Genetic Markers for Resistance to Ketosis in Dairy Cattle

by

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ABSTRACT

GENETIC MARKERS FOR RESISTANCE TO KETOSIS IN DAIRY CATTLE

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High-yielding dairy cattle are susceptible to ketosis, a metabolic disease which negatively affects the health, fertility and production of the cow. The goal of this research was to investigate candidate genes involved in the development of ketosis for single nucleotide polymorphisms (SNP) with the potential to be used as genetic markers for disease-resistance. A panel of 998 SNP markers were identified *in silico.* A preliminary sample of Canadian Holstein cows were genotyped for the new markers and producer-recorded cases of clinical ketosis and milk β-hydroxybutyrate, an indicator of ketosis, were used as the phenotypes for association analyses. Results of the analyses suggested that forty-five markers in thirty-one unique candidate genes were associated to the metabolic traits. This work is an initial step in the development of a genetic tool to improve ketosis-resistance and also indicates new pathways that may be related to the biological mechanisms underlying the disease.

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BHB	β-hydroxybutyric acid
CGAA	Candidate gene association analyses
DIM	Days in milk
DMI	Dry matter intake
DA	Displaced abomasum
EST	Expressed sequence tag
F:P	Fat-to-protein ratio
GEBV	Genomic breeding value
GPC	Glycerophosphocholine
GPC:PC	Glycerophosphocholine-to-phosphocholine ratio
GWAS	Genome-wide association study
KEGG	Kyoto Encyclopedia of Genes and Genomes
KET	Ketosis
LD	Linkage disequilibrium
LE	Linkage equilibrium
MAF	Minor allele frequency
NCBI	National Center for Biotechnology Information
NEB	Negative energy balance
NEFA	Non-esterified fatty acid
NMR	Nuclear magnetic resonance
SIFT	Sorting Intolerant from Tolerant
SNP	Single nucleotide polymorphism
QTL	Quantitative trait loci
QTN	Quantitative trait nucleotide
UTR	Untranslated region

CHAPTER ONE: Literature Review

1.1 Overview of Ketosis

The three weeks before and after parturition is a stressful time for dairy cows, as their bodies adapt to the physiological changes associated with the progression from a state of gestation to lactation (Drackley, 1999). Energy partitioning, mineral mobilization, and immune function are driven by homeorhesis in the periparturient dairy cow, which favours pregnancy and milk production over the normal, homeostatic maintenance of equilibrium in the cow's body tissues (Bauman and Currie, 1980; LeBlanc, 2010). During the periparturient - or transition - period, the cow is faced with the nearly impossible task of providing sufficient nutrients to her fetal calf, for her own demands during calving, and for the massive production of milk that occurs subsequently; this undertaking is further complicated by the depressed feed intake of the cow leading up to calving (Bertics *et al.*, 1992). Essentially, the cow will push her body to its metabolic limits, putting herself into negative energy balance (NEB), even to the point of causing a state of disease. The majority of metabolic and infectious disorders of dairy cattle occur during the transition period, and their occurrence signifies a poor adaptation to NEB (Duffield *et al.*, 2009; LeBlanc, 2010). One common form of transition period disease is the metabolic disorder ketosis (KET), which is traditionally defined as the elevation of ketone bodies in circulation exceeding normal limits, accompanied by the lack of appetite (Shultz, 1968; Kelton *et al.*, 1998). Increased ketone bodies in body fluids is a necessary, adaptive response to NEB; however, excess production is indicative of a poor transition to lactation, which has detrimental effects on the health and production of the cow (Shultz, 1968). While the changes that occur during the transition period have been the focus of many researchers over the past several decades, the mechanism which causes a maladaptive

response to NEB has not been determined, and KET continues to be a significant problem for dairy producers.

Pathogenesis

The concept of NEB was introduced previously as the inability of the dairy cow to meet the energy requirements for lactation from the feed she ingests. A significant reduction in dry matter intake has been well documented in the two weeks leading up parturition (Bertics *et al.,* 1992; Drum *et al.,* 1996; Olson, *et al.,* 2011). The energy balance is further challenged as the net energy requirement of the cow can increase by approximately fifty percent from two days prepartum to two days postpartum, when milk synthesis is initiated (Drackley *et al.,* 2005). As a ruminant, microbial fermentation of feed occurs before gastric digestion, which allows the cow to breakdown structural carbohydrates into volatile fatty acids (VFA) (Bell and Bauman, 1997). Glucose is also readily fermented by rumen microbes before it can be absorbed in the small intestines; therefore, the cow relies primarily on gluconeogenesis to meet her body's glucose requirements (Bell and Bauman, 1997). Gluconeogenesis occurs in the liver and kidneys, using propionate, a VFA, as the main substrate, but also lactate, glycerol and gluconeogenic amino acids (Reynolds *et al.*, 2003). Normally, endogenous production of glucose is sufficient to meet the needs of the cow; however, around parturition the demand for glucose, amino acids, and fatty acids drastically increases for the synthesis of milk lactose (Bell, 1995; Bell and Bauman, 1997). Bell (1995) estimated that even if all of the gluconeogenic substrates supplied by the cow's diet were directed towards gluconeogenesis, they would account for just sixty-five percent of the mammary requirement for glucose of 1,775 grams per day.

Partitioning of nutrients towards milk production requires metabolic changes at multiple tissues sites and is supported by hormonal control; some of these adaptations are presented in Table

1.1. The cow must mobilize body stores of lipid, glycogen, and protein to support lactation as well as for her own energy requirements. This shift towards catabolism of energy stores begins one to two weeks prepartum and peaks after calving (Reynolds *et al.,* 2003). Tamminga *et al.* (1997) estimated that the average cow will mobilize 30.9 kg of fat and 4.6 kg of protein in the first two months of lactation. Amino acids, lactate and glycerol are directed to the liver to fuel the increased rate of gluconeogenesis (Bell and Bauman, 1997). Up to eighty percent of the glucose that is produced is fated for the mammary gland and lactose synthesis (Bauman and Currie, 1980). To support the altered nutrient portioning, glucose is spared from use in peripheral tissues by decreased rates of lipogenesis and increased oxidation of non-esterified fatty acids (NEFA) that are released from adipose tissue (Bell and Bauman, 1997; Schäff *et al.,* 2013). The shift towards lipolysis raises the plasma NEFA concentration; these NEFAs are increasingly taken up by the liver and directed towards three different metabolic end-points: 1) complete oxidation to carbon dioxide, 2) partial oxidation to ketones, and 3) re-esterification to triglyceride (TG) (Reynolds *et al.,* 2003; Brickner *et al.,* 2009). TG synthesized by the liver can be exported to the blood stream as a component of very low density lipoproteins (VLDL) (Grummer, 1993). The massive influx of NEFA during the transition period is not fully matched by increased oxidative capacity or export of TG; therefore, more NEFAs are directed towards ketogenesis (McCarthy *et al.,* 2015).

Dairy cows are inherently inefficient at exporting TG from the liver as VLDL and do not match the rate of export to account for increased hepatic TG synthesis during lactation (Grummer, 1993). TG that is not removed from the liver accumulates within hepatocytes and may have an effect on the metabolic capacity of the liver (Bobe *et al.,* 2004; Schulz *et al.,* 2014). Reports of altered gene expression of enzymes involved in glucose and lipid metabolism and reduced enzyme activity have been reported in cows with high levels of liver TG (Rukkwamsuk *et al.,* 1999; Gross *et al.,* 2013;

Table 1.1: A summary of some of the metabolic adaptations to lactogenesis in dairy cattle, modified from Bauman and Currie (1980) and Ingvartsen and Andersen (2000) with information from Bell and Bauman (1997), Schäff et al. (2013), and Weber et al. (2013b).

Physiological Function	Metabolic Change	Tissue Site	Hormone
Milk synthesis	↑ blood flow ↑↑ uptake of nutrients (glucose, fatty acids, amino acids)	Mammary gland	↑ prolactin, estrogens, growth hormone
Lipid metabolism	↓ lipogenesis ↑ lipolysis ↑ absorption of fatty acids ↑ milk fat synthesis ↑ oxidation of fatty acids/ketones	Adipose tissue Liver Mammary Muscle/Periphery	↑ glucagon:insulin ↓ insulin sensitivity
Glucose metabolism	↓ glucose utilization ↑↑ gluconeogenesis	Peripheral tissues Liver	↓ insulin sensitivity ↑ glucagon:insulin
Protein metabolism	↑ mobilization of amino acids ↓ protein synthesis	Skeletal muscle	↑ glucagon:insulin

↑: increased rate ↓: decreased rate

Weber *et al.,* 2013a). A liver proteome analysis of cows with fatty liver did not detect changes in the gluconeogenesis pathway, but amino acid and lipid metabolism, as well as oxidative stress mechanisms were affected (Sejersen *et al.,* 2012). While the effect of TG infiltration is not clear, reduced metabolic potential of hepatocytes may advance the NEB during early lactation. Additionally, liver lesions can occur when the liver TG concentration reaches just fifteen percent (Schulz *et al.,* 2014).

The main theory for the inefficiency of complete oxidation is that the limited availability of citric acid cycle intermediates (i.e. succinyl-CoA, oxaloacetate) prevents acetyl-CoA, produced from β-oxidation, from being fully oxidized (Schultz, 1968; Aiello *et al.,* 1984; Drackley *et al.,* 2001; Li *et al.,* 2014). This increases the amount of acetyl-CoA directed towards the synthesis of the ketone bodies β-hydroxybutyrate (BHB), acetone, and acetoacetate, which has been well-documented during the transition period (Aiello *et al.,* 1984; Reynolds *et al.,* 2003). The formation of ketone bodies is part of the cow's adaptive response to NEB, as they can be used as an energy source in some peripheral tissues (Heitmann *et al.,* 1987). BHB is also a substrate for *de novo* milk fat synthesis, and may also regulate this process in mammary epithelial cells of ruminants (Bell, 1995; Zhang *et al.,* 2015). Additionally, BHB has an inhibitory effect on lipolysis in adipocytes, which can limit the excessive mobilization of TG (van der Drift *et al.,* 2013). Despite these adaptations, overproduction of ketone bodies elevates their concentration in circulation, resulting in hyperketonemia, which is associated with negative health effects in the dairy cow. High BHB and NEFA concentrations trigger the inflammatory response within hepatocytes, which links KET to oxidative stress-induced liver injury and may prolong the NEB (Shi *et al.,* 2014; Shi *et al.,* 2015). Furthermore, hyperketonemia impairs immune function by reducing leukocyte chemotaxis, inhibiting phagocytosis by neutrophils, and increases the risk of uterine and mammary gland

infections (Suriyasathaporn *et al.,* 1999; Hammon *et al.,* 2006; Grinberg *et al.,* 2008). Metritis, endometritis, and mastitis are associated with hyperketonemia in early lactation (Duffield *et al.,* 2009; Berge and Vertenten, 2014; Shin *et al.,* 2015). Additionally, hyperketonemia significantly increases the risk of displaced abomasum (DA) (Duffield *et al.,* 2009; Berge and Vertenten, 2014). Blood BHB has been recommended as a metabolic indicator to monitor DA, although the mechanistic connection between hyperketonemia and DA is not well-understood (LeBlanc *et al.,* 2005). Disease incidence increases the chance of an animal being culled from the herd. Compared to retained placenta, metritis and DA, cows that experienced KET during their first lactation had a higher risk of being culled (Dhakal *et al.,* 2015). McArt *et al.* (2012) reported that the risk of removal from the herd increased by 1.4 for every 0.1 mmol/L increase in BHB.

Hyperketonemia in early lactation also has an effect on the reproductive performance of the dairy cow. Reist *et al.* (2000) determined that cows with elevated ketone concentrations in the six weeks following calving began ovulating again later than cows with lower ketone levels. In addition to taking more time to come into estrus and being bred later, ketotic cows are 20-50% less likely to become pregnant at first insemination (Walsh *et al.,* 2007). Bovine oocytes that were fertilized *in vitro* in media supplemented with BHB were less likely to develop to the blastocyst stage (Sarentonglaga *et al.,* 2013). Hyperketonemia reduces the reproductive performance of dairy cows and increases the risk of reproductive disorders (i.e. endometritis, ovarian cyst), which are unfavorable to the producer (Shin *et al.,* 2015).

KET may also be a problem for the producer due to production losses. A loss of 0.5kg of milk per day is associated with every 10 µmol/L increase in BHB (McArt *et al.,* 2012). Duffield *et al.* (2009) reported decreased milk yield at the first test day and an average loss of 300 kg over the entire lactation for cows with high blood BHB levels. Duffield *et al.* (2009) also found elevated

BHB was associated with increased milk production in the second week of lactation; similar results have been reported elsewhere and may indicate that cows with a greater production capacity have an increased risk of developing hyperketonemia (Schulz *et al.,* 2014; Vanholder *et al.,* 2015). This explanation is supported by Fleischer *et al.* (2001) who found an association between KET and high milk yield in the previous lactation.

As a metabolic disease, the pathogenesis of KET is multifactorial and many tissues, organs, and pathways are altered in the ketotic cow. The development of NEB into hyperketonemia and subsequently KET is not fully-understood. An additional complication is that a cow can have elevated ketone levels with no clinical indication of disease.

Clinical Findings

KET can be classified as either clinical or subclinical disease based on the observation of clinical signs in addition to hyperketonemia. Elevated concentration of ketones can be recognized in the blood, urine, milk or on the breath of the cow (Kelton *et al.,* 1998). Signs of clinical KET may include decreased appetite, gaunt appearance and weight loss, reduced rumen motility, and dry manure (Schultz, 1968; Kelton *et al.,* 1998). Behavioural changes may also occur. Often the cow will seem dull and depressed, but she also may be highly excitable and uncoordinated; this form of the disease is called nervous KET (Schultz, 1968). The clinical signs of KET are non-specific and are not easily distinguishable from other transition period diseases. For this reason, feeding and social behaviours, as well as activity have been researched to find a correlation that can be used to identify ketotic cows (Itle *et al.,* 2014). An additional challenge for producers is identifying cows that are subclinically-ketotic. These cows also have hyperketonemia and experience the same health- and production-limiting effects as cows with clinical KET; however, there are no recognizable signs (Kelton *et al.,* 1998; Duffield *et al.,* 2009). Testing the ketone body

concentration and other metabolic indicators, is critical for the diagnosis and monitoring of both clinical and subclinical KET.

Diagnosis

Pathologically, KET is characterized by high NEFA and ketone and low glucose concentrations in the blood (Xia *et al.,* 2012). Research over the last four decades has explored the measurement of different ketone bodies, metabolites, hormones, and production parameters in various body fluids, with alternative diagnostic tools (Schultz, 1968; Duffield *et al.,* 1997; Seifi *et al.,* 2011; Klein *et al.,* 2012; Xia *et al.,* 2012). The measurement of ketones in the blood is the most widely accepted indicator of ketotic status in dairy cattle (Schultz, 1968; Duffield *et al.,* 1997; Xia *et al.,* 2012). BHB is the most abundant ketone body found in the blood stream, while acetone is the main ketone in milk (Enjalbert *et al.,* 2001). Plasma BHB has been considered the standard for measurement of hyperketonemia due to its abundance in circulation and improved stability during storage, relative to acetone and acetoacetate (Työppönen and Kauppinen as cited in Enjalbert *et al.,* 2001). Blood analysis is considered to be less variable for ketone measurement than urine and milk, which can be affected by diet changes and high somatic cell count; however, blood collection is more invasive and not practical for herd-based testing on farms (Schultz, 1968; Geishauser *et al.,* 2001). Nitroprusside tablets and powders, dipsticks and test strips for ketones have been developed for herd-screening using milk and urine as test substrates; however, their test sensitivity and specificity are limited (Geishauser *et al.,* 2001; Oetzel, 2004).

Some other biomarkers that have been proposed as indicators of KET include prepartum NEFA concentration, the milk fat-to-protein ratio (F:P), milk glycerophosphocholine-to-phosphocholine ratio (GPC:PC), and plasma sphingolipids (Ospina *et al.,* 2010; van Knegsel *et al.,* 2010; Klein *et al.,* 2012; Rico *et al.,* 2015). F:P is a production parameter related to a cow's

energy balance, that can be calculated at each test day and is a useful tool for producers. A high F:P has been shown to be associated with a higher chance of developing subclinical KET; however, on its own F:P is not a useful screening test (Duffield *et al.,* 1997). GPC:PC describes the rate that phospholipid is broken down to be used as an alternative energy source during NEB; this is a measure of a cow's response to NEB that is independent of KET status (Buitenhuis *et al.,* 2013). Additionally, the use of spectral data from tested milk has been shown to predict ketone body concentration (van Knegsel *et al.,* 2010). Milk BHB is routinely measured by herd recording services in multiple countries, including Canada and the Netherlands (Canadian Dairy Network, 2011; van der Drift *et al.,* 2012; Denis-Robichaud *et al.,* 2014). Denis-Robichaud *et al.* (2014) established thresholds for milk BHB; a concentration of BHB equal or greater than 0.20 mmol/L of milk is indicative of hyperketonemia with high accuracy (Area under the curve > 90%; Denis-Robichaud *et al.,* 2014).

A threshold for BHB is important for testing purposes, as ketogenesis is a necessary adaptation to NEB in early lactation and every cow will have increased levels. Duffield *et al.* (2008) determined blood BHB thresholds for hyperketonemia based on negative effects to health and production. A concentration greater than 1.2 mmol/L in the first week postpartum was associated with an increased risk of displaced abomasum (DA), while a concentration greater than 1.4 mmol/L was the best threshold for the development of clinical KET and this was when the greatest impact on milk yield was observed (Duffield *et al.,* 2008). Similar thresholds have been reported (LeBlanc *et al.,* 2005; Ospina *et al.,* 2010). While cows with clinical KET often have a greater blood BHB concentration than cows with the subclinical disease, cows presenting with clinical signs have been reported with a BHB concentration as low as 0.600 mmol/L (Suthar *et al.,* 2013). Furthermore, Oetzel (2004) has reported subclinical cases in practice with more than 3

mmol/L BHB. Subclinical KET is a challenge for producers who want to monitor the health of their herd, and also leads to variable reports of prevalence.

Epidemiology

The three weeks following calving is the most critical period for the onset of KET in dairy herds (Schultz, 1968, Zwald *et al.,* 2004). McArt *et al.* (2012b) determined that the peak prevalence of subclinical (BHB 1.2-2.9 mmol/L) and clinical KET (BHB>2.9 mmol/L) during this period was at five and seven days in milk, respectively. The prevalence of KET depends on the criteria used for classification, as well as factors such as, region, breed, stage of lactation, and parity. When estimated from producer and veterinary records, the prevalence of clinical KET was 3.3% in European herds and 4.5% in Canadian herds (Koeck *et al.,* 2012; Suthar *et al.,* 2013). Based on a blood BHB threshold of 3.0 mmol/L, the prevalence of clinical KET was determined to be 3.02% and 11.6% in dairy herds located in the United States and the Netherlands, respectively (McArt *et al.,* 2012b; Vanholder *et al.,* 2015). Zwald *et al.* (2004) used data from producer management software programs to estimate the mean lactation incidence rate for KET was 10% in American herds; this parameter takes into account the lactations at risk of being affected by KET (Kelton *et al.,* 1998). A previous meta-analysis by Kelton *et al.* (1998) reported a lactation incidence rate range of 1.3% to 18.3% for KET. In clinical practice, Oetzel (2004) observed variability between herds in the definition of clinical KET used for health recordings, as well as the overestimation of disease in small herds and underestimation in large herds. Measuring the prevalence of subclinical KET, or simply hyperketonemia, based on blood BHB, is recommended to get a better sense of the problem in a herd.

The prevalence of subclinical KET has been reported at much higher levels than the clinical form of the disease, in multiple studies. McArt *et al.* (2012b) determined a prevalence of 43.3%

for BHB concentration between 1.2 and 2.9 mmol/L in American dairy herds. Using the same threshold, the prevalence was determined to be 21.8% and 47.8% for European and Dutch herds, respectively (Suthar *et al.,* 2013; Vanholder *et al.,* 2015). A prospective cohort study of dairy herds in the United States determined that 40% of the herds sampled had a prevalence of hyperketonemia (BHB >1.2 mmol/L) greater than 15%; 8% of these herds had a prevalence higher than 35% (Ospina *et al.,* 2010). Evidently, hyperketonemia is a common problem for dairy cattle in early lactation, and there is a disconnect between the number of cows with elevated BHB levels and the cows who present with clinical signs of KET.

Increasing parity has been repeatedly shown to increase the risk of hyperketonemia in dairy cattle (Duffield *et al.,* 1997; Berge and Vertenten, 2014; Vanholder *et al.,* 2015). Cows in their second or greater lactation have a greater risk of developing KET than primiparous cows (OR=1.5 for parity 2 and OR=2.8 for parity 3 to 7; Berge and Vertenten, 2014). Wathes *et al.* (2007) described differing endocrine and metabolic profiles for primiparous and multiparous cows during the transition period. Additionally, Berge and Vertenten. (2014) and Vanholder *et al.* (2015) found that calving in the spring (April to June) increased the odds of KET.

In summary, the prevalence of clinical KET ranges from 3 to 12%, while subclinical KET ranges from 22 to 48%. Clinical and subclinical KET affects dairy herds globally, and continues to be a problem for producers despite the research that has been focused on developing treatment and prevention strategies for this disease.

Treatment

The traditional treatment for KET aims to increase glucose concentration by administering an exogenous dose of glucose, a glucocorticoid or providing gluconeogenic material for

endogenous glucose production (Schultz, 1968). A bolus of dextrose has been recommended as a short-term treatment for hypoglycemia and is useful for cows presenting with nervous signs; however, this treatment is not sufficient alone (Gordon *et al.,* 2013). Glucocorticoids, mainly dexamethasone or flumethasone, have also been proposed for the treatment of KET to improve hypoglycemia and to block the effect of insulin (Gordon *et al.,* 2013). This treatment option is supported by the finding that cows experiencing hyperketonemia have low cortisol levels (Forslund *et al.,* 2010). When used in combination with oral propylene glycol, a gluconeogenic precursor, cows injected with dexamethasone had lower BHB and higher glucose and insulin concentrations (van der Drift *et al.,* 2015). Conversely, Seifi *et al.* (2007) found that cows treated with isoflupredone acetate in a large field study were at a higher risk of developing subclinical KET relative to the control group. There may also be short-term side effects associated with glucocorticoid treatment, including reduced calcium levels and low milk production (Seifi *et al.,* 2007). An alternative hormone therapy that has been studied is a glucagon infusion, which is a gluconeogenic hormone and can subsequently enhance the production of insulin (Bobe *et al.,* 2004). A fourteen-day treatment by continuous intravenous infusion resulted in improved clearance of TG from the liver and increased glucose levels in nutritionally-induced ketotic cows; this also improved the condition of fatty liver (Hippen *et al.,* 1999). A low dose of insulin has been suggested as a treatment for KET in combination with other methods, but its use has been limited by its high-cost and limited supporting evidence for its efficacy (Hayirlil *et al.,* 2002; Gordon *et al.,* 2013).

The supplementation of gluconeogenic substrates, including propylene glycol and propionate, by oral drench, has been recommended as the basis of ketosis-treatment; this treatment is proposed to enhance gluconeogenesis, which will restore glucose and insulin levels (Gordon *et*

al., 2013). A large field study tested the usefulness of the propylene glycerol drench and determined that cows in the treatment group were less likely to develop a DA, die or be culled after developing subclinical KET (McArt *et al.,* 2012a). Likewise, Plantoni and Allen (2015) found that propylene glycol was more effective than glycerol infusion for treatment of KET. For practical purposes, providing gluconeogenic substrates as a top dress is less labour-intensive than administering a bolus to each cow; however, neither supplementary propylene glycol nor glycerol improved the disease status when provided as top dress in the diet (Lomander *et al.,* 2012). Reasons for this may be related to the difference in the speed of delivery when provided as an oral drench compared to a top dress (Lomander *et al.,* 2012).

The recommended treatment for KET of a propylene glycol drench, has not changed over the past six decades, due to limited evidence in the published literature (Gordon *et al.,* 2013). Alternative treatment options have focused solely on stimulating gluconeogenesis, despite the other metabolic pathways that have been linked to the pathogenesis of KET. Instead, research has looked into preventative methods of control in the areas of nutrition, medicine, and genetics.

Prevention and Control

Strategies to prevent the onset of KET in early lactation are based on controlling for factors which put cattle at a higher risk of developing this disease. Some of these risk factors include high colostrum and milk yield, second or higher parity, high body condition score in the dry period, and reduced DMI post-calving, and ketogenic feeds being used (Ingvartsen, 2006; Schulz *et al.,* 2014; Vanholder *et al.,* 2015). For the latter three risk factors, nutritional management have been proposed for control. Ketogenic feedstuffs include rations that have a high proportion of ketogenic amino acids, leucine, lysine, phenylalanine, tryptophan and threonine, or silage which a high butyrate concentration (Ingvartsen, 2006; Vicente *et al.,* 2014). Vicente *et al.* (2014) determined

that grass silage had higher levels of butyrate than corn silage and was linked to a higher risk of subclinical KET when fed to lactating cows. Aside from choosing appropriate forage for the lactating cows, nutritional management during the dry period has also been recommended for prevention of KET (Safdar, 2015). Over-conditioned dry cows, do not respond well to NEB and have increased BHB, NEFA, and weight loss post-calving (Schulz *et al.,* 2014). Splitting the dry cow group has been recommended in order to prevent over-feeding cows in the at the beginning of the dry period, while encouraging them to eat as they get closer to parturition, when their DMI drops (Ingvartsen, 2006; Overton and Waldron, 2004). Feeding cows more frequently and increasing the proportion of concentrate in the cows' diet has been proposed to increase intakes during the transition period and improve NEB (Asl *et al.,* 2011; Schulz *et al.,* 2014). As simple as this feeding strategy seems, intake is hard to monitor at the herd level and the mechanisms that control feed intake in ruminants are complex and not fully-understood (Allen, 2014).

Additional feeding strategies that have been studied include additives such as propylene glycol, propionate, niacin, dietary fat, specific fatty acids and ionophores (Overton and Waldron, 2004). As previously noted, topdressing with propylene glycol or glycerol to stimulate gluconeogenesis does not improve metabolic status in early lactation (Lomander *et al.,* 2012). Research regarding propionate as a preventative feed additive has had variable results and propionate is not palatable for the cow (Overton and Waldron, 2004). Variable results have also been reported for supplemental niacin, depending on the route of administration (Overton and Waldron, 2004; Morey *et al.,* 2011). As a feed additive niacin is ineffective, but has been shown to reduce lipolysis and NEFA concentration when the rumen is bypassed, either by encapsulation or infusion (Morey *et al.,* 2011). Niacin, converted to NAD+/NADH in cells, acts as a cofactor in enzyme reactions including mitochondrial respiration and the citric acid cycle (Depeint *et al.,*

2006). Niacin has also been shown to bind the G-protein-coupled receptor 109A-mediated pathway *in vitro* and may play a role in lipid mobilization in adipose tissue (Kenez *et al.,* 2014). The G-protein-coupled receptor 109A is expressed in the adipose, liver, muscle, and brain of cows and is activated by BHB in a negative-feedback loop that reduces lipolysis (Offermanns *et al.,* 2011; Titgemeyer *et al.,* 2011). In a clinical trial testing the effects of niacin supplementation, Morey *et al.* (2011) observed lower NEFA levels in the treatment group, but the same response was not seen in plasma BHB. Additionally, the cows who received the encapsulated niacin supplement had reduced prepartum DMI, which may limit the practicality of this strategy for controlling the occurrence of KET (Morey *et al.*, 2011).

Feeding a high fat diet is proposed to increase the energy density of the diet to reduce NEB and to decrease the level of NEFAs released, since dietary fat bypasses the liver and can provide energy to peripheral tissues, reducing the amount of mobilization of NEFA from adipose tissue (Pickett *et al.,* 2003). Grum *et al.* (1996) fed a high fat diet prepartum and observed a reduction in the amount of TG stored in the liver, and an increased rate of peroxisomal β-oxidation; however, DMI was reduced and the frequency of transition period disease was high in this study. Depressed DMI was also reported when an oral drench was used to provide fat to cows at calving (Pickett *et al.,* 2003). In this study, the fat drench had no effect on NEFA or BHB concentrations and the amount of TG infiltrating the liver was unchanged (Pickett *et al.,* 2003). The effect of conjugated linoleic acids on lipid metabolism has also been studied, since supplementation has been shown to reduce the percentage of milk fat (Overton and Waldron, 2004). Cows in NEB who received an encapsulated conjugated linoleic acid supplement had lower milk fat yields, but slightly higher milk yield and milk protein yields, with no changes in glucose, insulin, or NEFA concentration in circulation (de Veth *et al.,* 2006). Baumgard *et al.* (2002) saw similar results when conjugated

linoleic acid was infused into the abomasa of lactating cows, which may indicate a mammary-specific effect; the treatment reduced milk fat but did not improve whole-body energy balance.

Monensin is an ionophore with antimicrobial properties that is commonly used on dairy operations. Monensin is known to have an effect on rumen fermentation and increase the proportion of propionate being produced, which can then be directed towards glucose synthesis in the liver (Sauer *et al.,* 1989). Monensin has been studied as a feed additive as well as a bolus. Sauer *et al.* (1989) fed cows a concentrate containing monesin for one week before calving and noted lower ketone and milk fat concentrations postpartum. Similar results were also observed when a slow-release bolus was administered to cattle during the transition period (Duffield *et al.,* 1998). A more recent study out of Florida was conducted with the same treatment as Duffield *et al.* (1998); however, serum BHB concentrations were not different between the treatment and control groups (Melendez *et al.,* 2006). Topdressing with direct-fed microbials has also been proposed to alter rumen fermentation products in order to prevent hyperketonemia (Luan *et al.,* 2015).

Besides the feed additives and oral supplements described here, other preventative strategies for KET aim to regulate hormones that play a role in energy metabolism. Insulin and glucagon have both been researched as potential methods of control. Insulin is an anabolic hormone which limits lipolysis, increases the storage of glucose, and increases the use oxidation of ketones for energy; however, ketotic cows often present with insulin-resistance that allows for glucose to be spared (Gordon *et al.,* 2013). Slow-release insulin injected intramuscularly prepartum was successful in increasing DMI and decreasing serum NEFA and BHB levels (Hayirli *et al.,* 2002). While these results are desirable, even the lowest dose of insulin produced hypoglycemia in the treatment cows (Hayirli *et al.,* 2002). Glucagon was introduced previously as a potential treatment for KET and fatty liver by infusion. When administered subcutaneously

just after calving, the effects seen over the next two weeks were elevated glucose and reduced NEFA concentrations, with no negative effects on milk production or DMI (Nafikov *et al.,* 2006). A shift towards more efficient protein use was indicated in this study, but there was no change in BHB level or the TG content of the liver (Nafikov *et al.,* 2006).

Strategies that focus on nutritional and hormonal balance for controlling the frequency of KET events in a herd have been shown to be successful, in some circumstances, by controlling for risk factors of KET. An additional risk factor is the innate susceptibility to KET that can be passed on from a cow's sire and dam, which is explained by additive genetic variance. Genetic variation may explain the differences observed in how cows adapt to NEB, develop either clinical or subclinical disease, and respond to treatments. The identification of cows who are resistant to KET, based on genetic merit, is a potential, long-term solution to control for KET on farm.

1.2 Approaches for the genetic control of ketosis

For many years, the genetic improvement of dairy cows was focused exclusively on selection for greater milk-yield, in order to increase the economic value of a cow. This type of single-trait selection has had negative consequences on health and fertility traits (Jones *et al.,* 1994; Veerkamp *et al.,* 2003). Cows with high genetic merit for milk production tend to respond poorly to NEB, despite their greater feed intake during the transition period (Veerkamp *et al.,* 2003). In a multi-generational selection experiment, Jones *et al.* (1994) noted that the cows in the high-yielding selection group had greater milk production but also significantly elevated health and reproduction expenses. Recently, many countries have expanded their national selection indices to include more functional traits, in a shift away from single-trait selection, towards a more balanced breeding goal

(Miglior *et al.,* 2005). Genetic improvement of functional traits like ketosis-resistance can be accomplished by selecting genetically superior animals to include in a breeding program, based on information from their phenotypic records, relationships with other animals, and molecular markers in their genetic code.

Generally, it is difficult to implement phenotypic selection for health traits; this is due to the difficulty, cost, and variability of recording data on disease (Muir, 2007; Neuenschwander *et al.*, 2012). This is true for ketosis-resistance, which could be based on animals' health records, ketone levels in blood, milk, or urine, or other metabolic indicators. Health records logged by producers or veterinarians have the potential to be used for genetic evaluations when they are available, and are cost-effective due to voluntary recording systems and established data pipelines. However, in Canada only 40% of dairy producers keep records that are suitable for accurate evaluations (Koeck *et al.,* 2012). Producers in Canada record disease traits based on the clinical definition of KET (Kelton *et al.,* 1998). Subclinical and misdiagnosed cases could result in the underestimation of disease. In addition to the inconsistencies with phenotypic data, genetic evaluations for health traits are limited by low estimates of heritability. Heritability in the narrow sense, refers to the proportion of phenotypic variance of a trait that can be attributed to the genes that were transferred from parent to offspring, or the ratio of additive genetic variance to phenotypic variance (Falconer and Mackay, 1996). A trait with low heritability is therefore difficult to improve through phenotypic selection as there is little resemblance between relatives. Fitness traits such as disease resistance have low estimates of heritability and the value is population specific (Falconer and Mackay, 1996). Health records have been used to estimate the heritability of KET in Canadian, Norwegian, and American herds, which ranges between 0.02 and 0.16 (Zwald *et al.*, 2004; Heringstad *et al.*, 2005; Koeck *et al.,* 2012; Koeck *et al.,* 2014). The low heritability of KET estimated using health

records is in part due to the binary scale of recording disease (i.e 0= no disease, 1= disease) (Tveit *et al.,* 1992). This limitation fails to differentiate between the severities of cases, which could be valuable when the aim is to select for disease resistance. Metabolic indicator traits that are genetically correlated to KET may improve the reliability of the trait, and subsequently the response to selection; as previously discussed, plasma and milk BHB, F:P, BCS, and GPC:PC have been proposed as good predictors of KET (Klein *et al.,* 2013; Koek *et al.,* 2014; Ehret *et al.,* 2015).

The measurement of blood ketone concentrations for all animals in a herd would not be practical for producers due to high costs and labour requirements, as frequent sampling over multiple weeks would be required. Using milk sampled on test days to detect ketone levels would be a more convenient measure of hyperketonemia, but may not capture all of the variation due to limited frequency of measurements (van der Drift, 2012). The Canadian dairy industry has standards for herd recording that include milk analysis for BHB; however, the test days may be up to 50 days apart and may not fall within the first three weeks postpartum, when hyperketonemia is best detected (Canadian Dairy Network, 2011). A strong, positive genetic correlation between milk BHB and clinical KET has been estimated to be between 0.48 and 0.75 (Koeck *et al.,* 2014; Koek *et al.,* 2015). van der Drift *et al.* (2012) calculated estimates of heritability for blood and milk BHB to be 0.17 and 0.16, respectively. Similarly, Koeck *et al.,* (2014) estimated the heritability of milk BHB at the first test-day (4-40 DIM) to be 0.12, which was equal to the heritability for the fat to protein ratio. The heritability of plasma acetoacetate concentration was estimated to be 0.11 (Tveit *et al.,* 1992). Using indicator traits that are genetically correlated to disease occurrence and have improved heritability can be helpful when determining estimated breeding values for ketosis-resistance, which can then be used to rank and select for superior animals.

As of December 2016, metabolic disease resistance is included in national genetic evaluations in Canada; however, several European countries have already evaluated ketosis-resistance and included it in their selection criteria for several years. Changes in genetic trend due to selection for ketosis-resistance have been inferred from the health recordings of the Norwegian Red cattle population of Norway (Heringstad e*t al.*, 2005). In Norway, KET has been included in the total merit index at varying weight values from 1978 until 1996, when KET was merged into a trait called 'other diseases', with a lower index weight (Heringstad *et al.,* 2005). A decreasing genetic trend for KET – measured as the deviation from the average of sire posterior means for cows born in 1976 – was observed between 1980 and 1995, when the greatest relative weight was put on KET (Heringstad *et al.,* 2005). The genetic trend for Norwegian Red cattle was also studied through multiple five-generation selection experiments. Heringstad *et al.* (2007) established four selection groups: high milk production, low milk production, high protein yield, and low mastitis frequency. The mean EBV for KET per cow-generation increased in the high-yielding genetic group, had a flat line trend in the high protein group, and decreased in both the low-yield and low mastitis groups (Heringstad *et al.,* 2007). After five generations of selection the high production group had a mean EBV for KET that was 3% higher than the low production group, where a higher ketosis EBV is unfavourable (Heringstad *et al.,* 2007). These findings draw attention to another disadvantage of conventional selection for health traits; strong selection for these traits may negatively affect production traits, which will reduce the economic-potential of the progeny. Metabolic pathways, such as milk synthesis and fat mobilization, influence both KET and production traits, which limits the effectiveness of genetic selection. A more effective selection strategy requires the identification and understanding of the genes that control the observed

phenotype. Molecular information found in an animal's genetic code provides the opportunity to improve the selection response for the trait ketosis-resistance.

Genetic markers

The bovine genome is estimated to be made up of 3,000 Mb that are spread over thirty chromosome pairs – twenty-nine autosome pairs and a pair of sex chromosomes – and contain the instructions of over 20,000 protein-encoding genes (RefSeq Accession GCF_000003055.6). The sequencing and mapping of the bovine genome has provided an unparalleled tool for the genetic improvement of livestock: the selection of animals based on specific genes or regions of the genome that control economically-important traits, termed quantitative trait loci (QTL; Falconer and Mackay, 1996). QTL for congenital defects, milk production, conformation, reproduction, and health traits have been described in dairy cattle populations, and the list continues to grow (Andersson, 2001; Ashwell *et al.*, 2001; Dekkers, 2004; Songstegard *et al.*, 2013; Pant *et al.*, 2014; Yudin and Voevoda, 2015). QTL can be represented by SNP, insertions/deletions, duplications, copy number variants, and epimutations (Dekkers, 2004; Yudin and Voevoda, 2015). The strongest QTL that has been discovered in the bovine genome lies within BTA14, close to the *DGAT1* gene, which affects milk fat composition (Winter *et al.*, 2002). A SNP at position 232 is associated with lower milk fat when there is an alanine residue replaces a lysine, which reduces the ability of DGAT1 to bind to acyl-CoA (Winter *et al.*, 2002). The SNP in *DGAT1* is just one example of a functional mutation that is associated with an economically-important trait in dairy cattle, or a quantitative trait nucleotide (QTN; Mackay, 2001). QTN can be found within exons, introns, promoters, and untranslated regions (UTR) before and after the coding sequence of the gene. The *DGAT1* QTN described by Winter *et al.* (2002) is located within a protein-coding region of the gene and causes a change in the activity of the enzyme. QTN within non-coding regions

may alter the function and expression of the gene, the rate of mRNA transcription and processing, or alter transcription factor binding sites (Yudin and Voevoda, 2015). Furbass *et al.* (2006) described another QTN within bovine *DGAT1* in the form of variable number of tandem repeats in the 5'-UTR region of the gene that is also associated with milk fat content and milk yield. Although this genetic variant does not change the sequence of the amino acids that make up the protein, the expression of *DGAT1* is stimulated by different numbers of repeats (Furbass *et al.*, 2006). QTN, like those found in *DGAT1* that have direct effects on the trait phenotype, can provide useful information about an animal's genetic potential when the its genotype is known. In addition, genetic markers that are linked to a QTN are valuable for estimating genetic potential.

Falconer and Mackay (1996) described the optimal criteria for genetic markers: i) highly polymorphic, ii) high genome coverage, iii) neutral effects on other economic traits and overall fitness and iv) codominance. Markers can be in linkage equilibrium (LE) or disequilibrium (LD) with an unknown QTL. LE exists when the alleles of the QTL and the marker are assorted into haplotypes randomly; offspring have the same chance of inheriting a recombinant haplotype as an nonrecombinant one from a parent. In the case of LD, the marker is assumed to be close to the position of a QTL and offspring will inherit a nonrecombinant marker-QTL haplotype from a parent more often than if they were inherited by chance (Zondervan and Cardon, 2007). Therefore, a marker linked to a QTL will be indirectly associated with the trait that the QTL controls. Therefore, genetic markers can be useful for characterizing a trait, as long as the association with the unknown QTL is maintained.

The association of LD and LE markers is most likely dependent on the proximity of the marker to the true QTL; the further the marker is from the QTL, the higher the probability will be that a recombination event will occur during meiosis, which would break the association. LE-

markers make use of the LD that extends over long distances within a certain family because only one generation of recombination has occurred. Denser marker maps used for the detection of population-wide LD do not require a specific family structure (Dekkers, 2004). Direct markers are QTN, which code for the functional mutation; this should not change from generation to generation or across populations. While direct markers provide the best opportunity for genetic gain and are the easiest to implement into a selection program, the detection and development of these markers is more complex (Dekkers, 2004).

Historically, different strategies have been implemented to make use of the information that genetic markers provide. Gene-assisted and LD or LE-based marker-assisted selection incorporate one or more genes or markers into traditional genetic evaluations (Fernando and Grossman, 1989; Meuwissen *et al.,* 2013). This form of selection was shown to moderately increase genetic gain by improving the accuracy of EBV (Dekkers, 2004; Silva *et al.,* 2014). Genetic gain in dairy cattle was further improved by the implementation of dense marker panels and genomic selection. In contrast to gene- or marker-assisted selection, genomic selection estimates the breeding value of animals from the sum of the effects of thousands of markers that are spread across the genome (GEBV; Meuwissen *et al.,* 2001; Hayes *et al.,* 2009). Therefore, selection decisions can be made for animals that do not yet have records for traits or do not have progeny with records, reducing the generation interval substantially (Meuwissen *et al.,* 2001). Additionally, Hayes *et al.* (2009) reported increases in reliability of up to twenty percent for some traits, when comparing EBV and GEBV calculated at birth. Parker Gaddis *et al.* (2014) demonstrated that when pedigree and genomic information were blended, the mean reliability of sire predicted transmitting abilities for KET increased from 0.18 to 0.35 for unproven sires. Today,

the genomic selection of young dairy animals is common practice worldwide, although genomic selection has its own set of challenges (Hayes *et al.,* 2009; Chesnais *et al.,* 2016).

The goal of any form of genetic selection is to identify the animals with the greatest number of favourable alleles that will be passed on to their offspring (Yudin and Voevoda, 2015). In order to do this, reliable alleles for a trait must be known; QTL effects must be estimated in a reference population that has reliable phenotypic records (Hayes *et al.,* 2009). The two main strategies for identifying QTL and QTN are GWAS that investigate generic DNA markers spread out over the entire genome and candidate gene association analyses of variants within key functional genes (Andersson, 2001).

Genome-wide Association Studies

Under the assumptions of the infinitesimal model, quantitative traits are assumed to be under the control of many genes, each having a small impact on the phenotypic variation observed. GWAS aims to capture this variation by testing the association of many anonymous markers that are spread out across the entire genome with the trait of interest. Simply put, GWAS is a statistical test of phenotypic differences between animals from different marker genotype groups (Mackay, 2001).

Technology advances over the past two decades has made the genotyping of animals for thousands of SNP on arrays simple and inexpensive, which has led to a plethora of GWAS for numerous traits in livestock species. Ashwell *et al.* (2001) identified QTL for economically-significant traits in dairy cattle using just 155 microsatellite markers. The current standard for genotyping for research is an array with 50,000 SNP markers, while the use of high-density bovine arrays, with more than 777,000 markers, and whole genome sequencing is increasing. Imputation

methodologies have also contributed to the decreasing cost of genotyping large numbers of animals (Sargolzaei *et al.,* 2014).

To date, very few GWAS for metabolic traits in dairy cattle have been published in the scientific literature, and the results of these have not been confirmed (Tetens *et al.,* 2012; Buitenhuis *et al.,* 2013; Gaddis *et al.,* 2014; Tetens *et al.,* 2015). Comparison of the GWAS that have been performed is difficult due to differences in experimental design, including: population structure, marker density of the array used, and choice of phenotypic trait analyzed. Tetens *et al.* (2012) mapped QTL for F:P and energy balance – calculated as the difference between energy intake and energy demand for milk production and maintenance – in a modest-sized cohort of German Holstein bull dams. Nineteen and seven significant SNP on the BovineSNP50 BeadChip (Illumina Inc., San Diego, CA) for F:P and energy balance, respectively, were identified in this study; these were mapped to nine probable candidate genes, including *DGAT1* and genes that affect energy metabolism (glycolysis, gluconeogenesis, TCA cycle; Tetens e*t al.,* 2012). There were no SNP in common between F:P and energy balance in this study, which indicates mechanistic differences between these two measures of metabolic status (Tetens e*t al.,* 2012). Other GWAS have targeted the variation observed in milk metabolites to detect QTL in dairy cattle.

A Danish GWAS performed by Buittenhuis *et al.* (2013) genotyped 371 cows to detect QTL for thirty-one milk metabolites quantified using nuclear magnetic resonance (NMR). Eight SNP with genome-wide significance were detected using the BovineHD BeadChip (Illumina Inc., San Diego, CA)– more than 700, 000 markers – for metabolites including orotic acid, malonate, galactose-1-phosphate, glucose, urea, carnitine, and glycerophosphocholine (GPC; Buittenhuis *et al.,* 2013). No QTL for BHB or acetoacetate were detected in this study; however, the milk was sampled between 129 and 228 DIM, which is past the period when cows have the highest risk of

hyperketonemia (McArt *et al.*, 2012b; Buittenhuis *et al.*, 2013). Tetens *et al.* (2015) collected milk from 3 – 351 DIM to determine the GPC:PC by NMR to be used as the dependent measure for a GWAS in Holstein-Friesian cattle. These were mostly first lactation cows that were genotyped with the BovineSNP50 BeadChip (Illumina Inc., San Diego, CA, United States of America; Tetens *et al.*, 2015). SNP on BTA7, 23, and 25 were associated with GPC and the strongest signal on BTA25 was mapped to the *APOBR* gene, which encodes the apolipoprotein B receptor (Tetens *et al.*, 2015). The GWAS by Buittenhuis *et al.* (2013) also detected a strong signal on BTA25 associated with GPC. Both of these GWAS suggest that *APOBR* is a promising candidate gene, which could contain a QTN associated with metabolic status in dairy cows.

The limited number of QTL that have been detected for metabolic traits by GWAS demonstrates a need for additional studies. The lack of GWAS for metabolic traits is partially due to the difficulty of collecting accurate phenotypes for the large sample size that is required to estimate the effects of tens of thousands of SNP. An alternative approach for detecting QTL is the analysis of SNP within candidate genes that are chosen for a specific trait.

Candidate Gene Association Analyses

Ketosis-resistance is a multifactorial trait that is most likely influenced by many different biological pathways, genes, and their interactions. While GWAS aims to capture the effects of loci spread across the entire genome, it is often difficult to interpret any of these effects as being causative (Andersson, 2009). In contrast, candidate gene association analyses (CGAA) use *a priori* knowledge of the trait phenotype and gene function to narrow the focus of the study to regions of the genome where a QTN is likely to be found. CGAA are useful to fine-map regions surrounding previously-detected QTL or genes, that have been identified as having biological relevance to the trait. In CGAA SNP are discovered by looking for differences within the gene sequence of animals

at the high and low end of the trait spectrum. CGAA are typically low-throughput compared to GWAS; sequencing PCR amplicons of genes is more time-consuming than genotyping with microarrays, especially when many genes are being studied. However, recently, targeted imputation has been used to deduce sequence data from the whole genome sequence of founder animals (Raven *et al.,* 2016). Although CGAA are more laborious, these studies can be more informative than GWAS. CGAA allows for the identification of novel SNP and a more complete understanding of their effects on the gene product.

Over the past decade candidate gene investigation has led to the discovery of SNP for many traits in dairy cattle, including: milk yield and composition, clinical mastitis, somatic cell count, Johne's disease, fertility traits, body traits, BCS, energy balance and serum BHB (Olsen *et al.,* 2007; Wang *et al.,* 2008; Skelding *et al.,* 2010; Pant *et al.,* 2011; Alim *et al.,* 2012; Clempson *et al.,* 2012; Rincon *et al.,* 2012; Zhou *et al.,* 2013; Fontanesi, *et al.,* 2014; Pant *et al.,* 2014; Wang *et al.,* 2014; Mahmoudi *et al.,* 2015; Nani *et al.,* 2015; Tetens *et al.,* 2015; Usman *et al.,* 2015; Bagheri *et al.,* 2016; Raven *et al.,* 2016). Many of these studies provide evidence to suggest that the SNP identified are QTN. Wang e*t al.* (2014) described a putative QTN within an intron of the *CD46* gene that results in a novel splice variant and protein that is larger than the wild type. Dairy cattle that have either the CT over the CC or TT genotype at this locus were shown to be more resistant to mastitis (Wang *et al.,* 2014). *CD46* was selected as a positional candidate gene based on the gene product's role in infection control (Wang *et al.,* 2014). Like mastitis, KET is a complex health trait with many potential candidate genes; however, only a limited number of association analyses have been performed for genes related to KET or metabolic disease.

A study by Clempson *et al.* (2012) aimed to identify SNP within *insulin-like growth factor binding protein-2 (IGFBP2)* associated with fertility, milk production and metabolic status in a

sample of 430 dairy cattle. *IGFBP2* was chosen based on its involvement in the insulin-like growth factor system that is known to affect energy status and performance (Clempson *et al.,* 2012). The study identified a novel SNP within the gene by sequencing PCR amplicons of regions of the gene and determined that second lactation cows with the CC genotype tended to have a higher concentration of glucose and lower concentration of BHB than the TT genotype in the first week postpartum; however, these findings did not reach statistical significance ($0.15<P>0.05$; Clempson *et al.,* 2012). In this study, the strongest SNP effects were detected under the most metabolically stressful conditions, when multiparous cows were in the first week of lactation (Clempson *et al.,* 2012). These findings re-emphasize the difficulty of measuring the true metabolic status of the cow to determine her phenotype. There is a short window to accurately quantify a cow's response to NEB, which makes it difficult to differentiate between cows who can transition better than others; Mahmoudi *et al.* (2015) took this into consideration in the design of their association study of the γ subunit of the adenosine mono-phosphate-activated protein kinase gene (*AMPKγ1* or *PRKAG1*).

The AMPK signalling pathway is known to regulate energy balance in mammalian species and has been shown to be activated in bovine hepatocytes by both acetate and BHB (Kahn *et al.,* 2005; Li *et al.,* 2013; Deng *et al.,* 2015). Mahmoudi *et al.* (2015) genotyped and sampled cows who had at least three lactations, on the fifth DIM for serum BHB. A novel SNP in the 3'UTR of the gene was identified and it was determined that cows that were homozygous for the mutant allele had a higher BCS in the dry period, BHB postpartum and 305-day milk yield than the other genotypes (Mahmoudi *et al.,* 2015). The researchers propose that the SNP changes the binding affinity of a microRNA to the mRNA target sequence that is known to be in this region and affects the amount of AMPK translated (Mahmoudi *et al.,* 2015). Both Clempson *et al.* (2012) and

Mahmoudi *et al.,* (2015) successfully identified SNP for metabolic traits in positional candidate genes. CGAA can also benefit from information from GWAS, which can narrow down the genome to regions that are associated with the trait of interest.

Tetens *et al.* (2015) identified a candidate gene on BTA25 with strong association to GPC via the GWAS that was discussed earlier; the researchers re-genotyped with primers designed to cover the entire region of *APOBR*– exons, as well as introns. Twenty-nine polymorphisms were associated with GPC, with the top nonsynonymous SNP explaining 33 and 17% of the additive genetic variance and phenotypic variance, respectively (Tetens *et al.,* 2015). While this study strongly suggests that *APOBR* contains a causative mutation for GPC, strong pairwise linkage disequilibrium in this region (r^2= 0.96) limited the identification of the exact QTN among the 29 SNP detected in this study. Nevertheless, this study successfully identified a highly significant QTL for GPC and is a starting point for the investigation of the effect of this gene on the dynamic metabolism of the dairy cow in early lactation.

The process of detecting QTN is inherently valuable for the application of results for animal breeding, as well as for the information they contribute to the comprehension of complex phenotypes, like ketosis-resistance. KET continues to be a common disease of dairy cattle with a large economic impact on producers, and is most likely under-reported and treated. Forms of treatment and prevention for KET have seen no significant improvements over the propylene glycol drench, although advancements in detection tests have been made. The demand for genetic improvement of dairy cattle for metabolic traits has been acknowledged by several countries and evaluation services that now offer selection indexes for wellness traits. However, improvement of KET will be limited by the low heritability of the trait and reliability of EBV. Identifying genetic markers for KET will provide a genomic tool that will increase reliabilities and reduce the

generation interval. The results of few GWAS of metabolic traits have been published and these need to be validated independently to determine if the markers will maintain association with the QTL across populations. Even less work has been done to characterize SNP within candidate genes involved in metabolism and suggest evidence of functional QTN. This review of scientific literature has revealed a noticeable gap in the understanding of how dairy cows adapt to NEB during the transition period and the genetic factors that make cows susceptible to KET.

CHAPTER TWO: Rationale for Research

2.1 Introduction

Modern dairy cattle have been bred to maximize milk production and therefore, profitability. High-yielding dairy cows, however, are susceptible to metabolic stress during the transition period that can lead to diseases like KET (Veerkamp *et al.*, 2003). While research over the past two decades has led to various recommendations for early screening and treatment of ketotic cows, prevention of KET in early lactation would be the ideal solution. Management strategies for the prevention of KET, which were discussed in Chapter One, include the reduction of BCS in the dry period, nutritional additives, and hormone modulators. Despite the identification of these methods of prevention, the prevalence of ketosis on farms has not seen a reduction. Koeck *et al.* (2012) reported the prevalence of producer-recorded cases of KET at 4.5% in the Canadian Holstein population, while a later study estimated that 14% of this population had levels of milk BHB above the threshold for hyperketonemia in early lactation; both of these estimates are on the low end of the estimated ranges for clinical and subclinical KET summarized in Chapter One (Koeck *et al.*, 2014).

There is a global interest in breeding more robust dairy cattle with improved resistance to disease (Miglior *et al.*, 2005; Kargo *et al.*, 2014; Koeck *et al.*, 2015). In 2016, Zoetis Genetics (Kalamazoo, MI) released a commercial genetic evaluation system for dairy wellness traits, and Canadian Dairy Network (Guelph, ON), the dairy genetic evaluation service in Canada, has released a metabolic disease-resistance index (Canadian Dairy Network, 2016). The accumulation of phenotypic data for KET by producers and on-farm systems in Canada provides an excellent opportunity to identify and select for breeding cows that transition better and are less susceptible

to developing KET. Additionally, positive genetic correlations have been reported for KET and other transition period diseases and selection for ketosis-resistance may improve these traits indirectly (Koeck *et al.,* 2015; Jamrozik *et al.,* 2016). However, the low heritability of this trait is indicative of a low response to conventional selection and would benefit from the additional information provided by genetic markers.

To date, only a handful of association studies have been performed for metabolic traits, which have been limited by small samples and difficulty in obtaining reliable phenotypes for these traits. Additionally, the complexity of KET suggests that any QTL detected will most likely only explain a small amount of the genetic variance. In Chapter One, a review of the published association studies revealed that while the GWAS were able to detect QTL for metabolic traits, the markers on commercial panels may not be as informative as the SNP identified from the small-scale CGAA that were described. Raven *et al.* (2016) found that the accuracy of genomic predictions using high-density common SNP was improved when novel SNP from candidate genes were included. Despite these limitations, several promising candidate genes and QTL have been reported, as well as strong evidence for two potential QTN. These results are not only valuable as tools for selection, but also for providing new information about the underlying biology and molecular mechanisms associated with the disease. Clempson *et al.* (2012) and Mahmoudi *et al.* (2015) highlighted the importance of signalling networks, while Tetens *et al.* (2015) confirmed the relevance of hepatic apolipoprotein metabolism to the pathophysiology of KET. Further investigation of genes that are associated with KET is needed to provide a more complete picture of the metabolic adaptations that occur during the transition period and to explain a significant proportion of the genetic variance of this trait to aid in selection for disease-resistance.

2.2 Hypothesis

In this study, it was hypothesized that genetic variants within and surrounding genes that are linked to the development of KET can be utilized as markers for the prediction of KET in dairy cattle. Additionally, these variants can be identified *in silico* by making use of publicly available sequence databases, to create a cost-effective panel of SNP to be used for commercial breeding applications.

2.3 Research Objectives

The main objective of this research was to develop a set of novel markers for KET and determine their ability to predict resistance to this disease in dairy cattle through association analyses. The specific objectives included:

Objective One: Candidate Gene Investigation

A list of positional candidate genes was selected for *in silico* investigation by compiling evidence from what is currently known about metabolic changes that occur during the transition period and regions of the genome identified by GWAS. The identification of polymorphisms within these candidate genes was determined by mining public online cDNA and SNP libraries for sequence data, which were aligned in variant calling software to detect putative SNP to be included on a custom marker panel.

Objective Two: Association Analyses

The marker panel was utilized to genotype a sample of several hundred Canadian Holsteins with reliable disease records for KET, as well as milk BHB, an indicator of KET. Statistical

association between the cows' records and their genotype at each marker loci was tested to determine the efficacy of the markers.

CHAPTER THREE: *In silico* investigation of candidate genes for ketosis

3.1 Abstract

Genetic selection of dairy cattle that are resistant to developing the metabolic disease KET is a potential solution to the economic losses faced by producers, as well as the reduced herd health and welfare associated with this disease. Genetic evaluations for KET, a health trait with low heritability, would benefit from the additional information provided by genetic markers. The objective of this study was to identify novel SNP within candidate genes for KET to be incorporated onto a custom genetic marker panel. Investigating candidate genes provides the opportunity to discover functional SNP that are not currently included on commercial high-density marker panels. A list of 120 candidate genes, selected based on biological relevance, were selected for *in silico* investigation: a) genes which encode key enzymes and regulatory factors involved in metabolic pathways related to KET; b) genes that have been shown to be differentially expressed in ketotic animals; and c) genes that have been proposed by GWAS for KET. Within the candidate genes, putative SNP were identified by aligning sequence data from online cDNA libraries with the gene reference sequence. The variant calling program, Sequencher 4.9, was used to identify SNP and whether there was a corresponding amino acid substitution. SNP were prioritized for inclusion in the panel based on the predicted functional change of the encoded protein. A set of 998 SNP were incorporated onto a custom low-density panel for genotyping to determine their effectiveness as markers for KET.

3.2 Introduction

All modern dairy cattle that have been selected for high levels of milk production undergo metabolic stress during the periparturient period as they transition from late gestation to lactation. This shift in physiological state is accompanied by reduced feed intake and negative energy status that the cow must cope with. As the cow's demand for nutrients doubles to sustain lactogenesis, she must alter energy metabolism in the liver, adipose tissue, mammary gland, skeletal muscle, and peripheral tissues (Bauman and Currie, 1980; Drackley *et al.,* 2005). In response to NEB, ruminants mobilize body stores of protein and fat to oxidize as body fuel so that carbohydrates may be conserved to support milk synthesis (Tamminga *et al.,* 1997). Decreasing glucose and insulin concentrations mediate the major metabolic changes needed to stabilize blood glucose, which include: increased rate of gluconeogenesis, reduced lipogenesis and increased release of NEFA from adipose, increased uptake and metabolism of NEFA in the mitochondria of hepatocytes, and increased ketogenesis (Herdt, 2000). NEFA and ketone bodies are used as an alternate fuel source in the heart, kidney, skeletal muscle and mammary gland to further conserve glucose and restore energy balance (Heitmann *et al.,* 1987; Schaff *et al.,* 2013). However, cows that do not make the necessary metabolic adaptations are susceptible to hyperketonemia. Differences in metabolite profiles, nutrient partitioning and hepatic regulation of metabolism of ketotic and non-ketotic cows have been studied with the goal of describing the pathophysiology of KET, but the results of these studies have been inconclusive in explaining why some high-yielding cows develop KET while most do not (Loor *et al.,* 2005; Loor *et al.,* 2007; van Dorland *et al.,* 2009; Li *et al.,* 2012; Moyes *et al.,* 2013; Weber *et al.,* 2013; van Dorland *et al.,* 2014).

Genetic evaluations of KET and indicator traits including milk BHB have produced low estimates of heritability (0.02-0.16) that pose a challenge for the selection of cattle for ketosis-

resistance (Zwald *et al.,* 2004; Heringstad *et al.,* 2012; van der Drift *et al.,* 2012; Koeck *et al.,* 2012; Koeck *et al.,* 2014). Despite low heritability, several GWAS and candidate gene studies have successfully detected regions of the genome and identified several genes that are highly associated with metabolic traits (Clempson *et al.,* 2012; Tetens *et al.,* 2012; Buitenhuis *et al.,* 2013; Gaddis *et al.,* 2014; Mahmoudi *et al.,* 2015; Tetens *et al.,* 2015). Further, evidence of QTN for biomarkers of KET have been reported in three genes related to energy status – *insulin-like growth factor binding protein-2, adenosine mono-phosphate-activated protein kinase, and apolipoprotein B receptor* (Clempson *et al.,* 2012; Mahmoudi *et al.,* 2015; Tetens *et al.,* 2015). KET is a multifactorial disease that is most likely influenced by multiple loci and improving resistance to this disease would benefit from the identification of many associated genetic markers. A functional genomics approach for detecting genetic markers for complex traits has been effectively implemented in our lab in the past. Squires *et al.* (2014) developed and tested the association of a panel of SNP for boar taint by investigating candidate genes involved in the metabolism of boar taint compounds; this analysis identified SNP by comparing sequence data that is available through public, online databases, as well as sequencing cDNA from animal tissue. The purpose of this study was to propose a comprehensive list of candidate genes for KET and to categorize the polymorphisms within these genes to construct a panel of putative markers for selection to improve KET.

3.3 Methods

Selection of Candidate Genes

The initial step in the development of the marker panel was to designate which metabolic pathways may influence the onset of hyperketonemia in early lactation. Based on the understood

physiological adaptations to NEB, pathways involved in glucose and lipid metabolism, and ketogenesis were initially chosen for the analysis.

Additional pathway information came from the preliminary results of an association analysis of health trait EBV. Grossi (unpublished data, 2014) genotyped 1553 dairy cattle with the BovineSNP50 BeadChip (Illumina Inc., San Diego, CA) and subsequently submitted these to the Bovine HD BeadChip with approximately 700,000 SNP. The animals genotyped had estimated breeding values for health traits, including clinical KET. QTL were detected and mapped to potential candidate genes that were within 100kbp. The list of genes was inputted into the Database for Annotation, Visualization, and Integrated Discovery version 6.7 tool for functional analysis. The location of the QTLs was also obtained in order to determine if any genes with functional relevance were located nearby (<2 Mbp), although useful LD occurs at distances less than 100 kbp (Sargolzaei *et al.*, 2007).

The majority of the candidate genes were selected for investigation based on their proposed involvement in metabolic pathways related to the pathogenesis of KET. Enzymes and regulatory factors known to be involved in gluconeogenesis, fatty acid synthesis and degradation, the citric acid cycle and ketone synthesis and degradation were recognized by searching the species-specific Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. The KEGG pathway database is a useful, online tool that contains diagrams of the major physiological pathways and breaks them down to the molecular reactions, including the enzymes and cofactors that initiate each step.

Candidate genes that have been suggested in the scientific literature were also included in the analysis to confirm the results of other researchers; these studies have identified key genes by comparing the transcriptome, proteome and metabolome of transitioning cows (Loor *et al.*, 2005;

Loor *et al.,* 2007; van Dorland *et al.,* 2009; Li *et al.,* 2012; Moyes *et al.,* 2013; Weber *et al.,* 2013; van Dorland *et al.,* 2014; Akbar *et al.,* 2015).

In Silico SNP Determination

The candidate genes for KET were investigated *in silico* to categorize reported SNP and to detect novel SNP in order to compile a panel of putative markers. For each gene, the species-specific GenBank, UniGene and dbSNP databases that are maintained by the National Center for Biotechnology Information (NCBI) were mined for gene reference sequence, expressed sequence tags (ESTs) and SNP data, respectively. ESTs are short segments of cDNA of highly-expressed transcripts used for gene discovery and provide helpful information for organisms that do not have a completely-sequenced genome (Pontius *et al.,* 2003). The UniGene database collects ESTs for known genes, as well as those that are undiscovered; to be included, an EST must contain at least 100 quality base pairs and one overlapping sequence must be anchored at the 3' end (Pontius *et al.,* 2003). UniGene also provides information on which tissue the transcript is expressed in. dbSNP contains a collection of genetic polymorphisms at a specified location in the genome and therefore, can be mapped to genes of interest (Kitts and Sherry, 2011). dbSNP is constantly expanding to include public submissions of new SNP. The data from these sources are free and publically-available to download for anyone with a computer and an internet connection.

The downloaded sequence and SNP data for each gene was compiled and aligned by the variant calling software Sequencher v. 4.9, using the UMD 3.1.1 assembly for *Bos taurus*. Alignment with gene reference transcripts, as well as genomic reference sequences, allows for the identification of SNP within the coding region, introns and promoter regions. The software also provides translation of the gene sequence, which can be used to determine potential changes to the amino acid sequence and interpret the functional consequences of a polymorphism.

Marker Prioritization

During the *in silico* investigation, it became apparent that highly-polymorphic regions of genes, where SNP were located within twenty-five or less base pairs of each other, would be a challenge for the BeadChip genotyping platform. SNP in this region were prioritized for inclusion on the panel based on their predicted effect on gene products; the Sorting Intolerant From Tolerant (SIFT) algorithm was utilized to determine if an allele substitution would change the encoded amino acid, and if this change would alter protein function (Sim *et al.*, 2012). SIFT provides the consequence of the change (tolerated or deleterious) and a prediction score that takes into account whether a SNP is located within a highly-conserved region or not (Kitts and Sherry, 2011).

SNP Density

Visualization of the location and density of the SNP within the bovine genome was performed by creating a heat map with SNP1101 v. 1.0 (Sargolzaei, 2014).

3.4 Results and Discussion

Pathway Investigation

The metabolic pathways proposed to contain genes that may affect a cow's ability to transition successfully were: gluconeogenesis, fatty acid synthesis and degradation, citric acid cycle, ketone body synthesis and degradation, lipid mobilization and metabolism. Genes involved in regulation of these pathways were also considered.

Functional annotation of the genes that were identified by Grossi (unpublished data, 2014) resulted in thirty-one different pathways; the ten pathways with the highest number of genes

located close (<100 Kbp) to a QTL are shown in Table 3.1. The high prevalence of identified pathways involved in cell signaling in this table suggests that genes involved in regulation of metabolism may be more influential than enzymes involved in metabolic processes.

Other notable pathways that were highlighted include: PPAR signaling pathway (5 genes), pantothenate and CoA biosynthesis (4) and nicotinate and nicotinamide metabolism (4). The identification of the pantothenate and CoA biosynthesis and nicotinate and nicotinamide metabolic pathways suggests an important role of vitamins as co-factors in metabolic pathways related to KET. As discussed in Chapter 1.1., niacin supplementation has historically been used for the prevention and treatment of KET in dairy cattle, with variable results.

The PPAR signaling pathway plays a key regulatory role in the activation of metabolic pathways in the liver, skeletal muscle, and adipocytes. A variety of long-chain fatty acids and glucose have been reported as activators of several PPAR variants in ruminants, which in turn are transcription factors for genes involved in lipid metabolism (Bionaz *et al.,* 2013; Kahn *et al.,* 2013). Additionally, Cotter *et al*. (2014) found that knocking out the hepatic isotope (*PPARα*) suppresses ketogenesis and gluconeogenesis in neonatal mice.

The genes that were highlighted by the GWAS and annotated to the PPAR signalling pathway included: solute carrier family 27 (fatty acid transporter), members 1 and 6 (*SLC27A1* and *SLC27A6*), cytochrome P450, family 4, subfamily 1, polypeptide 11 (*CYP4A11*), apolipoprotein A-II (*APOA2),* and acyl-CoA synthetase long-chain family member 6 (*ACSL6).*

Gluconeogenesis

An increased rate of gluconeogenesis in the liver is one of the major, homeorhetic

Table 3.1: The top ten pathways identified from the functional annotation of candidate genes proposed by Grossi (unpublished data, 2014).

Pathway	Gene Count
Jak-STAT signaling pathway	12
Neuroactive ligand-receptor interaction	12
MAPK signaling pathway	12
Cytokine-cytokine receptor interaction	11
Purine metabolism	9
Fc gamma R-mediated phagocytosis	8
Systemic lupus erythematosus	8
Focal adhesion	8
Regulation of actin cytoskeleton	8
T cell receptor signaling pathway	7

adaptations that occur in ruminants during NEB (Reynolds *et al.*, 2003). Altered gene expression and activity levels of many gluconeogenic enzymes have been reported during periods of metabolic stress. Body condition, feed intake, liver fat content, BHB, NEFA, hormones and other factors have been proposed to regulate the expression of these enzymes during the transition period (Loor *et al.*, 2005; Hammon *et al.*, 2009; Weber *et al.*, 2013; Selim *et al.*, 2014; Akbar *et al.*, 2015). Weber *et al.* (2013) noted increased expression of *PCK1, PC, and G6PC* at different time points during the periparturient period. The relationship between expression of these genes and liver fat content has also been quantified, but with contradicting results; Hammon *et al.* (2009) reported upregulation of all genes, while Selim *et al.* (2014) reported downregulation, and Weber *et al.* (2013) reported an effect only for *PC.* Downregulation of *ALDOA* has been reported during the two weeks before and after calving (Loor *et al.*, 2005). Rukkwamsuk *et al.* (1999) compared the activity of phosphoenolpyruvate carboxykinase (PCK1), fructose, 1,6-bisphosphatase (FBP1), and glucose 6-phosphatase (G6P) from the liver of cows overfed to induce fatty liver and control cows around parturition. Activity of PCK1 was lower for overfed cows at all time points and FBP1 activity was lower before calving, suggesting that the degree of fatty liver can impact a cow's rate of glucose production by altering enzyme activity.

Mutations within genes that code for gluconeogenic enzymes or transcription factors of these genes could alter a cow's ability to synthesize glucose, furthering her NEB and increasing her risk of hyperketonemia. The genes involved in gluconeogenesis that were investigated for SNP are presented in Table 3.2. The genes selected enzymes of enzymes that catalyze each step in the synthesis of glucose from precursors; several enzymes are dual purpose and also catalyze the reverse reaction in the glycolysis pathway. *G6PC3* was also investigated, but there were

Table 3.2: Genes involved in hepatic gluconeogenesis and number of putative SNP detected *in silico.*

Gene	Gene name	Process in pathway	Number of SNP
PKLR	pyruvate kinase, liver and RBC	phosphoenolpyruvate → pyruvate	17
PC	pyruvate carboxylase	pyruvate → oxaloacetate	16
PCK1	phosphoenolpyruvate carboxykinase	oxaloacetate → phosphoenolpyruvate	8
ENO1	enolase 1 (alpha)	phosphoenolpyruvate ↔ 2-phosphoglycerate	3
ENO2	enolase 2 (gamma, neuronal)	phosphoenolpyruvate ↔ 2-phosphoglycerate	8
ENO3	enolase 3 (beta, muscle)	phosphoenolpyruvate ↔ 2-phosphoglycerate	6
PGAM1	phosphoglycerate mutase 1	2-phosphoglycerate ↔ 3-phosphoglycerate	3
PGAM2	phosphoglycerate mutase 2 (muscle derived)	2-phosphoglycerate ↔ 3-phosphoglycerate	5
PGK1	phosphoglycerate kinase 1	3-phosphoglycerate ↔ 1,3-bisphophoglycerate	9
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	1,3-bisphosphoglycerate ↔ glyceraldehyde-3-phosphate	6
TPI1	triosephosphate isomerase 1	glyceraldehyde-3-phosphate ↔ dihydroxyacetone phosphate	3
ALDOA	aldolase, fructose-bisphosphate A	diihyroxyacetone phosphate + glyceraldehyde-3-phosphate ↔ fructose-1,6-bisphosphate	13
ALDOB	aldolase, fructose-bisphosphate B	diihyroxyacetone phosphate + glyceraldehyde-3-phosphate ↔ fructose-1,6-bisphosphate	7
ALDOC	aldolase, fructose-bisphosphate C	diihyroxyacetone phosphate + glyceraldehyde-3-phosphate ↔ fructose-1,6-bisphosphate	7
FBP1	fructose-bisphosphatase 1	fructuose-1,6-bisphosphate → fructose-6-phosphate	10
GPI	glucose-6-phosphate isomerase	fructose-6-phosphate ↔ glucose-6-phosphate	7
G6PC	glucose-6-phosphatase catalytic subunit	glucose-6-phosphate → glucose	2
G6PC2	glucose-6-phosphatase catalytic subunit 2	glucose-6-phosphate → glucose	7

insufficient ESTs available to determine the presence of SNP. Three genes involved in gluconeogenesis have been proposed previously as candidate genes, due to their proximity to QTLs for metabolic traits. A GWAS for energy balance mapped a suggestive QTL to *ENO2* and *TPI1* (Tetens *et al.*, 2012). Grossi (unpublished data, 2014) detected a QTL for KET EBV that is 122,180 Kbp downstream of *PGAM2*.

Fatty Acid Synthesis and Degradation

During periods of NEB, the balance between the synthesis and degradation of fatty acids tips towards degradation as NEFA can provide energy-dense fuel for necessary body functions and milk production (Drackley *et al.*, 2001). Mobilization of fat from body stores increases the flux of NEFA into the liver where it is either esterified, or oxidized in mitochondria and peroxisomes to produce acetyl-CoA (Drackley *et al.*, 2005). The pool of acetyl-CoA can either enter the citric acid cycle or be diverted to ketogenesis. Functional mutations in any of the genes involved in the degradation of NEFA could influence the fate of NEFA coming into the liver. Additionally, genes that encode isoforms of enzymes involved in fatty acid oxidation in other tissues may be relevant as they could reduce the influx of NEFA (Schäff *et al.*, 2013). Table 3.3 contains a list of genes involved in fatty acid synthesis and degradation and the number of SNP detected *in silico*.

Li *et al.* (2012) noted that the hepatic expression of β-oxidation enzymes differed depending on the ketosis-status of dairy cattle; expression of *acyl-CoA synthetase long-chain family member 1 (ACSL1)* was upregulated in ketotic cows, while *carnitine palmitoyltransferase I* and *II (CPT1, CPT2)* and *acyl-CoA dehydrogenase, long chain (ACADL)* were downregulated. This finding suggests that the ketotic cow is able to activate the increased NEFA coming into the

Table 3.3: Genes involved in fatty acid synthesis and degradation with corresponding number of putative SNP detected *in silico.*

Gene	Gene name	Process in pathway	Number of SNP
ACSL1	acyl-CoA synthetase long-chain family member 1	fatty acid → acyl-CoA	20
ACSL3	acyl-CoA synthetase long-chain family member 3	fatty acid → acyl-CoA	9
ACSL4	acyl-CoA synthetase long-chain family member 4	fatty acid → acyl-CoA	10
ACSL5	acyl-CoA synthetase long-chain family member 5	fatty acid → acyl-CoA	25
ACSL6	acyl-CoA synthetase long-chain family member 6	fatty acid → acyl-CoA	22
ACSBG1	acyl-CoA synthetase bubblegum family member 1	fatty acid → acyl-CoA	8
ACSBG2	acyl-CoA synthetase bubblegum family member 2	fatty acid → acyl-CoA	17
CPT1A	carnitine palmitoyltransferase 1A	acyl-CoA transport into mitochondrion	19
CPT1B	carnitine palmitoyltransferase 1B	acyl-CoA transport into mitochondrion	5
CPT1C	carnitine palmitoyltransferase 1C	acyl-CoA transport into mitochondrion	4
CPT2	carnitine palmitoyltransferase 2	acyl-CoA transport into mitochondrion	18
ACOX1	acyl-CoA oxidase 1	acyl-CoA → trans-Δ^2-enoyl-CoA	2
ACOX3	acyl-CoA oxidase 3	acyl-CoA → trans-Δ^2-enoyl-CoA	7
ACADM	acyl-CoA dehydrogenase, medium cahin	acyl-CoA → trans-Δ^2-enoyl-CoA	9
ACADL	acyl-CoA dehydrogenase, long chain	acyl-CoA → trans-Δ^2-enoyl-CoA	9
ACADVL	acyl-CoA dehydrogenase, very long chain	acyl-CoA → trans-Δ^2-enoyl-CoA	5
ACADS	acyl-CoA dehydrogenase, short chain	acyl-CoA → trans-Δ^2-enoyl-CoA	12
ACADSB	acyl-CoA dehydrogenase, short/branched chain	acyl-CoA → trans-Δ^2-enoyl-CoA	10
PECR	Peroxisomal trans-2-enoyl-CoA reductase	trans-Δ^2-enoyl-CoA ↔ 3-L-hydroxyacyl-CoA	8
ECHS1	enoyl-CoA hydratase, short chain 1	trans-Δ^2-enoyl-CoA ↔ 3-L-hydroxyacyl-CoA	7

EHHADH	enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase	trans-Δ^2-enoyl-CoA ↔ 3-L-hydroxyacyl-CoA ↔ β-ketyoacyl-CoA	7
HADHA	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein)	trans-Δ^2-enoyl-CoA ↔ 3-L-hydroxyacyl-CoA ↔ β-ketyoacyl-CoA	14
HADHB	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit	L-hydroxyacyl-CoA ↔ β-ketyoacyl-CoA ↔ fatty acyl-CoA + CoA	14
ACAA1	acetyl-CoA acyltransferase 1	β-ketyoacyl-CoA ↔ fatty acyl-CoA + CoA	5
ACAA2	acetyl-CoA acyltransferase 2	β-ketyoacyl-CoA ↔ fatty acyl-CoA + CoA	7
GCDH	glutaryl-CoA dehydrogenase	glutaryl-CoA → trans-Δ^2-enoyl-CoA	6
ECI1	enoyl-CoA delta isomerase 1	cis-Δ^3-enoyl-CoA ↔ trans-Δ^2-enoyl-CoA	6
ECI2	enoyl-CoA delta isomerase 2	cis-Δ^3-enoyl-CoA ↔ trans-Δ^2-enoyl-CoA	6
ALDH2	aldehyde dehydrogenase 2 family		8
ADH4	alcohol dehydrogenase 4	1-alcohol ↔ aldehyde	10
ADH5	alcohol dehydrogenase 5	1-alcohol ↔ aldehyde	25
ADH6	alcohol dehydrogenase 6	1-alcohol ↔ aldehyde	22
ADH7	alcohol dehydrogenase 7	1-alcohol ↔ aldehyde	3
ALDH1B1	aldehyde dehydrogenase 1 family member B1	aldehyde ↔ fatty acid	4
ALDH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide	aldehyde ↔ fatty acid	7
ALDH2	aldehyde dehydrogenase 2 family	aldehyde ↔ fatty acid	8
ALDH3A2	aldehyde dehydrogenase 3 family member A2	aldehyde ↔ fatty acid	5
ALDH7A1	aldehyde dehydrogenase 7 family member A1	aldehyde ↔ fatty acid	10
ALDH3A2	aldehyde dehydrogenase 3 family member A2	aldehyde ↔ fatty acid	5
CYP4A11	cytochrome P450, family 4, subfamily A, polypeptide 11	fatty acid ↔ ω-hydroxy fatty acid	6
SCP2	sterol carrier protein 2	transporter	9
SLC25A20	solute carrier family 25 member 20	transporter	3

liver through increased expression of *ACSL1*, but the oxidative capacity of hepatocytes does not increase to match the rate of activation. If β-oxidation is inhibited, more fatty acyl-CoA would be available for triacylglyceride synthesis and this would contribute to fat deposition in the liver. Weber *et al.* (2013a) noted that cows with higher liver fat content had higher expression of *ACSL1*. Still, other studies have reported a positive correlation between expression of these fatty acid oxidation enzymes and serum BHB and the indicators of KET (Loor *et al.*, 2005; Weber *et al.*, 2013; Akbar *et al.*, 2015). The transcription-level response to increased NEFA uptake is not well-understood and differences could be due to genetic variation.

Cánovas *et al.* (2013) sequenced the RNA of several differentially expressed genes involved in fatty acid synthesis in order to characterize the variations associated with citrate content in cow's milk. Although Cánovas *et al.* (2013) worked on a different trait than KET, they detected SNP in *acyl-CoA synthetase, long-chain family member 5 (ACSL5), acyl-CoA synthetase short-chain family member 1, and ACSL1*. The GWAS performed by Grossi (unpublished data, 2014) also detected QTL surrounding several key genes including *carnitine palmitoyltransferase 2 (CPT2; 5), sterol carrier protein 2 (SCP2;* 2 QTL*), cytochorome P450, family 4, subfamily A, polypeptide 11 (CYP4A11;* 9 QTL*), enoyl-CoA delta isomerase 2 (ECI2;* 3 QTL*), acyl-CoA synthetase bubblegum family member 1 (ACSBG1;* 1 QTL), *ACADL* (2 QTL), *acetyl-CoA acyltransferase 1 (ACAA1;* 1 QTL), *enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase (EHHADH;* 1 QTL) and *acyl-CoA synthetase long-chain family member 6 (ACSL6;* 1 QTL). One QTL was also mapped to the promoter region of *ACSL6* (Grossi, unpublished data, 2014).

Citric Acid Cycle

Carbohydrates and fatty acids are completely oxidized in the mitochondria via the citric acid cycle. After calving, the capacity of the liver to convert acetyl-CoA derived from both

palmitate and propionate to CO_2 and chemical energy via the citric acid cycle decreases (McCarthy *et al.,* 2015). McCarthy *et al.* (2105) also noted that cows that had a high rate of palmitate metabolism prepartum maintained a high rate of oxidation throughout their transition; this relationship was apparent for propionate metabolism, as well. Genes that encode proteins involved in the citric acid cycle were mined for SNP, shown in Table 3.4, as cows that are more efficient at oxidizing fuel to energy may transition better. SNP within citric acid cycle genes have been reported previously, including three SNP associated with milk citrate content within *isocitrate dehydrogenase (NADP(+)) 1, cytosolic (IDH1;* Cánovas *et al.,* 2013). Grossi (unpublished data, 2014) detected a QTL for KET approximately 1 Mbp upstream of *succinate dehydrogenase, C subunit (SDHC).*

Synthesis and Degradation of Ketone Bodies

The synthesis of ketone bodies is an adaptive response to NEB, which provides glucose-sparing fuel to peripheral tissues (Herdt, 2000). Instead of joining oxaloacetate and entering the citric acid cycle, acetyl-CoA is partially oxidized to acetoacetate, acetone and BHB (Voet *et al.,* 2002). SNP within ketogenic genes may alter the function of the encoded enzymes and favour ketone body synthesis over other endpoints. Studies have shown that the expression of 3-hydroxy-3-methylglutaryl-CoA synthase 2, a rate-limiting enzyme of ketone body synthesis, is not altered due to metabolic status (Loor *et al.,* 2007; van Dorland *et al.,* 2009; van Dorland *et al.,* 2013). van Dorland *et al.* (2013) also reported no difference between expression of the ketogenic genes *3-hydroxybutyrate dehydrogenase 1 (BDH1),* and, *3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1),* between cows with a high metabolic load compared to those with a low load. Alternatively, nutritionally ketosis-induced cows exhibited upregulated expression of *acetyl-CoA acetyltransferase 1 (ACAT1;* Loor *et al.,* 2007). Grossi (unpublished data, 2014) identified a

Table 3.4: Genes involved in the citric acid cycle with corresponding number of putative SNP detected *in silico.*

Gene	Gene name	Process in pathway	Number of SNP
CS	citrate synthase	oxaloacetate + acetyl-CoA → citrate	11
ACO1	aconitase 1	citrate ↔ isocitrate	12
ACO2	aconitase 2	citrate ↔ isocitrate	11
IDH3A	isocitrate dehydrogenase 3 (NAD(+)) alpha	isocitrate ↔ α-ketoglutarate	8
IDH3B	isocitrate dehydrogenase 3 (NAD(+)) beta	isocitrate ↔ α-ketoglutarate	4
IDH3G	isocitrate dehydrogenase 3 (NAD(+)) gamma	isocitrate ↔ α-ketoglutarate	4
OGDH	oxoglutarate dehydrogenase	α-ketoglutarate ↔ succinyl-CoA	11
DLST	dihydrolipoamide succinyltransferase	α-ketoglutarate ↔ succinyl-CoA	2
DLD	dihydrolipoamide dehydrogenase	α-ketoglutarate ↔ succinyl-CoA	17
SUCLG1	succinyl-CoA ligase alpha subunit	succinyl-CoA ↔ succinate	10
SUCLG2	succinyl-CoA ligase beta subunit	succinyl-CoA ↔ succinate	13
SDHA	succinate dehydrogenase, A subunit	succinate ↔ fumarate	11
SDHB	succinate dehydrogenase, B subunit	succinate ↔ fumarate	3
SDHC	succinate dehydrogenase, C subunit	succinate ↔ fumarate	3
SDHD	succinate dehydrogenase, D subunit	succinate ↔ fumarate	4
FH	Fumarate hydratase	fumarate ↔ L-malate	8
MDH2	malate dehydrogenase 2	L-malate ↔ oxaloacetate	12
MUT	methylmalonyl-CoA mutase	S-methylmalonyl-CoA ↔ succinyl-CoA	14

QTL on BTA1 1500 Kbp upstream of *BDH1* and 7 QTL on BTA3 that are within less than 1000 Kbp of *3-oxoacid CoA-transferase 2 (OXCT2);* putative SNP identified in these genes and the other genes related to ketone body synthesis and degradation are presented in Table 3.5.

Hepatic Molecular Signalling Networks

Evidence of altered expression of genes involved in regulating metabolic processes in the liver of ketotic cows suggests that mutations in these genes may be useful targets for *in silico* SNP investigation (Loor *et al.,* 2007; Schoenberg *et al.,* 2011; Bionaz *et al.,* 2013; Akbar *et al.,* 2015). A selection of genes involved in molecular signalling networks are presented in Table 3.6; many of these are genes that encode cytokines, transcription factors, transporters, cofactors, and receptors that affect key metabolic genes upstream that are involved in the hepatic processes summarized previously. Additionally, *agouti-related neuropeptide (AGRP),* which is a neuropeptide primarily expressed in the hypothalamus, was included as a candidate gene based on evidence from differential hepatic gene expression studies of cows in NEB and also due to the link between *AGRP* expression and feeding behavior (Loor *et al.,* 2007; Borner *et al.,* 2013; Perikins *et al.,* 2014).

Many of the regulatory genes studied have been proposed as candidate genes based on their differential expression during the periparturient period, as well as their proximity to QTL. A powerful QTL found within *diacylglycerol O-acyltransferase 1 (DGAT1)* has been well studied as a genetic factor affecting milk composition; Tetens *et al.* (2012) also proposed *DGAT1* as a candidate gene for F:P, an indicator of KET. Another candidate gene was identified by Clempson *et al.* (2012) who reported trending association between SNP within *insulin like growth factor binding protein 3 (IGFBP3)* and metabolic status. *IFGBP3,* as well as *insulin like growth factor 1 (IGF1)* concentration have been proposed as early biomarkers of KET (Piechotta *et al.,* 2015). A

Table 3.5: Genes involved in ketone body metabolism with corresponding number of putative SNP detected *in silico.*

Gene	Gene name	Process in pathway	Number of SNP
ACAT1	acetyl-CoA acetyltransferase 1	acetyl-CoA + acetyl-CoA → acetyoacetyl-CoA	12
ACAT2	acetyl-CoA acetyltransferase 2	acetyl-CoA + acetyl-CoA → acetyoacetyl-CoA	8
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1	acetoacetyl-CoA + acetyl-CoA → 3-hydroxy-3-methylglutaryl-CoA	11
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2	acetoacetyl-CoA + acetyl-CoA → 3-hydroxy-3-methylglutaryl-CoA	16
HMGCL	3-hydroxy-3-methylglutaryl-CoA lyase	3-hydroxy-3-methylglutaryl-CoA → acetoacetate + acetyl-CoA	8
OXCT1	3-oxoacid CoA-transferase 3	acetoacteyl-CoA + CoA ↔ acetoacetate	5
OXCT2	3-oxoacid CoA-transferase 2	acetoacteyl-CoA + CoA ↔ acetoacetate	7
BDH1	3-hydroxybutyrate dehydrogenase, type 1	acetoacetate ↔ β-hydroxybutyrate	9
BDH2	3-hydroxybutyrate dehydrogenase, type 2	acetoacetate ↔ β-hydroxybutyrate	10

Table 3.6: Genes involved in the regulation of energy metabolism and the corresponding number of putative SNP detected *in silico.*

Gene	Gene name	Function	Number of SNP
DGAT1	diacylglycerol O-acyltransferase 1	lipid metabolism	4
FGF21	fibroblast growth factor 21	metabolic regulator	8
PPAR-α	peroxisome proliferator activated receptor alpha	transcription regulator	6
FOXA2	forkhead box A2	transcription regulator	13
HNF4A	hepatic nuclear factor 4 alpha	transcription regulator	9
IGF1	insulin like growth factor 1	signal transduction	6
IGFBP3	insulin like growth factor binding protein 3	signal transduction	6
ACSS2	acetyl-CoA synthetase short chain family member 2	lipid metabolism	8
DBI	diazepam binding inhibitor, acyl-CoA binding protein	lipid metabolism	5
FADS1	fatty acid desaturase 1	lipid metabolism	6
FADS2	fatty acid desaturase 2	lipid metabolism	6
LDLR	low density lipoprotein receptor	lipid metabolism	8
MSMO1	methylsterol monoxygenase 1	enzyme; cholesterol synthesis	9
SCD	stearoyl-CoA desaturase (delta-9-desaturase)	enzyme; fatty acid desaturation	4
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	lipid metabolism	6
FN1	fibronectin 1	lipid metabolism	9
ELOVL6	ELOVL fatty acid elongase 6	lipid metabolism	4
ANGPTL4	angiopoietin like 4	signal transduction	5
FABP3	fatty acid binding protein 3	transporter	5
IL6	interleukin 6	cytokine	5
LPIN1	lipin 1	lipid metabolism	5
NEU3	neuraminidase 3	lipid metabolism	14
SLC27A2	solute carrier family 27 member 2	transporter	11
PLG	plasminogen	lipid metabolism	9
VDR	vitamin D (1,25-dihydroxyvitamin D3) receptor	lipid metabolism	5
AGRP	agouti related neuropeptide	lipid metabolism	5
APOA1	apolipoprotein A1	transporter	12
EPO	erythropoietin	protein binding/transport	7

HSD3B1	hydroxyl-delta-5-steroid dehydrogenase 3 beta- and steroid delta-isomerase 1	lipid metabolism	7
KLB	klotho beta	metabolic regulator	13
FGFR2	fibroblast growth factor receptor 2	metabolic regulator	6
FGFR1	fibroblast growth factor receptor 1	metabolic regulator	8
APOBR	apolipoprotein B receptor	transporter	6

QTL associated with malonate concentration in milk was mapped close to *fibronectin 1* (*FN1)* (Buitenhuis *et al.,* 2013). Tetens *et al.* (2015) reported associations between SNP in the gene encoding apolipoprotein B receptor and milk biomarkers. Aside from identifying the PPAR signalling pathway through functional annotation of the GWAS performed by Grossi (unpublished data, 2014), QTL were detected near *plasminogen (PLG)* and *erythropoietin (EPO).*

Array Development

A total of 998 SNP were classified and incorporated onto an Illumina 8K array in order to create a custom panel of markers located within genes related to KET. These putative SNP are located in 120 candidate genes for KET spanning 28 chromosomes; BTA3 contains the highest number of SNP (96). The density of the SNP is demonstrated as a heat map in Figure 3.1.

3.5 Conclusions

During the transition period, dairy cows must be able to tread the line between hypoglycemia and hyperketonemia by altering tissue mobilization and nutrient utilization. Many animals are not able to achieve this balance. Candidate genes related to the metabolic adaptations to NEB were investigated in order to characterize the genetic variation in these key genes. Short sequence tags were collected and aligned *in silico* to detect 998 SNP within 120 genes. The proposed SNP were incorporated onto an array to create a panel of putative markers for KET that will be subsequently used for association studies. Association testing of the panel will also provide proof of concept for *in silico* SNP detection in dairy cattle using public-access cDNA libraries rather than sequencing technologies.

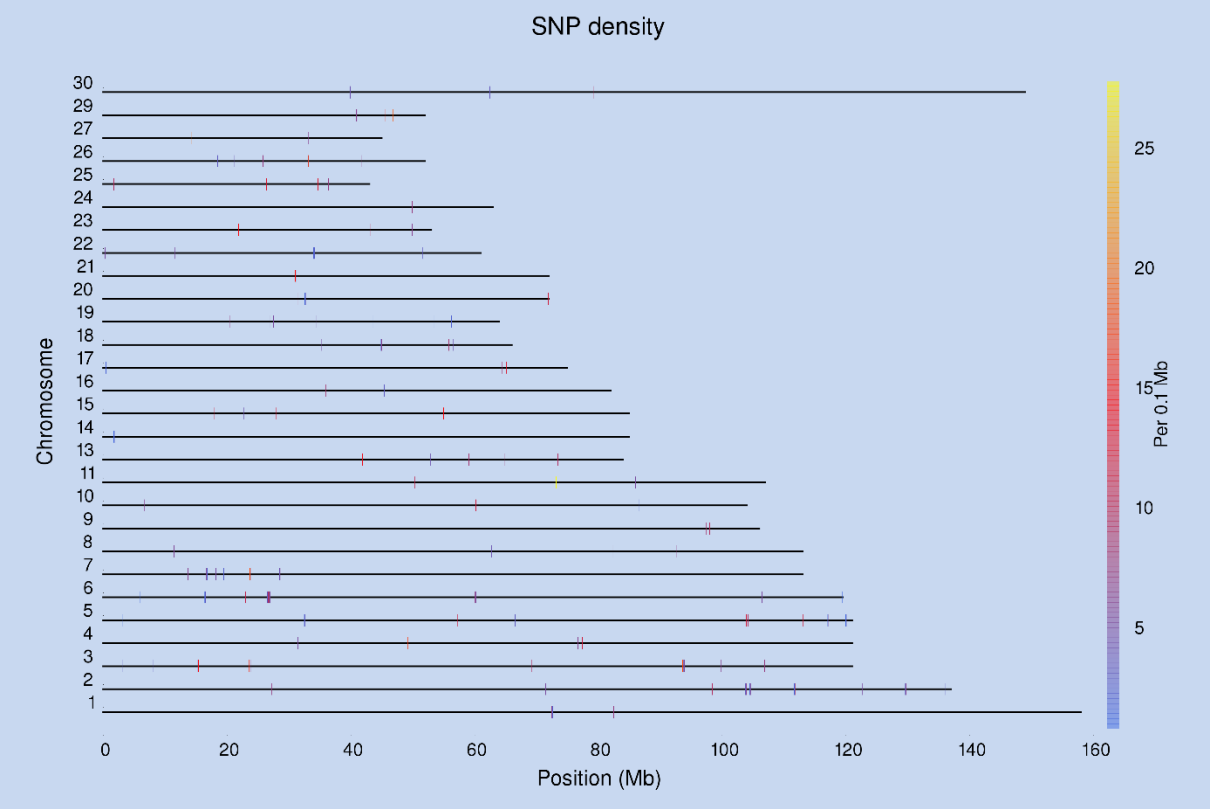


Figure 3.1: Heat map showing the distribution of the 998 putative SNP making up a novel marker panel for ketosis in dairy cattle. Colours represent the number of SNP located within a bin set to 100 Kbp. The X chromosome was coded as Chromosome 30. No markers were located on Chromosome 12 and 28.

CHAPTER FOUR: Candidate gene association study of ketosis and milk β-hydroxybutyrate

4.1 Abstract

A custom marker panel of 998 SNP located within candidate genes for KET was previously developed. This study aimed to test the association of these putative SNP with KET and milk BHB in cows. Canadian Holsteins (n=535) that had been previously genotyped with the BovineSNP50 BeadChip (Illumina Inc., San Diego, CA) and with records for producer-recorded health events and milk BHB, were re-genotyped with the custom markers for KET. Association analyses were conducted separately for each trait, and also by creating subsets of cows based on whether their records were from the first lactation or from the second to fifth lactation. A single SNP mixed animal model was implemented by fitting the additive genetic relationship matrix to account for the polygenic effect. Approximately 60% of the SNP on the marker panel were not segregating in the sample of cows. A total of 5 SNP reached chromosome-wise significance (FDR<0.05), while 40 others were suggestive of association with KET or milk BHB (FDR<0.20). Five SNP, within the genes *ACADL*, *FABP3*, and *MSMO1*, were found to be associated with both traits, which provides better evidence of true association with KET. The preliminary analyses of the custom marker panel revealed SNP with the potential to be used as genetic markers for KET and milk BHB after further validation. The results of the study are also useful in providing insight on new areas of research for these traits. A number of SNP involved in genes that affect cholesterol metabolism were detected by the analyses, highlighting the relationship between KET and a pathway not often focused on in the study of KET. Additionally, a novel, deleterious mutation within the fibronectin 1 gene was found to be associated with increased levels of milk BHB.

4.2 Introduction

KET is a multifactorial disease that is the result of a maladaptive response to NEB in early lactation. While essentially all cows undergo NEB after calving, not all cows develop KET. van Dorland *et al.* (2013) observed variable metabolic loads and differential gene expression in the liver of cows that had been pre-selected for high milk fat; despite being selected for high F:P, an indicator of KET, the susceptibility to KET was different for cows within this group (van Dorland *et al.,* 2013). Many studies suggest that altered metabolic regulation that contributes to KET differs at the cow level (van Dorland *et al.,* 2009; Cánovas *et al.,* 2013; Khan *et al.,* 2013; Weber *et al.,* 2013; van Dorland *et al.,* 2013). Association analyses that have been conducted for metabolic traits - energy balance, F:P, GPC:PC, blood BHB, BCS, milk metabolites, EBV – have identified regions of the genome, candidate genes and probable QTN that explain some of the variation between cows (Clempson *et al.,* 2012; Tetens *et al.,* 2012; Buitenhuis *et al.,* 2013; Gaddis *et al.,* 2014; Mahmoudi *et al.,* 2015; Tetens *et al.,* 2015).

Further exploration of candidate genes for KET could contribute to our understanding of the genetic architecture of this trait. CGAA, which are often limited by scope, are commonly conducted by comparing the sequence of a few genes expressed in key tissue sites for polymorphisms associated with a disease. The construction of a custom array of markers for candidate genes specific to the trait of interest would allow for the investigation of many key genes simultaneously, as well as identify probable QTN with biological significance. Chapter Three described the detection of SNP within 120 candidate genes proposed to be related to KET. The objective of this study is to test the association of these putative genetic markers with metabolic traits. In Canada, producer-recorded disease data and milk test day records provide reliable sources of information on health traits for dairy cattle that have been used successfully for genetic

evaluation in the past (Neuenschwander *et al.,* 2012; Koeck *et al.,* 2015). Producer-collected KET records and milk BHB, an indicator trait with high genetic correlation to KET, were used as phenotypes for the analyses after adjusting for fixed effects. Records of primiparous and multiparous cows were analyzed as separate traits, as studies propose that cows respond to NEB differently in their first lactation compared to later lactations, and for genetic analysis these traits are considered to be separate but related traits. (Chapinal *et al.,* 2012; Koeck *et al.,* 2015; Jamrozik *et al.,* 2016).

4.3 Methods

Genotypes

Animal samples were made accessible through coordination with previous Canadian Dairy Network (Guelph, ON) initiatives. All cows had previously been genotyped with the BovineSNP50 BeadChip (Illumina Inc., San Diego, CA) and their 50K genotypes were also provided by Canadian Dairy Network (Guelph, ON). A sample of 653 Canadian Holstein cows were re-genotyped with the low density array containing the 1K putative KET markers. These cows came from 5 herds located within Quebec and Ontario with an annual KET disease reporting frequency greater than 1% to ensure herds with inconsistent recording were avoided. Additionally, cows were chosen from large herds with high annual frequency of KET. Cow selection was not randomized, but based on creating a sample with close to equal numbers of cases and control for KET (322 cases and 331 controls). A case animal was defined as a cow with at least one reported case of KET throughout their production lifetime. A pedigree file consisting of 24,260 individuals was traced back 7 generations.

Phenotypes

Health and milk recording data from April 2007 to December 2015 were obtained from Canadian Dairy Network (Guelph, ON) and matched to the genotyped cows. Only records from the first five lactations were considered. Disease records are reported by producers as a binary trait; a score of 0 being no case, and a score of 1 representing at least one case of KET reported within the first 100 days postpartum. Each cow with a KET record also had a milk BHB record. Milk BHB records are the predicted from first test-day (5-40 DIM) milk samples that have been analyzed by a mid-infrared spectrometer (MilkoScan FT+, Foss, Hillerød, Denmark). A log transformation - $\log_e$(milk BHB + 1) - was applied to milk BHB phenotypes to normalize the distribution. In the data set there were 12 and 13 herd-year classes for lactation 1 and later lactations, respectively. There were 16 classes of season-year for both lactation 1 and later lactations. Seasons were defined as 1 = January to March, 2 = April to June, 3 = July to September, 4 = October to December. There were 11 levels of age at first calving for first parity animals. DIM of test-day records was defined as 7 classes (5 days each). There were 195 first lactation and 445 lactation 2-5 records, after randomly removing records for cows with data from more than one lactation. Summary statistics for this data set are described in Table 4.1.

Each phenotype was adjusted for environmental effects in SAS v. 9.4 (SAS Institute Inc., Cary, North Carolina, United States of America) by fitting univariate linear models. Although KET is a binary trait, a threshold model was not fitted for this trait as studies have shown that linear and nonlinear methods of evaluation for categorical traits are comparable (Meijering, 1984; Kadarmideen and Dekkers, 2001). Additionally, first and later (2-5) lactations were treated as separate traits for KET and milk BHB, as outlined by Koeck *et al.* (2015).

Table 4.1: Summary statistics for phenotypic records – frequency of disease for KET and mean values for milk BHB and log transformed-milk BHB, with standard deviation in parentheses.

Phenotype	Lactation	
	1 n =195	2-5 n = 445
KET, %	35.90	23.15
Milk BHB, mmol/L (SD)	0.09421 (0.05347)	0.1147 (0.06261)
$\log_e$ Milk BHB, mmol/L (SD)	0.08887 (0.04789)	0.1064 (0.05358)

Representations of the four models used to adjust phenotypes for environmental effects are:

$$KET1_{ijkn} = HY_i + SY_j + AGEC_k + e_{ijkn},,$$

$$KET2\text{-}5_{ijln} = HY_i + SY_j + P_l + e_{ijln},$$

$$\log_e BHB1_{ijkmn} = HY_i + SY_j + AGEC_k + DIM_m + e_{ijkmn},$$

$$\log_e BHB2\text{-}5_{ijlmn} = HY_i + SY_j + P_l + DIM_m + e_{ijlmn},$$

where the response variables $KET1_{ijkn}$ and $KET2\text{-}5_{ijln}$ are the KET record of the n^{th} cow in first lactation or later lactation, respectively, and $\log_e BHB1_{ijkmn}$ and $\log_e BHB2\text{-}5_{ijlmn}$ are the log-transformed milk BHB record of the n^{th} cow in first lactation or later lactation, respectively. The fixed effects included: HY_i = effect of the i^{th} herd-year, SY_j = effect of the j^{th} season-year, $AGEC_k$ = effect of the k^{th} age at first calving. P_l = effect of the l^{th} parity (4 levels), DIM_m = effect of the m^{th} days in milk at time of sampling. The random residual effect for each model is the adjusted phenotype of the n^{th} cow which is represented here as e_{ijkn}, e_{ijln}, e_{ijkmn}, and e_{ijlmn}.

Association Analyses

After adjusting for environmental effects, the residuals of each model, which consist of genetic and error components, were used as the response variable to test for association with the 1K putative markers. Phenotypes used for association analyses included adjusted phenotypes ($\log_e$BHB and KET) for first lactation, later lactations, and combined lactation cows (first and later lactations together). Unadjusted KET records were also analyzed for first lactation, later lactations, and combined lactation cows. The rationale for using records without adjusting for environmental effects was that the model was that estimation of some effects was limited by the binary nature of

the trait, as well as over-paramaterization of the model, which increases the standard error and reduces the ability to detect associations. Comparison of the results of the association analyses using adjusted phenotypes and unadjusted records for KET revealed that similar peaks were apparent, with the peaks found using unadjusted records being larger and resulting in a higher number of SNPs reaching significance. While using unadjusted records can be biased due to confounding environmental effects, the similarity between the location of peaks supported the decision to use these records.

SNP with minor allele frequency (MAF) lower than 1%, call rate less than 90%, or excess of heterozygosity greater than 15% were removed for quality control. Additionally, animals with genotyping call rate less than 90% were not included in the analyses.

Using the software SNP1101 v. 1.0 (Sargolzaei, 2014), the following univariate single SNP mixed model was applied for the analyses:

$$y_i = \mu + b \text{ x SNP} + a_i + e_i$$

where y_i is the phenotype of the i^{th} individual; μ is the overall mean; SNP is the genotype of a SNP in the individual that has been coded as 0, 1, 2 for genotype AA, AB, BB; *b* is the regression coefficient of y_i on SNP, which is the allele substitution effect; a_i is the random additive genetic (polygenic) effect of the i^{th} individual with $a_i \sim N(0,\mathbf{A}\sigma^2_a)$, where **A** is the additive genetic relationship matrix among individuals and σ^2_a is the additive genetic variance; e_i is the random residual effect of the i^{th} individual's record with $e_i \sim N(0,\mathbf{I}\sigma^2_e)$, where **I** is an identity matrix and σ^2_e is the error variance. The expectations are $E(a_i) = 0$ and $E(e_i) = 0$. Individual SNP variances were calculated as described by Su *et al.* (2014), although this method does not take into account linkage disequilibrium; therefore, the sum of the SNP variances would be inflated.

SNP1101 v. 1.0 (Sargolzaei, 2014) was also used for mapping and plot creation. The analyses were performed using the cows' 50K genotypes in addition to the genotypes of the 998 SNP of interest. This was done in order to visualize better the peaks and also the location of the candidate gene SNP within the denser map.

False discovery rate (FDR) at chromosome-wise level was used to correct for multiple testing. A false discovery rate q-value less than 5% for a SNP was considered to be the threshold for significant association with the traits, while a q-value less than 20% was considered suggestive.

4.4 Results

Marker Panel

After removing SNP to meet quality control criteria, 336 of the 998 SNP included on the custom marker panel were considered for the association analysis. The range of minor allele frequencies was between 0.012 to 0.493. These SNP are found on within or near 94 unique candidate genes located on all autosomes except BTA12 and BTA28. BTA3 contained the highest number of sufficiently-polymorphic SNP (40).

Ketosis

The results of the association analyses for KET and each group of cows are presented in Table 4.2. When the residuals of the model that adjusted disease records for binary traits was used for the association analyses, no SNP were found to be significant at the chromosome-level, after correcting for multiple testing, although, 2 SNP were identified as being suggestive of association

Table 4.2: SNP associated (chromosome-wise FDR < 0.05) and suggested to be associated (chromosome-wise FDR < 0.20) with ketosis.

SNP ID	Chr[1]	Gene	Minor Allele Frequency	Allele Substitution Effect	p-value	Variant Consequence
All Parities Adjusted Phenotypes (*n* = 535)						
SNP584	2	ACADL	0.0562	0.282	7.30xe^{-3} [b]	3' UTR
SNP402	23	ECI2	0.0366	0.325	1.31xe^{-2} [c]	3' UTR
All Parities Unadjusted Records (*n* = 535)						
SNP402	23	ECI2	0.0366	0.467	1.45xe^{-3} [a]	3' UTR
SNP127	15	APOA1	0.342	-0.179	3.78xe^{-3} [a]	L → M
SNP825	6	ADH6	0.335	-0.161	5.54xe^{-3} [c]	synonymous
SNP827	6	ADH6	0.331	0.160	6.10xe^{-3} [c]	synonymous
SNP311	2	FABP3	0.147	0.194	8.27xe^{-3} [c]	synonymous
SNP584	2	ACADL	0.0563	0.305	9.15xe^{-3} [c]	3'UTR
SNP828	6	ADH6	0.389	0.138	1.59xe^{-2} [c]	synonymous
SNP323	2	HMGCL	0.364	-0.138	1.67xe^{-2} [c]	3'UTR
SNP451	25	MDH2	0.346	0.141	1.90xe^{-2} [c]	3' UTR
SNP128	15	APOA1	0.0239	0.409	1.99xe^{-2} [b]	synonymous
SNP816	6	ALDH1C	0.328	0.132	2.10xe^{-2} [c]	5' UTR
SNP317	2	HMGCL	0.374	0.129	2.62xe^{-2} [c]	G → S
SNP207	18	AGRP	0.116	-0.137	9.54xe^{-2} [c]	T → P
First Parity Unadjusted Records (*n* = 196)						
SNP903	7	ACSL6	0.305	0.332	3.81xe^{-3} [b]	5' UTR
SNP253	19	ALDH3A2	0.340	0.302	5.49xe^{-3} [b]	5' UTR
SNP449	25	MDH2	0.321	-0.315	6.67xe^{-3} [b]	3' UTR
SNP466	26	SCD	0.266	-0.311	9.63xe^{-3} [b]	synonymous
SNP171	17	MSMO1	0.418	0.258	1.24xe^{-2} [c]	5' UTR
SNP451	25	MDH2	0.371	0.269	1.29xe^{-2} [b]	3' UTR
SNP402	23	ECI2	0.0368	0.615	2.08xe^{-2} [c]	3' UTR
SNP249	19	ALDH3A2	0.360	0.242	2.18xe^{-2} [c]	3' UTR
SNP389	23	MUT	0.124	-0.314	3.08xe^{-2} [c]	5' UTR
Later Parity Unadjusted Records (*n* = 410)						
SNP939	8	ALDH1B1	0.306	-0.202	4.16xe^{-3} [b]	C → R
SNP532	27	FGFR1	0.1029	0.296	5.32xe^{-3} [b]	3' UTR
SNP179	17	MSMO1	0.456	-0.191	5.86xe^{-3} [b]	3' UTR
SNP174	17	MSMO1	0.442	0.177	8.70xe^{-3} [b]	3'UTR
SNP207	18	AGRP	0.120	-0.186	4.81xe^{-2} [b]	T → P
SNP1	10	SLC27A2	0.249	-0.147	4.87xe^{-2} [c]	3' UTR
SNP178	17	MSMO1	0.419	-0.127	5.70xe^{-2} [c]	3' UTR

[1]The chromosome that harbours the SNP in the previous column

[a] chromosome-wise false discovery rate (FDR) q-value less than 0.05

[b] chromosome-wise false discovery rate (FDR) q-value less than 0.10

[c] chromosome-wise false discovery rate (FDR) q-value less than 0.20

with the trait (FDR <20%). The 2 suggestive SNP were detected within the genes *ECI2* and *ACADL*.

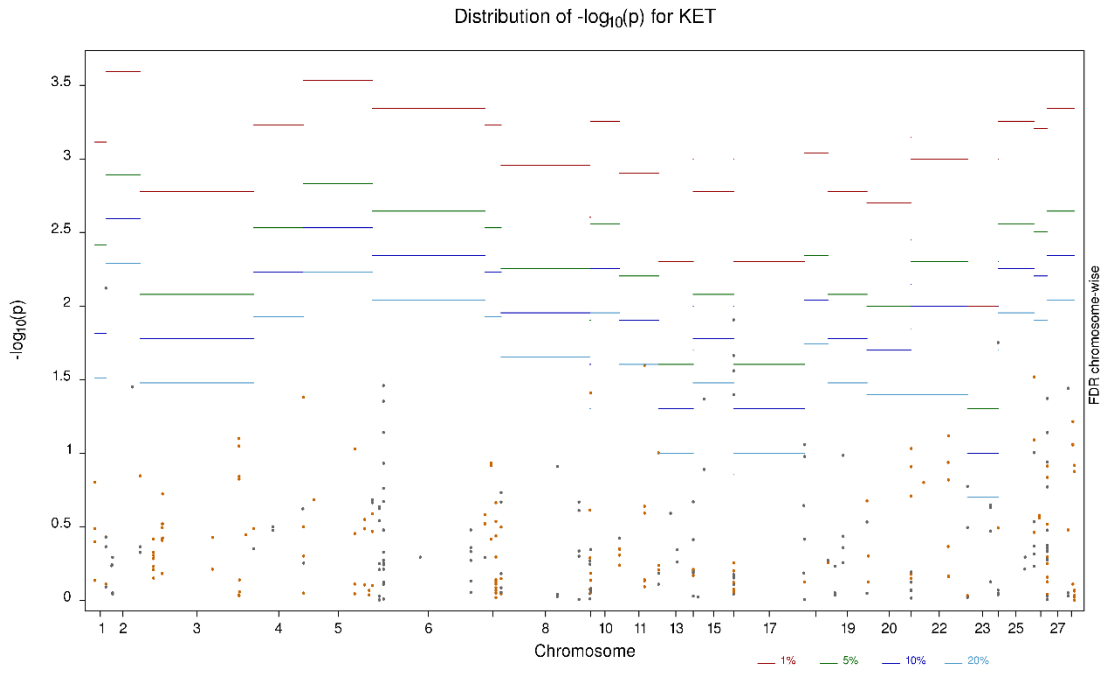
Due to the limited ability of the model to adjust for environmental effects, the analysis of unadjusted health records was also implemented; this resulted in the detection of 2 significant and 11 suggestive SNP. The SNP that reached chromosome-wide significance were located within *ECI2* and *APOA1*. Suggestive SNP were found within *ADH6, FABP3, ACADL, HMGCL, ADH1C, APOA1, MDH2* and *AGRP*. Manhattan plots for the analyses of unadjusted records and adjusted phenotypes for KET are presented in Figure 4.1; these plots show common peaks on BTA2, 6, 15, 21, 23, 25, and 26. Similar results were noted when the peaks for unadjusted and adjusted KET records were compared for the primiparous cows and multiparous cows.

For first parity cows, the analysis of unadjusted KET records yielded no significant SNP, but 9 that were suggestive of association. The SNP were located within the candidate genes *ACSL6, ALDH3A2, MDH2, SCD, ECI2, MUT* and *MSMO1*. No significant and 7 suggestive SNP were found to be associated with KET in later lactation animals. *ALDH1B1, FGFR1, MSMO1, AGRP* and *SLC27A2* contain suggestive SNP for KET in later lactation cows. Manhattan plots of the analyses for first and later lactation cows are presented in Figure 4.2.

Milk β-hydroxybutyrate

Association analysis for the trait log-transformed milk BHB across all lactations resulted in no SNP reaching a significant level of association after correcting for multiple testing; however, 10 SNP were suggestive. The suggestive SNP are located within 5 candidate genes: *MSMO1, DLD, PLG, ACSBG1, OXCT1*. Association analysis of just the first parity cows for this trait was also conducted and resulted in 3 significant and 7 suggestive SNP. *FN1* contains a nonsynonymous and a synonymous SNP and *FABP3* contains a synonymous SNP that was shown to be associated to

a.



b.

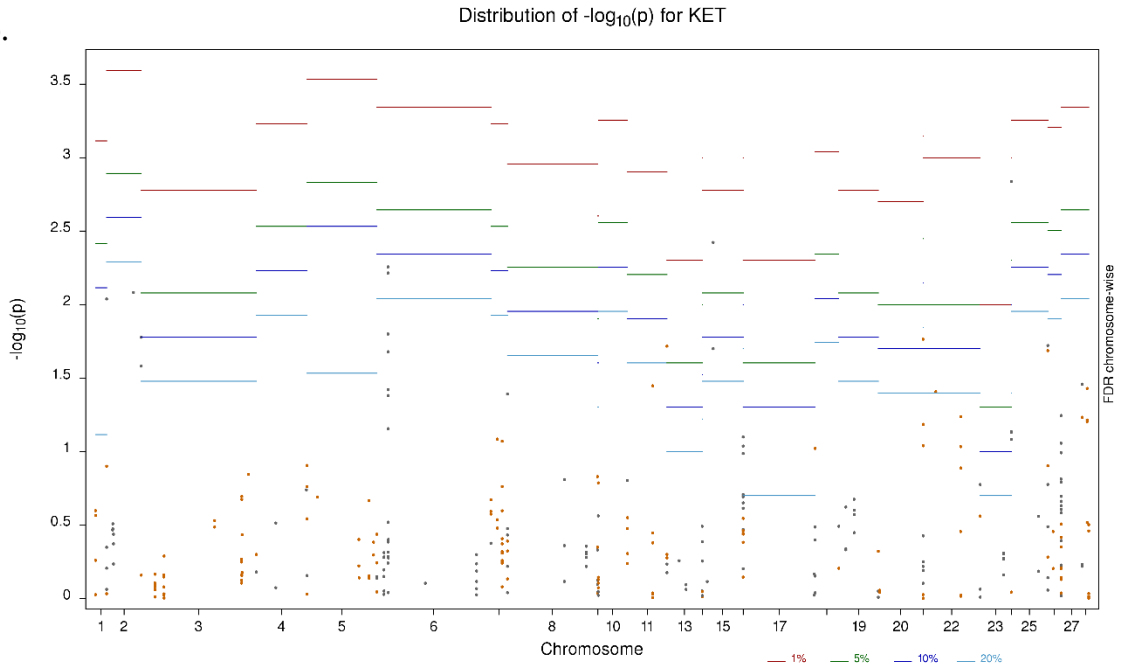
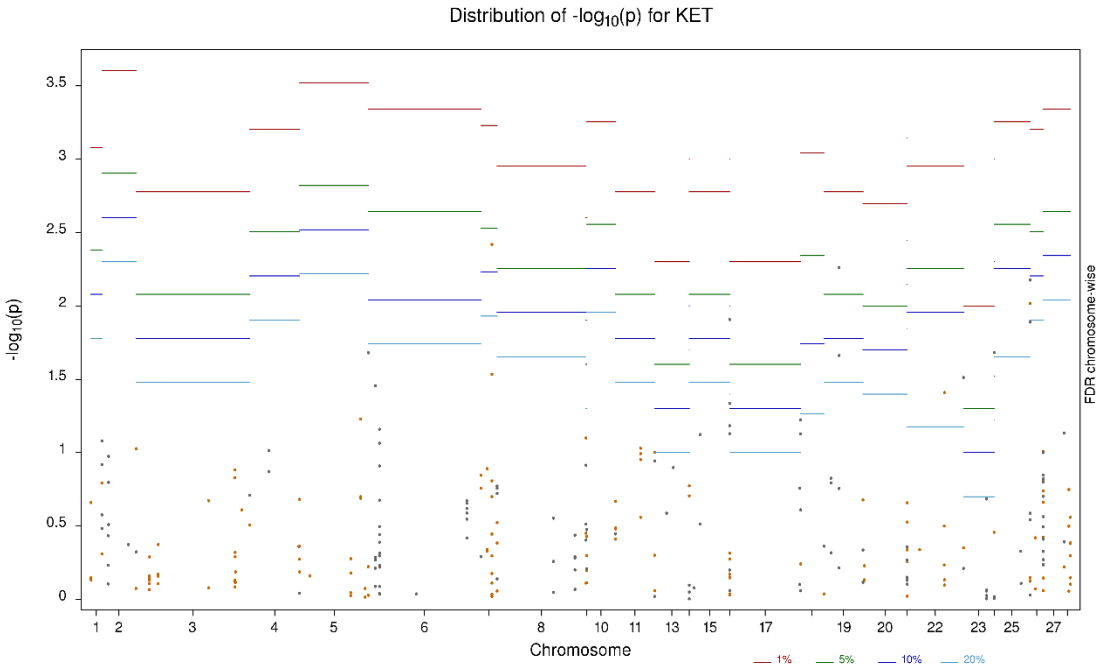


Figure 4.1: Manhattan plots for the results of association analyses with ketosis phenotypes adjusted for environmental effects (a) and unadjusted records (b).

a.



b.

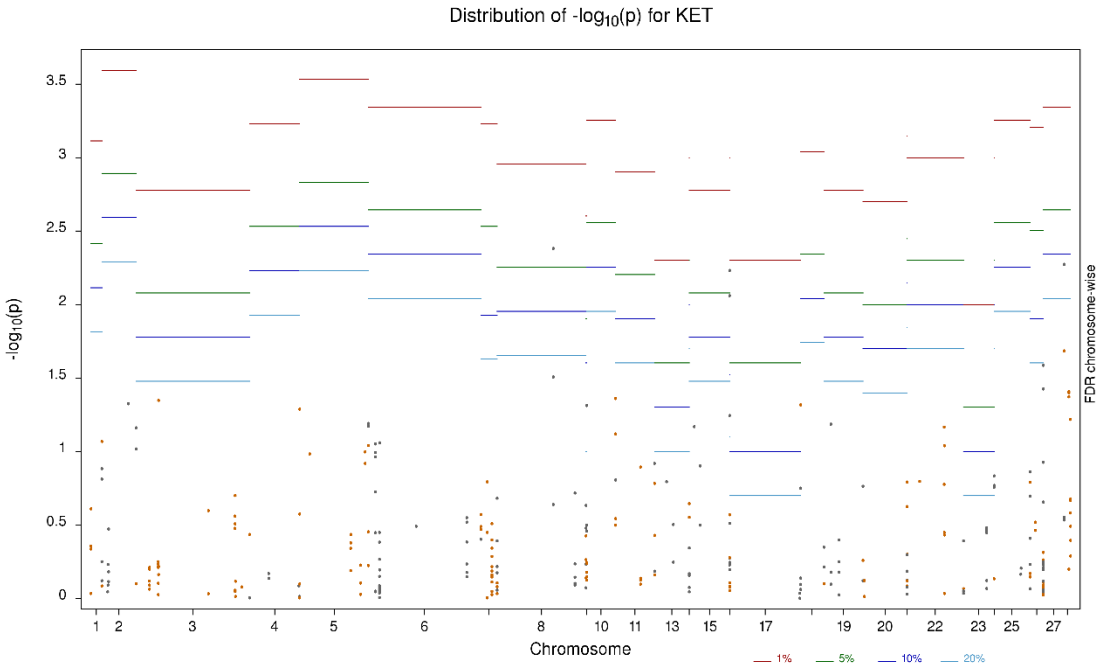


Figure 4.2: Manhattan plots for the results of association analyses with ketosis records in first parity (a; n=195) and parity 2 – 5 cows (b; n=410).

milk BHB. The genes that harbour suggestive SNP included *ACADSB, PECR, BDH1*, *DGAT1*, and *ACADL*. No significant and 8 suggestive SNP were detected by analyzing cows from later lactations. These SNP were located within the genes: *CS, CPT1B, IGF1, PCK1, DLD* and *HNF4A.* The results of each association analysis of milk BHB are presented in Table 4.4. Manhattan plots depicting the results of each analysis are shown in Figure 4.3 for all cows and Figure 4.3 for cows separated by first and later parity.

Association analyses were also performed using the cows' 50K genotypes in combination with the 998 genotypes of the custom panel for unadjusted ketosis records and adjusted BHB phenotypes of cows from all parities. Figure 4.5 depicts the Manhattan plots for both traits with the candidate gene SNP represented in a contrasting colour to show the location of these SNP within the denser 50K SNP map. These plots demonstrate the presence of candidate gene SNP near peaks that are easier to visualize with the dense SNP map overlain. For KET, candidate gene SNP can be observed within clear peaks on BTA2, 6, and 26. Alternatively, peaks on BTA1, 3, and 17 harbour candidate gene SNP for the trait BHB. Detailed results of these analyses are not presented as the objective of this study was to determine the association of the custom markers with KET and milk BHB.

4.5 Discussion

Records

The estimated frequency of KET in herds that record this disease in Canada is 4.5%, although the prevalence of subclinical disease has been estimated to be at least three times higher (Koeck et al., 2014; Koeck et al., 2015). The low frequency of disease-recording makes this a

Table 4.3: SNP associated (chromosome-wise FDR < 0.05) and suggested to be associated (chromosome-wise FDR < 0.20) with log-transformed milk BHB. Back transformed estimates of the allele substitution effect are presented in mmol/L units.

SNP ID	Chr[1]	Gene	Minor Allele Frequency	Allele Substitution Effect	p-value	Variant Consequence
All Parities Adjusted Phenotypes (*n* = 535)						
SNP171	17	MSMO1	0.375	0.0142	$1.43xe^{-2}$ [c]	5' UTR
SNP699	4	DLD	0.433	-0.0131	$1.51xe^{-2}$ [b]	synonymous
SNP974	9	PLG	0.202	-0.0158	$2.08xe^{-2}$ [c]	synonymous
SNP335	21	ACSBG1	0.234	0.0147	$2.71xe^{-2}$ [c]	synonymous
SNP271	20	OXCT1	0.327	0.0124	$2.87xe^{-2}$ [c]	synonymous
SNP178	17	MSMO1	0.448	-0.0117	$3.16xe^{-2}$ [c]	3' UTR
SNP 332	21	ACSBG1	0.222	0.0139	$3.96xe^{-2}$ [c]	3' UTR
SNP179	17	MSMO1	0.438	-0.0119	$4.02xe^{-2}$ [c]	3' UTR
SNP174	17	MSMO1	0.420	0.0112	$5.27xe^{-2}$ [c]	3' UTR
SNP333	21	ACSBG1	0.319	0.0113	$6.68xe^{-2}$ [c]	synonymous
First Parity Adjusted Phenotypes (*n* = 196)						
SNP287	2	FN1	0.4504	0.0254	$2.05xe^{-3}$ [a]	R → T
SNP285	2	FN1	0.495	-0.0230	$5.28xe^{-3}$ [a]	synonymous
SNP311	2	FABP3	0.120	0.0382	$5.22xe^{-3}$ [a]	synonymous
SNP390	23	ACADSB	0.146	0.0343	$7.01xe^{-3}$ [b]	5' UTR
SNP297	2	PECR	0.170	-0.0279	$1.76xe^{-2}$ [b]	synonymous
SNP170	1	BDH1	0.244	0.0252	$2.12xe^{-2}$ [c]	5' UTR
SNP584	2	ACADL	0.0524	0.0365	$8.09xe^{-2}$ [c]	3' UTR
SNP107	14	DGAT1	0.379	0.0165	$8.26xe^{-2}$ [c]	A → E
SNP106	14	DGAT1	0.382	0.0158	$9.39xe^{-2}$ [c]	A → T
Later Lactation Adjusted Phenotypes (*n* = 410)						
SNP768	5	CPT1B	0.458	-0.0189	$9.10xe^{-2}$ [c]	5' UTR
SNP790	5	IGF1	0.371	0.0164	$1.73xe^{-2}$ [c]	5' UTR
SNP83	3	PCK1	0.323	-0.0173	$1.85xe^{-2}$ [c]	synonymous
SNP699	4	DLD	0.434	-0.0146	$2.86xe^{-2}$ [c]	synonymous
SNP80	3	PCK1	0.321	-0.0161	$2.91xe^{-2}$ [c]	3' UTR
SNP776	5	CS	0.429	-0.0142	$3.30xe^{-2}$ [c]	synonymous
SNP103	13	HNF4A	0.0183	-0.0487	$6.38xe^{-2}$ [c]	3' UTR
SNP101	13	HNF4A	0.0183	-0.0499	$9.07xe^{-2}$ [c]	3' UTR

[1]The chromosome that harbours the SNP in the previous column

[a] chromosome-wise false discovery rate (FDR) q-value less than 0.05

[b] chromosome-wise false discovery rate (FDR) q-value less than 0.10

[c] chromosome-wise false discovery rate (FDR) q-value less than 0.20

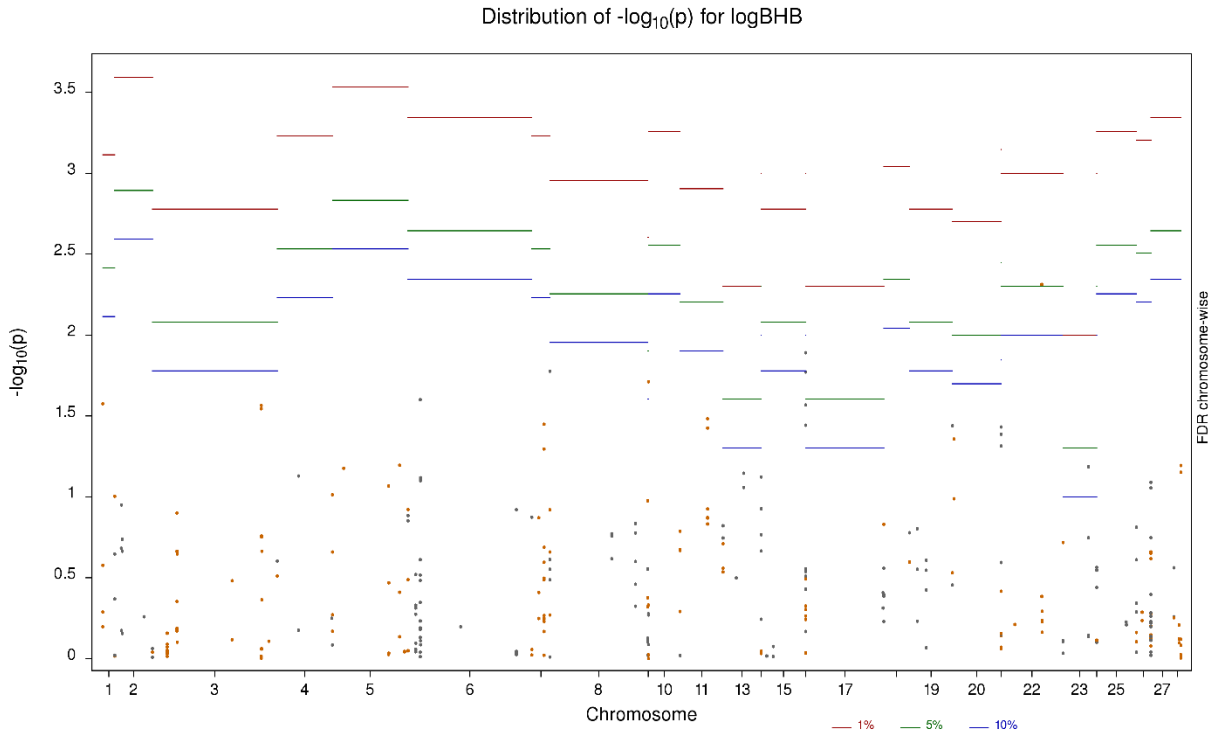
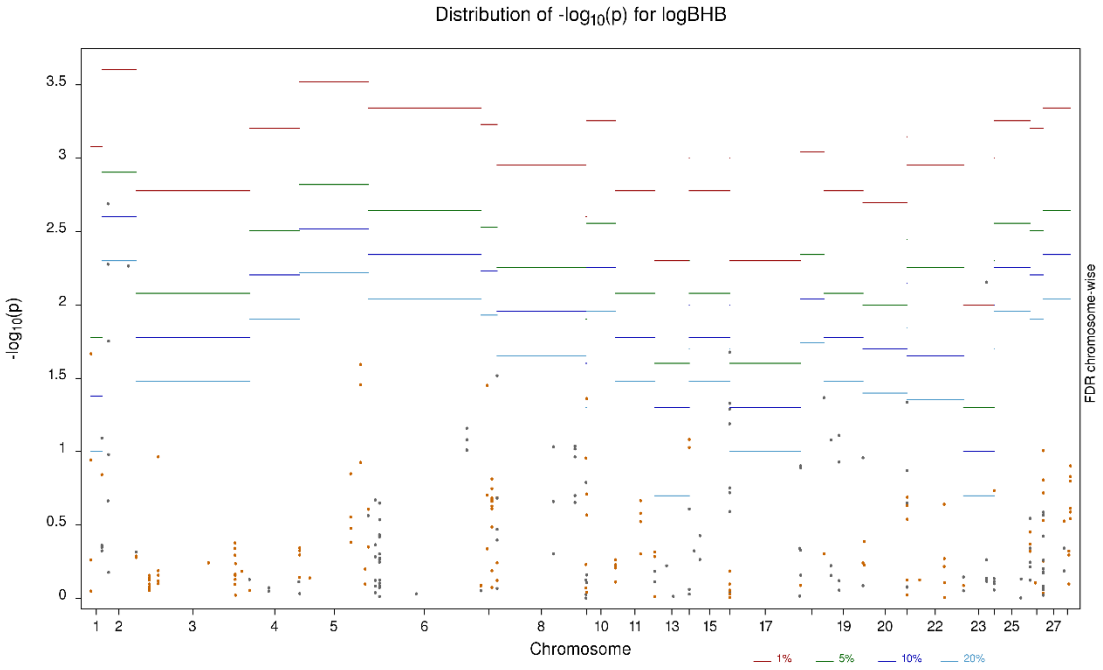


Figure 4.3: Manhattan plot showing the results of the association analyses with log-transformed milk β-hydroxybutyrate adjusted for environmental effects.

a.



b.

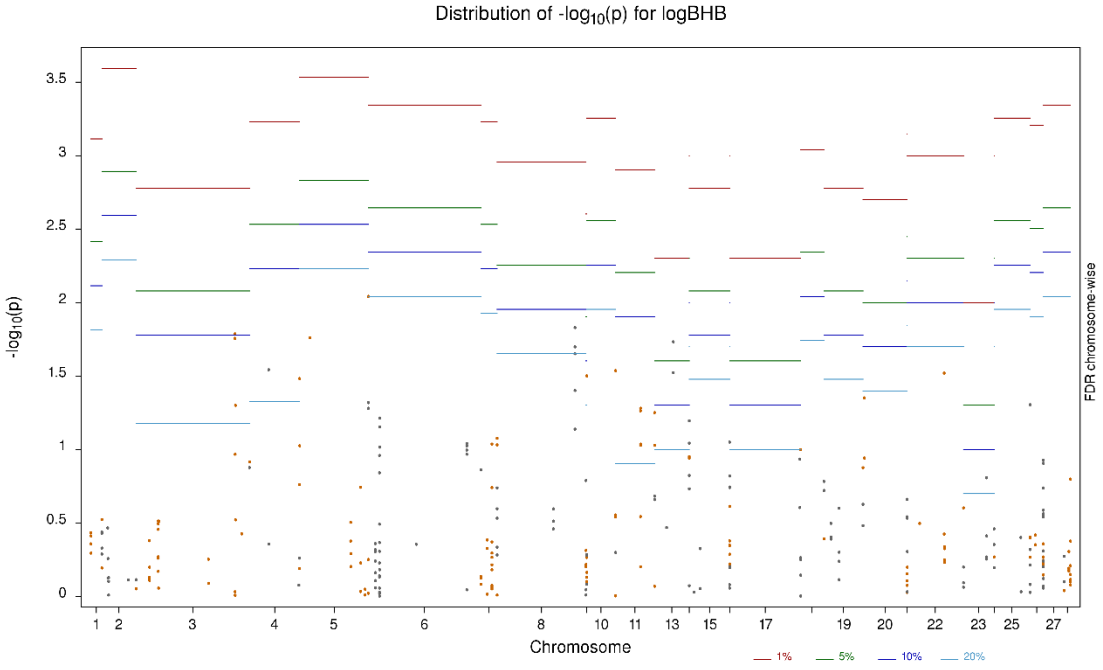


Figure 4.4: Manhattan plots of the results of the association analyses with log-transformed milk β-hydroxybutyrate adjusted for environmental effects in first parity cows (a; n=195) and parity 2 – 5 cows (b; n=410).

a)

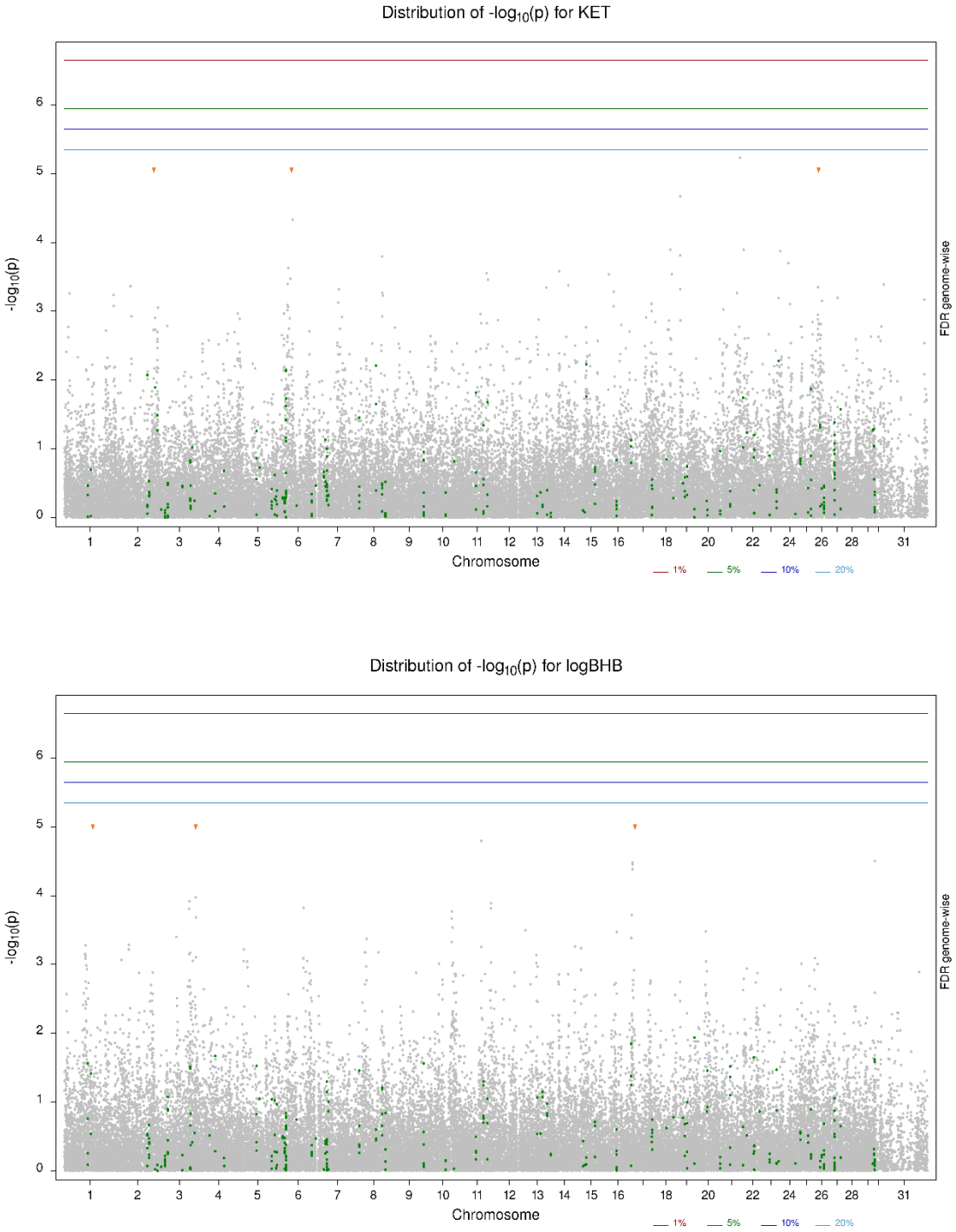


Figure 4.5: Manhattan plots for 50K + 998K SNP association analyses for unadjusted ketosis records (a) and log-transformed milk β-hydroxybutyrate adjusted for environmental effects (b). The candidate gene SNP are presented as green dots while the SNP on the 50K panel are grey. Orange arrows indicate obvious peaks that harbour candidate gene SNP.

difficult trait to study and to use for genetic evaluation; however, this is also a trait that would greatly benefit from the added information of molecular markers. In the study, putative SNP within candidate genes related to KET were tested to detect associations between a cow's genotype and metabolic traits in Canadian Holsteins. This study made use of the cow health records and test-day milk analysis data that is reported in Canada and includes cases of KET and predicted milk BHB. These were existing datasets that greatly reduced the labor time and costs associated with phenotype collection and genotyping. An additional advantage to using genotyped cows for the analysis was the utilization of direct records on each animal and even multiple records on an animal from successive lactations. Ideally, repeated records could be used to better model the health and milk data and also to fit SNP effects along the lactation curve. The available records were limited by the small sample of cows that had complete health and milk records, as well as 50K genotypes. To improve the chances of capturing more variation in the sample, cows were chosen non-randomly to increase the proportion of animals that had had a case of KET at some point in their productive life; 36% of first parity and 23% of later lactation records were disease cases. The inflation of disease frequency in the sample introduces bias and therefore, the results of the analyses were most likely over-estimated. Alternatively, de-regressed EBV for KET and milk BHB, with the added information from ancestors and correlated traits, would be interesting to use for future analyses. For dairy cattle traits, de-regressed evaluations are the most common response variable for GWAS (Macciotta *et al.,* 2015).

Marker Panel Results

In addition to testing the association of candidate gene SNP with metabolic traits, the results of the study also provide proof of concept for the detection of SNP *in silico* using bovine cDNA libraries. These are mostly novel SNP that were not previously included on commercial

SNP chips and are located within and near candidate genes related to KET. Chapter Three outlined the criteria for the selection of the 998 SNP used for the analyses. Previously, Squires *et al.* (2014) identified 97 SNP for boar taint using similar methods with porcine databases. For three pig breeds 63 to 86% of the SNP were found to be polymorphic (MAF>0.05) after genotyping more 3,474 pure-bred boars (Squires *et al.*, 2014). In this study, 653 Holstein cows from Ontario and Quebec were genotyped with the custom panel of SNP. Surprisingly, just 34% were polymorphic (MAF>0.01), although 78% of the genes of interest contained at least one polymorphic SNP. A high proportion of fixed SNP could be the result of increased relatedness in the small sample, breed differences, or poor SNP selection criteria. The level of inbreeding is a measure of relatedness, as an inbred offspring is the result of mating individuals that are related (Miglior and Burnside, 1995). The pedigree for the sample of cows analyzed contains 24,260 animals with an average inbreeding coefficient of 2.25%, which is lower than the inbreeding of 8% for Canadian Holsteins that was estimated by Stachowicz *et al.* (2011). There were 237 unique sires and 213 dams of the 653 cows sampled. The number of sires and dams in the data set may be an indicator of bias in the pedigree of the cows; a high proportion of full- and half-siblings reduces the amount of genetic variation in the sample. Li *et al.* (2015) performed a GWAS using data on both Chinese and Danish Holstein populations to increase the power to detect QTL for milk fat traits. QTL segregating in both populations also provide more evidence of causal genes and mutations. Using cows from different populations or even different breeds would be useful to determine if any fixed SNP may be rare variants that do not segregate in the sample of cow used for the analyses. Despite the large number of non-segregating SNP, development of the marker panel allowed for the analysis of several hundred markers within candidate genes for KET. Unlike a typical GWAS, these are not anonymous, common markers, but SNP found in genes proposed to have an effect on metabolic

adaptation during the transition period. Unlike a typical CGAA, which may focus on identifying SNP in a limited number of genes by sampling and sequencing cows with extreme phenotypes, 94 candidate genes are represented on the custom marker panel, and 31 of the genes contained one or more SNP for the metabolic traits under investigation in this study. Figure 4.6 presents a visualization of the 31 SNP-containing genes and their roles in hepatic metabolic processes.

Ketosis Results

Results of the association analyses indicated that SNP within 18 unique genes that are involved in fatty acid (*ACADL, ECI2, ADH6, ACSL6, ALDH1C, ALDH3A2, ALDH1B1)* and ketone body metabolism *(HMGCL),* the citric acid cycle (*MUT, MDH2)* and regulation of energy metabolism (*APOA1, AGRP, MSMO1, FGFR1, SLC27A2, SCD, FABP3)* were associated to KET. Notably, no significant or suggestive SNP were detected within any candidate genes involved in gluconeogenesis. The analysis of unadjusted KET records for all available cows produced the best results compared to using adjusted phenotypes or separating cows by first and later lactation; this finding is presumably due to the inclusion of more animals with records, which has an impact on allele frequency. The 2 significant SNP detected, SNP402 and SNP127, are found within genes that have a role in lipid metabolism, *ECI2* and *APOA1*. Both SNP402 and SNP127 are novel mutations that were identified *in silico* and were not included in any SNP databases at the time of detection. SNP402 and SNP127 explained 8.5 and 8.0% of the phenotypic variance, respectively.

ECI2 encodes enoyl-CoA delta isomerase, an enzyme that converts a cis-double bond to a trans-double bond so that β-oxidation may continue (Voet *et al.*, 2002). Disruption of this process would reduce fatty acid oxidation in the liver and increase accumulation of TG in hepatocytes. The marker panel included 5 other SNP within *ECI2,* but SNP402 was the only one that met the quality control criteria. The observed frequency

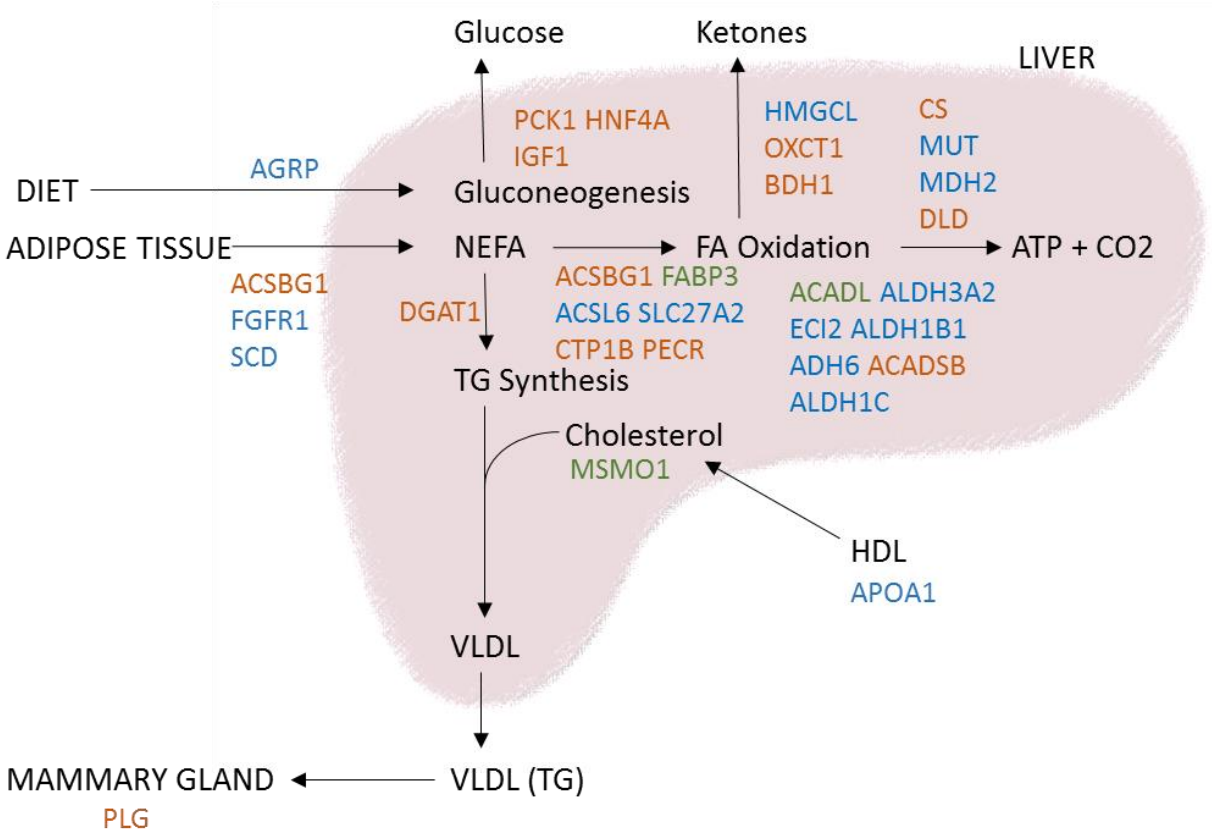


Figure 4.6: Visual representation of 30 candidate genes containing SNP associated or suggested to be associated to the metabolic traits investigated in the marker-trait association analyses. Genes containing SNP for ketosis and milk β-hydroxybutyrate are shown in blue and orange, respectively. The three genes shown in green contain SNP for both traits. *Fibronectin 1* is not shown, although two SNP were detected in this gene. as this gene's connection to hepatic energy metabolism is not well-understood.

of the minor allele for SNP402 was just 3.7%; if a MAF of 5% had been chosen as the threshold for quality control this rare variant would have been overlooked. The estimated allele substitution effect for SNP402 was the highest of any analysis; the estimate was 0.467 and 0.615 for the unadjusted records of all cows and first parity cows, respectively. Although these estimates are most likely inflated, their relative size compare to many of the other allele substitution effects is notable. Considering that the trait used was unadjusted records where 1 is equal to a case of KET and 0 is a non-case, two copies of the minor allele would effectively predict that the cow would have a case of KET. SNP402 is located within the 3'UTR of *ECI2*; this SNP is 21 bp downstream of the stop codon of the gene. Being in the untranslated region of the gene makes it difficult to interpret if changes to protein translation or function may be a consequence of this mutation; although, SNP within 3'UTRs should not be discounted because of this. Clop *et al.* (2006) described the functional effect of a SNP within the 3'UTR of the myostatin gene in Texel sheep that creates a target site for microRNA that inhibit protein translation and contribute to muscular hypertrophy, or double muscling. Further investigation of the 3'UTR of *ECI2* should be conducted to determine the consequence of a mutation in this region.

The second SNP that was significantly associated to KET, SNP127, is located within the third exon of the gene *APOA1*, which encodes apolipoprotein A-1. Apolipoproteins are the amphiphilic protein component of lipoproteins that are involved in lipid transport throughout the body. Apolipoproteins and associated proteins have been increasingly studied to determine their involvement in the development of fatty liver and other functional traits. Decreased serum concentrations of APOA1 and other apolipoproteins have been shown to be associated with KET, DA, fatty liver, milk fever, and downer cow syndrome (Oikawa and Katoh, 2002; İleri-Büyükoğlu *et al.,* 2009). The expression of apolipoprotein B100 and E are inhibited by high concentrations of

NEFA, which has been suggested to limit the assembly of low density lipoproteins and promote fat accumulation in the liver (Liu *et al.,* 2014). A causative mutation within *APOBR* for milk GPC, a proposed biomarker for KET, was detected by Tetens *et al.* (2014), while a SNP within the promoter region of *apolipoprotein A-II* has been linked to body traits in cattle (Zhou *et al.,* 2013). A loss of function mutation within apolipoprotein B causes cholesterol deficiency in Holsteins (Menzi *et al.,* 2016). There has been less focus on APOA1 in relation to KET and fatty liver development, seemingly because APOA1 is a component of high density lipoprotein and chylomicrons; the study of fatty liver syndrome has been focused mainly on the very low density lipoproteins that export TG from the liver. The SNP detected within *APOA1* in this study is the first, to my knowledge, to be detected within this gene. The residues of APOA1 form repeating α helices that coat the lipoprotein; formation of the helices is dependent on the polarity and order of the amino acids (Voet e*t al.,* 2002). The mutation at SNP127 results in the translation of a methionine instead of a leucine, which are both non-polar residues and result in a neutral substitution, as predicted with SIFT software. Functional studies should be conducted to confirm this prediction. In addition to apolipoprotein formation, APOA1 has been linked to intracellular signalling. Induction of cAMP production by APOA1 has been observed in human fibroblasts and Chinese hamster ovary cells (Haider *et al.,* 2004). cAMP subsequently activates protein kinases that phosphorylate various enzymes, including those that stimulate β-oxidation and gluconeogenesis and inhibit lipogenesis (Voet *et al.,* 2002). The SNP detected within *APOA1* suggests that the relationship between this apolipoprotein and lipid homeostasis should be studied further.

There was little overlap seen between the SNP detected using all cows, and each subset by lactation. Particularly, the primiparous and multiparous subsets did not share any common SNP,

although SNP within the gene *MSMO1* were detected in both groups. Variable results could be attributed to the distribution of genotypes in the small subsets. Alternatively, this could indicate that different genes impact a cow's metabolism in her first lactation compared to later lactations. The increased risk of developing KET in later lactations has been well-documented, but the root of the metabolic variances between heifers and cows has not been established (Berge and Vertenten, 2014; Vanholder *et al.*, 2015). Chapinal *et al.* (2012) suggest that physiological differences between heifers and multiparous cows allow them to cope with NEB uniquely. Additionally, separate genetic evaluations for heifers and cows have been proposed (Koeck *et al.*, 2015; Jamrozik *et al.*, 2016). The results of this study could suggest that in the first parity, SNP within genes that encode metabolic enzymes (*ACSL6, ALDH3A2, MDH2, ECI2, MUT)* play a significant role in the development of KET, while in later parities, regulatory factors may contribute to a cow's health status more (*FGFR1, MSMO1, AGRP, SLC27A2)*. The results of the analyses of milk BHB may support this trend.

Although no SNP reached statistical significance using first lactation cows, the suggestive SNP potentially explained a large amount of phenotypic variance; the top four SNP explained 20.2, 18.7, 17.8 and 16.4% of the phenotypic variance for the trait KET. One of the notable genes that contained a suggestive SNP for this group is *ACSL6,* which encodes acyl-CoA synthetase long-chain family member 6; Grossi (unpublished data, 2014) also identified a QTL for KET in the promoter region of this gene. The SNP detected in this study, SNP903, was approximately 10kbp upstream of the previously identified QTL. Interestingly, the loci detected by Grossi (unpublished data, 2014) was included on the marker panel, but was not polymorphic in current sample of animals. ACSL6 is part of the family of thiokinases that are responsible for activation of fatty acids in the cytosol before they are oxidized in mitochondria (Voet *et al.*, 2002). A SNP in the promoter

region could affect the transcription of ACSL6, and therefore, the fate of fatty acids mobilized during early lactation. Grossi (unpublished data, 2014) also detected multiple QTL close to the genes *ECI2* and *ACADL*, which were found to harbour significant and suggestive SNP in this study. A SNP within *ACADL* that is associated with liver fat accumulation has also been identified in humans (Mirkov *et al.*, 2012). Li *et al.* (2012) reported decreased abundance of *ACADL* mRNA and protein levels in the liver of ketotic cows. *ACADL* encodes long-chain acyl-CoA dehydrogenase, the enzyme that catalyzes the initial step of oxidation for long-chain fatty acids. This result reiterates the importance of enzymes that control the fate of fatty acids in the liver to the development of KET.

Milk β-hydroxybutyrate Results

The candidate gene association study of milk BHB resulted in the identification of 3 SNP associated to the trait and 23 suggestive SNP in 17 genes. These genes were selected for their functional relevance to the development of KET; SNP were detected within genes for fatty acid metabolism (*ACADSB, ACADL, ACSBG1, PECR, CPTIB),* ketogenesis (*OXCT1, BDH1),* TCA cycle (*CS, DLD),* gluconeogenesis (*PCK1),* and metabolic regulatory factors (*MSMO1, PLG, FABP3, IFG1, DGAT1, FN1, HNF4A).* In agreement with the results for KET, different SNP were identified when cows were analyzed by first lactation or later lactation. The strongest associations were observed in the analysis of the first lactation subset; 3 SNP on BTA2 reached chromosome-wise significance. Two of these SNP were located within the fibronectin 1 gene and one was located in the fatty acid binding protein 3 gene.

Fibronectin 1 is an extracellular matrix protein that has been linked to many signaling pathways and interactions (Loor *et al.*, 2007; Voet *et al.*, 2012). Although the mechanistic relationship with KET is not yet clear, *FN1* is differentially-expressed in the liver tissue of ketotic

cows and a QTL for milk malonate has been mapped to this gene (Loor *et al.,* 2007; Buittenhuis *et al.,* 2013). The consequence of the nonsynonymous mutation at SNP287 is the translation of a threonine residue instead of arginine. Threonine is a neutral, polar amino acid, while arginine is basic; the substitution is predicted to be deleterious. SNP285, also located within *FN1*, is a synonymous mutation, likely in LD with SNP287. The proportion of phenotypic variance explained by SNP287 and 285 was 16.9 and 14.2%, respectively; however, the calculation of SNP variance in this analysis did not take into account LD and therefore the proportion of variance explained is confounded for these SNP due to their close proximity. Although the impact of these SNP on the phenotype is not easily explained, the results of this analysis provide additional evidence of the relationship between fibronectin 1 and KET that should be looked into further.

Another significant SNP for milk BHB, SNP311, lies within the first exon of *FABP3.* Fatty acid binding proteins facilitate intracellular fatty acid transport and can affect the concentration of fatty acids within cells (Kulig *et al.,* 2010). Fatty acid binding proteins have been reported to interact with metabolic enzymes, including those involved in fatty acid activation, which would impact fatty acid utilization (Glatz and van der Vusse, 1996). Again, a gene that impacts the metabolic fate of fatty acids that come into the liver has been identified by this CGAA. Additionally, by binding and reducing the availability of fatty acids and other lipophilic compounds, fatty acid binding proteins can impact signalling pathways (Glatz and van der Vusse, 1996). SNP for milk fat content have been identified in Jersey cattle, buffalo, and goats (Kulig *et al.,* 2010; Dixit *et al.,* 2015; Dubey *et al.,* 2106). Loor *et al.* (2007) found that *FABP3* was differentially-expressed by ketotic cows. In this study, SNP311, a synonymous mutation, was found to be significantly associated with milk BHB and explained 15.8% of the phenotypic variance.

Several SNP identified in the analysis were located within candidate genes close to QTL associated with EBV for KET that Grossi (unpublished data, 2014) detected. Suggestive SNP within *BDH1, PLG, ACSBG1,* and *ACADL* support the findings of Grossi (unpublished data, 2014). As discussed previously, *ACADL* also harbours a significant SNP for the trait KET; association with two distinct, but related traits, provides evidence of a SNP within *ACADL* that contributes to a cow's susceptibility to KET. The results of the analyses of KET and milk BHB revealed 4 more SNP in common between the two traits; SNP311 in *FABP3* and SNP171, 174, and 178 within *MSMO1.* For all common SNP, the estimated direction of the allele substitution effect was consistent for both traits; if the SNP was associated with an increased chance of developing KET (closer to 1), it was also associated with an increase in milk BHB. SNP171 is located in the promoter region of the methylsterol monooxygenase 1 gene, while SNP174 and 179 lie in the 3'UTR. *MSMO1* is an enzyme embedded in the membrane of the endoplasmic reticulum that is involved in biosynthesis of cholesterol (Voet e*t al.,* 2002). After parturition there is an increased demand for cholesterol that is met by increased expression of genes involved in biosynthesis (Kessler *et al.,* 2014; Gross *et al.,* 2015). It has been hypothesized that cholesterol is in high demand after calving to form lipoproteins necessary for the export of TG from the liver (Kessler *et al.,* 2014). Further, Loor *et al.* (2007) reported differential gene expression of *MSMO1* in the liver of cows with ketosis. Along with *MSMO1* and *APOA1,* various other identified genes - *ACSBG1, DGAT1, FABP3, HNF4A* – are involved in cholesterol metabolism. This highlights a pathway that is often overlooked when considering the pathogenesis of KET and may be an area of future research.

Dense Marker Map

The lack of SNP that reached genome-wide significance for the analysis using the combination of the 50K panel and the 998 candidate gene SNP, demonstrates the limitations of studying traits with low heritability in a sample size that is numerically-challenged. Although the aim of this study was to estimate associations between the custom marker panel for KET and metabolic traits, the analysis was also performed using the available 50K genotypes; this allowed for the SNP of interest to be visualized alongside the denser marker map and the identification of clearer peaks. For KET, peaks were observed on BTA2, 4, 6, 7, and 25. BTA2 contains the genes *ACADL*, *FABP3* and *HMGCL* that were found to harbour SNP for KET. *ADH6 and ALDH1C* are located on BTA6 and *MDH2* was the only gene on BTA25 that contained suggestive SNP for KET. The peaks on BTA2, 6, and 25 contain a number of the candidate gene SNP. Peaks on BTA1, 3, 6, 10, 17, and 20 were observed for milk BHB; candidate gene SNP were seen near the peaks on BTA1, 3, and 17. *BDH1, PCK1,* and *MSMO1* were the only candidate genes with suggestive SNP located on BTA1, 3, and 17, respectively. Grossi (unpublished data, 2014) found strong signals on BTA3, 9, and 24 when GWAS was conducted for KET EBV using high-density markers (777K); only the peak on BTA3 for milk BHB observed in this study supports these findings.

4.6 Conclusions

The association analyses of SNP within genes involved in the pathogenesis of KET detected 45 SNP in 31 unique genes for metabolic traits. The results revealed novel mutations with the potential to be used for genetic markers and identified genes and pathways that could lead to a better understanding of disease susceptibility. Various SNP involved in cholesterol metabolism were detected in the study and suggest that this pathway may play a key role in the development of KET in early lactation. Additionally, the impact of the deleterious mutation within *FN1* should

be explored further, as there is currently no mechanistic basis for the involvement of this gene with KET. These preliminary results are promising considering the limitations of working with traits that have low heritability in a small sample of animals; validation studies should be planned to confirm the findings of this project and to determine the effects of potential rare variants that were not segregating in this sample. Identifying and understanding the genetic factors that predispose cows to KET would benefit the dairy industry as a whole by providing producers, breeding services, and veterinarians a tool to forecast a cow's susceptibility to this disease.

CHAPTER FIVE: General Discussions

The successes of genetic improvement for production traits in livestock have revolutionized agriculture as a whole and have inspired many to focus now on the improvement of functional traits such as fertility, lameness, and disease-resistance (Berry *et al.*, 2011). This year saw the release of the Canadian Dairy Network (Guelph, ON) Metabolic Disease Resistance selection index, as well as the CLARIFIDE Plus (Zoetis Genetics, Kalamazoo, Michigan, United States of America) genetic evaluation for wellness traits in dairy cattle. One wellness trait that has a large impact on farms in Canada is KET. The economic losses associated with KET include veterinarian and treatment costs, reduced milk production, delay in conception, early culling, and death (Guard, 2008; McArt *et al.,* 2015; Gohary *et al.,* 2016). Various economic models have been used to estimate the cost of KET on farm; estimates for a case of clinical KET range from $187 to $300 CAD, while subclinical KET ranges from $78 to $378 CAD (Geishauser *et al.*, 2001; Guard, 2008; McArt *et al.,* 2015; Raboisson *et al.,* 2015; Gohary *et al.,* 2016). Each of these economic models also included the relative risk and associated costs of a cow with subclinical KET developing subsequent disease. Genetic improvement of KET has been limited due to low heritability, unclear disease definitions and phenotypes, and an antagonistic relationship with milk production. Further, the underlying biological mechanisms that lead to the development of KET are not well-understood.

The goal of any selection program is to increase the frequency of the beneficial alleles of a gene underlying a trait; traditional selection criteria is based on the phenotype of an individual and their relatives, which changes the frequency of alleles, albeit blindly (Falconer and Mackay, 1996). To date, few of the underlying genes that control economic traits have been identified. This study aimed to shed some light on the genes that contribute to an animal's susceptibility to KET

with two main objectives: 1) to search within candidate genes for putative markers and 2) to test the association of these markers with metabolic traits. This strategy allowed for the fine-mapping of many key genes without the need to sequence each gene individually and phenotype many cattle, but was also restricted by the quality of sequence data available in online collections. The sequence data that was attained for the *in silico* investigation is submitted by anonymous researchers world-wide who study a variety of populations and breeds. Additionally, the reference sequence, *Bos taurus* UMD 3.1.1, is based on the genomic DNA of Hereford cattle. While this is useful to identify novel and rare genetic variation, not all the polymorphisms found *in silico* translated to the Canadian Holstein cows sampled in this study; in fact, just 30% of the SNP were sufficiently polymorphic. It was expected that a number of SNP included on the panel would not be segregating, as sequence data in online databases comes from a variety of sources. Continued use of the marker panel to genotype cattle would be beneficial to confirm the presence of polymorphisms in other populations and in different breeds. Future studies that aim to employ the same strategy for SNP detection may find it difficult to sort through the large quantity of sequence and SNP data that is added to online databases daily. For example, at the beginning of SNP discovery for this study the gene *glucose-6-phosphatase* had 13 SNP categorized in the dbSNP database and currently, this number has grown to more than 700. With such a large number of reported SNP in the database it is difficult to determine which SNP are true mutations. Further, the imminent release of an improved bovine genome assembly will impact all genetic studies and require re-alignment to verify the presence and location of new and old SNP. Despite the challenges of identifying SNP *in silico,* hundreds of new markers were found to be segregating in this study; this provided proof of concept for the detection of SNP by *in silico* sequence alignment for cattle. Other deliverables of the study included: detection of multiple SNP – including novel,

rare, and deleterious variants – associated with metabolic traits, confirmation of QTL reported by earlier studies, and trend identification for the types of genes and metabolic processes that likely effect a cow's susceptibility to KET. The results of this study lead to a fresh set of research questions related to the mutations, genes, and pathways linked to KET.

The first major research question that should be addressed is: are the results of the association analyses applicable to independent populations? Validation of SNP effects will be necessary if the marker panel is to be used for commercial applications. Ideally, validation would come after the reference population increases – either by more genotyping with the new SNP or by imputation of the genotypes. All of the cows genotyped in the current study had been previously genotyped with the BovineSNP50 BeadChip (Illumina Inc., San Diego, CA), which allows for imputation of their genotypes for the new 998 SNP. Imputation accuracy of the new SNP is expected to be very high (more than 90%) based on previous work (Larmer *et al.*, 2014). Increasing the size of the genotyped reference population will improve the confidence of the results of the association analyses. Validation studies should be conducted by calculating genomic EBV based on an animal's genotypic information and the estimated allele substitution effects for all SNP (50K+998 SNP) and just the 50K and comparing the gains in reliability from the inclusion of all SNP. Reliability is the square of the accuracy, calculated as the correlation between the genomic EBV and the true EBV divided by the square root of the reliability of the EBV (Hayes *et al.*, 2009). Validated SNP would provide a tool for the selection of cattle less susceptible to KET by providing increased reliability to evaluations of metabolic traits. It would also be beneficial to assess the correlation between the KET markers and milk production traits to ensure that selection for resistance to KET does not have antagonistic effects on cow profitability. A validated panel of SNP would also be helpful for developing prevention, monitoring, and treatment strategies for

cattle identified as being at-risk for KET. Additionally, a tool to identify KET-susceptible cattle would be beneficial for other researchers; it is difficult to study the metabolic changes that occur before the onset of KET when there is no accurate way to predict which cows will be affected.

The second major research question that arises from the results of this study is: how do the mutations detected by this study affect the traits they are associated with? This question could be answered by performing functional studies of the biological effects of mutations associated with KET and milk BHB. Possible research to quantify the functionality of SNP could include assessment of differences in gene expression, protein expression, metabolic activity, binding affinity, chromatin accessibility and exon-splicing of the mutant and wild-type alleles. Functional characterization of SNP is beneficial for providing evidence for potentially causative mutations as well as mechanistic insight at the molecular level. Results of this study could fuel the hypotheses of dozens of functional studies. Further, these studies could be useful to determine the effects of mutations in different tissues that the gene may be expressed; this is especially important for regulatory factors and transporters. Real *et al.* (2014) described the effects on drug metabolism for a known QTN within the bovine adenosine triphosphate-binding cassette transporter G2 gene. Although the QTN was previously reported to affect the quality and quantity of milk, these researchers found that selection for this QTN would also have an impact on a cow's ability to process various drug substrates (Real *et al.,* 2014). This study cautions that even when markers are found for a specific trait, there could be unwarranted effects in other tissues.

The results of the current study highlighted the importance of genes that control the utilization of fatty acids in the liver and also genes involved in cholesterol metabolism, which asks the question: how do these processes contribute to the development of KET in early lactation? Several SNP were found in genes that may affect the accumulation of TG in hepatocytes. The

effect of elevated liver fat content on energy metabolism has been previously studied, with contrasting results (Rukkwamsuk *et al.,* 1999; Sejersen *et al.,* 2012; Gross *et al.,* 2013). Additionally, the etiologies of fatty liver and KET has long been speculated to be related (Grummer, 1993). Kessler *et al.* (2014) reported that enzymes that control cholesterol biosynthesis are most likely upregulated at the onset of lactation to meet the increased demand for cholesterol to form lipoproteins to export liver TG. The links between cholesterol metabolism, TG accumulation in hepatocytes, and KET should be investigated further. The results of this work also suggest that genes involved in behaviour would also be important to investigate. A SNP within the gene encoding agouti-related neuropeptide was suggested to be associated with KET for both first and later parity cows. Expression of this neuropeptide in the hypothalamus has been linked to differences in adaptive behavior during starvation, including feeding behavior (Borner *et al.*, 2013; Perkins *et al.*, 2014). Understanding the impact of mutations in other genes that drive behaviour that would impact a cow's energy state in early lactation would also be helpful.

A fourth research question that warrants further investigation is: how does the development of KET differ between the first and later lactations? The work for this thesis reported different SNP and genes depending on lactation status for KET and milk BHB. The physiological differences between a primiparous and multiparous cow's ability to adapt to NEB are not well-understood and literature that compares these groups is limited. Swali and Wathes (2007) suggested that primiparous cows mobilize body tissue to a lesser extent than multiparous cows because they are retaining nutrients for their own growth and development. This hypothesis is supported by the finding that primiparous cows birth calves that are smaller in size than the offspring of multiparous cows, suggesting that energy partitioning in first parity cows favours maternal growth over fetal growth (Swali and Wathes, 2007). Endocrine and metabolic profiles

for primiparous and multiparous cows also show several key differences. First, primiparous cows tended to have higher concentrations of insulin-like growth factor I and insulin than multiparous cows, presumably because dairy heifers are still putting down their own body tissues as they are gestating (Wathes *et al.,* 2007). Wathes *et al.* (2007) also reported a negative correlation between insulin-like growth factor I and NEFA. Low levels of insulin-like growth factor I and binding protein have been suggested to be predictors of ketosis in multiparous cows (Piechotta *et al.,* 2015). Gene expression studies would also be helpful to explain the different strategies that primiparous and multiparous cows employ to cope with NEB.

This thesis describes the process of developing a novel panel of putative genetic markers for KET and the preliminary analyses of these markers using producer-recorded health reports and milk test-day data. These markers were selected by fine-mapping a comprehensive list of candidate genes *in silico*, making use of online cDNA libraries. Association analyses revealed SNP for both traits of interest, although the number of SNP in common was less than expected. This thesis confirmed the presence of QTLs in regions of the genome that had been previously identified, as well as suggested new markers for metabolic traits. The results of this work have the potential to lead to a new tool for the identification of disease-resistant cattle and also further research on the molecular mechanisms underlying KET.

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