Energy Status and Reproductive Performance in Dairy Cattle in New Zealand

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Abstract

Blood and milk parameters other than ketones have been used extensively to monitor the risk of negative energy balance (NEB) and reproductive inefficiency in dairy cows. However, few such studies have been conducted in New Zealand. The aim of this study was to investigate the benefits of using milk parameters as a monitoring tool in addition to blood beta-hydroxybutyrate (BHB) and non-esterified fatty acids (NEFA) to better predict risk of submission within three weeks of planned start of mating (PSM).

A prospective study was conducted in 135 dairy cows from five different pasture-fed, spring-calving commercial dairy farms in Manawatu district of New Zealand from July to November, 2014. Blood samples were analyzed for NEFA and BHB, both pre-calving (up to 2 weeks before expected calving date) and post-calving (2 weeks after calving). Fat, protein, lactose, BHB, and urea concentrations were measured in milk samples taken 2 weeks post-calving, repeating approximately at 4 week intervals until 6 weeks after PSM. The reproductive outcome variable tested was whether or not the cows were inseminated in the first 3 weeks of PSM.

NEB was seen in 57/135 cows pre-calving (based on blood NEFA [BN1] ≥ 0.4 mmol/L) and 47/135 cows post-calving (based on blood BHB [BB2] ≥ 1.2 mmol/L) giving 95% confidence interval, while 21.5% of the cows showed negative reproductive outcome. Strong correlations between milk components values meant that of the 20 milk component tests, data from only 10 were included in the logistic regression.

Milk BHB sampled in the second month after calving (B2) was the only near significant variable in the final logistic regression model ($p = 0.061$) with the mean values for cows submitted in the first 3 weeks of the breeding season being 0.05 mmol/L, and for cows which were not submitted 0.09 mmol/L. Overall, this study could not provide any evidence on advantage of adding milk parameters to blood information in improving the accuracy in predicting 3-week submission rate. However, the relatively low power of this study and limited negative outcomes suggest this need to be confirmed by taking more animals and choosing other reproductive outcome such as 3-week or 6-week in-calf rate.

Key words: NEB, BHB, NEFA, milk parameters, 3-week submission rate

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List of Abbreviations

ATP	Adenosine tri-phosphate
BCS	Body condition score
BHB	Beta-hydroxybutyrate
CB	Cross breed
CNS	Central nervous system
CPT-I	Carnitine palmityltransferase-I
DA	Displaced abomasum
F	Friesian
GnRH	Gonadotrophin releasing hormone
IGF-I	Insulin-like growth factor-I
J	Jersey
LH	Luteinising hormone
LIC	Livestock Improvement Corporation Ltd
MFAT	Ministry of Foreign Affairs and Trade
MUAEC	Massey University Animal Ethics Committee
NEB	Negative energy balance
NEFA	Non-esterified fatty acid
NZDS	New Zealand Development Scholarship
NZVP	New Zealand Veterinary Pathology
PSM	Planned start of mating
PVD	Purulent vaginal discharge
ROC	Receiver operating characteristics
RP	Retention of placenta
RR	Relative risk

SCK	Subclinical ketosis
TAG	Triglycerides
TCA	Tri-carboxylic acid
VFA	Volatile fatty acid
VLDL	Very low-density lipoprotein

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Chapter 1: Literature Review

1.1 Introduction

The expansion of the dairy industry has resulted in increased herd size and individual animal production (DairyNZ/LIC, 2013). Two of the key factors which influence the productivity of a dairy farm are the proportion of animals which are affected by disease and longevity of the animal (Wathes et al., 2008). Cattle which are maintained disease-free in a herd for long periods of time are more productive than animals which survive in the herd for short periods or which get multiple diseases.

In modern systems, production diseases, which are often subclinical, are frequently the most economically important diseases (Blood et al., 1978; Ott et al., 1999). Therefore, an efficient farm needs a system where-by production diseases can be monitored and predicted in advance using simple, fast, and cheap diagnostic methods (Hamann and Krömker, 1997). One key advance in this area has been the development of herd testing which can provide information, through measuring the components of milk, on many production-related issues from subclinical mastitis to protein/energy balance. One of the key areas which can be evaluated using herd testing is energy balance as this can have a significant impact on milk components (De Vries and Veerkamp, 2000).

Energy balance is the difference between the energy intake and the sum of energy required for maintenance and milk production (Patton et al., 2008). Energy status in dairy cows is of utmost importance for it has a direct relationship with milk production, metabolic disease risk, and reproductive performance. Events occurring during the transition period (3 weeks pre-partum to 3 weeks post-partum) have a direct impact on the energy balance of the cow during the production cycle. This means that the transition period is a critical period in the annual cycle of a dairy cow (Block, 2010).

One of the key impacts of prolonged negative energy balance (NEB) is reproductive performance as the severity and length of NEB in early lactation is closely linked to reproductive outcomes (Buckley et al., 2003; Drackley and Cardoso, 2014; Patton et

al., 2007). After parturition, milk production increases swiftly, but there is a lag in the increase in feed intake as the cow adapts to the metabolic and physiological changes associated with parturition and the onset of lactation, so all dairy cows are in NEB during early lactation (Grummer et al., 2004; Ingvartsen and Andersen, 2000). Nutritional management during the transition phase is one of the key drivers determining the extent and severity of NEB (Drackley and Cardoso, 2014; Patton et al., 2007). Another is genetics; selection of cows for increased milk production has increased the risk of prolonged severe NEB (Veerkamp, 1998).

Determining the actual energy status of an individual cow is a complex process, requiring the determination of energy inputs (feed and mobilisation of body energy reserves) and energy outputs (maintenance and milk production- the energy of which is dependent on milk fat, protein, and lactose content) (Lucy et al., 1991; Patton et al., 2008). An alternative is to measure metabolites which are associated with energy status, such as beta-hydroxybutyrate (BHB) and non-esterified fatty acids (NEFA) in blood. These results can then be used on an individual or a herd basis to estimate the severity of NEB.

The aim of this review is to look at energy balance in the dairy cow, to assess its relationship to reproductive performance, and to identify additional monitoring that could be evaluated alongside the monitoring of NEFA and BHB concentrations to improve our predictions of future reproductive performance.

1.2 New Zealand dairy system

The New Zealand dairy industry is based on feeding pasture, which means that most farms are seasonally calving so that peak milk yield coincides with maximal grass growth (Holmes et al., 2007). Since the New Zealand dairy system relies on pasture, which, on average, provides nearly 90% of the feed intake (FAO et al., 2014), it is generally a low input system (Yang et al., 2010). However, there is variation in systems across New Zealand, with some farms importing more than 30% of their feed. Such systems are rare, with only 3% of herds purchasing feed for year-round feeding. Nevertheless, most herds do purchase feed as herds which purchase no feed account for only 15% of all dairy herds (and only 10% of national milk production) (FAO et al.,

2014). In dairy systems, where pasture is the main source of feed, cows are often at risk of being underfed; i.e. the pasture provided is less than the cow could eat (Butler, 2014). Reduced pasture growth can therefore result in a significant reduction in productivity. For example, in New Zealand, total milk solid production was 1.6% lower in the 2012/13 season than in 2011/12, because the drought reduced pasture production (DairyNZ/LIC, 2013). If, during spring, pasture production is lower than anticipated and it is not compensated for by increased supplementary feed, it is likely to lead to increased NEB during this critical period. Similarly, if the stocking rate on a farm increases, without a concomitant increase in pasture production or supplement purchase, the risk of prolonged NEB increases.

Although the New Zealand system is generally low input, milk solids production per cow is increasing. Over the last 10 years, the average milk solids production per cow has increased by 31 kg (DairyNZ/LIC, 2013). Much of this gain has been due to the increase in genetic potential for milk yield. This is likely to have increased the extent of NEB as cows with high genetic merit for milk production tend to preferentially direct nutrients into the mammary glands rather than in maintaining body condition (Butler, 2014; Pryce et al., 2001). Thus, higher producing cows tend to lose more weight during early lactation than lower producing cows (Rauw et al., 1998). This propensity has been linked to reduced fertility across all types of dairy system (Mee, 2012). To understand this link we need to understand the normal energy metabolism processes in a dairy cow.

1.3 Energy metabolism in dairy cows

1.3.1 Glucose metabolism

Unlike non-ruminants, dairy cattle do not get most of their required glucose through absorption from the digestive tract. Instead, the carbohydrate components of the diet, from long chain polysaccharides, such as cellulose, through to starches and monosaccharides, get fermented by rumen microbes into short chain volatile fatty acids (VFA) (Aschenbach et al., 2010; McArt et al., 2013); these are utilised in the liver for the production of glucose via a process called gluconeogenesis, which supplies the

body with nearly 90% of its glucose requirements (Nafikov and Beitz, 2007; Yost et al., 1977).

Of the VFAs formed in the rumen, only propionate, valerate, and isobutyrate can act as precursors for glucose synthesis (Aschenbach et al., 2010), with propionate being the most important (Reynolds, 2005; Yost et al., 1977; Young, 1977). The two other major VFAs formed in the rumen are acetic acid and butyric acid, both of which can be converted to acetyl coenzyme A (acetyl-CoA), which is then used in the tricarboxylic acid (TCA) cycle to help generate adenosine tri-phosphate (ATP) (Van Houtert, 1993),

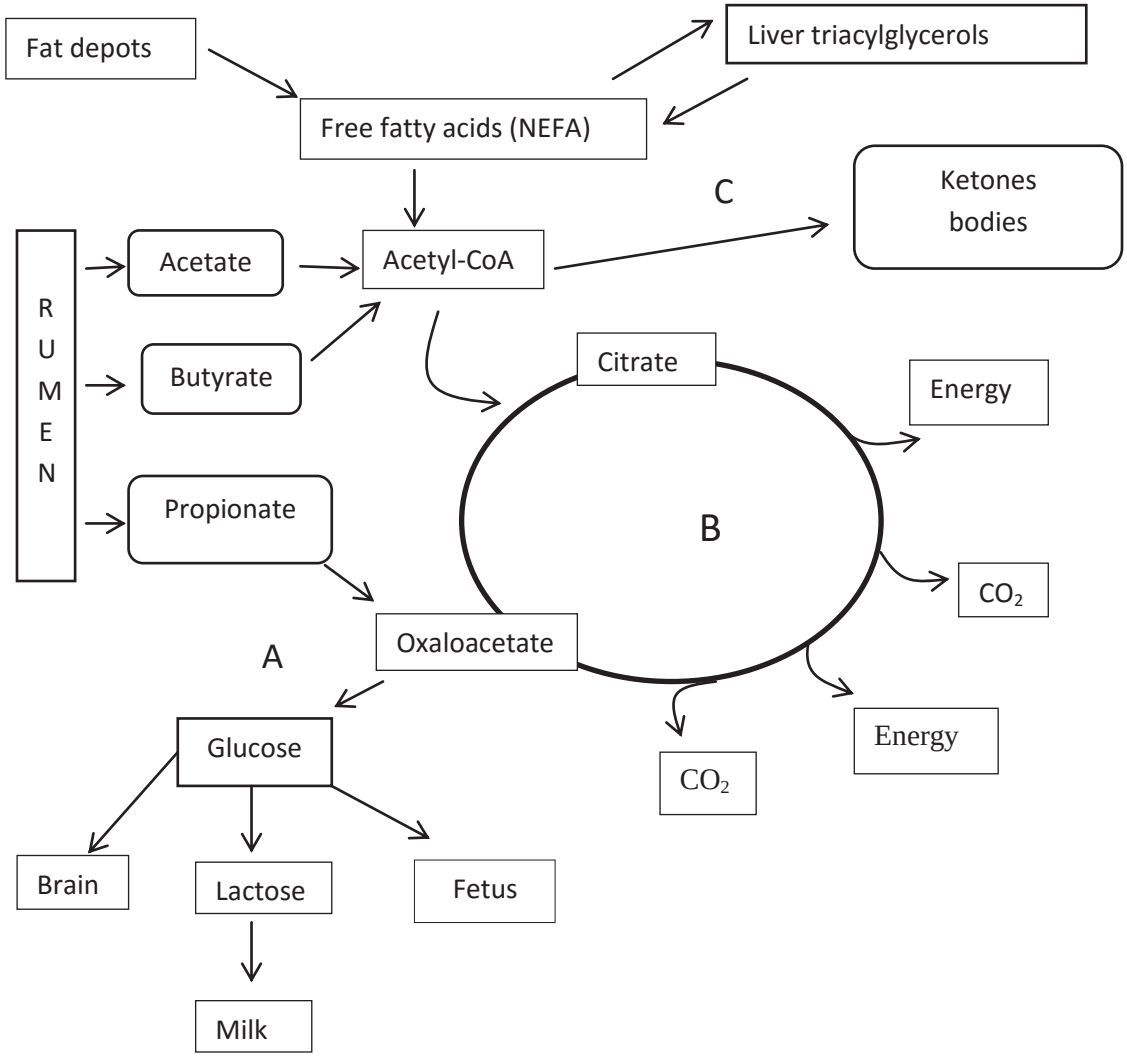

***Figure 1.1**: Metabolism of carbohydrate and fat in ruminant in relation to gluconeogenesis (A), tricarboxylic acid (TCA) cycle (B) and ketogenesis (C). Adapted from (Reece, 2009).*

or converted to other fats (e.g. milk fat) (Bauman and Griinari, 2003). The metabolism of the three main rumen VFAs is summarised in Figure 1.1.

Insulin and glucagon are the two major hormones that control and regulate the circulating concentrations of glucose. Insulin increases tissue utilisation of glucose and its conversion to the storage carbohydrate glycogen as well as promoting lipogenesis (fat production), while the principal role of glucagon is to increase blood glucose concentration, mainly by stimulating glycogenolysis (Brockman, 1978).

1.3.2 Fat metabolism

NEB has major effects on fat mobilisation, as the decreasing concentration of blood glucose promotes lipolysis which leads to the release of non-esterified free fatty acids (NEFA). NEFA may be used by muscle as a fuel source, taken up by the liver (Herdt, 2000), or used in the mammary glands for milk fat synthesis (Chilliard and Ferlay, 2004). In the liver, NEFA can be: completely oxidised to provide energy (ATP) via the TCA cycle; partially oxidised via β-oxidation to form ketone bodies (acetone, acetoacetic acid, and beta-hydroxybutyrate [BHB]); converted to triglycerides (TAG) and stored in the liver; or transformed to very low-density lipoproteins (VLDLs) and transported out of the liver (Drackley et al., 2001). The presence of NEFA and ketone bodies in the circulation is part of the normal process of adaptation to energy demands (such as lactation) as they can act as a fuel source for important tissues. However, their over-production is detrimental for both health and production (McArt et al., 2013). In addition, high levels of circulating NEFA can overwhelm the liver's capacity to oxidise them and then inhibit hepatocyte gluconeogenesis, thereby promoting NEB (Li et al., 2012).

Ruminants have only a limited ability to transport triglycerides, formed by the re-esterification of excess NEFA, out of the liver as VLDLs (McArt et al., 2013). Triglycerides thus readily accumulate in the liver when NEB is sustained and NEFA production is enhanced for long periods.

The utilisation of the products of oxidation of NEFA depends on the availability of oxaloacetate. This combines with acetyl-CoA (from NEFA) to form citrate. However, when an animal is in NEB, the availability of the precursors of oxaloacetate (pyruvate

or propionate) is less and then production of acetyl-CoA increases due to oxidation of fat (Reece, 2009). So, excess acetyl-CoA accumulates as acetoacetyl CoA, which is then further degraded to ketone bodies such as acetoacetate, beta-hydroxybutyrate, and acetone via a process called ketogenesis (Figure 1.1).

1.4 Negative energy balance

1.4.1 NEB and adaptation process

During the periparturient period, the energy requirements of a dairy cow increase significantly because of high demands from the developing fetus, uterus, and mammary glands (Gerloff, 2000). After calving, glucose demands are extremely high as milk contains 40-50 g/L of lactose (Pandya and Haenlein, 2009). All of these changes mean that the metabolisable energy requirement of a dairy cow increases two- to three-fold during the transition period (Aschenbach et al., 2010; Drackley et al., 2001). At the same time, during late pregnancy and early lactation, there is a substantial decrease in voluntary feed intake due to endocrine and metabolic changes (Grummer et al., 2004; Ingvartsen and Andersen, 2000). Hayirli et al. (2002) reported that the reduction in dry matter intake in the final 3 weeks of gestation could be as much as 30%. This decrease can be even greater after parturition (Drackley and Cardoso, 2014), further reducing the availability of glucogenic precursors.

The alteration in energy requirements (and balance) results in significant changes in metabolism during the periparturient period, with increases in hepatic gluconeogenesis, fatty acid mobilisation from adipose stores, amino acid mobilisation from muscles and decreasing peripheral use of glucose being the key adaptations (Bell, 1995). The liver is crucial for this process, being the organ where distribution and allocation of glucose, amino acids, lactic acid, NEFA, and ketone bodies occurs (Herdt, 2000). The role of hormones such as insulin, glucagon, and growth hormone, and neuro-regulatory substances, e.g. epinephrine and norepinephrine, in shifting the fuel supply of the body from glucose towards lipid sources is also crucial (Brockman and Laarveld, 1986). In addition, muscles shift their fuel use towards ketones and also act as a source of amino acids for gluconeogenesis (Drackley et al., 2001).

1.4.2 Regulation of hepatic fatty acid metabolism

The metabolism of fatty acids is regulated by hormones (e.g. insulin, glucagon), and enzymes such as malonyl-CoA and carnitine palmityltransferase-I (CPT-I) (Figure 1.2). During NEB, less glucose gets transported to and metabolised in the pancreatic β-cells, which results in reduced insulin production (Newgard and McGarry, 1995). A low

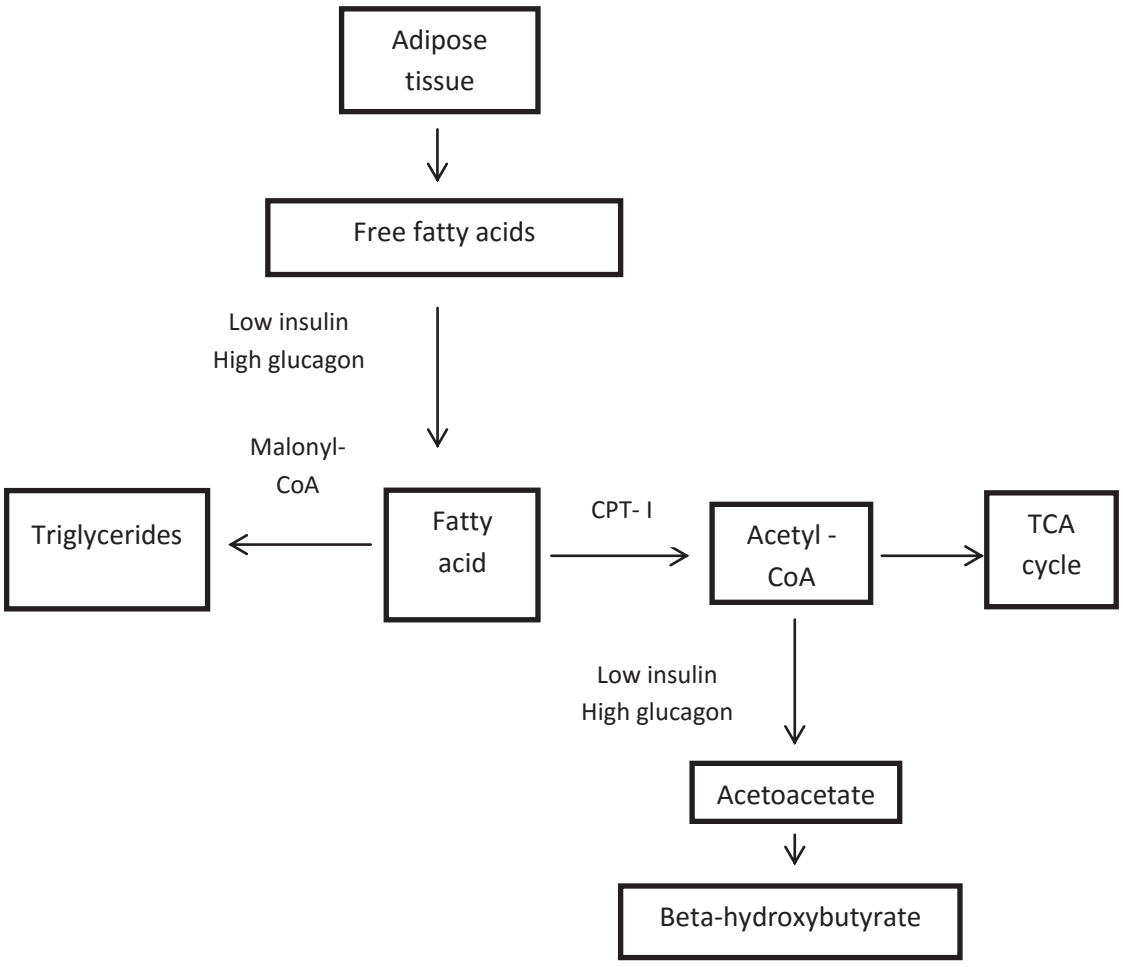

***Figure 1.2**: Metabolism of hepatic fatty acid regulated in various steps by insulin/glucagon ratio, malonyl-CoA and CPT-I. Adapted from Holtenius and Holtenius (1996).*

insulin/glucagon ratio facilitates increased mobilisation of adipose tissue and the subsequent release of free fatty acids (in contrast, increased availability of insulin, glucose or other glucogenic compounds decreases the oxidation of long chain fatty acids [Jesse et al., 1986]). The free fatty acids released by the adipose tissue get transported to the liver where they are either re-esterified into triglycerides (regulated by malonyl-CoA) or converted to acetyl-CoA (regulated by CPT-I) (Figure 1.2). CPT-I is

the enzyme responsible for catalysing the initial step of fatty acid oxidation, the transportation of NEFA into the mitochondria (McGarry and Foster, 1980).

The concentration of malonyl-CoA is high in animals in positive energy balance, because citrate is over-produced by the TCA cycle. Once transported out of the mitochondria, this excess is converted to acetyl-CoA and then, irreversibly, into malonyl-CoA (Herdt, 2000). This increased concentration of malonyl-CoA stimulates fatty acid synthesis and suppresses fatty acid oxidation and ketone body formation by inhibiting the activity of CPT-I (McGarry et al., 1978).

By contrast, during NEB, citrate production by the TCA cycle is reduced, as is the subsequent production of malonyl-CoA, stimulating CPT-I (Herdt, 2000). The role of increased glucagon compared to insulin is also important during this process. CPT-I induces rapid entry of NEFA into the mitochondria where they get metabolised into ketone bodies or enter the TCA cycle, both of which require conversion of NEFA to acetyl-CoA (Herdt, 2000; McGarry and Foster, 1979). The reduced insulin concentration facilitates the metabolism of acetyl-CoA into acetoacetate in mitochondria, which, when it enters the cytosol, gets further metabolised into BHB and other ketone bodies (Herdt, 2000).

Increased concentrations of ketones act as a signal to reduce both oxidation of glucose and transport of free fatty acids into the liver, thus ketone concentrations regulate both glucose utilisation and their own production (Holtenius and Holtenius, 1996). Ketone bodies are utilised in the hepatic, renal, mammary, and other peripheral tissues in lactating cows, and also in the placenta and fetus in pregnant cows (Heitmann et al., 1987). Prolonged NEB can lead to over-production of ketone bodies, which accumulate because low concentrations of insulin impede their utilisation in the peripheral tissues (Hove, 1974), thus causing a metabolic condition, ketosis.

1.5 Ketosis

Ketosis occurs as a result of excess lipid metabolism in response to an insufficient availability of carbohydrate. Ketosis can be clinical (with signs ranging from lethargy and inappetence to central nervous system [CNS] disturbances), or subclinical, but high

concentrations of circulating ketone bodies are found in both cases. Ketosis has often been divided into type I or type II based on pathogenesis.

1.5.1 Type I ketosis

This is common in high producing dairy cows during the first 3 to 6 weeks post-calving (Cook et al., 2006; Oetzel, 2007) when glucose demand surpasses the capacity of the liver for gluconeogenesis. The body goes into NEB due to low blood glucose and insulin (Holtenius and Holtenius, 1996); resulting in activation of CPT-I and increased ketogenesis as described earlier. In this type of ketosis, most of the NEFA get converted into ketone bodies and very little gets re-esterified into triglycerides. Type-I ketosis is therefore characterised by high blood ketone concentrations and the relative absence of fatty liver (Herdt, 2000).

1.5.2 Type II ketosis

This occurs immediately post-partum in cows which have been over-fed during the dry period, causing an increase in plasma glucose and insulin concentration during that time. Stress factors promote rapid fatty acid mobilisation and NEFA accumulation in the liver cytosol, which gets re-esterified into triglycerides, producing “fatty liver” (Holtenius and Holtenius, 1996). The re-esterification of NEFA utilises glycerol which requires glucose (Pethick et al., 2004), further reducing carbohydrate availability.

Although, this classification of ketosis has a long history in the literature (Herdt, 2000; Holtenius and Holtenius, 1996), its value has been questioned (Gordon et al., 2013). Firstly, most ketosis in US herds occurs within the first 10 days of calving and is often accompanied by other diseases. Additionally, the clear biochemical distinction between the two types of disease is not reflected in reality; few cows that have ketosis also have excess insulin and glucose and the amount of fat in the liver varies widely between animals (Herdt, 2000).

Gordon et al. (2013) stated that defining ketosis as subclinical or clinical (based on the presence/absence of clinical signs) was more useful than trying to define whether there was fatty liver or not. They also stated that ketosis should be defined as hyperketonaemia as there was no significant association between the serum

concentration of ketone bodies and the presence of clinical signs. This is relevant in New Zealand's pasture-based condition because, so far, there have been no reported data on the ranges of blood BHB concentration seen in cows with clinical or subclinical ketosis (Compton et al., 2014).

1.6 Effects of negative energy balance

NEB experienced by the animal while recovering from the stress imposed by calving, and high energy requirement of early lactation, is associated with hormonal (insulin, glucagon) and nutritional disturbances. Such disturbances have been linked to metabolic complications (such as ketosis, displacement of abomasum, fatty liver), reduction in milk production, poor reproductive performance, and changes in the range of blood and milk metabolites.

1.6.1 Effects of NEB on reproduction

Following calving, in order for cows to conceive successfully the uterus needs to undergo effective involution i.e. there needs to be restoration of the endometrium and deeper layers of the uterus, elimination of bacterial contamination of the uterine lumen, and atrophy of the uterine tissues with reduction in size. Additionally, ovarian cyclicity needs to resume, with all phases of the follicular cycle (selection, recruitment, and ovulation) needing to occur. Under New Zealand conditions, these processes need to be completed within 60 days of calving so that a calving index of ~365 days can be maintained (Harris and Kolver, 2001).

Both of these processes can be significantly affected by NEB.

1.6.1.1 Effects on ovary

NEB is associated with a delay in the resumption of oestrus cyclicity following calving, impaired quality of the ovulated oocyte, and reduced function of the post-ovulation corpus luteum (Drackley and Cardoso, 2014; Wathes et al., 2007).

Although follicular waves resume within 5-7 days of calving, irrespective of the energy status of the cow (Butler, 2003), this is not sufficient for fertility to be normal. In order for ovulation to occur, fully functional dominant follicles are required. These follicles

need to be able to produce sufficient oestradiol to stimulate the release of gonadotrophin releasing hormone (GnRH) from the hypothalamus, which will then stimulate pulsatile luteinising hormone (LH) release by the pituitary gland and, finally, ovulation (Sunderland et al., 1994). It is likely that many of the effects of NEB on the ovary are mediated by its impact on the concentration of Insulin-like growth factor-I (IGF-I) and insulin, both of which are reduced by NEB (Newgard and McGarry, 1995; Spicer et al., 1990). IGF-I is responsible for stimulating follicle cell proliferation and the LH surge (Wathes et al., 2007), while, insulin promotes steroidogenesis (oestradiol production) by stimulating receptors present in the granulosa cells (Butler, 2014). In addition to impacts on insulin and IGF-I, the hypoglycaemia, which can result from NEB, desensitises the glucosensor in the caudal brainstem, reducing the pulsatility of LH (Cates and O'Byrne, 2000; Murahashi et al., 1996).

NEB also leads to high NEFA concentrations in both serum and follicular fluid (Leroy et al., 2004), which interacts with the membrane of oocyte and delays its maturation (by hindering the meiosis process), and lowering the weight of the corpus luteum and progesterone concentration (Jorritsma et al., 2004). It also diminishes proliferation of granulosa cells and steroidogenesis (Vanholder et al., 2005), by inducing apoptosis (cell death).

1.6.1.2 Effects on uterus

Increased NEB is associated with a delay in involution following parturition. Wathes et al. (2009) suggested that this delay resulted from the increased inflammation of the uterus seen in post-calving cows with NEB, combined with reduced numbers of phagocytic mononuclear cells (whose role is to remove the contaminating bacteria). Thus, although there is an increased inflammatory response, it is less effective than that seen in normal cows.

In addition, increased concentrations of NEFAs can affect the inflammatory response directly by causing physical damage to the size and number of mononuclear cells or, indirectly, by modifying intracellular signalling, inducing oxidative stress or altering the biosynthesis of lipid mediator (Contreras and Sordillo, 2011). Indeed, these effects may be the underlying cause of the reduced production of mononuclear cells.

These impacts on reproductive tract function will lead to longer intervals between calving and first observed oestrus and poorer conception rates when cattle are inseminated. Under New Zealand conditions, these effects are likely to result in reduced submission rate (the proportion of cattle submitted for insemination within a specific period [e.g. 3 weeks] of the start of the breeding season) and 6-week In-calf rate (the proportion of cattle that get pregnant in the first 6 weeks of the breeding season), and, particularly if the herd has a short breeding season, increased empty rate (Burke and Fowler, 2007).

1.7 Measuring energy status

Energy balance can be calculated by estimating the energy requirement for maintenance and milk production and subtracting energy intake. Calculation of energy requirements for maintenance and milk production need regular liveweight measurements as well as monitoring milk fat, protein, and lactose content (Lucy et al., 1991; Patton et al., 2008). To calculate energy intake, feed intakes need to be accurately measured and all feeds analysed; on pasture-based farms, measurement of intake, particularly at an individual cow level, can be extremely difficult (Leslie et al., 2003).

Another alternative is measuring liveweight on a regular basis. The advent of automated liveweight measurement means that this is much simpler to do than it has been in the past. Continued liveweight loss over time is indicative of negative energy balance, while liveweight gain indicates positive energy status (Jorritsma et al., 2003). However, relying completely on liveweight, disregarding the effects of breed, stage of lactation, parity, and gestation, could give a misleading representation of energy status (Alawneh, 2011).

Body condition score (BCS) is a non-invasive way of assessing energy reserve of the animal, which is more precisely the metabolisable energy which gets stored in the muscle and fat of the animal body (Edmonson et al., 1989). It can be considered that animal with high BCS are obese and in positive energy balance while those in low BCS are wasted and might be in NEB. However, some herd-level factors (such as feed type, stocking rate etc) and cow-level factors (such as BCS at calving, parity, season of

calving etc), which tend to affect BCS among animal (Roche et al., 2009), confounds the accuracy of BCS in representing energy status of the animal. Therefore, change in body condition score might be more appropriate than BCS alone.

BCS loss post-calving is also representative of the energy loss; and associations between BCS loss, reproductive performance, and other metabolic diseases are well proven. For example, Roche et al. (2007) reported associations of BCS loss post-calving with reproductive performance indicators such as submission rate and in-calf rate. Similarly, Roche et al. (2009) showed that there were associations between BCS and many periparturient metabolic diseases (such as ketosis, milk fever, displaced abomasum, and fatty liver).

Change in individual cow BCS represents a reduction in plasma glucose concentration, but differing BCS between cows does not necessarily represent different plasma glucose concentrations (Bernabucci et al., 2005). A cow with high BCS, which may be considered to be in positive energy balance, in fact has a higher risk of metabolic disease occurrence due to higher mobilisation of fat reserves (Busato et al., 2002). Furthermore, BCS is a subjective measure with limited ability to detect small, but biologically important changes. By the time sufficient detectable BCS change has occurred due to NEB, the negative consequences of that NEB on productivity and disease could already have occurred (Russel and Wright, 1983). Thus, BCS is not suitable as a sole assessment of the likely impact of NEB, particularly if we want to put in place management measures to prevent the impact of increased NEB.

This means that monitoring the concentration of certain blood metabolites (i.e. NEFA, BHB) is preferred to assess the immediate response to the change in energy balance. As discussed previously, changes in these metabolites are closely related to physiological and biochemical changes in the body, and are thus able to represent the short-term effects of energy balance changes during the periparturient period (Bowden, 1971). However, these are not the only parameters that change in response to energy balance; changes in energy balance also affect milk parameters such as fat, protein (Grieve et al., 1986; Heuer et al., 2000), lactose, urea (Reist et al., 2002; Steen et al., 1996), and BHB (Denis-Robichaud et al., 2014).

1.7.1 Energy status measurement and blood parameters

Blood NEFA and BHB represent the extent of fatty acid mobilisation from the adipose tissues (Adewuyi et al., 2005) and oxidation of fat in the liver (LeBlanc, 2010), respectively. Looking at the order in which these two metabolites are produced during fat metabolism, it can be assumed that high concentrations of BHB in the blood tend to represent more severe NEB than high concentrations of NEFA.

1.7.1.1 NEFA

Pre-calving (i.e. 2 to 14 days before) measurement of NEFA concentrations is an effective measurement of the risks of NEB and metabolic diseases (particularly Type-II ketosis) post-partum (Cook et al., 2006; Leslie et al., 2003; Oetzel, 2004). However, post-partum, the use of NEFA is not so clear cut as the normal process of mobilising body fat as part of the adaptation to lactation results in increased NEFA concentrations in early lactation (particularly in high yielding dairy cattle), reducing its specificity (Cook et al., 2006). Nevertheless, post-partum NEFA concentrations were found to predict metabolic diseases as accurately as pre-partum NEFA or post-partum BHB (Ospina et al., 2010).

The threshold NEFA concentration used for NEB detection pre-partum ranges from 0.2 to 0.5 mmol/L (Drackley, 2000; McArt et al., 2013). Use of pre-partum blood NEFA threshold of > 0.4 mmol/L and > 0.5 mmol/L were found to have optimal sensitivity and specificity in detecting NEB, respectively (Leslie et al., 2003). In the late pre-calving period, NEFA values increase in normal cattle up to 1.2 mmol/L on the day of calving; they then decrease (Drackley [2000] stated that at 1 week and 3 weeks after calving, NEFA concentrations should not exceed 0.7 mmol/L and 0.3 mmol/L, respectively), while Ospina et al. (2010) reported that post-partum NEFA concentrations of > 0.6 mmol/L were associated with increased risk of displaced abomasum, clinical ketosis, metritis, and retained fetal membranes.

Although NEFA concentrations are strongly associated with metabolic status, there are some factors which reduce the reliability of blood NEFA concentration for assessing

NEB. For example, the time of feeding determines when NEFA concentration reaches its maximum concentration which consequently determines the sampling time (Cook et al., 2006). This effect, however, may not be significant in all cases (Ospina et al., 2010), and may be of limited importance under New Zealand's pasture-based conditions where cows have access to feed all the time.

The large variation in NEFA concentrations with time, particularly in the late dry period, can also increase the complexity of interpretation; indeed Oetzel (2004) suggested that samples collected within 48 hours of calving should be discarded.

Finally, NEFA values are elevated by stress, such as that associated with the handling required for sampling (Drackley, 2000). These factors mean that elevated NEFA concentrations needs to be interpreted with care.

1.7.1.2 BHB

Of the three major ketone bodies (acetone, acetoacetate, and BHB) formed in the liver, the concentrations in blood and milk are highest for BHB, so this is the ketone body which is usually tested for in cows that are in NEB (Ospina et al., 2013).

The recommended time of blood sampling in order to monitor energy status varies from 1-2 weeks post-partum to 5-6 weeks (Compton et al., 2014; Compton et al., 2015; Verkerk and Guiney, 1999). The optimal timing depends upon the indicator (occurrence of disease, reduction in milk production or fertility) which is used to define NEB (or SCK) in cows and the threshold level used.

Although most studies recommend the use of post-partum BHB concentrations as a measure of SCK or NEB (as pre-partum BHB values are lower and more easily affected by factors other than energy balance), some studies have suggested that pre-partum BHB measurement can be of value. For example, Chapinal et al. (2012) reported that pre-partum blood BHB concentrations of ≥ 0.6 mmol/L were associated with reduced milk production.

The threshold values for post-partum BHB used by seven studies (and their rationale for their choice) are summarised in Table 1.1. Two methods for determining BHB thresholds are found in the literature: (1) analysing the distribution of BHB results and

determining the threshold based on the values which lie at the high end of that distribution; and (2) analysing the BHB threshold value which is associated with an increased risk of reduced productivity. The latter is preferable, as it is only through its impact on subsequent productivity that SCK/NEB becomes important.

***Table 1.1**: Examples of post-partum cut-off point for blood BHB used by published studies.*

Cut-off point	Reference	Rationale behind selecting the cut-off point
1 mmol/L	Ospina et al. (2010)	Highest combined sensitivity and specificity, from the ROC curve for predicting DA, CK, metritis, and RP.
	Verkerk and Guiney (1999)	Increased risk of prolonged post-partum anoestrus interval.
≥ 1.2 mmol/L	Nielen et al. (1994)	Based on distribution of the blood BHB data which were skewed around 1.2 mmol/L.
	Sakha et al. (2007)	Based on an apparent break (at > 1 to 1.2 mmol/L) in the distribution of the blood samples with different concentrations.
	Compton et al. (2015)	Increased risk of purulent vaginal discharge (PVD) at 5 weeks post-calving and reduced 6-week pregnancy rate.
≥ 1.4 mmol/L	Duffield et al. (2009)	Sensitivity and specificity for detecting clinical ketosis
	Compton et al. (2014)	Used as a high reference value for SCK since no BHB concentration ranges for pasture-grazed cows available.

DA= Displaced abomasum, SCK= Subclinical ketosis, RP= Retention of placenta
ROC curve= Receiver Operating Characteristic curve, analyses sensitivity versus 100- specificity.

In New Zealand pasture-fed herds, the recommended cut-off point of post-partum BHB concentration has varied between ≥ 1 (Verkerk and Guiney, 1999) to 1.4 mmol/L (Compton et al., 2014). A recent large-scale study by Compton et al. (2015) concluded, based on the risk of endometritis and 6-week pregnancy rate, that the threshold in New Zealand should be 1.2 mmol/L.

As it is closely associated with NEB, it is unsurprising that blood BHB has been associated with poorer reproductive outcomes (e.g. Verkerk and Guiney, 1999; Compton et al., 2015). As well as indirect NEB-related effects, BHB can have significant direct effects on fertility; for example, increased BHB impairs the developmental competence of oocytes as seen in the reduction in the number of blastocysts (Leroy et al., 2006) and suppressing glycolytic pathways in the ovary (Sarentonglaga et al., 2013).

As for NEFA, there are diurnal fluctuations in blood BHB concentrations relative to the time of feeding (Nielsen et al., 2003; Ospina et al., 2010), principally due to the conversion of ruminal butyric acid to BHB (Cook et al., 2006; Oetzel, 2004). So, in concentrate-fed cattle, it is better to evaluate BHB concentrations 4-5 hours after the major feed of the day (Cook et al., 2006). Again, as with NEFA, such diurnal variations are minimal in pasture-based herds because of the availability of feed at all times (Mahrt et al., 2014), but may be important if significant feed is given at specific times (such as after milking).

Although NEB and BHB are usually closely linked, in some circumstances BHB may lack sensitivity and specificity as a test for NEB. This can occur because BHB is just one of the many possible pathways of NEFA metabolism, and there can be a time lag between metabolism of NEFA and its conversion to BHB (Ospina et al., 2013). Moreover, BHB is not solely formed as part of the process of utilisation of excess acetyl-CoA; it is also formed in the rumen as a result of hydroxylation of butyrate, particularly if feeds such as butyric silage are fed. These increases can interfere with the interpretation of BHB results, particularly pre-partum where low concentrations of BHB are expected (Reist et al., 2000).

Thus, although both BHB and NEFA provide good guides to energy status, they both have limitations. Additional tests, such as milk parameters, may be useful alongside BHB and NEFA in order to provide more information and better prediction of the impact of NEB, particularly as milk testing is routinely undertaken on many farms as a management tool.

1.7.2 Milk parameters and energy status

1.7.2.1 BHB

Plasma BHB and milk BHB are highly correlated (r^2 = 0.68, $p < 0.001$ [Verkerk and Guiney, 1999]; r = 0.89, $p < 0.01$ [Denis-Robichaud et al., 2014]), although milk BHB concentrations are consistently lower than those of plasma. Milk BHB can therefore be used as an alternative to plasma BHB for monitoring energy balance.

The cut-off point of milk BHB for detecting hyperketonemia was defined at ≥ 0.07 mmol/L by Enjalbert et al. (2001) and ≥ 0.2 mmol/L by Geishauser et al. (2000) and Denis-Robichaud et al. (2014). All authors reported that milk BHB had a high specificity and sensitivity for determining hyperketonemia. It is likely that the difference in the milk BHB cut-off points across studies was, at least partly, due to variation in the blood BHB thresholds which the studies used to define hyperketonemia.

Nevertheless, although milk BHB can be useful for predicting blood BHB, it has no advantage over the measurement of plasma BHB, unless milk samples are to be taken for other reasons such as a routine herd test. However, the value of such tests may be limited because the usual monthly schedule means that such tests are unlikely, for most cows, to occur when the risk of hyperketonaemia is greatest (Denis-Robichaud et al., 2014).

1.7.2.2 Urea

In animals in NEB, plasma urea concentrations can increase significantly because of increased utilisation of body protein as a source of energy. However, elevated urea concentrations can also occur because of excess intake of crude protein, particularly rumen-degradable protein (Jorritsma et al., 2003). In the latter circumstances, detoxification in the liver of absorbed ruminal ammonia to urea can increase NEB because it requires energy (Norouzi et al., 2010), but the effect is relatively small.

Urea molecules are small and easily pass through cell membranes, such as those of the mammary glands (Řehák et al., 2009). Plasma urea is thus highly correlated with milk urea (r = 0.82, $p < 0.001$) (Butler et al., 1996). As for milk BHB, the key advantage of

milk urea is that it can be easily and non-invasively tested for alongside other tests in a routine herd test (Roy et al., 2011).

The issue with urea measurement as a measure of energy status is that although urea can be elevated as part of the process of response to NEB, the main process affecting NEB is protein (and non-protein nitrogen) intake, with increased intake being associated with increased urea. Thus, milk urea lacks specificity as a measure of energy status, and much of the published association between reproduction and urea concentration has focussed on protein intake not NEB as the underlying cause of the reduced fertility (Laven and Drew, 1999; Laven et al., 2007).

A further issue is that most of the data on urea and fertility have come from housed cows. In pasture-based systems, we lack good data on what determines abnormal milk urea concentrations. Cows on pasture-based diets are exposed to high levels of systemic urea because their diets, particularly in spring, contain high levels of non-protein nitrogen (nitrate) from pasture; however, there are only limited changes in dietary intakes of nitrate between dry cows and early lactation, as both groups are grazing in spring pasture. This means that pasture-based cows tend to have higher milk urea concentrations but are probably better adapted to such levels (Laven et al., 2007). This also means that the limited increases in urea concentration associated with most NEB may have less effect, and can also be more difficult to detect because differences in pasture nitrate intake have much more impact on milk urea than NEB does.

1.7.2.3 Fat

Milk fat is measured routinely in dairy herds via herd tests. During NEB, milk production decreases due to low availability of glucose, and very low density lipoproteins (VLDLs) which are formed in the liver as a result of fat mobilisation are taken up by the udder. These two factors cause elevation of milk fat (De Vries and Veerkamp, 2000). Therefore, milk fat is negatively correlated with energy balance (Reist et al., 2002), with increases in milk fat paralleling increases in blood ketone concentrations (Duffield et al., 2009).

Duffield et al. (1997) showed that a 1% increase in milk fat was associated with a more than two-fold increase in the risk of subclinical ketosis, while Denis-Robichaud et al. (2014) reported that the threshold value of milk fat which indicated hyperketonaemia was ≥ 4.2%. However, because milk fat was strongly influenced by nutritional, genetic, and environmental factors, the predictive value of this threshold was poor. To counter this some studies have suggested that change in milk fat percentage, or, perhaps milk fat to protein ratio, should be the preferred measure (De Vries and Veerkamp, 2000).

The lack of specificity of increased milk fat as a measure of energy status means that it can be difficult to establish whether the relationship between the milk fat and reproductive performance is related to energy balance. In some cases it is clear that it is not; for example, Norouzi et al. (2010) reported that cows with a milk fat concentration between 2.8% and 2.99% had a 14% higher fertility rate than those with milk fat below 2.8%. In some cases there may be an association; for example, Heuer et al. (1999) reported that a high fat to protein ratio was associated with a higher risk for ovarian cysts and a lower first service conception rate.

1.7.2.4 Protein

Like milk fat, milk protein is also routinely measured as part of herd testing on commercial farms. Milk protein has often been used alongside milk fat to create a fat: protein ratio. Indeed, Krogh et al. (2011) recommended using the ratio instead of milk fat alone as they believed that protein percentage was "rather stable and cow-specific" and could therefore be used as a method of adjusting for a "cow-effect". However, Madouasse et al. (2010) concluded that the tendency of milk protein to decrease more rapidly during early lactation compared with milk fat caused an initial increase followed by a decrease in the milk fat to protein ratio, which made the ratio ambiguous to interpret at the time when information about NEB is most needed.

Furthermore, utilising a single value as a ratio loses information compared to using the two values together; this review will therefore focus on the impact of energy status on the milk protein percentage alone.

Even though milk protein yield in milk is more related with the genetics of the animal compared to nutrition, there are some evidences that support milk protein synthesis in

mammary gland highly related with energy availability (Bionaz et al., 2012). Reduction in milk protein due to the deficiency in energy and proteins supply caused by feed restriction (Gross et al. (2011) also supports the role of energy balance for milk protein yield. Improved energy balance minimizes protein utilization for energy, facilitating more protein availability for milk protein yield (van Knegsel et al., 2005). Moreover, milk protein is reduced in high producing cows suffering from NEB because milk protein synthesis requires there to be sufficient glucose available to spare the amino acids used for milk protein synthesis, which would otherwise be used for gluconeogenesis (Yang et al., 2010).

Several studies have shown an association between low milk protein and fertility. Opsomer et al. (2000) identified low average milk protein on the first 100 days of lactation as a risk factor for resumption of ovarian activity, and low milk protein at 55 days post-partum as a risk factor for delayed ovulation. In another study, lower pregnancy rate to first service was observed in cows reaching the nadir of milk protein relatively late in the lactation (Buckley et al., 2003). Similarly, cows in which the ovarian activity did not resume within 7 weeks of calving had significantly lower milk protein concentrations in their third week of lactation (Konigsson et al., 2008). As energy balance is probably the major factor influencing milk protein percentage in the individual cow, it is likely that these findings reflect the influence of NEB on fertility.

1.7.2.5 Lactose

Milk lactose production is a very good indicator of metabolic condition of a cow as this milk component is derived from the plasma glucose (Bickerstaffe et al., 1974). The average lactose content of milk is around 4.8%, and represents about 70% of the glucose used by mammary glands (Reynolds, 2005).

However, although lactose production is closely associated with glucose availability, it is one of the tightest controlled milk parameters, with lower production of lactose being reflected in lower milk volume rather than altered concentration (Mirzaei-Aghsaghali and Fathi, 2012). Nevertheless, lactose percentage has been associated with NEB (Reist et al., 2002), and both Reksen et al. (2002) and Smith et al. (2014)

reported that lactose concentration was associated with the time to resumption of luteal activity.

1.8 Conclusion

Ketosis is one of the most common subclinical diseases seen in dairy herds. It causes significant losses in terms of reduced milk production, herd health, and reproductive performance. Ketosis is a consequence of NEB, and better monitoring of energy status could allow management actions to be taken earlier in the progress of the disease. As seen in this review, many studies have evaluated the potential for blood and milk parameters other than ketones as predictors of energy status or of the risk of NEB negatively affecting reproductive performance.

However, few such studies have been undertaken under New Zealand conditions. The aim of this study was to identify whether adding additional monitoring to pre- and post-partum BHB measurement would better improve the prediction of the impact of NEB on reproductive performance (as measured using 3-week submission rate).

CHAPTER 2

Assessment of Milk Parameters to Improve Association of Blood Parameters with 3-Week Submission Rate

Chapter 2: Assessment of Milk Parameters to Improve Association of Blood Parameters with 3-Week Submission Rate

2.1 Introduction

Two key metabolites that are often assessed as part of the process of estimating negative energy balance (NEB) in the dairy cow are non-esterified fatty acid (NEFA) and beta-hydroxybutyrate (BHB). During NEB, the primary source of energy for tissues (i.e. glucose) is reduced, so the cow's metabolism adapts itself to the increasing energy demand of lactation by mobilising body fat, thus producing free fatty acids (such as NEFA) and ketone bodies (such as BHB), which are utilised as alternative energy sources. However, over-production of such blood metabolites can have deleterious effects on milk production and reproductive performance. Generally, complete oxidation of NEFA in the liver via TCA cycle or re-esterification helps to minimise such negative effects. But the accumulation of intermediary products in the liver overwhelms its capacity to neutralise them. When this occurs, metabolic diseases (ketosis, displacement of abomasum, fatty liver), decreases in milk production, reduced fertility, and alterations in the milk constituents become apparent.

Threshold values for pre-calving NEFA (≥ 0.4 mEq/L [Leslie et al., 2003]) and post-calving BHB (≥ 1.2 mmol/L [Compton et al., 2015]) have been shown to be associated with NEB and reduced productivity. However, the link is far from complete with some animals in NEB not having elevated NEFA or BHB and many animals with increased NEFA and BHB having normal productivity and disease risk. Additional information alongside the measurement of NEFA and/or BHB could improve the value of such measurement. In particular, the use of milk constituent testing alongside blood testing could be valuable. Milk constituents have been shown to be associated with productivity and disease, so their measurement, in addition to blood NEFA and BHB, could add significantly to the value of such blood samples. Furthermore, data such as milk fat and protein are already regularly recorded on farm, and additional tests on such samples does not add to time for farm and veterinary staff.

The aim of this study was to evaluate whether the use of milk constituents data alongside NEFA and BHB added value to the latter when predicting reproductive outcomes, specifically 3-week submission rate (i.e. the risk of being inseminated in the first 3 weeks of the breeding season).

2.2 Materials and methods

2.2.1 Farms and animals

All procedures were approved by the Massey University Animal Ethics Committee (MUAEC).

The study was undertaken from July to November, 2014 on five commercial dairy farms in the Manawatu district in the southern North Island of New Zealand. This was a convenience sample based on locality and farm staff being willing to assist with the sampling process. All the herds were principally pasture-based, spring-calving herds. Four of the five farms milked twice daily, with one milking once-a-day.

From each of the selected farms, the aim was to obtain 30 early calvers (whose expected calving date was within 2 weeks of the day of enrolment). Inclusion criteria for the cows were: i) calving for at least the second time; ii) apparently healthy and no medication history in the past 30 days; and iii) no record of ketosis in past lactations.

In July 2014, on each of the farms, up to 30 cows were selected from the group of cows which were closest to parturition at the time of enrolment ('in the springer mob'). The cows were run through the milking parlour and the first 30 cows, which met the criteria, were selected and tail-painted for easier identification. The details of the five farms and their sample size are given in Table 2.1. The total number of cows selected was 146.

2.2.2 Sampling regimen

2.2.2.1 Blood collection and analysis

All cows were blood sampled at enrolment (i.e. up to 3 weeks before the expected date of calving), and then 2 weeks after calving (Table 2.2). Up to 10 ml of blood was taken via coccygeal venepuncture into evacuated tubes with no anticoagulant

(Vacutainer, Becton-Dickinson). Pre-calving samples were collected in the afternoon from all of the five farms, while the post-calving samples were collected during afternoon milking in four of the farms milking twice-a-day and during morning milking in the single farm milking just once-a-day.

Table 2.1: *Details of the five farms used in the experiment.*

Farm	Herd size	Sample size	Shed type	Breed	Milking
Farm A	415	30	Herringbone	F, J	Twice a day
Farm B	280	24	Herringbone	F, J	Once a day
Farm C	398	30	Rotary	J	Twice a day
Farm D	240	30	Herringbone	F, J, CB	Twice a day
Farm E	650	32	Rotary	F × J	Twice a day

F = Friesian, J = Jersey, CB = Cross breed

The blood samples were then sent to New Zealand Veterinary Pathology (NZVP) (Palmerston North, New Zealand) for testing of BHB and NEFA using commercial kits on the Roche Modular P800 clinical chemistry analyser (Roche Diagnostics, Auckland, New Zealand). If samples were not able to be submitted on the same day, they were refrigerated overnight at 1-4 °C.

2.2.2.2 Milk collection and analysis

Milk samples were collected pre-milking by hand-stripping when the sampling day was not a herd test day, and using a milk meter when it was. The first milk sampling occurred 2 weeks after calving, and was then repeated at approximately 4 week intervals until 6 weeks after planned start of mating (PSM) (Fig 2.1). Herd testing did not include analysis of milk BHB and urea, so a separate milk sample was collected using hand-stripping pre-milking on all occasions.

Milk BHB (mmol/L) and urea (mmol/L) concentration were analysed by NZVP using commercial kits on the Roche Modular P800 clinical chemistry analyser. Milk samples were first centrifuged at 1700 *g* for 6 minutes prior to testing in order to remove the milk fat.

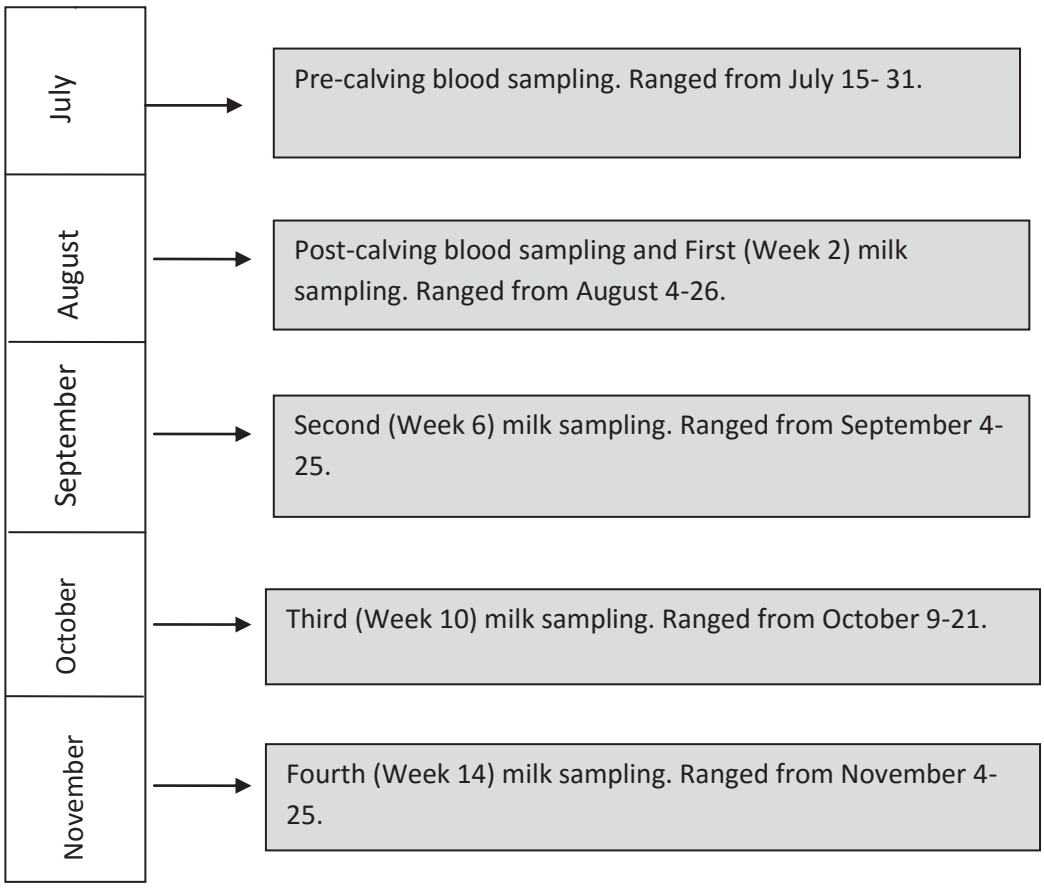

Fig 2.1: *Time-line showing sampling frequency*

Analysis of milk fat (%), protein (%), and lactose (%) concentrations were undertaken by Livestock Improvement Corporation Ltd (LIC) (Hamilton, New Zealand). Samples were tested using Infrared (IR) spectroscopy on either a Milkoscan auto-analyser (Foss, Denmark) or a Bentley FTS.FCM (Bentley Instruments, USA).

Table 2.2: *Experimental design of the study.*

Sample	Parameters measured	Pre-calving sampling time	Post-calving sampling time
Blood	BHB, NEFA	2-3 weeks before expected calving date (BB1 and BN1)	2 weeks after calving (BB2 and BN2)
Milk	BHB, urea, fat, protein and lactose	–	At week 2 (B1, U1, F1, P1, L1), week 6 (B2, U2, F2, P2, L2), week 10 (B3, U3, F3, P3, L3), and week 14 (B4, U4, F4, P4, L4) post-calving.

2.2.3 Data handling

Data were handled using Microsoft Excel 2010 and included the creation of blood and milk variables, reproductive outcome variables, as well as identifying anomalies in the records such as incomplete data or aberrant values. From the measurements of blood and milk constituents, four and 20 different variables were created, respectively.

The outcome variable was created by calculating the interval between the PSM for each farm and the first insemination date of each cow from those farms. This variable was expressed as a categorical variable so that cows which were inseminated in the first 3 weeks of the breeding season were denoted by “1” and cows which were not inseminated in that period were denoted by “0”.

Cattle in which oestrus synchronisation was used in order to inseminate the cow were categorised as “0”, irrespective of whether they were inseminated in the first 3 weeks or not.

2.2.4 Statistical analysis

All analyses were performed using SPSS version 22 (IBM, USA).

Relative risk (RR) analysis was used to determine whether cows which were identified as at-risk of significant NEB pre-partum (NEFA ≥ 0.4 mmol/L) were more likely to have elevated BHB values (≥ 1.2 mmol/L) post-partum.

The blood and milk variables data were tested for normality using the Shapiro-Wilk's test. As almost all of the measures were not normally distributed ($p < 0.05$), Spearman's rank order correlation test was used to identify correlations between variables.

The results of the correlation were then used to minimise the number of variables put forward to the logistic regression. Highly correlated variables ($r > 0.6$) were identified and one of the pair removed. This removal was based on: 1) keeping the variable which was correlated with most variables (i.e. protein 2 and 1 were strongly correlated but keeping protein 2 removed two more variables); and 2) keeping the earliest of the pair (i.e. urea 1 was kept rather than urea 2, 3, or 4).

The remaining milk variables were then used alongside all four blood variables and farm as explanatory variables in a logistic regression with submission category as the outcome variable. A backward step-wise elimination procedure was then conducted until only significant variables ($p < 0.1$) were retained. Two models were created. In the first model, the blood parameters were retained in the model, irrespective of their p-value. In the second, all variables were subject to backward elimination. The means for the variables which were retained in the final model were obtained for the risk of 3-week submission rate.

2.3 Result

2.3.1 Enrolled animals and collected samples

Of the 146 dairy cows that were initially enrolled, 11 were excluded (Table 2.3). Two were excluded because they were heifers and nine because no reproductive data were recorded. Of those nine, three died, two were culled and four were sold. Data from 135 animals were available for the final analysis.

The timing of the blood and milk sampling along with the timing of calving are shown in Table 2.3. Over the five farms the median date of first insemination was 28 October, 2014 (minimum 11 October, 2014, maximum 11 December, 2014).

F2 and P2 data were excluded for Farm A and C due to an error in sampling; milk as collected after the cows had been milked led to aberrantly high results. L2 records could not be obtained from farm B (n = 28) due to mechanical error at the laboratory. Overall, 5% of blood and milk data (162 out of 3240 measurements used in the final analysis) were missing or excluded due to technical or mechanical error.

***Table 2.3**: Timing of sampling and calving for the five farms included in the study.*

Farm	Sample size	Cows excluded from final analysis	Cows submitted within 3 weeks of PSM	Pre-calving blood sampling time	Calving period	Post-calving blood sampling	Time from pre-calving sample to calving			Time from calving to post-calving sample			Milk sampling	
							Median (days)	Max (days)	Min (days)	Median (days)	Max (days)	Min (days)	Start date	End date
Farm A	30	2	18	15/07	17/07-10/08	05/08-25/08	12	26	2	13.5	26	8	05/08	04/11
Farm B	30	2	23	31/07	01/08-17/08	20/08-25/08	5	17	1	15	23	6	20/08	19/11
Farm C	24	1	14	17/07-23/07	17/07-15/08	04/08-28/08	5	23	0	15	30	5	04/08	10/11
Farm D	32	6	23	18/07	20/07-14/08	06/08-11/08	9	27	2	12	18	4	06/08	06/11
Farm E	30	0	28	24/07	24/07-14/07	13/08-26/08	7	21	0	15	32	9	13/08	25/11
Total	146	11	106											

All the dates mentioned were in the year 2014.
Max= maximum, Min= minimum

2.3.2 Association between NEFA pre-calving and BHB post-calving

In the pre-calving period, 57/135 cows (42%) were at risk of NEB (BN1 ≥ 0.4 mmol/L) as shown in Table 2.5. Farms C and D both had more than 50% of their cows above this threshold.

In the post-calving period, using a cut-off point of ≥ 1.2 mmol/L, 47/133 cows (35%) had NEB based on their BB2 results (Table 2.5). Farms C and E both had more than 50% of their cows above this threshold.

There was no significant association between NEFA pre-calving and BHB post-calving (r_s = 0.121, p = 0.167). Furthermore, cows which had elevated pre-calving NEFA were not more likely to have elevated BHB post-calving than cows which did not have elevated NEFA pre-calving (Table 2.4); relative risk 1.15 (95% confidence interval 0.72-1.82).

Table 2.4*: Proportion of cows with elevated non-esterified fatty acids (NEFA) pre-calving which also had elevated beta-hydroxy butyrate (BHB) concentration post-calving.*

	BN1 > 0.4 mmol/L	***BN1 < 0.4 mmol/L***	Total
BB2 ≥ 1.2 mmol/L	21	26	47
BB2 < 1.2 mmol/L	34	52	86
Total	55	78	133

BN1= pre-calving blood NEFA concentration; BB2= post-calving blood BHB concentration

***Table 2.5**: Farm-wise mean, maximum, and minimum value of the blood measurements pre- and post-calving along with the farm-wise incidence of NEB in the pre-calving (BN1 ≥ 0.4 mmol/L) and post-calving (BB2 ≥ 1.2 mmol/L) samples.*

Farm	Sample size (n)	BB1 (mmol/L)			BN1 (mmol/L)			Number of samples with BN1 ≥ 0.4 mmol/L (*n*)	BB2 (mmol/L)			Number of samples with BB2 ≥ 1.2 mmol/L (*n*)	BN2 (mmol/L)		
		Mean	Max	Min	Mean	Max	Min		Mean	Max	Min		Mean	Max	Min
Farm A	28	0.35	0.50	0.20	0.27	0.60	0.10	6	1.03	1.60	0.70	9	0.39	0.80	0.20
Farm B	28	0.80	1.10	0.60	0.22	0.80	0.10	6	0.92	1.60	0.60	3	0.41	0.80	0.20
Farm C	23	0.57	1.70	0.30	0.62	1.30	0.20	17	1.38	3.80	0.70	14	0.28	0.50	0.10
Farm D	26	0.53	0.80	0.20	1.14	2.00	0.40	26	0.96	1.90	0.60	6	0.76	1.40	0.40
Farm E	30	0.26	0.60	0.10	0.13	0.50	0.00	2	1.18	2.50	0.70	15	0.34	0.60	0.20
Total	135							57				47			

BB1= Pre-calving blood BHB concentration; BN1= Pre-calving blood NEFA concentration; BB2= Post-calving blood BHB concentration; BN2= Post-calving blood NEFA concentration.

2.3.3 Three-week submission rate

Two cows (one each from Farm D and Farm E) were treated using an oestrus synchronisation programme and were therefore categorised as not being submitted within 3 weeks of PSM.

Overall, 106/135 (78.5%) cows were inseminated within 3 weeks of PSM. The number of cows, for each farm, which were not inseminated during that period, as well as the median interval between PSM or calving and insemination, are shown in Table 2.6. The median duration between PSM and insemination was 15 days, ranging from 0 days to 55 days. Likewise, the median duration between calving to insemination was 89 days, which varied from a minimum of 62 days to a maximum of 132 days. And the median duration between calving to PSM was 76 days, varying from 54 days to 86 days.

Table 2.6*: Farm-wise distribution of cows not inseminated within 3 weeks, interval from PSM to insemination, interval from calving to insemination and interval from calving to planned start of mating (PSM).*

Farm	Number of cows not inseminated within 3 weeks	Interval from PSM-insemination			Interval from calving-insemination			Interval from calving-PSM		
		Median	Max	Min	Median	Max	Min	Median	Max	Min
Farm A	10/28	18	48	1	95	125	65	76.5	86	62
Farm B	5/28	17	29	5	83	95	62	66	70	54
Farm C	9/23	19	51	1	93	132	64	78	85	56
Farm D	3/26	13	40	4	90	120	71	78	85	60
Farm E	2/30	13	55	0	89	122	74	78	85	64
Total	29/135	15	55	0	89	132	62	76	86	54

2.3.4 Correlation between blood and milk parameters

A selection of the correlations between the measured parameters is shown in Table 2.7. One hundred and fifty-five pairs of milk and blood components measurements were either positively or negatively correlated ($r_s > 0.17$). Of these 19 were strongly correlated ($r_s > 0.6$).

All the milk urea measurements were strongly correlated as were the protein measurements ($r_s > 0.6$). F4 was strongly correlated with P2, P3, and P4 ($r_s > 0.75$). Figures 2.1-2.4 illustrate some of the associations between the measured variables.

***Table 2.7**: Correlation between blood and milk variables seen in the Spearman's rank-order correlation test.*

	BB1	BN1	BB2	BN2	B1	U1	F1	P1	L1	B2	U2	F2	P2	B3	F3	L3	B4	L4
BB1	1																	
BN1 r_s	**.318****	1																
p	**.000**																	
BB2 r_s	**-.180***	.049	1															
p	**.039**	.574																
BN2 r_s	.139	**.311****	**-.235****	1														
p	.109	**.000**	**.006**															
B1 r_s	**.203***	**.331****	**.635****	-.026	1													
p	**.019**	**.000**	**.000**	.763														
U1 r_s	**-.559****	**-.199***	**.493****	**-.449****	.160	1												
p	**.000**	**.021**	**.000**	**.000**	.065													
F1 r_s	**-.423****	-.034	.114	.005	-.084	**.378****	1											
p	**.000**	.697	.195	.957	.340	**.000**												
P1 r_s	**-.271****	**-.393****	-.097	-.149	**-.294****	.171	**.324****	1										
p	**.002**	**.000**	.269	.089	**.001**	.050	**.000**											
L1 r_s	.049	**-.287****	**-.173***	-.071	**-.192***	-.159	-.141	**.238****	1									
p	.575	**.001**	**.048**	.422	**.028**	.069	.109	**.006**										
B2 r_s	.055	.024	.091	**-.172***	**.212***	-.056	-.117	-.053	-.091	1								
p	.527	.783	.296	**.048**	**.014**	.524	.182	.549	.302									
U2 r_s	**-.448****	**-.290****	**.352****	**-.514****	.077	**.704****	.123	-.019	**-.196***	.143	1							
p	**.000**	**.001**	**.000**	**.000**	.376	**.000**	.162	.833	**.025**	.100								
F2 r_s	-.059	.082	-.095	-.140	-.027	-.046	.197	.150	.093	.174	-.012	1						
p	.583	.448	.382	.196	.802	.674	.071	.172	.399	.107	.914							
P2 r_s	**-.343****	**-.495****	**.274***	**-.510****	-.132	**.472****	**.410****	**.724****	.183	-.090	**.537****	.133	1					
p	**.001**	**.000**	**.010**	**.000**	.223	**.000**	**.000**	**.000**	.094	.409	**.000**	.216						
B3 r_s	-.027	**.210****	.170	-.098	**.263****	.065	-.139	**-.433****	-.137	**.336****	**.216****	.041	**-.465****	1				
p	.754	**.015**	.050	.262	**.002**	.459	.113	**.000**	.120	**.000**	**.012**	.701	**.000**					
F3 r_s	.019	.042	.016	-.027	.146	-.060	**.199***	.060	-.061	**.223****	.029	**.648***	.105	**.281****	1			
p	.829	.631	.858	.759	.095	.492	**.023**	.499	.490	**.010**	.744	**.000**	.332	**.001**				
L3 r_s	.061	-.097	-.147	-.045	**-.224***	.027	.093	**.359****	**.296****	**-.350****	-.067	-.018	**.441****	**-.577****	**-.425****	1		
p	.488	.270	.096	.613	**.011**	.764	.297	**.000**	**.001**	**.000**	.453	.873	**.000**	**.000**	**.000**			
B4 r_s	**.276****	.167	.105	-.136	**.341****	-.121	**-.196***	**-.346****	-.130	**.378****	-.012	-.018	**-.346****	**.522****	**.175***	**-.421****	1	
p	**.001**	.054	.234	.122	**.000**	.167	**.026**	**.000**	.141	**.000**	.890	.868	**.001**	**.000**	**.045**	**.000**		
L4 r_s	-.087	-.138	**-.237****	.092	**-.344****	-.116	.101	**.266****	**.450****	**-.311****	**-.233****	-.070	.157	**-.361****	**-.272****	**.616****	**-.444****	1
p	.321	.114	**.006**	.297	**.000**	.187	.255	**.002**	**.000**	**.000**	**.008**	.518	.145	**.000**	**.002**	**.000**	**.000**	

Note: Strongly correlated variables U3, U4 (with U1, U2) and L2, P3, F4, P4 (with P2) were excluded from the table as their result were similar to those shown by the correlated variable.

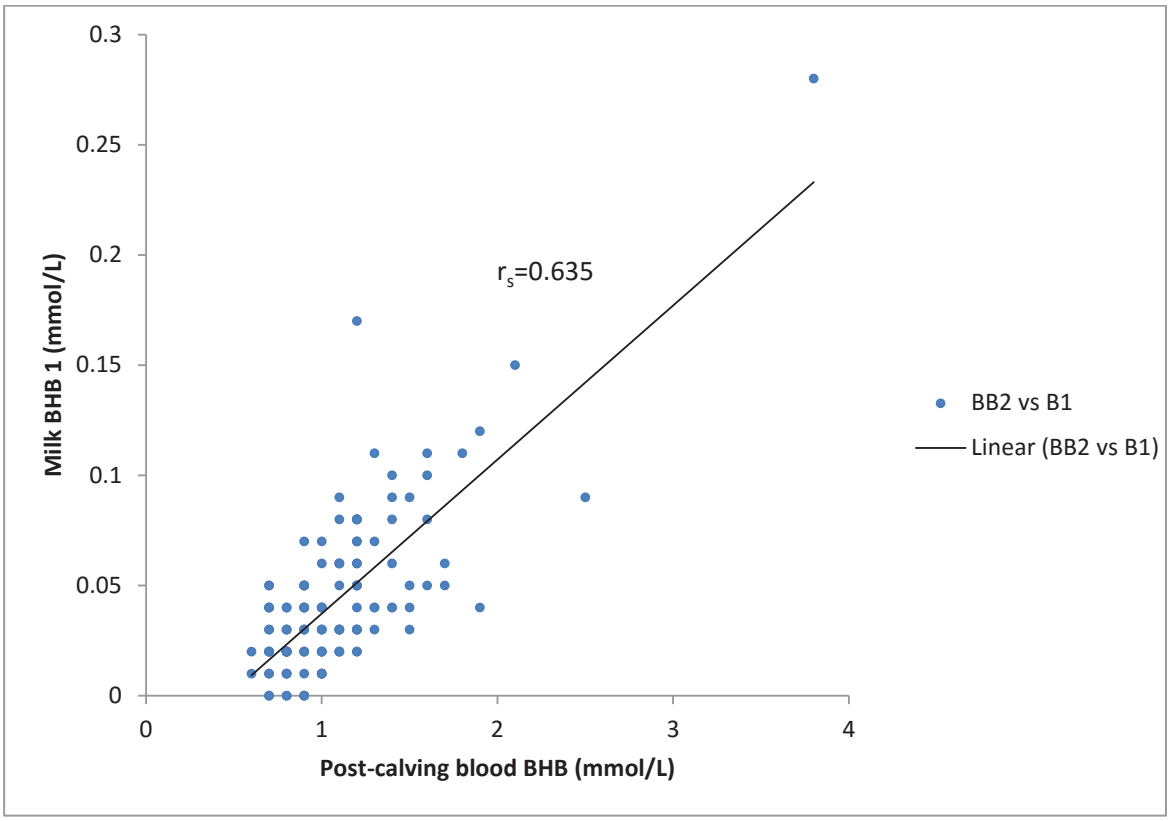

Figure 2.2: *Positive correlation between BB2 and B1.*

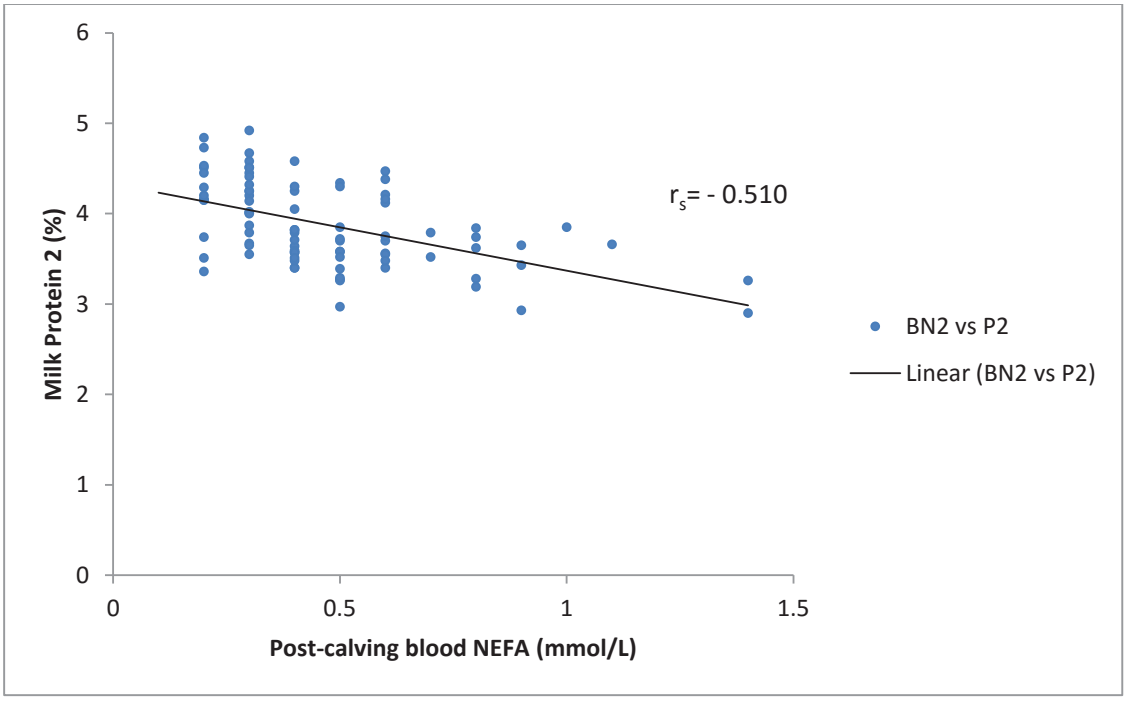

Figure 2.3: *Negative correlation between BN2 and P2.*

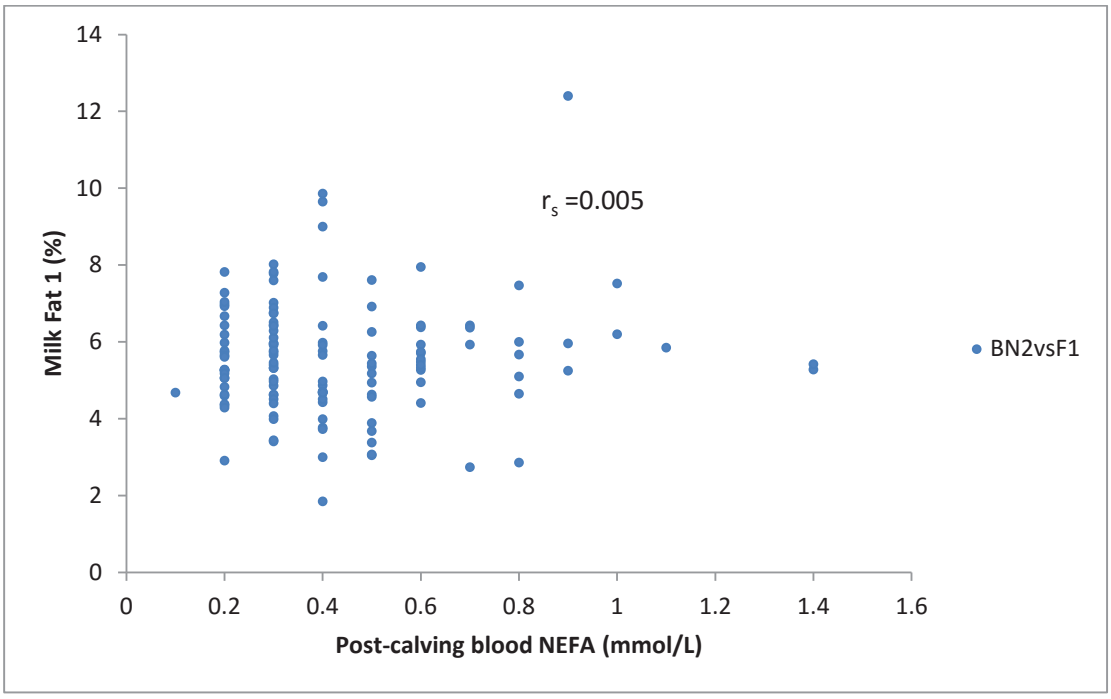

Figure 2.4: *Non-significant positive correlation between BN2 and F1.*

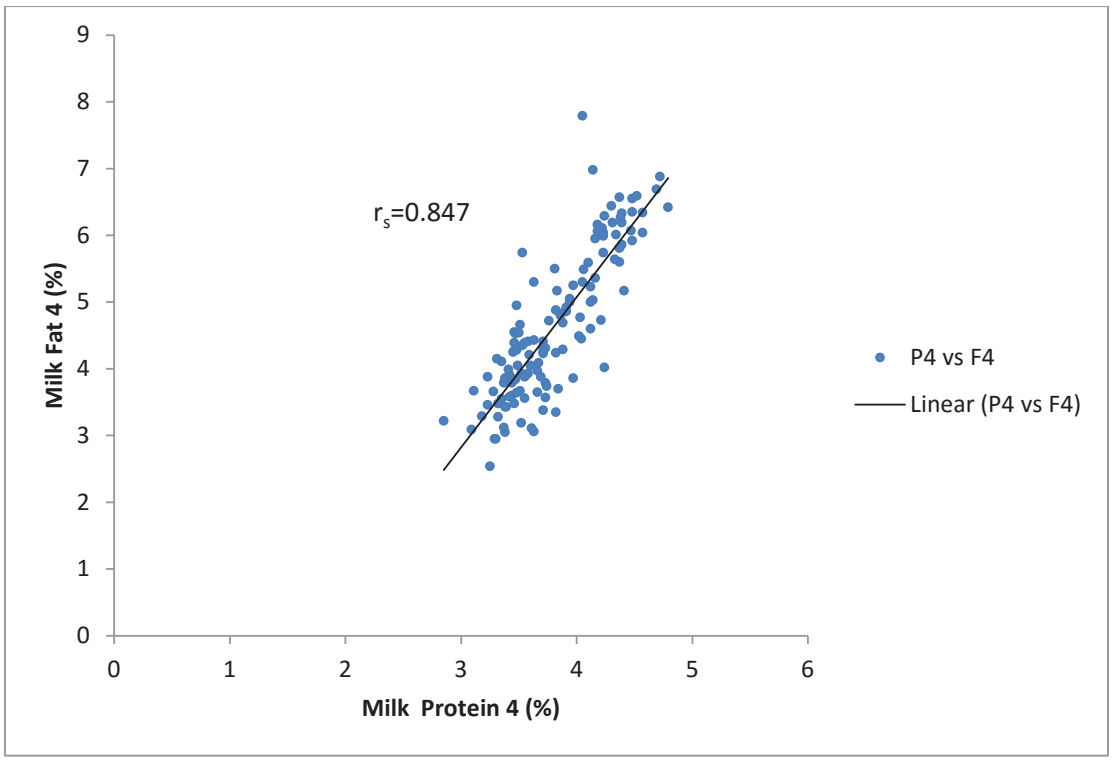

Figure 2.5: *Very strong and positive correlation between P4 and F4.*

2.3.5 Association of the variables with the reproductive outcome

The strong correlation between milk variables meant that only 10/20 variables were included as explanatory variables in the logistic regression model. The removed variables were B1, U2, U3, U4, F3, F4, P1, P3, P4, and L2.

The first multivariable logistic regression model included all four blood and 10 milk parameters, alongside farm. None of the blood or milk components were significantly associated with the risk of submission within the first 3 weeks of PSM (Table 2.8) ($p > 0.05$). Model diagnostics performed by plotting residuals against probability of the regression model (fig 2.6) suggests that the assumptions made were valid.

***Table 2.8**: Logistic regression model for predicting the risk of submission in the first 3 weeks of breeding.*

Variable	Estimates	S.E.	Wald	df	Significance	Odds Ratio
BB1	8.164	4.881	2.797	1	.094	3510.486
BN1	-.365	2.286	.025	1	.873	.694
BB2	-3.739	2.213	2.855	1	.091	.024
BN2	1.463	2.768	.279	1	.597	4.318
U1	.259	.534	.236	1	.627	1.296
F1	.172	.433	.159	1	.690	1.188
L1	-2.867	3.702	.600	1	.439	.057
B2	-29.496	20.842	2.003	1	.157	.000
F2	-.436	.460	.896	1	.344	.647
P2	2.323	2.288	1.030	1	.310	10.203
B3	-2.748	17.076	.026	1	.872	.064
L3	1.685	3.017	.312	1	.576	5.395
B4	-6.958	19.364	.129	1	.719	.001
L4	-.132	4.938	.001	1	.979	.877
Farm			1.922	3	.589	
Farm (1)	-1.052	3.007	.122	1	.726	.349
Farm (2)	-3.807	2.769	1.891	1	.169	.022
Farm (3)	-2.274	2.914	.609	1	.435	.103
Constant	2.794	26.940	.011	1	.917	16.354

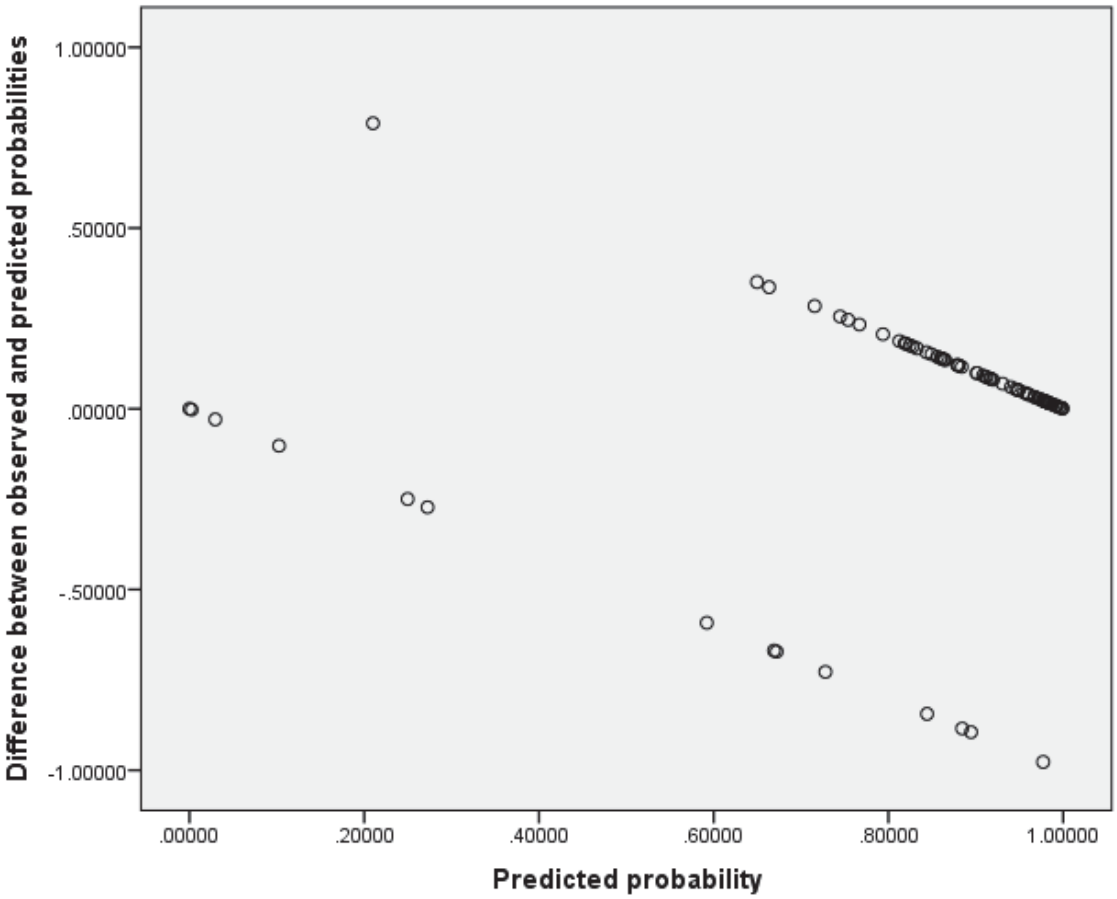

***Fig 2.6**: Residuals vs probability of the regression model.*

2.3.6 Impact of milk variables on the prediction of reproductive outcome

The second logistic regression model was created with the blood parameters and the farm forced into the model while the milk variables were available for backward stepwise removal. B2 was only the milk parameter retained in the model (p = 0.061); the odds ratio was very low (~3×10^{14}) (Table 2.9).

Table 2.9: *Variables which remained in the model for predicting the risk of submission in the first 3 weeks of breeding after backward stepwise elimination. Blood variables and the farm were forced in the model.*

Variable	Estimates	S.E.	Wald	df	Sig.	Odds Ratio
BB1	4.907	3.403	2.079	1	.149	135.242
BN1	-.316	1.863	.029	1	.865	.729
BB2	-2.341	1.358	2.971	1	.085	.096
BN2	.251	1.943	.017	1	.897	1.286
Farm			8.152	3	.043	
Farm (1)	-4.692	1.674	7.861	1	.005	.009
Farm (2)	-4.269	2.418	3.115	1	.078	.014
Farm (3)	-2.852	2.771	1.059	1	.303	.058
B2	-31.277	16.685	3.514	1	.061	.000
Constant	6.109	2.399	6.484	1	.011	449.786

In the final logistic regression model, all parameters were available for backward stepwise elimination; in this model, only farm and B2 were retained, although again the p-value for the latter was high (p = 0.062), but the odds ratio was still very low (Table 2.10).

Table 2.10: *Variables remained in the model for predicting the risk of submission in the first 3 weeks of breeding after using backward stepwise elimination.*

Variable	Estimates	S.E.	Wald	df	Sig.	Odds ratio
Farm			7.300	3	.063	
Farm (1)	-3.052	1.173	6.766	1	.009	.047
Farm (2)	-.670	.937	.511	1	.475	.512
Farm (3)	-.872	1.086	.645	1	.422	.418
B2	-28.025	15.015	3.484	1	.062	.000
Constant	3.962	1.120	12.518	1	.000	52.558

The mean of B2 for animals which were submitted for insemination within 21 days of PSM was then compared to that of animals which were not (Table 2.11).

Table 2.11: *Mean of milk beta-hydroxybutyrate at the second milk sampling.*

Submission	Mean	N	Std. deviation
0	0.09	28	0.14
1	0.05	105	0.03
Total	0.06	133	0.07

2.4 Discussion

This study is the first study in New Zealand that has been aimed at identifying whether the inclusion of additional milk information alongside blood parameters would improve the accuracy of prediction of the impact of NEB on reproductive performance (which in this study was the probability of submission within 3 weeks of PSM).

However, in this study, no significant association between 3-week submission rate and any blood parameter was found. The only significant finding was that milk BHB concentration in the second month after calving was significantly lower in cows which were submitted in the first 3 weeks after PSM.

These data thus suggest that adding milk parameters to NEFA and/ or BHB blood results may not increase the predictive value of blood results.

Three-week submission rate was chosen for this analysis because it could easily be collected and analysed within the time limits available for this thesis, and had been shown in pasture-based cows to be associated with severity of NEB, as measured by severity of body condition score (BCS) loss between calving and nadir (Roche et al., 2007). The lack of significant findings in this study suggests that, in this dataset, there was no association between the outcome variable and energy status.

This might have occurred due to the reproductive outcome not being related to the level of NEB, the lack of power of the study (i.e. inadequate number of not-submitted cows), or the choice of reproductive outcome not being powerful enough to differentiate between the cows with different energy status.

2.4.1 Association of outcome with factors other than energy

One of the reasons for the lack of significance seen in this study might be that factors other than energy were the major determinants of risk of submission in the first 3 weeks in this dataset. There was no association between NEB as determined by blood parameters and risk of submission in the first 3 weeks. Overall 3-week submission rate

was 78.5% (106/135); for cows with elevated pre-partum NEFA (BN1 ≥ 0.4 mmol/L) or elevated post-partum BHB (BB2 ≥ 1.2mmol/L) the equivalent figures were 73% (41/56) and 77% (36/47), respectively.

Furthermore, there was little consistency between pre- and post-partum diagnosis of NEB, which may suggest that the energy deficiency seen in these cattle was not severe enough to cause reproductive effects as it did not persist from pre- to post-partum.

A lack of association between NEB and the length of anovulatory anoestrus has been reported in several studies (e.g. Spicer et al. [1990]; Snijders et al. [2001]). In pasture-based cattle, Pedernera et al. (2008) were able to alter NEB nadir and NEFA and BHB concentrations by feeding two different diets, but found no effect on days to first luteal phase or days to first AI.

One factor which may have reduced the impact of energy balance on submission rate in this study is that all of the study cows were early calvers, which, in the pastoral New Zealand situation, maximises the chances of being submitted for insemination in the first 3 weeks as the longer calving to PSM interval of early calving cows means that they have longer to recover from the homeorrhetic changes associated with calving. As shown in Table 2.6, the minimum and maximum duration between calving and PSM was 54 and 86 days, respectively. In a study conducted in New Zealand, cows which were calved > 40 days before at the start of PSM had a higher submission rate than those that were calved ≤ 40 days (88.2% vs 78.9%, $p < 0.01$) (McDougall, 2001). Similarly, the presence of late calvers in the herd was found to reduce the submission rate in another pasture-based study in New Zealand (Macmillan et al., 1975).

As the cows were early calvers, it is likely that the key influence on 3-week submission rate in these cows was risk of heat detection (as they were at low risk of being in anovulatory anoestrus at the PSM). This is principally a farm/staff effect, with large differences in heat detection efficiency between farms driving the difference in their submission rate. Thus even though submission rate is related to the resumption of ovarian cyclicity which is closely associated with NEB (Wathes et al., 2007), errors in heat

detection could easily have obscured the impact of NEB on risk of submission, particularly as the number of cows not inseminated in the first 3 weeks was relatively small.

2.4.2 Power of the study

An alternative suggestion for the lack of association between NEFA and BHB and 3-week submission rate was the relatively low statistical power of the study. For this study the power to detect a difference was determined (Ellis, 2010), firstly by the number of cows which had abnormal BHB and/or NEFA (n = 56 and 47, respectively), and by the number of cows which were not submitted within the first 3 weeks (n = 29). Thus for a relative risk analysis, a 20% difference could have been detected with 80% power in this study, far larger than is biologically important.

For the logistic regression, as the probability of non-submission was 22%, with 135 animals the logistic regression would have detected an odds ratio of 1.8 for an individual with a NEFA or BHB concentration of one standard deviation above the mean (0.45 and 0.40 for BN1 and BB2, respectively) using a one-tailed test with a significance level of 5% and a power of 80% (Hsieh, 1989). Increasing the proportion of negative outcomes, such as by choosing an outcome such as 6-week in-calf rate, where the proportion would have been nearer 50% than 3-week submission rate, would have decreased the detectable odds ratio to a minimum of 1.5 when 50% of cows had a negative outcome.

Nevertheless, the actual detectable odds ratio is reasonable and shows that there were sufficient animals to detect a biologically important impact of NEB. However, more individuals would have increased the sensitivity of the study; for example, Compton et al. (2015) showed a significant association between BHB and vaginal discharge (53/522 cows had purulent vaginal discharge) and BHB and 6-week in-calf rate (113/565 were not in-calf). In those cases, the detectable odds ratios would have been < 1.5 and < 1.4, respectively.

2.4.3 Choice of reproductive outcome variable

As discussed earlier, the choice of 3-week submission rate limited the number of negative outcomes and thus reduced power compared to an alternative such as 6-week in-calf rate, which would have had more negative outcomes. However, increasing power is not the only rationale for using an alternative outcome variable.

As discussed earlier, particularly when comparing across farms, heat detection efficiency (which is a farm/staff effect not directly related to NEB) can obscure the impact of NEB. Therefore, the use of other reproductive outcome measures where heat detection is less important could have provided a better test of this studies hypothesis.

Alternative reproductive indicators such as 3-week or 6-week in-calf rate are less dependent on heat detection, as they include the impact of conception rate as well as submission rate, although heat detection is still a crucial factor (MacMillan, 2012). However, these measures require pregnancy diagnosis, which is usually undertaken in January/February, too late to be included in this thesis. However, further analysis using these measures is ongoing.

2.4.4 Association of submission rate with other predictors of NEB rather than NEFA and BHB concentrations

As discussed in the literature review, NEFA and BHB can lack sensitivity as measures of NEB; it may be that in this study this lack of sensitivity resulted in no apparent effect of severity of NEB on submission rate. This is consistent with the findings of McDougall et al. (1993), who reported that although cows with anovulatory anoestrus had evidence of significantly greater NEB (based on BCS, blood glucose, and blood urea) there were no differences between the groups in blood NEFA or BHB concentrations. Similarly, Patton et al. (2007) found no association between plasma NEFA in the first 4 weeks of lactation and calving to ovulation interval or the conception rate in between these cows. In contrast, Burke et al. (2010), in a study conducted in New Zealand, did find a trend ($p = 0.055$) towards increased blood NEFA being associated with increased calving to ovulation interval, although they failed to find a significant effect of increased BHB.

Alternative measures of NEB, such as insulin like growth factor-I (IGF-I) and insulin (Newgard and McGarry, 1995; Spicer et al., 1990), may be significantly better at predicting the delay in resumption of ovulatory activity (Beam and Butler, 1998; Butler, 2003; Pedernera et al., 2008). However, such tests are significantly more expensive than NEFA or BHB, which is why this study focussed on improving the predictive value of NEFA/BHB by adding milk testing.

2.4.5 Sampling time

As discussed in the literature review, the timing of sampling relative to calving can have a significant impact on the expected value of NEFA/BHB in normal animals, particularly the former. It is recommended that pre-partum NEFA concentrations should be measured 14 and 2 days before calving (Cook et al., 2006; Leslie et al., 2003; Oetzel, 2004), as concentrations close to calving tend to elevate (Oetzel, 2004). In the present study, of the 135 cows which were enrolled, nine were sampled < 2 days before calving. Of these cows, six had NEFA ≥ 0.4 mmol/L (two each with 0.4, 0.7 and 1 mmol/L), but only two were not inseminated within 3 weeks of PSM. Incorporating (average NEFA = 0.454 mmol/L) or removing (average NEFA = 0.448 mmol/L) such NEFA values didn't make much difference in the average pre-calving NEFA concentration. Twenty cows were sampled > 14 days before calving, of which nine had NEFA ≥ 0.4 mmol/L, while seven failed to inseminate.

On the other hand, post-partum BHB concentration is generally measured within 2 weeks of calving (Duffield et al., 2009). In this study, 60 cows were sampled > 14 days post-calving, of which 21 cows had BHB ≥ 1.2 mmol/L and 14 were not inseminated within 3 weeks of PSM.

Pre-calving sampling was complicated by the need to predict calving time; which even in cows with known calving dates and apparent preparatory can be unpredictable (Thomas, 2012). This problem can be particularly difficult to overcome in smaller herds where fewer cows are calving at any one time (Oetzel, 2004).

2.4.6 Variation of milk components with stage of lactation and stage of milking

With the progress in the stage of lactation, energy balance and milk yield tend to differ which together has its contribution in fluctuating the concentration of the milk components (Auldist et al., 1995). However, in this study, almost all the four milk sampling points lied within the early stage of lactation (interval between the most early calving date and furthest milk sampling point was 132 days). Therefore, the effect caused by variation in the milk components with the stage of lactation had minimal effect over the result.

The sampling points had little effect on the fluctuation of these milk components, except milk urea and fat (Annex 3). Protein, urea and BOHB value were rather consistent along the entire four sampling points. Milk fat value went on decreasing along the sampling points; the only major variation seen was in the second sampling (from 5.58% to 4.95%). This might have occurred because milk fat is directly related to the energy balance and could have decreased as the body energy improved. The decreased value in further measurements might underestimate the presence of NEB and its association with the risk of submission.

Similarly, slight increase in urea concentration in the second measurement could have occurred because of the increase in feed intake of lush green spring pasture (Moller et al., 1993). Otherwise, there was not much variation in their concentration along the four different measurements. Because of their association with the increased protein intake rather than the energy status, such increase might overestimate the presence of NEB.

In this experiment, hand stripped foremilk as well as conventional herd test samples via machine milking were used for the analysis of milk components. Foremilk is generally not recommended to represent the accurate milk constituent (Nielsen et al., 2005) as there is tendency to have lower value (BHBA and Fat) or higher value (urea, protein and lactose) for some milk constituents in foremilk sample compared with the rest of the milking. Conventional herd test samples tend to provide most representative sample from each quarter avoiding the variation between foremilk and in-between milk or post-stripped milk. However, in this study it was not possible to get the herd test sample each time as

three of the private farms conducted herd-test every three months. Herd test samples could only be utilized for testing fat, protein and lactose concentration due to the limitations of LIC. Therefore, hand-stripped milk samples were compulsory for BOHB and urea as they needed to be submitted separately to NZVP for milk BOHB and urea analysis. Besides, post-stripping showed excessively high fat and protein value; therefore, it was not used.

Milk fat increases significantly as the milking progresses with foremilk sample having low fat concentration (Nielsen et al., 2005). Since high fat percentage represents NEB, foremilk samples might underestimate the presence of NEB. On the other hand, milk protein decreases towards the end of milking and during NEB (Nielsen et al., 2005). Foremilk sample in this case might again underestimate the presence of NEB. For lactose, such effect might be minimal as they tend to stay constant during the beginning of milking. Urea tends to decrease as the milking process reaches its end and high value represents NEB. Therefore, foremilk urea samples might overestimate the presence of NEB.

2.4.7 Correlations between parameters

Of the 20 different milk parameters which were measured, only 10 were used for the final model. This was because there were many strong correlations ($r_s \geq 0.6$) (Table 2.7), which meant that many of the parameters measured, did not add additional information.

Milk BHB (B1) was not used in the model as it was correlated ($r_s = 0.64$) with post-calving blood BHB (BB2), consistent with the findings of Enjalbert et al. (2001) and Denis-Robichaud et al. (2014). The only other significant correlation ($r_s = 0.2$) between milk and blood BHB was that between BB1 and B4 ($r_s = 0.28$), which may have occurred as when days in milk increases, energy balance stabilises slowly causing lower and less variable ketone production.

All of the milk urea measurements were correlated to each other, and therefore, U1 was used as the sole representative of urea measurements in the model. In a New Zealand

study (Auldist et al., 1998), urea concentration varied with the time of the year and stage of lactation, as milk urea is principally a measure of protein and non-protein-nitrogen intake. All the milk samples in the current study were taken between 4 August, 2014 and 25 November, 2014, so the variation in stage of lactation and time of year was limited, limiting the variation in urea concentrations. These data strongly suggest that if urea is to be used as a predictor of future reproductive performance, only a single sample is needed, with the best timing being based on when a prediction of performance is needed rather than at a specific time post-calving.

Milk protein measurements were also strongly correlated with each other. Milk protein shows a downward trend in the early lactation and thereafter increases as the lactation progresses (Auldist et al., 1998; Ng-Kwai-Hang et al., 1982). The latter is a result of the concentrating effect of the decrease in milk yield with the increase in days in milk. Milk protein content is also dependent on the energy supply (Eicher et al., 1999). In early lactation, the available energy is used to increase milk yield rather than the protein content. However, when the energy balance improves, the surplus energy is also used to increase the protein content of the milk later in the lactation (Coulon and Rémond, 1991). As with urea, the limited time span of this study meant that no major variations in protein concentration were noted, and the same conclusion applies that only one measure of protein is required if it is to be used as a predictor. These data suggest that P2 (taken in between 4-25 September, 2014) may be the best measure as this also reduces the need for other non-protein samples (i.e. L2 and F4). This finding needs confirmation in a further study on more farms.

Except for F2 and F3 (r_s = 0.648), milk fat measurements were not strongly correlated, although they were correlated. These differences are likely to occur because milk fat is more sensitive to changes in energy balance than milk urea and protein (Reist et al., 2002), being initially high when NEB is greatest and then decreasing; in this study mean F1 was 5.57%, thereafter mean fat concentrations decreased, ranging from 4.65-4.71%. This suggests that multiple fat measurements may be of value if milk fat is to be used for predicting future reproductive performance.

All the milk BHB measurements were correlated with each other (r_s > 0.2), but the correlations were not strong enough for one measurement to replace the others. As already mentioned, BHB in milk and blood are correlated and are therefore highly influenced by the changes in energy balance of the body with the progress in days in milk. This means that BHB in milk should decrease as the days in milk progresses. However, in this study, the mean milk BHB values ranged from 0.03-0.06 mmol/L, lower than the threshold point for ketosis recommended by Enjalbert et al. (2001) of 0.07 mmol/L. The lack of correlation between different measurements suggests that multiple milk BHB measurements may be needed to predict the likely reproductive outcome.

Like milk fat, milk lactose measurements also showed strong correlations for only two time points, i.e. L3 and L4 (r_s = 0.616), although all time points were significantly correlated (r_s > 0.2). This lack of strong correlation supports the value of taking multiple measurements of lactose. However, there was not much variation in the mean milk lactose concentration across the four measurements (4.84-5.04%), consistent with the findings of Gurmessa and Melaku (2012) that milk lactose was stable with respect to stage of lactation.

2.4.8 Milk BHB as a predictor of submission rate

In the model the only near significant association found was B2; with cattle which were not submitted having a mean of 0.09 mmol/L, and submitted cows a mean of 0.05 mmol/L. Although this difference is in the expected direction (higher BHB, lower reproductive performance), in an exploratory study such as this one, it is possible to get apparently significant effects which are statistical anomalies because of the multiple comparisons which make up the exploratory analysis. This finding therefore needs to be fully explored in more studies.

2.5 Conclusion

Based on the blood NEFA and BHB, the prevalence of NEB was pre-calving (42%) and post-calving (35%) among the sampled cows. Failure of insemination within 3 weeks of PSM was found in 29/135 cows. This study was unable to show any significant association between these blood parameters and the risk of submission within 3 weeks of PSM.

Multiple strong correlations meant that of the 20 milk measurements (four measurements of five different parameters [protein, fat, BHB, urea, lactose]), only 10 were included in the model. Of these 10 measures, only milk BHB in the second month post-calving was found to be associated with risk of submission. None of the other four parameters showed any association with this reproductive outcome. Moreover, this study could not provide any evidence that incorporating additional milk information to the blood measurements helps to improve their accuracy in predicting the 3-week submission rate.

Further analysis of this dataset should be undertaken using reproductive outcome indicators such as 3-week or 6-week in-calf rate to see whether this finding is specific to submission rate.

Further study with more cows across more farms would also be of value to confirm the lack of findings in this study.

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APPENDIX 1: Mean, median, and standard deviation of the blood and milk variables

Variables	Valid data	Missing values	Mean	Median	Mode	Std. Deviation
BHBB1	135	0	0.49	0.51	0.30	0.25
NEFAB1	135	0	0.45	0.31	0.10	0.45
BHBB2	133	2	1.09	1.00	1.20	0.40
NEFAB2	133	2	0.43	0.40	0.30	0.24
BHB1	133	2	0.04	0.04	0.02	0.04
Urea1	133	2	5.92	5.80	4.20	2.01
Fat1	131	4	5.57	5.44	4.68	1.48
Protein1	131	4	3.82	3.81	3.66	0.41
Lactose1	131	4	5.03	5.06	5.06	0.22
BHB2	133	2	0.06	0.05	0.04	0.07
Urea2	133	2	7.03	7.10	7.40	2.77
Fat2	89	46	4.68	4.60	5.27	1.35
Protein2	89	46	3.87	3.79	3.40	0.46
Lactose2	105	30	4.84	4.82	4.73	0.32
BHB3	135	0	0.03	0.03	0.00	0.03
Urea3	135	0	5.80	5.40	3.90	2.18
Fat3	134	1	4.71	4.63	3.28	1.56
Protein3	134	1	3.73	3.64	3.52	0.43
Lactose3	131	4	4.94	5.04	5.08	0.37
BHB4	133	2	0.05	0.05	0.05	0.03
Urea4	133	2	5.38	4.90	4.30	1.64
Fat4	133	2	4.65	4.41	3.79	1.10
Protein4	133	2	3.81	3.73	3.71	0.41
Lactose4	133	2	5.04	5.05	5.11	0.15

APPENDIX 2: Test of normality of the blood and milk variables using Shapiro-Wilk's test

Variables	Shapiro-Wilk		
	Statistic	df	Sig.
BOHBB1	.919	52	.002
NEFAB1	.774	52	.000
BOHBB2	.817	52	.000
NEFAB2	.827	52	.000
BOHB1	.773	52	.000
Urea1	.957	52	.060
Fat1	.889	52	.000
Protein1	.956	52	.052
Lactose1	.987	52	.855
BOHB2	.875	52	.000
Urea2	.924	52	.003
Fat2	.978	52	.438
Protein2	.966	52	.140
Lactose2	.941	52	.013
BOHB3	.834	52	.000
Urea3	.972	52	.253
Fat3	.984	52	.702
Protein3	.969	52	.200
Lactose3	.863	52	.000
BOHB4	.884	52	.000
Urea4	.971	52	.237
Fat4	.962	52	.094
Protein4	.974	52	.304
Lactose4	.981	52	.570

Significance > 0.05 = normal distribution

APPENDIX 3: Variation of milk components along the different sampling points