconeogenesis exacerbating the fat mobilization to serve as alternative source of energy while glucose is prioritize for milk production. Because the increased in milk production, these cows would also be avoiding fat accumulation in the liver and the associated negative impact on milk yield, reproductive performance, and culling (Herdt, 1988; Rukkwamsuk et al., 1999; Bobe et al., 2004).

The differences in milk composition throughout lactation were less constant than milk yield. Milk fat content increased in cows with HYK diagnosis in wk1, but only during the first 2 DHI tests. As previous reports suggest, variations in milk fat content are generally minor, ranging from 0.10% to 0.66% (van der Drift et al., 2012; Vanholder et al., 2015). Similar to our finding, Santschi et al. (2016) observed an increase in milk fat content on the first DHI test of 0.36% and 0.18% in cows diagnosed with HYK in wk1 and wk2, respectively. This effect of HYK in milk fat content is likely the exacerbated fat mobilization experienced by cows with HYK soon after calving. According to Xu et al. (2015), there are indications of oxidative stress and liver dysfunction in cows with HYK type II (peak of BHB concentration on d 7) in contrast to cows with HYK type I (HYK diagnosed later in the lactation), which can be a reason for the different fat content observed in cows according to the time of diagnosis. Nevertheless, when we observed milk fat content in the entire lactation, we found it to be higher for cows diagnosed with HYK in wk2, contrary to what happened in the first 2 months of lactation. A more active mammary gland with more

de novo fatty acids (C6:0, C8:0, C10:0, C12:0, C14:0) and medium-chain fatty acid (C15:0) synthesis may be behind the increase in fat content in the entire lactation among cows diagnosed with HYK in wk2 (Mann et al., 2016). A milk fatty acids profile could clarify this differences in milk fat content in the short and long term. Another milk component also related to oxidative stress and liver apoptosis damage is MUN (Song et al., 2016). It has been previously reported that HYK cows produce between 4.6% to 16.6% less MUN compared with non-HYK cows (Santschi et al., 2016). In our study we observed a reduction of 8.9% in the first 2 months of lactation. The decrease in MUN is indicative of both, low dietary protein and protein digestibility, indicating an inefficient use of nitrogen, and also low protein biosynthesis in the liver likely as a result of reduced feed intake, oxidative stress, and liver dysfunction, all related to HYK type II (Song et al., 2016). Hence, it is expected that cows with a better metabolic adaptation during the transition period have better nitrogen efficiency for milk production (Nousiainen et al., 2004).

The estimate of the association between HYK in wk1 and pregnancy within 150 DIM suggested a negative association only among cows diagnosed in wk1. It is worth noting that originally we had estimated the minimum sample size to be 360 cows to observe a significant meaningful difference on pregnancy rate between groups. We enrolled 362 cows but 20 cows were excluded from the analyses. The difference in pregnancy by the end of the 150 d of follow up period was 0.5% higher than expected and the prevalence of HYK was 2.5% higher than expected. However, these larger values were not enough to reduce the required sample size to the number of cows that we analyzed (n=342). This determined a power of 76.1% for pregnancy rate.

The reproductive results were in agreement with previous reports that cows diagnosed with HYK from 3 to 7 DIM had 30% lower pregnancy at 1st service than cows positive after the 8th day in milk (McArt et al., 2012). Reproductive efficiency is highly related to energy availability, and the onset of lactation requires a large amount of energy (Herdt, 2000). Moreover, the partitioning of energy to milk yield occurs at the expense of reproductive performance (Dawuda et al., 2002; Butler, 2003). According to Butler and Smith (1989), it is the duration and severity of the energy deficit that results in poor reproductive performance mediated through lower ovulation. Therefore, it is possible to infer that those cows with HYK in wk1 were at a higher risk of reproductive impairment because of a poor transition with an elevated nutritional deficit after calving.

Herd removal was larger in cows with HYK diagnosed in wk1 than in wk2. The association between HYK in wk1 and herd removal can be mediated through multiple diseases. In our study we observed a larger LSCC in cows with HYK in wk1, indicating higher risk of clinical mastitis and a potential reason of culling of these group of cows. Although in the published literature, the effect of HYK on intramammary infections is controversial, in a meta-analysis was reported that the odds of developing subclinical mastitis infections (i.e., somatic cell count >200,000 cells/mL) was 1.42 (95% CI: 1.26 to 1.60) times higher for HYK+ than HYK- cows (Raboisson et al. 2014). It has been argued that elevated concentrations of circulating ketone bodies can directly affect the immune response by impairing chemotaxis, oxidative burst, and phagocytosis (Hoeben et al., 1997; Sartorelli et al., 1999; Suriyasathaporn et al., 2000), all necessary mechanisms used by the host to control intramammary infections. Furthermore, high levels of BHB in blood have been

associated with oxidative stress (Song et al., 2016) that can further compromise the immune response increasing the risk of diseases, death or culling. The HYK status in wk2 did not show evidence of an association with herd removal, and disease incidence (i.e., mastitis, metritis, retained placenta, left-side displaced abomasum, and milk fever) occurring in the week before diagnosis (i.e., 1st wk of lactation) was not a confounder variable as it did not influence the estimates. Besides diseases, herd removal is also affected by other factors such as the cost of replacing the animal, reproduction problems, and milk production (Fetrow et al., 2006). Thus, the previously affected outcomes (i.e., milk volume, milk composition, and reproductive efficiency) might be mediators on the association between HYK in wk1 and herd removal.

HYK type I occurs because milk production is elevated and the demand for glucose exceeds the capacity for glucose production. The blood concentrations of glucose and insulin are low and the concentration of ketone bodies is high (Holtenius and Holtenius, 1996). An increase in DMI is sufficient to reduce ketone body concentrations and avoid further negative outcomes. While Holtenius and Holtenius (1996) described HYK type I to occur between 3 to 6 weeks postpartum, we observed cows with production outcomes similar to the described for cows with type I as soon as in wk2. A shift of the timing when HYK type I occurs could be possible considering the evolution of dairy cattle in recent decades. Even though genetic pressure has recently changed toward a more health-related selection, for many decades the focus of selection was solely on increasing milk production (Miglior et al., 2017). Thus, nowadays, it would not be unexpected to observe this pathophysiology earlier in lactation. In a recent study, Ruoff et al. (2017) found that

multiparous cows between 3 to 6 weeks postpartum did not have hypoglycemia as suggested in type I, while cows with HYK diagnosed in the first 2 weeks postpartum were already showing hypoglycemia. In the same study it was discussed that the concept of type I and II might not be transferable to modern commercial dairy farms. However, based on our results, the distinction created by Holtenius and Holtenius (1996) still is useful but needs to be accommodated to the modern and more intensive dairy systems. In such a case, the elevated BHB concentration in wk2 would be caused by elevated demands of energy for milk synthesis rapidly adjusted by the gradual increase in feed intake.

Due to the observational nature of the study, further research should be performed to confirm or refute our results. Our findings should not be extrapolated to other BHB thresholds as the results are expected to change, which would require a specific evaluation. Lastly, the incorporation of measurements such as fat infiltration in the liver, and insulin and glucose concentrations could be considered in future studies in order to help to elucidate the etiology of HYK at different time points in early lactation.

## 5.6 Conclusion

Our study findings indicate that HYK diagnosed during wk1 of lactation are indicative of a negative performance in terms of milk yield, reproduction and herd removal. Meanwhile, these associations were less severe when HYK is diagnosed during wk2 of lactation. Further research is warranted in order to understand the underlying cause of the differences observed in this study.

Table 5.1: Descriptive characteristics and univariate analyses of dairy cows at baseline in the first two weeks postpartum

| | Week 1 | | | Week 2 | | |
|---|---|---|---|---|---|---|
| | HYK+[1] [N (%/SD)][3] | HYK-[2] [N (%/SD)] | *P*-value[4] | HYK+ [N (%/SD)] | HYK- [N (%/SD)] | *P*-value |
| BCS at dry | | | | | | |
| < 3.75 | 4 (11.4) | 31 (88.6) | 0.19 | 1 (3.4) | 28 (96.6) | 0.15 |
| ≥ 3.75 | 68 (22.5) | 234 (77.5) | | 31 (13.1) | 206 (86.9) | |
| Calving season | | | | | | |
| Fall | 30 (20.1) | 119 (79.9) | 0.49 | 25 (18.9) | 107 (81.1) | 0.18 |
| Spring | 20 (26.7) | 55 (73.3) | | 11 (16.4) | 56 (83.6) | |
| Summer | 27 (20.6) | 104 (79.4) | | 31 (26.7) | 85 (73.3) | |
| Calving ease score | | | | | | |
| 0 | 14 (23.3) | 46 (76.7) | 0.92 | 8 (30.8) | 18 (69.2) | 0.49 |
| 1 | 26 (24.6) | 80 (75.4) | | 17 (22.2) | 78 (77.8) | |
| 2 | 31(19.0) | 132 (80.0) | | 36 (25.0) | 108 (75.0) | |
| 3 | 6 (23.1) | 20 (76.9) | | 6 (12.0) | 44 (88.0) | |
| Parity | | | | | | |
| 2 | 19 (12.9) | 128 (87.1) | 0.001 | 21 (16.8) | 104 (83.2) | 0.15 |
| ≥ 3 | 58 (27.9) | 150 (72.1) | | 46 (24.2) | 144 (75.8) | |
| Previous gestation length | | | | | | |
| < 285 | 12 (20.3) | 47 (79.7) | 0.97 | 6 (11.3) | 47 (88.7) | 0.07 |
| ≥ 285 | 63 (21.6) | 229 (78.4) | | 61 (23.6) | 198 (76.4) | |
| Previous days dry | | | | | | |
| < 60 | 50 (19.2) | 211 (80.8) | 0.006 | 46 (19.1) | 195 (80.9) | 0.12 |
| ≥ 60 | 28 (34.6) | 53 (65.4) | | 21 (28.4) | 53 (71.6) | |
| Previous 305ME (kg) | 13,517 (± 2,308) | 14,242 (± 2,195) | 0.024 | 13,743 (± 2,599) | 14,152 (± 2,181) | 0.28 |
| Previous lactation length | | | | | | |
| < 305d | 34 (18.5) | 150 (81.5) | 0.16 | 26 (15.2) | 145 (84.8) | 0.006 |
| ≥ 305d | 43 (25.1) | 128 (74.9) | | 41 (28.5) | 103 (71.5) | |

[1] HYK+ = Cows diagnosed as positive to hyperketonemia (BHB≥ 1.2mmol/L).

[2] HYK- = Cows diagnosed as negative to hyperketonemia (BHB< 1.2mmol/L).

[3] Number and percentage of cows per group.

[4] P-value from Chi-square/ANOVA analyses

Table 5.2: Estimated marginal means of milk yield and components for 10 months of lactation.

| Outcome | HYK Positive (SE) | HYK Negative (SE) | Mean diff. per day (95% CI) [1] | *P*-value |
|---|---|---|---|---|
| Milk (kg/d) | | | | |
| Week 1 | 42.4 (1.6) | 46.1 (0.8) | -3.7 (-6.67, -0.83) | 0.013 |
| Week 2 | 43.7 (1.4) | 45.2 (1.0) | -1.0 (-4.04, 1.05) | 0.24 |
| Fat (%) | | | | |
| Week 1 | 3.9 (0.1) | 3.9 (0.0) | 0.02 (-0.21, 0.17) | 0.83 |
| Week 2 | 4.1 (0.1) | 3.8 (0.1) | 0.29 (0.08, 0.51) | 0.007 |
| Protein (%) | | | | |
| Week 1 | 3.1 (0.0) | 3.1 (0.0) | 0.00 (-0.08, 0.07) | 0.87 |
| Week 2 | 3.1 (0.0) | 3.1 (0.0) | 0.00 (-0.06, 0.05) | 0.88 |
| MUN (ml/dl) [2] | | | | |
| Week 1 | 11.5 (0.2) | 11.7 (0.1) | -0.18 (-0.63, 0.26) | 0.41 |
| Week 2 | 11.6 (0.2) | 11.5 (0.1) | 0.12 (-0.37, 0.63) | 0.62 |
| LSCC [3] | | | | |
| Week 1 | 3.8 (0.3) | 3.3 (0.1) | 0.49 (-0.06, 1.04) | 0.083 |
| Week 2 | 4.2 (0.3) | 3.6 (0.2) | 0.82 (0.25, 1.39) | 0.004 |

[1] Mean difference of HYK positive compared to HYK negative cows (reference) with 95% confidence interval.

[2] MUN = Milk urea nitrogen.

[3] LSCC = Linear score of milk somatic cell count.

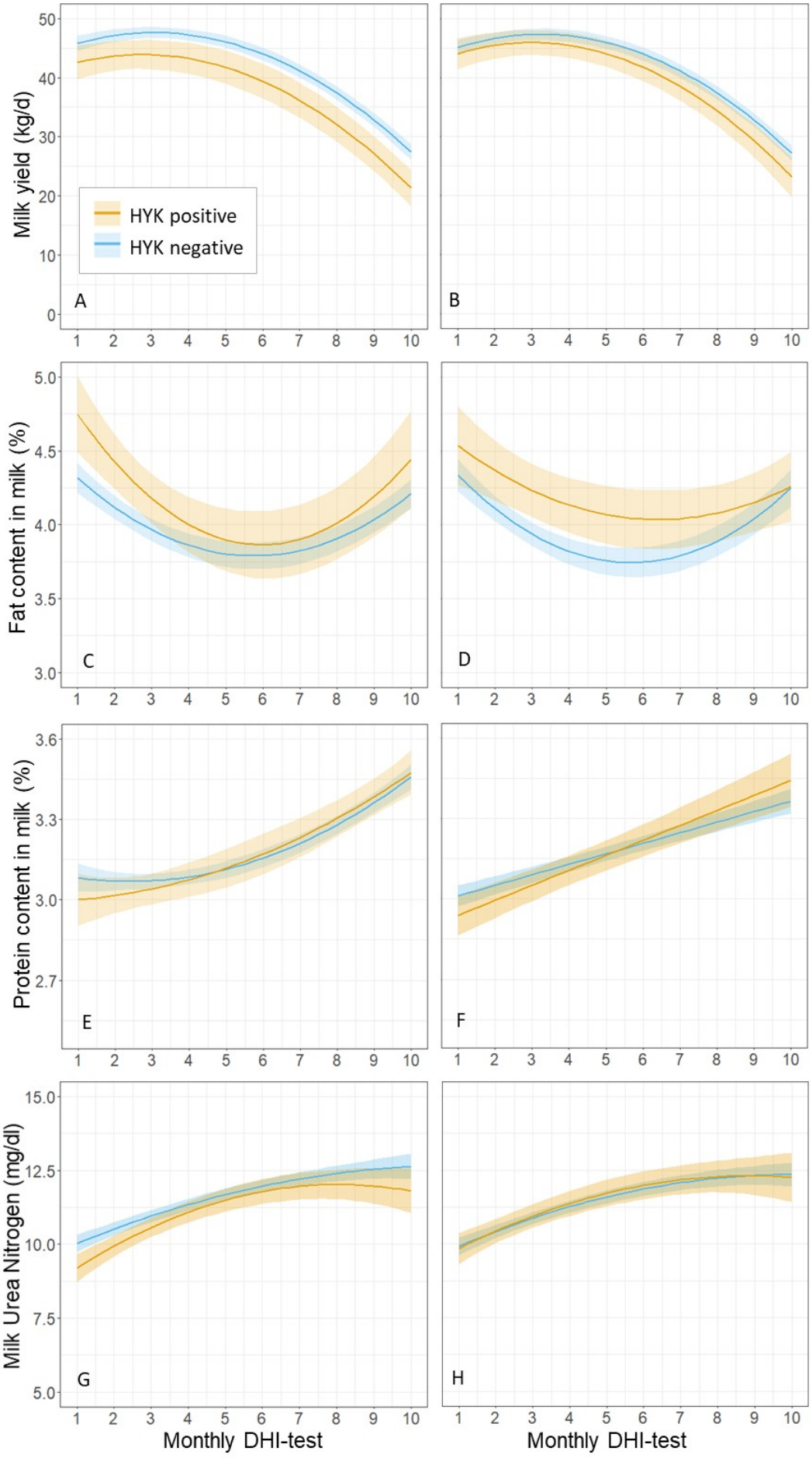
Milk yield (kg/d)
HYK positive
HYK negative
A
B
Fat content in milk (%)
C
D
Protein content in milk (%)
E
F
Milk Urea Nitrogen (mg/dl)
G
H
Monthly DHI-test
Monthly DHI-test

Figure 5.1: Estimated marginal means and 95% confidence intervals by monthly DHI-test for: daily milk volume (kg) according to diagnosis of hyperketonemia (HYK) in wk1 (A) and wk2 (B) after calving.; daily fat content in milk (%) in wk1 (C) and wk2 (D); daily protein content in milk (%) in wk1 (E) and wk2 (F); daily milk urea nitrogen (mg/dl) in wk1 (G) and wk2 (H);. Cows per group: A) HYK positive (HYK+) = 75, HYK negative (HYK-) = 274; B) HYK+ = 62, HYK- = 243; C) HYK+ = 75, HYK- = 274; D) HYK+ = 62, HYK- = 243; E) HYK+ = 80, HYK- = 287; F) HYK+ = 62, HYK- = 243; G) HYK+ = 79, HYK- = 287; H) HYK+ = 66, HYK- = 254.

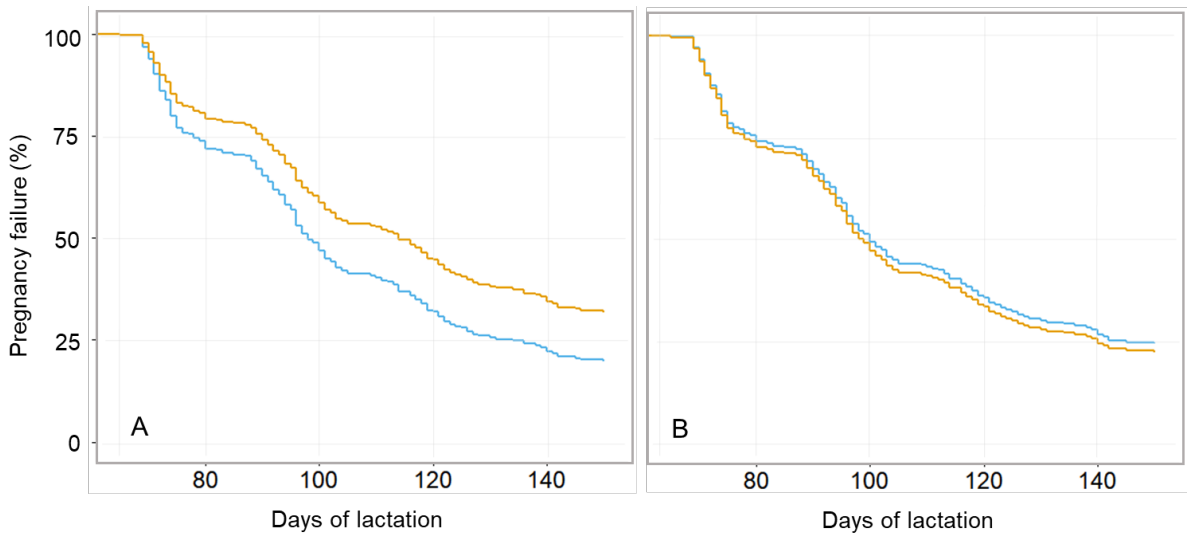


Figure 5.2: Cox Proportional-Hazard curves for time to pregnancy diagnosis within 150 days in milk, according to diagnosis of hyperketonemia (HYK) and moment of diagnosis.
A: Diagnosis of HYK in the 1st week of lactation. Orange line represents HYK positive cows (n=78) and blue line represents HYK negative cows (n=264). B: Diagnosis of HYK in the 2nd week of lactation. Orange line represents HYK positive cows (n=60) and blue line represents HYK negative cows (n=262).

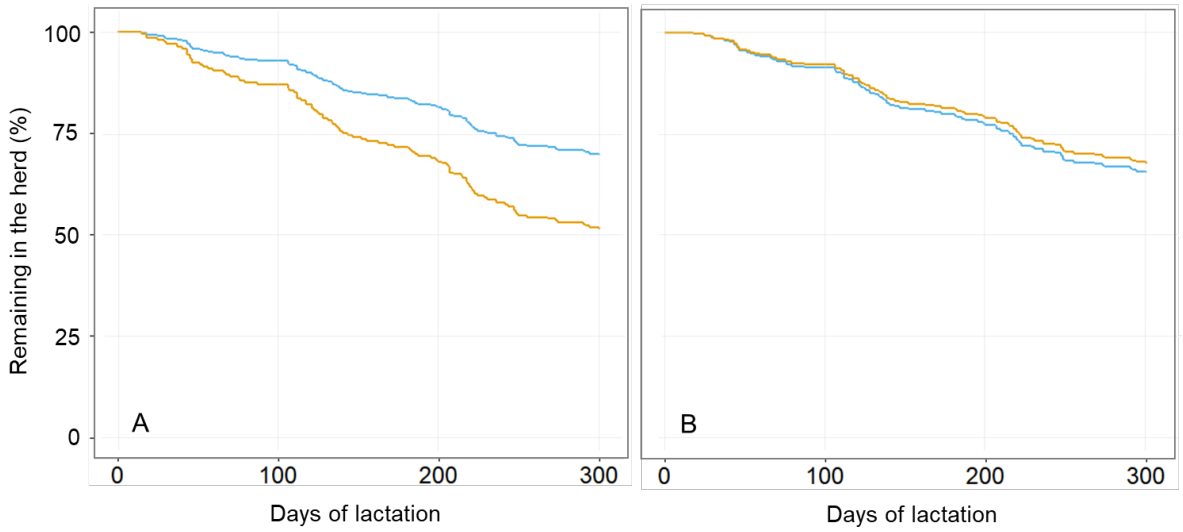


Figure 5.3: Cox Proportional-Hazard curves for time to herd removal up to 300 days in milk, according to diagnosis of hyperketonemia (HYK) and moment of diagnosis. A: Diagnosis of HYK in the 1st week of lactation. Orange line represents HYK positive cows (n=78) and blue line represents HYK negative cows (n=264). B: Diagnosis of HYK in the 2nd week of lactation. Orange line represents HYK positive cows (n=60) and blue line represents HYK negative cows (n=262).

# CHAPTER 6 – Summary of Results, Implications, and Future Directions

## 6.1 Introduction and objectives

Metabolic disorders are a key problem in the transition period of dairy cows. During this period, higher concentrations of circulating ketone bodies indicates that the cow is utilizing body reserves to fulfill the energy requirement of her new physiological state (Herdt, 2000).

Hyperketonemia (HYK) is defined as the increase of β-hydroxybutyrate (BHB), over a threshold of 1.2 mmol/L in blood. Hyperketonemia is a frequent metabolic disease affecting between 14% to 30.5% of the dairy cattle in the first three weeks post-calving (Mann et al., 2016; Chandler et al., 2018). Hyperketonemia has been associated with other metabolic and infectious diseases during early lactation such as retained placenta, milk fever, mastitis, metritis, displaced abomasum, and lameness. (Duffield et al., 2009; McArt et al., 2012a; Berge and Vertenten, 2014). Additionally, HYK has been associated with an increased likelihood of herd removal (McArt et al., 2012a; Roberts et al., 2012), and impaired reproductive performance (Walsh et al., 2007).

Despite the increase on our knowledge of hyperketonemia, there is an important margin for improvement on the epidemiology of the disease in terms of diagnostic, prevention, and control. For instance by understanding the performance of diagnostic tools under certain field and laboratory conditions. The role of risk factors in the development such as energy balance during the transition period on the dynamics of BHB concentration and HYK in early lactation, and in the future performance of hyperketonemic cows. Also by better understanding which is the best moment of disease diagnosis and its impact on a complete lactation.

## 6.2 Implications and Opportunities for Future Research

### *6.2.1 Implications*

The overarching goal of this thesis was to better understand the epidemiology of HYK to guide implementation of optimized decisions to diagnose and mitigate the occurrence of the disease. To this we had the following specific objectives:

1. Evaluate the test characteristics of a specific device to measure β-Hydroxybutyrate concentration in blood in reference to the laboratory assay, and the best practices to maintain an appropriate diagnostic performance in farm settings.

2. Describe the temporal patterns of β-Hydroxybutyrate concentration, development of hyperketonemia in early lactation, and changes in milk yield and

composition based on body condition score, as both a single measurement and its change over the late dry period.

3. Recognize the differential impact of hyperketonemia according to the moment of diagnosis during early lactation on milk yield and composition, reproductive performance, and herd removal.

4. Explore the role of milk yield during the first week after parturition, on the relationship between hyperketonemia diagnosed in early lactation and reproductive performance.

Based on the described results in the thesis we can draw the following conclusions:

1. The NVET is an acceptable alternative to the laboratory assay to measure BHB in whole blood with or without anticoagulant or thawed serum samples using the default calibration slope setting for screening purposes given the acceptable accuracy and precision.

2. Change in BCS during the dry period is a risk factor of variations of BHB and risk of HYK in the first two weeks postpartum, which should be primarily considered over BCS measured at a single time point when implementing prevention strategies. Specifically, cows with a loss in BCS larger than 0.5 points during the late dry period, as well as older cows are more likely to have a higher concentration of BHB and a greater incidence of hyperketonemia in early lactation.

Moreover, these cows also are more likely to experience a reduction in milk at the first monthly milk test day.

3. Hyperketonemia diagnosed during the first week of lactation is indicative of a future negative performance in terms of milk yield, reproduction and health. Meanwhile, cows that are diagnosed with HYK during the second week seem to be less affected than HYK negative cows.

4. Milk yield in early lactation plays a role in the association of HYK with reproductive performance. Among cows with low milk yield in the first week of lactation, HYK+ cows are less likely to becoming pregnant. Conversely, reproductive performance was similar among mid- and high-yielding cows, independent of HYK status in early lactation. The results suggest that milk yield could be considered in addition to the measurement of BHB concentration when monitoring dairy cows in early lactation.

Taken together, the implications of this body of work point to measures that can be utilized to diagnose, prevent and mitigate HYK in a comprehensive manner. During the prepartum period, specifically in the 3 weeks before calving, management should focus on preventing loss of BCS over 0.5 points to reduce the risk of HYK in early lactation. Cows that fail to avoid this loss of BCS have the peak of BHB concentration at the end of the 1$^{st}$ week of lactation. However, as we observed, not all hyperketonemic cows develop negative outcomes. Therefore, after calving,

diagnostics should be performed during the first week of lactation and especially on cows with the lower milk yield (i.e., lower quantile) as these are the cows with the higher risk of having negative performance. Cows positive to hyperketonemia during the second week are less affected by the disease in terms of health and productivity. Cows positive to HYK but with mid- and high-milk production level in early lactation also are less affected by the disease. Diagnostics are recommended to be performed using blood samples which can include whole blood, plasma or serum as the accuracy remains high. For practical purposes, samples can be collected and storage frozen, and tested all together at the earliest convenience.

### *6.2.2 Future Research*

This thesis addressed essential elements to prevent, diagnose and control hyperketonemia in a comprehensive manner. However, in order to deeply optimize decision-making at the field level, it is necessary to further develop some of the concepts involved in this body of work.

Our study of the changes of BCS in the late dry period helped us to identify cows that are at a higher risk of developing HYK and the moment when HYK is more likely to occur. This study was limited by only being in 1 herd and needs to be repeated to improve external validity. In addition, future research needs to focus on strategies to prevent loss of BCS during the late dry period as well as on the benefits of intervening these cows for diagnosis of HYK. Furthermore, a longer follow-up of the exposed

cows to postpartum BHB concentration could allow us to understand the length of the effect that loss of BCS has after calving.

A reevaluation of the optimal time to diagnose HYK is relevant and timely. In our study of the effect of HYK according to the week of diagnosis, we determine the importance that have the time of diagnosis of HYK. We identified the diagnosis during the first week of lactation as a crucial moment because of its association with a drop in milk yield, reproductive performance and higher risk of culling. However, a limitation of the study was that we only evaluated the exposure the first two weeks after calving. Future research is needed to determine with more granularity the exact time in which HYK is no longer causing negative effects on health and production to establish a clear timeframe for a screening protocol of HYK in which cows may benefit the most from treatment. Or as an alternative, evaluating and proposing different thresholds to different times periods after calving based on the association with negative heath and productive outcomes.

In addition, based on our results and previous research we believe that the differences between cows diagnosed with HYK at different time points during early lactation are a consequence of a different pathophysiology process. In order to elucidate this underlying mechanism of disease we are currently characterizing variations in the metabolic profile of dairy cows in the late dry period to identify potential novel biomarkers of hyperketonemia according to the moment of HYK. This work is

necessary to address the unclear role that metabolites play on the future performance of dairy cows. This will allow for additional insights into the pathophysiology of HYK to support decision-making at the prevention level.

From the study exploring the role of milk yield on the association between HYK and reproductive performance, we observed results that merit further investigation. A similar study with a higher enrollment of cows and daily measurements of milk yield is warranted. Also, it may be considered the inclusion of other health and productive outcomes such as metabolic diseases and long-term milk production as outcomes of interest. If results of this study are confirmed and cows with low milk yield in early lactation are effectively those at a higher risk of negative health and productive performance, intervention studies could be conducted to determine the benefit of targeting this group of cows for treatment. If proven successful, screening cows using both BHB concentration and milk-yield in early lactation to guide treatment decisions would provide help to stakeholders (i.e., veterinarians and producers) to improve the welfare and productivity of dairy cows while reducing costs and stress associated with unnecessary treatments. An evaluation that must be accompanied is an economic analysis to determine the feasibility of targeted treatment at the field level.

The studies in this thesis only included Holstein-Friesian dairy cows, which is a limitation in terms of external validity. Due to the genetic differences between breeds related to fat mobilization and BHB concentration postpartum, as mentioned in

chapter 1, extrapolation of results to other breeds needs to be done with caution. Moreover, the inclusion other breeds is scarce and is encouraged in future research.

To conclude, we hope that with this thesis, producers and veterinarians have more tools to take decisions to prevent, diagnose and control hyperketonemia. In addition, we hope that future research continue working on narrowing the efforts and resources towards those cows that may benefit the most from early diagnosis and control measures.

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