The Management of Hypocalcaemia in UK Dairy Herds

by
Eleanor J. M. Garnett
BSc (Hons), Nottingham Trent University

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School of Veterinary Medicine and Science
Sutton Bonington Campus
College Road
Sutton Bonington
Leicestershire
LE12 5RD
United Kingdom

DECLARATION

I hereby declare that this thesis has been composed by myself and has not been submitted for any other degree, in Nottingham or elsewhere. The work presented herein is my own, and all work of other authors is duly acknowledged. I also acknowledge the assistance given to me during the design and execution of the experiments contained in this thesis and during its preparation.

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"Look, Eleanor, moo cows!"

"They're not 'moo cows', they're Friesian heifers"

- Age 3

ABSTRACT

Periparturient milk fever is a widespread metabolic disease within the dairy industry, with a number of potential impacts on the affected animals' health and productivity in the subsequent lactation that can have a considerable economic impact on farmers. This thesis aimed to investigate current attitudes of both UK dairy farmers and veterinarians towards milk fever and subclinical hypocalcaemia, as well as investigating the feeding of rumen-protected rice bran during the dry period as a milk fever preventative, due to its reported potential as a calcium binder, within a commercial UK dairy herd. A retrospective study was also carried out in order to investigate associations between recorded milk fever events and other outcomes from farm records obtained from 78 UK dairy herds. The levels of agreement between three different blood sample types (serum, lithium heparin plasma, and lithium heparin whole blood) were also considered with regard to testing for concentrations of calcium, magnesium and phosphorous.

Questionnaires were distributed to UK dairy farmers and veterinarians in order to assess current attitudes towards milk fever and its prevention. When choosing a prevention strategy, farmers were most concerned with its efficacy, with its ease of use being their second priority. Veterinarians placed a greater importance on metabolic disease than farmers, and it appears that vets may have an important role in education on the subject of hypocalcaemia.

A retrospective study of over 59,000 lactations from 78 UK dairy herds found associations between recorded events of milk fever and an increased risk of dystocia, and an increased risk of a cow exiting the herd during the first 30 days of the lactation. Twin pregnancies were associated with a 30% reduction in the risk of milk fever.

Rumen-protected rice bran was trialled against a control feed on a UK dairy herd during the dry period as a potential milk fever preventative. Serum calcium concentrations in the control group were significantly higher pre-calving than in the group that received rumen-protected rice bran. Cows fed

rumen-protected rice bran were more likely to experience an elevated NEFA concentration post-calving than the control group. No feed-related differences were found in the subsequent 100 day yields.

Limits of agreement were examined to investigate whether bovine serum, plasma and whole blood samples can be interchangeably used for the analysis of calcium, phosphorous and magnesium concentrations. Serum and plasma results appeared to show high levels of agreement for all three of the analytes. Whole blood results were more variable.

In conclusion, the findings of this thesis provide an insight into the current attitudes within the UK dairy industry towards hypocalcaemia and its prevention. This thesis has also provided information on the effects and practicalities of feeding rumen-protected rice bran during the dry period in a commercial dairy herd and highlights the need for further research on the subject.

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representing data to a maximum value of 1.5 x IQR, with outlier points beyond these limits displayed as dots)

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LIST OF ABBREVIATIONS USED WITHIN THIS THESIS

BCS	Body condition score
BHB	Beta hydroxy butyrate
CAT	Clot activator tube
CI	Confidence interval
CP	Crude protein
Cr	Creatinine
d	Days
DCAB	Dietary cation-anion balance
DCAD	Dietary cation-anion difference
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
ECS	Extracellular space
EDTA	Ethylenediaminetetraacetic acid
g	Grams
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
ICS	Intracellular space
IgG	Immunoglobulin G
IQR	Interquartile range
IV	Intravenous
Kg	Kilograms
l	Litres
LDA	Left-displaced abomasum
LH	Lithium heparin
M/D	Energy density

ME	Metabolisable energy
meq	Milliequivalents
MF	Milk fever
mg	Milligrams
MJ	Mega Joules
ml	Millilitres
mmol	Millimoles
MP	Metabolisable protein
MPB	Metabolisable protein from bypass protein
MPE	Metabolisable protein from rumen energy
MPN	Metabolisable protein from rumen nitrogen
NDF	Neutral detergent fibre
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
OR	Odds ratio
PTH	Parathyroid hormone
RF	Rumen fermentable
RFM	Retained foetal membranes
RNA	Ribonucleic acid
RU	Rumen undegraded
SC	Subcutaneous
s.d.	Standard deviation
TMR	Total mixed ration
TP	Total protein
UKAS	United Kingdom Accreditation Service
UV	Ultraviolet

CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

Nutrition for dairy cows during the transition period, typically defined as the three weeks before and after calving (Grummer, 1995; Mulligan & Doherty, 2008), is critically important for the prevention of periparturient metabolic disease. This includes the management of calcium status. Calcium has a number of vital roles within the body, including in bone structure, in blood coagulation (Lovelock & Porterfield, 1952), in the transmission of nerve impulses (Campbell *et al*., 2008), in the activation of enzyme reactions, and in the contraction of muscle cells (Campbell *et al*., 2008). The demand for calcium in the dairy cow suddenly increases at parturition, largely due to the onset of lactation and the subsequent loss of calcium into the milk. The calcium homeostatic mechanism usually maintains serum calcium concentrations within a narrow range, supplying an adequate quantity of calcium to maintain normal cellular function. However, the sudden change in demand for calcium at calving disrupts this mechanism and some cows do not adapt quickly enough. This can result in clinical cases of the metabolic disease known as milk fever, which has immediate health risks, as well as downstream negative consequences for health and production. Subclinical cases of hypocalcaemia can also occur. These often go undetected but even whilst clinical signs are not manifested, it is speculated that even mild hypocalcaemia may still have deleterious effects on herd health and productivity (Curtis *et al*., 1983, 1985; Reinhardt *et al*., 2011).

Hypocalcaemia is an economically important metabolic disorder, both in terms of the cost of treatment of clinical milk fever and also in relation to the loss of productivity associated with both clinical and subclinical cases (Horst *et al*., 1997). In 2005 it was estimated that the economic cost of a clinical case of milk fever is around £200 (Husband, 2005), and subclinical cases will also have a financial impact due to the effects of hypocalcaemia on future productivity.

Oetzel (2013) estimates that each case of subclinical hypocalcaemia in the USA will cost a farmer $125 on average, taking into account decreased milk yield and increased incidence of other clinical diseases. Considering the number of cows likely to be affected by subclinical hypocalcaemia within a herd when compared to those affected by clinical milk fever, Oetzel (2013) estimates that overall subclinical cases within a herd will cost the farmer four times the amount spent on clinical cases. It is therefore important for farmers to be able to manage their herd in order to minimise the incidence of the disease. At present there are three main strategies employed for reducing incidence of hypocalcaemia: the targeted supplementation of calcium peripartum, the dietary cation-anion difference (DCAD) approach, and the use of low calcium diets. However, all of these strategies currently have negative aspects to them and in recent years there has been increasing research into the potential use of calcium binders in order to improve the calcaemic status of dairy cows around calving and into the start of their lactation.

1.2. Physiological basis of peripartum hypocalcaemia

1.2.1. Calcium homeostasis

Calcium has a number of important roles within the body and is required for many vital intercellular and extracellular functions. In the case of ionised calcium, the biologically active form, these roles include neuromuscular transmission, vascular smooth muscle tone and muscle contraction (Schenck *et al*., 2011). In an adult cow, blood calcium concentrations are maintained at around 8.5-10mg/dl (2.12-2.90mmol/l) (Goff, 2004). Total blood calcium levels of <1.4mmol/l are generally considered to indicate cases of clinical milk fever (DeGaris & Lean, 2009), with blood calcium levels between 1.4-2.0mmol/l being described as subclinical hypocalcaemia (Goff, 2008). Dietary calcium requirements vary according to bodyweight but a 650kg cow, for example, would require approximately 25g/day (Husband, 2005). Full-term pregnancy increases the calcium demand by an additional 13.9g/day (Husband, 2005)

and the colostrum produced in early lactation adds a demand of 20-30g/day onto the maintenance requirement (Goff, 2004). Lactation is even more demanding, with a cow yielding 40l of milk per day needing an additional 73g of calcium per day in addition to her maintenance requirements (Husband, 2005).

The plasma pool of an adult cow typically contains 3g of calcium, with around 9g of calcium in all of the extracellular fluid (Goff, 2004). The fluid within bone canaliculi will contain 6-15g of additional calcium, with the actual size of this calcium store depending on the acid-base status of the cow (Goff, 2004). This store of calcium is comparatively very small, with 99% of the body's calcium reserves (7.8-8.5kg) being stored in bone (Goff, 2014b). Absorption of calcium from the diet normally occurs by passive diffusion in the small intestine if sufficient dietary calcium levels are provided (Moreira *et al*., 2009). However, calcium can also be withdrawn from bone reserves in times of hypocalcaemia in order to prevent blood calcium concentrations from decreasing considerably. This carries a risk of lactational osteoporosis, usually resulting from a loss of 9-13% of skeletal calcium (Goff, 2004), but this condition can be reversed later in the lactation without being detrimental to health in the long-term.

Calcium homeostasis is regulated by parathyroid hormone (PTH), 1,25-dihydroxyvitamin D and calcitonin, which is secreted when blood calcium levels are elevated (Figure 1.1) (DeGaris & Lean, 2009). PTH is released by the parathyroid gland when blood calcium levels decline, and this leads to the increased renal reabsorption of calcium (Horst *et al*., 1997). This only allows for the recovery of small amounts of calcium as urinary calcium excretion is relatively low (Goff, 2008). If the calcium deficit is small then this increased reabsorption in the kidney will be sufficient to restore normal plasma calcium levels, and PTH will return to its baseline level. However, in cases of more

severe hypocalcaemia, the release of PTH will continue in order to stimulate the mobilisation of calcium stored in bone (Horst *et al*., 1997; El-Samad *et al*., 2002). When PTH concentrations increase, salts are removed from the bone matrix by the process of osteocytic osteolysis (El-Samad *et al*., 2002). This occurs to meet short-term calcium needs but if high levels of PTH continue to be present then osteoclasts in bone will be activated and the process of osteoclastic bone resorption will be initiated (El-Samad *et al*., 2002).

Intestinal absorption of calcium occurs by both active transport across epithelial cells and passive transport between epithelial cells (Goff and Horst, 1993). Active transport of calcium across intestinal epithelial cells is stimulated by the hormone 1,25-dihydroxyvitamin D (also referred to as calcitriol) (El-Samad *et al*., 2002), which is produced from vitamin D by the kidney. This hormone production occurs in response to increased levels of PTH in the blood (Goff, 2008) and its secretion increases both the intestinal absorption and bone resorption of calcium (DeGaris & Lean, 2009).

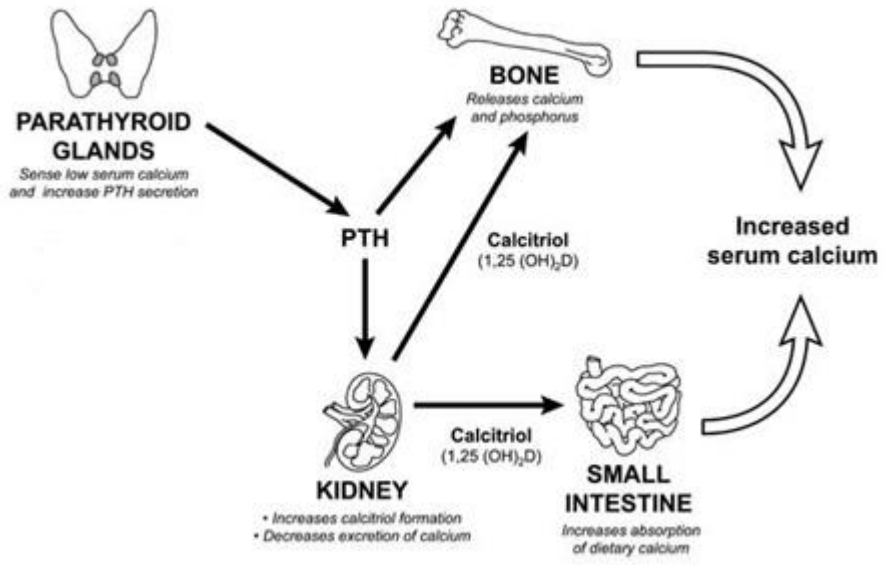


Figure 1.1. The mechanism of calcium homeostasis (a*dapted from Linus Pauling Institute, http://lpi.oregonstate.edu/infocenter/vitamins/vitaminD/dendocrine.html*)

The thyroid gland is also involved in calcium homeostasis. If blood calcium rises above a set level, the hormone calcitonin is released by the thyroid. This inhibits bone resorption of calcium and enhances the release of calcium by the kidney in order to lower blood calcium levels (Campbell *et al*., 2008).

Oestrogen also has a role, as its concentration increases during the final days of gestation. High levels of oestrogen reduce DMI, bone resorption and intestinal absorption of calcium, and also reduce responsiveness to PTH, exacerbating the problem of hypocalcaemia (Bigras-Poulin & Tremblay, 1998).

A large proportion of fully-grown adult cows appear to successfully adapt to the onset of lactation, increasing both intestinal absorption and bone resorption of calcium in order to meet the greater demands required for the production of colostrum and milk. However, in some cases this rapid adaptation does not occur soon enough due to the breakdown of regulatory mechanisms, and such cows experience hypocalcaemia (El-Samad *et al*., 2002). Hypocalcaemic cows are reported to actually have increased levels of PTH and 1,25-dihydroxyvitamin D circulating within the blood, but it is proposed that the target cells are less responsive to this hormone in affected animals (Block, 1994).

1.2.2. Clinical signs of milk fever

Cases of milk fever are typically associated with a number of clinical signs. Cows may exhibit cold extremities, a low temperature, poor appetite, mild bloat and constipation (due to gastrointestinal stasis caused by a reduction in smooth muscle function). Affected animals may become unsteady on their feet and an inability to stand will eventually occur, sometimes with an accompanying characteristic S-shaped bend in the neck.

1.2.3. Diagnosis of milk fever and subclinical hypocalcaemia

Most diagnoses of milk fever are made through observations of clinical signs. Clinical observations are the quickest way of diagnosing milk fever and so are generally most effective in facilitating rapid treatment of the condition.

Clinical chemical parameters can also be used, both for the diagnosis of clinical milk fever and subclinical hypocalcaemia. Cows with blood calcium concentrations of 1.4-2.0mmol/l are generally considered to have subclinical hypocalcaemia (Houe *et al*., 2001), with a diagnosis of clinical milk fever typically applying to those with blood calcium concentrations of <1.4mmol/L (Roche & Berry, 2006).

Calcium in the blood exists in three different fractions: ionised (free calcium), complexed/chelated, and protein-bound (Schenck & Chew, 2008). Ionised calcium concentrations in plasma indicate the levels of the physically active form of calcium within a cow. However, analysis of these concentrations is subject to problems associated with sample handling, analytical performance and the standardisation of analysers (Pfitzenmeyer *et al*., 2007). Larsen *et al*. (2001) investigated the suitability of measurements of ionised calcium in plasma for the diagnosis of subclinical hypocalcaemia in dairy cattle. Due to the potential loss of carbon dioxide during sample handling and transportation, ionised calcium measures were only used when the pH measured between 7.2 and 7.6 at the time of analysis, and this excluded a number of samples from the study. Samples with an acidic pH will favour the dissociation of calcium from proteins and so the ionised calcium concentration will appear to increase, with an alkaline pH having the opposite effect (Schenck & Chew, 2008). The aerobic collection of serum will lead to an increased pH, affecting ionised calcium concentrations, and so anaerobic sample collection is preferred (Schenck & Chew, 2008). Ionised calcium concentrations can be obtained from whole blood or plasma, although both

pH and ionised calcium levels are more stable in serum and can be stored for longer (Schenck & Chew, 2008). Heparin in samples can also have an effect, as its presence dilutes samples. Lithium heparin leads to an underestimation of ionised calcium (Lyon *et al*., 1995), with zinc heparin having the opposite effect, and it is important to take this into consideration during sample collection (Schenck & Chew, 2008).

Larsen *et al*. (2001) found a positive correlation between total calcium and adjusted ionised calcium in plasma samples ($P<0.001$) and so total blood calcium concentrations may be a reliable indicator of ionised calcium levels when diagnosing hypocalcaemia. However, this approach assumes that total calcium measurements directly correlate to ionised calcium concentrations and in some clinical conditions this has been shown not to be the case (Schenck & Chew, 2008). In dogs it has previously been suggested that total calcium levels should be 'corrected' in relation to total protein concentrations, due to the fact that a proportion of total calcium will be protein-bound and therefore not ionised. However, Schenck & Chew (2008) now argue that this is unreliable due to diagnostic disagreement between the correction formula and ionised calcium measurements in some clinical conditions. This is supported by Björkman *et al*. (2009), who found that corrected values for total calcium concentrations consistently failed to give an accurate representation of true calcium status.

1.2.4. Incidence and prevalence

Typically the incidence of milk fever in dairy cows is considered to be 5-10% per lactation (Sørensen *et al*., 2002; Mulligan *et al*., 2006a; DeGaris and Lean, 2008; Hayes *et al*., 2012), with Husband (2005) estimating a typical UK incidence of 4-9%. However, there is considerable variation between herds in terms of milk fever incidence, with reported incidence rates amongst some herds being as high as 60% in some UK herds in September and October

(Andrews et al., 2004). Parker-Gaddis *et al*. (2012) suggest that milk fever may not have a high priority amongst farmers and their study suggests that incidence levels within herds may be under-reported.

The prevalence of subclinical hypocalcaemia amongst herds is not currently known (Reinhardt *et al*., 2011). The subclinical incidence of hypocalcaemia in the national herd in the USA has been estimated at 50% in second lactation or greater cows by Oetzel (2013), and in New Zealand it was found by Roche *et al*. (2002) that incidence was 30-40% in grazing cattle. Houe *et al*. (2001) suggest that for every clinical case of milk fever within a herd, there will be another 3-6 cases of subclinical hypocalcaemia. Goff *et al*. (1996) found that the mean blood calcium concentration was <6.5mg/dl (<1.63mmol/l), and from this data suggest that subclinical hypocalcaemia is likely to be a widespread problem. Subclinical hypocalcaemia may not be obviously visible but does lead to a reduction in smooth muscle function (Houe *et al*., 2001; Roche and Berry, 2006).

1.2.5. Predisposing factors

It is normally around the time of calving and the onset of lactation that hypocalcaemia is seen in dairy cows. However, not all animals are equally at risk of suffering from milk fever, as factors such as breed and parity have an influence on the likelihood of a newly calved cow suffering from the disease.

- #### 1.2.5.1. Breed

Increased susceptibility to milk fever has been seen in certain breeds. Channel Island breeds have been reported to have a higher incidence of milk fever compared to Holstein-Friesian cattle (Roche & Berry, 2006; Horst *et al*., 1997), with Lean *et al*. (2006) finding a 2.25 times greater risk of milk fever in Jersey cows compared to Holstein-Friesians. Swedish Red and White cattle have been seen to be at significantly greater risk of milk fever than Swedish

Friesians (Bendixen *et al*., 1987), and Finnish Holstein-Friesians had a higher risk of milk fever than Finnish Ayrshires (Schnier *et al*., 2004).

The reasons for this increased risk in some breeds have not been fully elucidated. However, Goff *et al*. (1995) found a reduced number of intestinal 1,25-dihydroxyvitamin D_3 receptors in Jerseys compared to Holsteins of the same age. This would lead to reduced sensitivity to 1,25-dihydroxyvitamin D_3, meaning that the post-parturient response of increasing bone calcium resorption and intestinal calcium absorption would be impaired (Horst *et al*., 1997).

- 1.2.5.2. Age and parity

The risk of hypocalcaemia has been seen to increase with age (Reinhardt *et al*., 2011). Milk fever is very rare in heifers, reported at an incidence rate of 0.2% by Koeck *et al*. (2012). This is due to an existing upregulation of calcium homeostasis mechanisms (Martín-Tereso *et al*., 2011) but the risk of hypocalcaemia has been seen to increase with each subsequent calving (Horst *et al*., 1997; Bigras-Poulin & Tremblay, 1998), with a marked increase in the incidence of milk fever after the third lactation (Curtis *et al*., 1984). A number of studies have shown that an inverse correlation of serum calcium concentrations and age exists (Chan *et al*., 2006). However, the proposed reasons behind this vary, with some findings being made in association with reduced bone resorption or intestinal absorption efficiency (Anderson, 1991) and others suggesting a link to age-related dry matter intake (Marquardt *et al*., 1977). A negative curvilinear relationship between serum calcium values and age has been seen and, based on this model, Bigras-Poulin & Tremblay (1998) postulate that a two-year-old cow would have its blood calcium level reduced by 0.3mmol/l after five years. Reinhardt *et al*. (2011) found a correlation between increasing age and increasing incidence of subclinical hypocalcaemia, with serum calcium levels below the normal range being

recorded in 41% of second-lactation cows, compared to 25% of heifers. This is supported by the study of Chan *et al*. (2006), which found that primiparous cows had significantly higher serum calcium concentrations than multiparous animals. The study of Reinhardt *et al*. (2011) also found subclinical hypocalcaemia of a degree capable of compromising immune function (Kimura *et al*., 2006) in 47% of cows in their second lactation onwards. Heifers are still growing and therefore already have an upregulated calcium homeostatic system and so are able to successfully adapt to the calcium demands of the first lactation.

Older cows tend to have a higher milk yield, although this is partly due to on-farm selection reasons and the increased risk of culling for cows with lower yields. A higher milk yield increases the demand for calcium (Horst *et al*., 1997). The efficiency of intestinal calcium absorption and the resorption of calcium from bone decrease with age. Also, the production of 1,25-dihydroxyvitamin D_3 is seen to be impaired (Horst *et al*., 1997) and a reduction in sensitivity to it is also seen as the numbers of intestinal receptors for 1,25-dihydroxyvitamin D_3 decline (Horst *et al*., 1997). The combination of these age-related impairments leads to a reduced ability to effectively respond to acute challenges to calcium homeostasis (Horst *et al*., 1997). It has also been postulated that periparturient hypocalcaemia might be considered normal in older cows, due to decreased bone calcium content as a consequence of the ageing process (Reinhardt *et al*., 1988).

Cows that have previously had milk fever have an increased susceptibility to the condition in subsequent lactations. Roche and Berry (2006) found that cows were 2.2 times more likely to be affected by milk fever if they have previously been diagnosed when compared to those with no history of the condition.

- 1.2.5.3. Body condition

Body condition scoring (BCS) can be used to assess an animal's proportion of body fat, using palpation, and can have an impact on the incidence of metabolic disorders (Roche *et al*., 2009). Using a BCS scale of 1-5 (thin to fat), it has been seen that over-conditioned cows with a score of 4 or greater during the dry period have an increased likelihood of developing milk fever (Heuer *et al*., 1999). The reasons behind this are not fully understood. However, it has been suggested that the onset of hypocalcaemia may be linked to the reduced dry matter intake (DMI) seen in over-conditioned cows immediately prior to calving (Hayirli *et al*., 2002) or to the increased milk yield seen in such cows, owing to their comparatively greater body reserves (Roche *et al*., 2005).

Kim and Suh (2003) found that cows with a drying-off BCS of 3.8 lost significantly less condition than cows with a BCS of 4.3 and also had a reduced incidence of milk fever, and, as such, body condition score management has been used as a tool for reducing the risk of milk fever (Sørensen *et al*., 2002). Mulligan *et al*. (2006a) make the recommendation of a target BCS of 2.75 at drying-off and 3.0 at calving, although a drying-off BCS of 3.25-3.50 has been regarded as an industry standard (Contreras *et al*., 2004). However, it is important to consider the various criticisms of BCS evaluation. Both inter- and intra-assessor repeatability of measurement of body condition scoring is important (Vasseur *et al*., 2013), as the technique is subjective and is not considered to be particularly sensitive (Fischer *et al*., 2015). Small but rapid changes in body condition occur in early lactation in dairy cows but Fischer *et al*. (2015) report that these are rarely detected during BCS palpation, and Dugdale *et al*. (2012) report that BCS in horses is more accurate in estimating body fat in non-obese animals but is less useful in obese animals.

1.3. Physiological consequences of hypocalcaemia and the importance of its prevention

Due to the role of calcium, particularly in smooth muscle function and the immune system, hypocalcaemia can have a number of physiological consequences for affected cows (Grohn *et al*., 1989). These consequences have an impact on health and production, as well as affecting profitability within the herd. However, in some instances it is not clear that the relationship between milk fever and another disease is causal. For example, whilst an association between cases of milk fever and ketosis has been seen, this may not be cause and effect but may be reflective of other common underlying factors (Klerx & Smolders, 1997).

1.3.1. Risk of culling

Milk fever significantly increases the risk of culling up to 100-days post-calving, with Hayes *et al*. (2012) reporting that 10.9% more milk fever cows were culled in this time period when compared to unaffected animals (P=0.001). This resulted in cows suffering from milk fever being four times more likely to be culled than cows that did not show the clinical condition (Hayes *et al*., 2012). However, the proportion of milk fever cows culled immediately after diagnosis due to recumbency, compared to the number culled due to other factors occurring following hypocalcaemia, such as displaced abomasum or infertility, is not clear.

1.3.2. Negative energy balance and dry matter intake

A negative energy balance (NEB) can have other health and reproductive implications for affected cattle, including fatty liver syndrome (Bertics *et al*., 1992) and delayed ovulation (Jorritsma *et al*., 2003). It is a metabolic disorder that affects high-yielding cows, which can impair health and affect fertility for a number of months (Wathes *et al*., 2007).

Hypocalcaemia leads to a reduction in gastrointestinal motility, which in turn can lead to a reduced dry matter intake (DMI) (Mulligan *et al*., 2006b). In normocalcaemic cows, DMI is typically reduced anyway prior to calving by around 30% (Hansen *et al*., 2003). However, a reduced feed intake has been associated with even subclinical hypocalcaemia (Hansen *et al*., 2003), meaning that DMI can be affected even without clinical signs of milk fever. This is thought to be due to the associated depressed smooth muscle function, and rumen motility has been shown to be significantly reduced by hypocalcaemia (Daniel, 1983). Hypocalcaemia has been seen to have an effect on rumination (Hansen *et al*., 2003). In theory it is likely that hypocalcaemia will affect oesophageal movements during regurgitation, although this has not always been observed experimentally (Hansen *et al*., 2003). A reduction in jaw movement seen in cows with subclinical hypocalcaemia may be reflective of a reduction in the activity of skeletal muscle (Hansen *et al*., 2003). However, it has been suggested that it is smooth muscle function that suffers a greater reduction due to hypocalcaemia (Hansen *et al*., 2003).

A reduced DMI, leading to the risk of a cow entering negative energy balance (NEB), has a number of potential effects on cow health. Hypocalcaemia is a risk factor for incidence of left displaced abomasum (LDA) (Shaver, 1997). Massey *et al*. (1993) found that cows that had subclinical hypocalcaemia at calving were at 4.9 times greater risk of developing LDA, and this is supported by a study that induced hypocalcaemia in ruminants and found a reduction in gastrointestinal motility (Daniel, 1983). A reduction in rumen motility leads to a reduction in dry matter intake, reducing rumen fill and consequently increasing the risk of LDA (Mulligan *et al*., 2006b). Fourichon *et al*. (1999) reported a reduction in milk yield associated with cases of displaced abomasum, meaning that the occurrence of the condition potentially has an economic impact throughout that lactation.

Ketosis can occur following a reduction in DMI (Baird, 1982). Cows that have had milk fever are at an increased risk of ketosis (Klerx & Smolders, 1997), although it is not clear if this is a direct effect of milk fever. Ketosis has been linked to an increased calving to first service interval (Melendez *et al*., 2003), which has an economic impact within the herd. Cows with ketosis have also been seen to be over fifty times more likely to suffer from LDA (Melendez *et al*., 2003). However, it has been suggested that it is unclear whether ketosis is a cause or effect of an increased risk of this (Melendez *et al*., 2003).

Incidence of fatty liver is also associated with a reduced DMI. This in turn increases the risk of conditions such as mastitis, LDA, retained foetal membranes and poor fertility (Bremmer *et al*., 2000).

1.3.3. Fertility

Milk fever has been linked to a number of conditions in dairy cattle that relate to fertility, including retained foetal membranes (Klerx & Smolders, 1997), endometritis (Whiteford & Sheldon, 2005), and dystocia (Mulligan *et al*., 2006b). Both clinical and subclinical hypocalcaemia have been seen to have an effect on reproduction, although it is not always clear as to whether the effects on fertility are directly due to hypocalcaemia or if any reproductive problems occur as a secondary consequence of other conditions. Uterine involution in hypocalcaemic cows has been seen to take longer than in cattle with normal blood calcium concentrations (Kamgarpour *et al*., 1999; Whiteford and Sheldon, 2005). Hypocalcaemic cows have an extended calving to first ovulation interval (Risco et al., 1994; Kamgarpour et al., 1999) and have smaller follicles (Kamgarpour et al., 1999). Jonsson and Daniel (1997) found that inducing hypocalcaemia in sheep led to a reduction in blood flow to the ovaries. Hayes *et al*. (2012) found that cows that had been diagnosed with milk fever took 13 days longer to become pregnant when compared to unaffected animals. Milk fever can lead to a reduction in DMI and this can

cause a more severe negative energy balance, which will have a detrimental effect on fertility (Wilde, 2006). However, Fourichon *et al*. (1999) conducted a meta-analysis to look at the effects of disease on reproductive health and their findings suggest no link between milk fever and impaired reproduction.

1.3.4. Retained foetal membranes

Retained foetal membranes (RFM) is diagnosed when foetal membranes have not been expelled after twenty-four hours post-partum (Sheldon *et al*., 2008). Cows with milk fever are at an increased risk of developing RFM (Klerx & Smolders, 1997). Animals that have suffered dystocia, which can be linked to milk fever, have an increased risk of RFM (Melendez *et al*., 2003). However, cases of RFM may have other causes and may be multifactorial.

RFM has a negative impact on health and production. It has been suggested that there may be a link between cases of retained placenta and a reduced milk yield (Rajala and Gröhn, 1998). However, in this particular study it was noted that many cases of retained placenta were not promptly treated and, as such, the effects of this condition may have been overstated.

Cows with RFM have been seen to be significantly more likely to develop metritis (Melendez *et al*., 2003). This has an impact on fertility as it is associated with an increased number of services required for conception (Melendez *et al*., 2003). Placenta retention is linked to a reduced likelihood of conception at first service. Melendez *et al*. (2003) found that cows that had suffered from retained foetal membranes (RFM) were 60% less likely to conceive at first service. Hayes *et al*. (2012) reported that cows suffering from RFM had a 24.5% lower 100-day in-calf rate than cows that had not experienced RFM ($P<0.001$). Cows that had suffered RFM were also significantly less likely to be pregnant at 200-days post-calving, with 19.9% fewer diagnosed as being in-calf compared to non-RFM cows ($P=0.001$).

Potter *et al*. (2010) found that RFM was the most important risk factor for cows developing clinical endometritis, due to the necrotic material remaining in the uterine lumen providing an ideal environment for bacterial growth. RFM is linked to an increased risk of metritis, which in turn leads to an increased risk of abnormal oestrus (Klerx & Smolders, 1997).

1.3.5. Metritis and endometritis

Clinical metritis is the infection of the cavity, lining and deep layers of the uterus and is characterised by abnormal uterine enlargement and the presence of purulent uterine discharge detected in the vagina, but without detectable systemic illness, within 21 days post-calving (Sheldon *et al*., 2008). Endometritis does not affect the deeper layers of the uterus but instead is a localised infection of the uterine lining, and so enlargement of the uterus does not occur (Sheldon *et al*., 2008).

Hypocalcaemia has been linked to increased incidence of endometritis. Although the reasons for this correlation are not fully understood, it has been suggested that hypocalcaemia may predispose cows to develop uterine infection due to a delay in expulsion of the lochia (Whiteford and Sheldon, 2005), and this can have a detrimental effect on fertility. This has an impact on reproductive success, as cows with endometritis have a delay in the number of days before the first service and also an increased number of services required for conception (Gilbert *et al*., 2005). Endometritis leads to infertility whilst the infection is present and cows that have suffered from uterine infections will have impaired fertility in the future (Sheldon and Dobson, 2004). However, Cheong *et al*. (2011) did not find hypocalcaemia to be a significant risk factor for endometritis and Potter *et al*. (2010) also found the risk to be very low when compared to other factors. Despite the link to hypocalcaemia appearing to be insignificant, in this study the highest risk factor was found to be placenta retention. Whilst the causes of retained

placenta are considered to be multifactorial (Han and Kim, 2005), the condition has been linked to hypocalcaemia (Klerx & Smolders, 1997).

Both retained foetal membranes and metritis have been linked to delayed uterine involution, and this has been seen to lead to an increased number of services required for conception (Melendez *et al*., 2003). Metritis is associated with an increased risk of conception failure and subsequent culling (Bell & Roberts, 2007). Uterine infection has also been shown to lead to a reduction in DMI post-calving, leading to a drop in milk yield in early lactation (Bell & Roberts, 2007).

1.3.6. Dystocia, uterine prolapse and stillbirth

Milk fever leads to reduced uterine motility and it has been reported that cows with milk fever are three times more likely to exhibit dystocia when compared to normocalcaemic cows (Mulligan *et al*., 2006b). Dystocia has the direct effect of prolonging calving and increasing the risk of stillbirths, but also has an impact on the start of the lactation and has been seen to significantly reduce milk yield in early lactation, particularly amongst multiparous cows (Rajala and Gröhn, 1998). Cows with dystocia have been seen to be 2.2 times more likely to suffer from retained foetal membranes, which in turn has been associated with reduced fertility and a reduced pregnancy rate at first service (Melendez *et al*., 2003). Cases of uterine prolapse have also been associated with hypocalcaemia. Risco *et al*. (1984) found that serum calcium levels were significantly lower in cows with uterine prolapse than in those without. Whilst milk fever may increase the risk of dystocia and thus indirectly increase the risk of stillbirths, Markusfeld (1987) found no direct link between milk fever and stillbirth.

1.3.7. Mastitis

Milk fever has been linked to an increase in the concentration of plasma cortisol (Horst and Jorgensen, 1982), which may lead to increased immunosuppression and could increase the risk of clinical mastitis (Berry *et al*., 2007). Kimura *et al*. (2006) also found a link between reduced intracellular calcium storage and immunosuppression.

The impaired smooth muscle contraction associated with hypocalcaemia can also increase the risk of mastitis, due to the reduced effectiveness of the closure of teat sphincters (Goff and Horst, 1997), enhancing the risk of the invasion of bacteria. A link has been seen between incidence of milk fever and the increased risk of mastitis, with Curtis *et al*. (1983) suggesting that milk fever cows are eight times more likely to contract the condition.

1.3.8. Effect on milk yield

Milk yield can be reduced due to a negative energy balance associated with a reduction in DMI, which can occur following cases of retained foetal membranes, displaced abomasum and ketosis, all potentially linked to hypocalcaemia (Wilde, 2006). It has been postulated that a clinical case of milk fever may reduce the yield during that lactation. However, Rajala-Schultz *et al*. (1999) suggest that it is difficult to find evidence of this effect due to cows with higher yields being those at greater risk of milk fever, meaning that in theory such cows might yield below their potential, although this may still be higher than for healthy animals with a naturally lower milk yield.

1.4. Treatment and prevention strategies

If identified quickly enough, clinical cases of milk fever can often be treated. However, cows that have experienced milk fever are at a greater subsequent risk of a number of other conditions and so prevention of clinical and subclinical hypocalcaemia is important. There are three main strategies of

milk fever prevention in dairy cows: targeted supplementation of calcium peripartum, the dietary cation-anion difference (DCAD) approach, and the use of low calcium diets. Recent research into the use of dietary calcium binders suggests that these may be a useful means of achieving a low calcium diet.

1.4.1. Treatment of existing clinical cases

When clinical cases of milk fever are diagnosed, treatment is required as soon as possible to minimise the potential negative effects of recumbency. The fastest method of treating clinical milk fever is to administer calcium salts intravenously, most commonly in the form of calcium borogluconate (Goff, 2008), although rapid IV infusions of calcium do carry the risk of cardiac arrest (Horst *et al*., 1997; Goff, 2008) and relapse occurs in approximately 25% of treated animals (Horst *et al*., 1997). Subcutaneous calcium salts can be provided, although absorption rates can vary due to potentially compromised peripheral blood flow during recumbency (Goff, 2008; Oetzel, 2013). Calcium salts can also be injected intramuscularly, but only low doses of calcium can be provided at each injection site to minimise the risk of tissue necrosis, and so this method of treatment is not widely recommended (Goff, 2008).

1.4.2. Targeted peripartum calcium supplementation

High oral doses of soluble calcium can be provided both before and after calving and have been seen to reduce incidence of hypocalcaemia when used prophylactically (Pehrson *et al*., 1998). This can be administered in a number of different forms, including oral drenches, gels and boluses. Two main types of calcium supplementation include the use of calcium chloride and calcium propionate, as discussed in more detail in sections 1.4.2.1 and 1.4.2.2.

- #### 1.4.2.1. Calcium chloride

Calcium chloride is 36% calcium and so, when used in a drench or gel, provides a highly concentrated dose that can be rapidly absorbed (Horst *et al*.,

1997). The high concentration of calcium provided in this form increases blood calcium levels. It also has an acidifying effect on urine (Goff and Horst, 1994) and it has been suggested that its acidifying effect on the blood may be beneficial, as this may lead to an increased rate of calcium absorption from the gastrointestinal tract (Pehrson *et al*., 1998), due to the increased responsiveness of tissues to PTH (Goff and Horst, 1994). However, it is also possible that metabolic acidosis can be induced to a level that is detrimental (Goff & Horst, 1997), reducing DMI (Goff *et al*., 1996). Calcium chloride is also very caustic and has been reported to cause muscosal damage to the forestomach and abomasum (Wentink & van den Ingh, 1992). Its usage may also lead to the ulceration of existing abrasions in the mouth (Goff & Horst, 1994). Calcium chloride is typically administered in a dose of 50g to provide the best absorption (Oetzel, 2013).

- 1.4.2.2. Calcium propionate

Calcium propionate has been used as an alternative form of oral calcium supplementation around calving (Pehrson *et al*., 1998) and is considered to be more palatable than calcium chloride (Goff & Horst, 1993). However, it is a less concentrated form of calcium, meaning that larger volumes need to be administered (Horst *et al*., 1997), usually in doses of 75-125g (Oetzel, 2013). Goff and Horst (1994) found that it has a less rapid effect than that of calcium chloride, although its effect was more sustained. It has also been suggested that the gluconeogenic properties of propionate may be beneficial in reducing the risk of ketosis (Goff & Horst, 1994), as its administration has been shown to improve the energy balance post-calving (Goff *et al*., 1996), although the propionate levels supplied in this treatment are relatively low (Pehrson *et al*., 1998). Melendez *et al*. (2003) did not find a reduced incidence of ketosis when supplying propionate peripartum. It has also been suggested that propionate may actually increase the incidence of LDA (Goff *et al*., 1996). The use of calcium propionate has been shown to reduce incidence of milk fever in

Jersey cows (Goff *et al*., 1996). However, Goff *et al*. (1996) also found that there was no increase in blood calcium concentrations in the Holstein cows also given calcium propionate. Calcium propionate administration has been shown to have no effect on the pH of blood or urine (Goff & Horst, 1994), meaning that whilst it does not have a beneficial effect on increasing calcium absorption, it also does not cause detrimental severe metabolic acidosis (Goff & Horst, 1993). It has also been seen to have no effect on reducing peripartum disorders and does not appear to increase milk yield or fertility (Melendez *et al*., 2003).

- 1.4.2.3. Issues surrounding peripartum calcium supplementation

One major weakness of the calcium bolus approach is the need to accurately predict calving times. Manufacturers of calcium boluses typically recommend that the first dosage is given before calving, which may be 12 hours before calving (Oetzel, 2013) and sometimes as early as 24 hours before expected parturition. However, this is not always easy to predict and so some cows may calve without the 'protection' of this calcium supplementation. This strategy is also labour-intensive, as it requires all cows to be individually handled, and this may be time-consuming and stressful to the animals. It could also be seen as more of a 'treatment' strategy than a true preventative, as supplementing calcium around calving can in fact delay the adaptation of the calcium homeostatic mechanisms, and so can actually exacerbate issues of calcium homeostasis at this time (Agger, 2003).

Oral calcium administration can also be associated with the risk of overdose, with 250g of soluble calcium being fatal in some cows (Goff, 2008). Liquid drenches also carry the risk of aspiration pneumonia, particularly in recumbent animals (Goff & Horst, 1994). Oil-based gels have been marketed with the aim of being more palatable than water-based products, although the calcium supplied in this way has been shown to be less readily absorbed,

and this may be due to the oil acting as a calcium-binder and forming an insoluble calcium soap (Goff & Horst, 1994).

- 1.4.2.4. Supplying combinations of different forms of calcium

Different forms of calcium can be combined in order to maximise the benefits and minimise the negative effects of the individual compounds. This approach is seen in commercial boluses, some of which aim to supply both fast and slow-release forms of calcium. It has been proposed that a combination of prophylactic treatment with both calcium chloride and calcium propionate may be beneficial (Goff *et al*., 1996). The acidification of the blood associated with calcium chloride supplementation may serve to improve intestinal absorption and bone resorption of calcium if supplied at parturition, whilst calcium propionate supplementation around twelve hours post-partum may have additional benefits due to its gluconeogenic precursors and sustained release of calcium.

1.4.3. Dietary cation-anion difference (DCAD) / dietary cation-anion balance (DCAB)

Manipulating the dietary cation-anion difference (DCAD) has been used as a strategy for the prevention of milk fever. Initially DCAD was calculated as (Na + K) – Cl per 100g of dry matter (Hu *et al*., 2007) but the importance of S has since been noted and DCAD is typically now defined as the milliequivalents of (Na + K) - (Cl + S)/kg of dry matter (Block, 1994). As this difference decreases, pH is also reduced (Block, 1994). This relationship is not linear, as shown in Figure 1.2. Initially a decrease in DCAD will have a very small effect on urine pH and this would not be detectable through testing with a pH meter. However, the fall in pH will becoming steeper within the range of 7.5 to 5.5, as shown in Figure 1.2 (Husband, 2005).

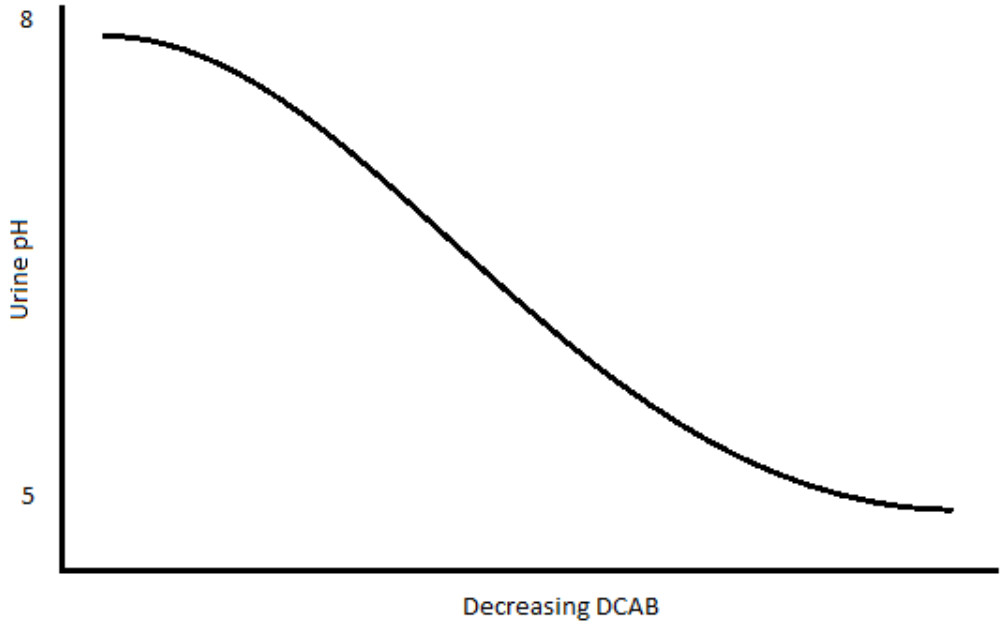


Figure 1.2. The non-linear relationship between decreasing DCAB and urine pH (Husband, 2005).

Other minerals can have an effect on the acid-base balance of blood but it is Na^+, K^+, Cl^- and S^{2-} ions that have the greater relevance to blood pH manipulation in dairy cows (Block, 1994). Inducing mild metabolic acidosis in this way has been shown to elevate blood calcium levels. The mechanism by which this occurs is not understood (Bethard *et al*., 1998). However, Horst *et al*. (1997) suggest that acidosis increases the responsiveness of tissues to PTH, which would have the effect of increasing resorption of calcium from bone.

Practically, the addition of anionic salts to the ration can be used to reduce the DCAD of the diet (Thilsing-Hansen *et al*., 2002b; Husband, 2005). The required quantity of salts will vary due to the DCAD of the existing dry cow ration and also according to the salt itself (Thilsing-Hansen *et al*., 2002b). The recommended length of the feeding period of anionic salts varies widely between studies, typically from 21 to 45 days (Thilsing-Hansen *et al*., 2002b), although Oetzel (1996) suggests that feeding for 10 days may be adequate. Urine acidification can be achieved within just 3 to 5 days of anionic salt

supplementation but such a short period of feeding may be unreliable, due to some cows calving earlier than predicted, and so supplementation for the entire transition period has been recommended (Husband, 2005). From a practical perspective, anionic salt supplementation is only viable if the DCAD of the existing diet is below 250meg/kg (Horst *et al*., 1997). Forage analysis can be carried out but DCAD values will vary considerably between silage crops. Anionic salt supplementation in itself can be expensive, increasing feed costs on farms using this strategy (Bethard *et al*., 1998).

Thilsing-Hansen *et al*. (2002a) compared the findings of experiments where feeds of varying DCAD values were fed and found a clear tendency towards reduced incidence of hypocalcaemia when lower DCAD feeds were provided. Incidence of milk fever was found to be below 20% in cattle fed negative DCAD diets, compared to milk fever incidence rates of 0-80% in cows provided with positive DCAD feeds (Thilsing-Hansen *et al*., 2002a).

Blood pH has an impact on incidence of hypocalcaemia and so the manipulation of the DCAD can be used to potentially prevent occurrence of milk fever (Block, 1994). If a negative DCAD can be achieved then mechanisms of calcium homeostasis have been seen to be more efficient (Block, 1994). The effects of a negative DCAD diet may be due to increased mobilisation of calcium from bone or increased intestinal calcium absorption (Block, 1994). However, a negative DCAD value can be difficult to achieve due to the variable macromineral concentrations of common forages (Mulligan *et al*., 2006a). High levels of dietary potassium should be avoided as this will have the effect of raising blood pH, reducing the effectiveness of the calcium homeostatic mechanism (Goff & Horst, 1997).

It has been suggested that a negative DCAD diet must include a high dietary calcium level in order to compensate for the high levels of urinary calcium

excretion associated with this hypocalcaemia prevention strategy (Block, 1994; Oetzel, 1996). However, it has also been argued that adopting a low calcium diet alongside the feeding of anionic salts may be of greater benefit, as it has been suggested that the calcium restriction principle may complement the DCAD strategy (Schonewille *et al*., 1994). If calcium supplementation is to be provided then the addition of limestone may not be suitable for use alongside a DCAD diet as this would have an alkalinising effect and would reduce the efficacy of the anionic salt provision (Goff & Horst, 1998).

DeGroot *et al*. (2010) found that feeding anionic salts pre-partum actually increased milk production and DMI post-calving when compared to a control diet. However, this study also reported no increase in plasma calcium concentrations post-partum, and so the level of supplementation provided in this experiment does not appear to have had any benefit in terms of reducing the risk of hypocalcaemia.

If a negative DCAD dietary strategy is being used with anionic salt supplementation then urinary pH should be used as a monitoring tool (Huber, 1976; Husband, 2005), with acidification of urine to a pH value of between 5.5 and 6.5 being expected within three to five days, compared to the normal alkaline pH of ruminant urine of >8 (Hu *et al*., 2007). Urine pH testing is cheaper than that of blood and may be more sensitive to detecting small pH changes (Goff & Horst, 1997). Urine can be collected through a free-catch or following manual stimulation of the perineum, or through the use of urinary catheters. However, urine pH may be an impractical monitoring strategy for grazing herds (Husband, 2005). The DCAD strategy is best employed when dry cows are able to be housed but Husband (2005) suggests that careful paddock management and the avoidance of fertiliser-usage may allow for low DCAD grazing to be used.

However, whilst it can be effective at preventing milk fever, the DCAD strategy is not without its drawbacks. Anionic salts are widely considered to have poor palatability (Thilsing-Hansen *et al*., 2002a). Inducing even mild metabolic acidosis in cattle has been shown to lead to a reduction in DMI (Huber, 1976; Hu *et al*., 2007). This reduced DMI and potential subsequent negative energy balance has been shown to increase plasma NEFA at calving (VandeHaar *et al*., 1999), and has also been linked to an increased risk of displaced abomasum (Cameron *et al*., 1998). The associated reduction in DMI with feeding anionic salts means that it is not recommended to use the DCAD strategy with heifers (Moore *et al*., 1997), meaning that dry cow groups on farms using this strategy may need to be split according to parity.

The majority of research into the manipulation of DCAD in dairy cattle diets has been into transition cows and so research into the effects moving into lactation has been limited (Roche *et al*., 2005b). The supplementation of anionic salts should be ceased at parturition, as a negative DCAD in early lactation has been shown to reduce milk yield (Escobosa *et al*., 1984; Tucker *et al*., 1988). The potential effects of a DCAD diet on other minerals should also be considered. Short-term supplementation of anionic salts prepartum has been shown to have no effect on selenium metabolism during late pregnancy (Gant *et al*., 1998). The potential impact of the DCAD diet in late gestation on calf health is also to be considered. Morrill *et al*. (2010) found that feeding the DCAD diet to dry cows had no effect on calf IgG levels.

A partial DCAD approach can be successfully used for the prevention of milk fever (Overton, 2004) and can be less labour intensive and less costly than a full DCAD diet. A partial DCAD diet involves the feeding of low potassium forages, such as unfertilised grass and straw, in order to reduce the DCAD without having to supplement with anionic salts (Hutjens & Aalseth, 2005).

Magnesium chloride supplementation may also be used with this approach, supplied either in the feed or drinking water (Hutjens & Aalseth, 2005). Reducing dietary potassium to dry cows is considered advantageous in the prevention of milk fever, even without the stricter monitoring that would be required when a full DCAD strategy is in use (Lean *et al*., 2006).

1.4.4. Low calcium diets

The restriction of dietary calcium pre-calving has been used as a milk fever prevention strategy and cows fed high calcium diets before calving have been seen to be at increased risk of developing milk fever post-calving (Belyea *et al*., 1974). Cows with a high dietary calcium intake will rely more on intestinal absorption of calcium than on resorption from bone (Belyea *et al*., 1974). Feeding a low calcium diet and inducing a negative calcium balance will lead to the activation of mechanisms of calcium homeostasis (Mulligan *et al*., 2006a), increasing calcium absorption from the small intestine and resorption from bone. The decline in blood calcium levels associated with a reduced dietary calcium intake leads to increased production of PTH, stimulating both the resorption of calcium from bone and the production of 1,25-dihydroxyvitamin D by the kidney (Goff, 2008). Cows fed a low calcium diet have been shown to be more resistant to challenges to calcium homeostasis than cows fed high calcium diets and have demonstrated a more rapid return to normal blood calcium levels (Belyea *et al*., 1974; Green *et al*., 1981). Restricted calcium diets have been shown to have no effect on dry matter intakes (Belyea *et al*., 1974; Moreira *et al*., 2009).

However, in order for calcium restriction to be an effective strategy the dietary intake of calcium must be below 20g per day (Wiggers *et al*., 1975; Houe *et al*., 2001), as higher levels of dietary calcium will not cause the required increase in PTH production (Goff, 2008). In practice this low dietary level is difficult to achieve whilst also supplying adequate energy and protein

(Green *et al*., 1981), due to the calcium content of widely used commercial feedstuffs (Thilsing-Hansen and Jørgensen, 2001; Mulligan *et al*., 2006a). The calcium content of forage will also vary considerably between farms, and even between paddocks, which adds further difficulties to the practical use of this strategy (Mulligan *et al*., 2006a). Calcium is also relatively high in grass, typically 0.4-0.8% on a dry matter basis (Pond *et al*., 2005), which is a common diet ingredient, particularly in the UK.

Low calcium diets may not be appropriate in all situations. Oba *et al*. (2011) propose that cows fed negative DCAD diets actually require high levels of calcium supplementation to compensate for the increased responsiveness to PTH, and thus increased levels of calcium mobilisation, brought about by the decrease in blood pH. It is also important when using this prevention strategy that cows are switched to a high calcium ration post-calving (Goff, 2008).

1.4.5. Use of calcium binders

The feeding of calcium binders has been proposed as a possible means of reducing the bioavailability of dietary calcium, mimicking the strategy of utilising low calcium diets (Thilsing-Hansen and Jørgensen, 2001). Calcium binders provided in the ration can irreversibly bind with dietary calcium, meaning that it is not available for absorption by the animal and passes out in the faeces (Goff, 2008). This means that higher levels of calcium can be provided than would otherwise be needed for the low calcium strategy, overcoming the difficulties associated with the formation of such rations, as even on a dietary calcium intake of 30-50g per day the desired hormonal response can still be achieved (Goff, 2008).

Wilson (2003) fed a vegetable oil supplement to dairy cows in order to act as a calcium binder and this led to the upregulation of calcium homeostasis and a reduction in the number of cases of milk fever. However, feeding high-fat

diets pre-partum has been shown to reduce DMI (Douglas *et al*., 2004). At present, there are two main alternative calcium binder options to consider: zeolite and rice-bran.

- 1.4.5.1. Use of zeolite

Synthetic zeolite A is an aluminosilicate that has been commercially used as a water-softener, due to its ion exchange properties, which allow it to bind to calcium (Cook *et al*., 1982). It also has EU approval for use in feedstuffs (EFSA, 2004). Early research into using zeolite as a calcium binder for milk fever prevention required around 1kg/day of zeolite to be consumed, although with chemical modification this has more recently been reduced to nearer 0.5kg/day (Goff, 2014b).

Cows fed synthetic zeolite A for the three weeks prior to calving have been reported to have significantly higher blood calcium levels at calving than cows in a control group (Thilsing-Hansen *et al*., 2003) and this has been proposed to be due to the activation of calcium homeostatic mechanisms, brought about by the shortage of dietary calcium (Thilsing-Hansen *et al*., 2002a). Supplementation with clinoptilolite, a naturally occurring zeolite, has also been seen to be effective in the prevention of parturient hypocalcaemia. Katsoulos *et al*. (2005) found that cows supplemented with 2.5% clinoptilolite had significantly reduced incidence of milk fever compared to the control cows that did not receive the supplement. Experimentally, zeolite supplementation has been shown to have a positive impact on calcium status in multiparous cows when fed in the two weeks prior to calving, with Thilsing-Hansen *et al*. (2003) reporting a mean efficacy of 58% in the prevention of hypocalcaemia. However, no additional benefit has been seen when feeding is increased to four weeks pre-partum (Thilsing-Hansen *et al*., 2003). Thilsing-Hansen *et al*. (2002a) found that the inclusion of zeolite in the ration improved the stabilisation of blood calcium post-calving. However, in this

experiment the mean serum calcium concentration remained above-hypocalcaemia in both the treatment and control group. The animals fed zeolite showed a reduced DMI pre-calving. The TMR fed during this study supplied 66g calcium per day and also included calcium carbonate supplementation peripartum, with 90g calcium provided on the day of calving and on the two days post-partum. Similarly, the work of Thilsing-Hansen & Jorgensen (2001) supplied dry cows with 38g of calcium per day within the TMR, increasing this to 45g per day in the final week before calving.

Zeolite is not calcium-specific (Thilsing *et al*., 2006) and it is possible that it may also bind to ions other than calcium (Grabherr *et al*., 2009). Its addition to feed has been linked to a decrease in serum magnesium levels (Thilsing-Hansen *et al*., 2003) and a decrease in inorganic phosphate levels in the blood (Thilsing-Hansen *et al*., 2003; Grabherr *et al*., 2009). Karatzia *et al*. (2011) found that with clinoptilolite supplementation the levels of phosphorous were only altered in the rumen, not in blood concentrations. When zeolite supplementation was given at a dose level of 700g, a more marked level of hypophosphataemia was seen (Thilsing-Hansen *et al*., 2002a). Chronic cases of hypophosphataemia are linked to poor productivity, although the short-term effects are unknown (Pallesen *et al*., 2008). However, this potential problem can be alleviated through phosphorous supplementation, although this reduces the beneficial impact of zeolite on calcium stability (Pallesen *et al*., 2008). This is potentially due to the capacity of aluminium to bind to phosphorous, reducing the availability of zeolite for calcium binding, and thus reducing the pre-partum stimulation of mechanisms for calcium homeostasis (Pallesen *et al*., 2008). Katsoulos *et al*. (2005) supplemented cows with clinoptilolite and found no significant effects on serum concentrations of phosphate or magnesium after long-term zeolite supplementation.

It is also important to consider potential effects of zeolites on trace elements. However, high doses of zeolite supplementation have been seen to have no effect on levels of copper, zinc, iron and manganese (Grabherr *et al*., 2009). The potential persistence of the mineral-binding effect after the cessation of supplementation and into the early lactation is also a risk and little research has been published on this possibility.

The potential impact of feeding zeolite supplements on dry matter intake (DMI) should also be considered. A reduced DMI pre-calving, leading to a negative energy balance, can lead to an increased risk of displaced abomasum (Cameron *et al*., 1998). A significant reduction in DMI was seen with zeolite supplementation by Grabherr *et al*. (2009). The reasons for this are not known but it has been suggested that it may be due to poor palatability of zeolite (Grabherr *et al*., 2009) or may be a consequence of hypophosphataemia, which has previously been seen to lead to a reduction in DMI (Gartner *et al*., 1982).

Zeolite supplementation has been found to have no effect on milk yield (Thilsing-Hansen *et al*., 2002a) but little research has been published on its effects on milk composition (EFSA, 2004). However, a reduction in milk fat yield has been seen following the inclusion of zeolite in the pre-calving ration (Thilsing-Hansen et al., 2002a). Further research is also needed into the potential for aluminium from zeolite to transfer into milk from a consumer health perspective (EFSA, 2004). Zeolite is not expected to have a negative environmental impact, due to the natural occurrence of zeolites in clay, but it is difficult to fully assess the potential detrimental health effects of zeolite supplementation for dairy cattle due to the limited data available (EFSA, 2004).

- 1.4.5.2. Rice bran

It is possible for dietary fibre to reduce calcium absorption within the digestive tract. This has been seen in humans, with the ingestion of wheat bran being linked to a reduced ability to absorb dietary calcium, which is considered to be due to its phytate content (Weaver *et al*., 1991; Harrington *et al*., 2001). Phytate is thought to reduce calcium availability by binding to it, due to its ability to chelate cations, and forming complexes in the small intestine that cannot be absorbed (Selle *et al*., 2009). Wheat bran consumption has been successfully used to induce a negative calcium balance in humans (Harrington *et al*., 2001). The ingestion of rice bran in particular has been linked to a reduced urinary calcium output in humans (Jahnen *et al*., 1992), which indicates a reduced intestinal absorption of dietary calcium. *In vitro* studies have confirmed the ability of a variety of brans (including rye, soy, wheat, oat and rice brans) to bind with calcium, with rice bran being shown to have the highest calcium binding capacity (Siener *et al*., 2001).

One problem with feeding bran to ruminants for its potential calcium binding properties is that bran and its phytic acid content are rumen-degradable (Clark *et al*., 1986). However, it is possible to treat bran in order to prevent this degradation. One method is to use a fat coating in order to provide rumen-protection. However, this can lead to a reduced DMI (Douglas *et al*., 2004) and dilutes the phytic acid content of the bran, potentially reducing its ability for calcium binding (Martín-Tereso *et al*., 2010). Alternatively, a high fat content can lead to calcium binding in itself, due to the formation of insoluble calcium soaps (Steele, 1984; Palmquist *et al*., 1986).

An alternative method for rumen-protecting rice bran is to use a formaldehyde treatment (Martin-Tereso *et al*., 2009). Whilst formaldehyde does have a known carcinogenic effect, it is also naturally occurring in feedstuffs and its transfer into the feed when used to rumen-protect rice bran

is negligible and falls within the natural occurrence range (Martin-Tereso *et al*., 2009). When treated in this way, Martin-Tereso *et al*. (2009) suggest that the rice bran product could have a phytic acid content capable of binding 7g of dietary calcium/kg of product.

Feeding rumen-protected rice bran to ruminants may be effective in reducing hypocalcaemia in two ways: its addition to the ration reduces the intake of calcium due to its own low calcium content, potentially reducing the intake of some feeds higher in calcium, and also due to its potential as a calcium binder (Martin-Tereso, 2010). The work of Martín-Tereso *et al*. (2010) found that calcium intake decreased when rumen-protected rice bran was supplied to non-lactating pregnant cows. However, a significantly reduced DMI was also seen, corresponding with the time period over which the rice bran treatment diet was supplied, and so in this case it cannot be determined whether the decreased calcium intake was a direct or indirect effect of the treatment diet.

Martín-Tereso (2010) found that cows consuming rumen-protected rice bran during the dry period had higher average blood calcium concentrations than untreated control animals in the subsequent lactation. The treatment group demonstrated a rapid initiation of recovery towards normocalcaemia, suggesting that the calcium homeostatic mechanisms were not suffering a lag time.

Martin-Tereso (2010) suggests a need for further research into the use of rumen-protected rice bran as a milk fever preventative, with the recommendation to trial the use of the product for 2-3 weeks pre-calving, removing the feed at parturition. It is suggested that there is the need to experimentally test varied dosages of the product in order to assess efficacy at reducing milk fever incidence and calcaemic status post-calving. Martin-Tereso (2010) also recommends experimental comparison between the use of

rumen-protected rice bran and a DCAD diet, and the use of a combination of rumen-protected rice bran and oral calcium supplementation at calving. It is also important that the product is trialled on commercial herds, due to the work of Martin-Tereso *et al*. (2011) having been carried out on an experimental farm and using tie-stall housing.

Rumen-protected rice bran, when used as a feed product during the dry period, has a number of potential advantages over other methods of milk fever prevention. Formaldehyde-treated bran has been seen to have no effect on DMI and, unlike the use of DCAD diets, it does not induce metabolic acidosis, thus avoiding related negative health effects, such as an impaired insulin response (Bigner *et al*., 1996). The defatting of rice bran reduces its susceptibility to rancidity (Martin-Tereso, 2010), improving its practicality for storage. It also has practical benefits in its application, such as its apparent lack of detrimental effects on heifers, which allows for its safe inclusion in the ration for all parities of dry cows (Martin-Tereso, 2010). Rumen-protected rice bran also has advantages over zeolite in that it is a rich source of dietary phosphorous and magnesium (Martin-Tereso, 2010), with the blood concentrations of both having been shown to be reduced following the dietary inclusion of zeolite.

1.5. Conclusion

The transition period, from the pre-calving dry cow to the newly-calved fresh cow, is an important stage in the lifecycle of a dairy cow. The hormonal regulation system for maintaining blood calcium concentrations must adapt to the onset of lactation and the increased demands for calcium placed on the body at this time. Many cows fail to adapt to this change in calcium demands quickly enough and thus become hypocalcaemic, or experience clinical milk fever. This condition has a number of effects on the animal, which can include the risks of entering a negative energy balance and ketosis, the increased risk

of developing mastitis (Curtis *et al*., 1983), reproductive problems such as the increased risk of RFM, metritis, endometritis and dystocia, reduced milk yields, and the increased risk of culling (Hayes *et al*., 2012). Not only do these conditions have welfare implications for individual animals, but they can also have a large economic impact on dairy farmers (Husband, 2005; Oetzel, 2013).

There are several main approaches to milk fever and subclinical hypocalcaemia prevention that are currently available to farmers. These can be broadly split into 'treatment' type approaches, such as the targeted supplementation of calcium around calving, and options that would be seen more as true 'preventatives', such as the more diet-based approaches. This includes DCAD diets and the use of low calcium dietary strategies, although both have practical drawbacks, as previously discussed. The feeding of calcium binders within the diet is a fairly recent development, and this approach aims to provide the benefits of feeding a low calcium diet whilst mitigating some of the practical issues with implementing such a strategy.

Zeolite has been proposed as a suitable dietary calcium binder and has experimentally been reported to increase blood calcium levels around calving (Thilsing-Hansen *et al*., 2003). However, there are potential drawbacks with using this approach, including the risk of hypophosphataemia (Grabherr *et al*., 2009), and issues of reduced DMI, suspected to be due to poor palatability. The feeding of rice bran as a milk fever preventative has been proposed as an alternative, due to its high phytate content and its ability to bind to dietary calcium (Weaver *et al*., 1991). The issue of rumen degradation of bran and its phytic acid content has previously been an issue with this approach. However, in recent years, research into formaldehyde treatments of bran have enabled the development of a 'rumen protected' rice bran, which is proposed to have a calcium binding capacity of 7g of calcium per kilogram of product (Martin-Tereso *et al*., 2009). Research in experimental herds has shown that feeding

rumen-protected rice bran to dry cows can increase the blood calcium concentrations of treated animals in the subsequent lactation when compared to untreated control animals (Martin-Tereso, 2010). However, the research to date is limited and further research is required into the use of this concept and product.

1.6. Research aims and objectives

This thesis focuses on milk fever and subclinical hypocalcaemia in the UK dairy industry, and aims to investigate the following topics:

- the attitudes of farmers and veterinarians within the UK towards milk fever and subclinical hypocalcaemia, and their current practices with regard to its prevention
- risk factors for a case of clinical milk fever and the potential associations between milk fever and other events during a lactation in recent years in UK dairy herds
- the potential for the feeding of rumen-protected rice bran during the dry period to improve the calcaemic status of cows around calving for the prevention of milk fever
- the impact of feeding rumen-protected rice bran during the dry period on the energy status of cows in the early lactation
- the impact of feeding rumen-protected rice bran during the dry period on the milk yield of cows in the subsequent lactation

CHAPTER 2: THE ATTITUDES OF UK DAIRY FARMERS AND VETERINARIANS TOWARDS MILK FEVER AND ITS PREVENTION

2.1. Introduction

Hypocalcaemia in dairy cows is considered to be an economically important condition to the industry (Horst *et al*., 1997; Oetzel, 2013) and has a negative impact on animal welfare, including the reported increased risk of retained foetal membranes (Klerx & Smolders, 1997) and mastitis (Curtis *et al*., 1983). There are several approaches to preventing clinical milk fever and subclinical hypocalcaemia, detailed in Chapter 1. However, there appears to be very little information about the opinions and attitudes of farmers and veterinarians about milk fever and its prevention. Whilst estimates of milk fever incidence have been published (Mulligan *et al*., 2006a; DeGaris & Lean, 2008), there is limited evidence about what methods farmers choose to prevent milk fever and the motivators behind those decisions. It is also important to consider the role that veterinarians play in the prevention of milk fever in UK dairy herds and to obtain their views on its management.

The aim of this chapter is to investigate farmers' and veterinarians' current practices in relation to treating and preventing milk fever. Thus, two cross-sectional surveys were designed to distribute to UK farmers and veterinarians to investigate current practice on farms in relation to the identification, treatment and understanding of milk fever and subclinical hypocalcaemia.

The main objectives of this chapter are:

- To understand the current preferences for milk fever prevention strategies by UK dairy farmers
- To understand farmer motivations for choosing an approach to prevention of milk fever
- To gain insight into the awareness of milk fever prevention strategies by UK veterinarians and their main recommendations

- To understand the main herd health concerns of both UK dairy farmers and veterinarians, to look at potential differences of opinions between the two groups, and to see whether issues relating to milk fever feature within those concerns

2.2. Materials & methods

The design and reporting of both cross-sectional surveys followed STROBE guidelines (STROBE, 2007).

2.2.1. Questionnaire

Both the farmer and veterinarian cross-sectional surveys (appendix A) were conducted online using Survey Monkey (www.surveymonkey.com, Portland, Oregon, USA). The purpose of the survey was clearly stated to all participants, who were free to withdraw from completing the questionnaire at any time, and it was stated that all responses would be anonymised during (University of Nottingham, reference number 290110121). The survey questions took a number of different formats, including free-text responses, multiple choice questions, and ranking questions. Whilst some questions differed completely between the two questionnaires, some were designed to enable a degree of comparison between the attitudes and beliefs of both groups.

Farmers were asked to provide information on their herd size, breed(s) of their animals, dry period lengths, housing arrangements, calving patterns, and milk fever incidence within their herd. Vets were asked to indicate the type of practice they work in, their number of years in practice, the proportion of their work time spent with dairy herds, and any postgraduate qualifications they may have. An outline of the main areas covered by each survey is shown in Table 2.1.

Table 2.1. Main topics covered by farmer and veterinarian questionnaires

Farmer survey	**Veterinarian survey**
Background information *e.g. herd size, calving pattern, health concerns within herd, dry period husbandry, estimated milk fever incidence*	Background information *e.g. practice type, experience, qualifications*
Milk fever prevention *e.g. main strategy chosen, factors influencing choice of strategy*	Herd health/production *e.g. main health/production issues considered important by vets, main health/production issues perceived to be important to farmers*
Herd health/production *e.g. knowledge of conditions linked to hypocalcaemia*	Milk fever prevention *e.g. awareness of options, recommendations to farmers*
	Understanding of farmer awareness and farmer motivators for milk fever and its prevention

2.2.2. Distribution and publicity

The survey for farmers was open during April and May 2012. It was distributed through an advert in British Dairying magazine and was also publicised through social media in May 2012.

The questionnaire for veterinarians was distributed through the mailing list of a commercial farm animal nutritional analysis service to vets who had previously submitted blood samples for analysis. Using this method, the survey was sent to 191 e-mail addresses of either individual veterinarians or practices in the summer of 2014, and completed by respondents between May and July of that year.

2.2.3. Inclusion criteria

Respondents to the farmer survey were required to be UK-based and to have dairy herds containing more than 20 adult animals. Responses from non-UK resident farmers and farmers with <20 or beef-only herds were excluded.

Respondents to the veterinarian survey were required to work with dairy cattle, either in mixed or specialist practice. Respondents who worked solely in small animal practice were excluded from the analysis.

2.2.4. Potential confounders

Milk fever risk varies by breed, as discussed in Chapter 1. Whilst the breed of animals farmed by individual farmer respondents was noted, the survey population is not necessarily balanced to reflect the breed profile of the UK dairy herd as a whole.

2.2.5. Missing data

In both the farmer and veterinarian surveys, a number of respondents did not complete the full set of questions. The answers of those who did not complete the full questionnaire have still been included for analysis up to the point at which they withdrew, and the number of responses included for each question is stated within each section of the results.

2.2.6. Data analysis

Categorical classifications of open answers were retrospectively added to the results of some questions within the survey for ease of data analysis.

Farmers were asked about the breeds of cows within their herds and these were retrospectively classified into 'black & white' (95%+ of herd were Holsteins/Friesians/Holstein-Friesian crosses), 'Channel Island' (95%+ of herd were Jerseys/Guernseys), and 'other' (other breeds or combinations of breeds/crosses). Herd sizes were classified into the following size groups: small (100 cows or fewer), medium (101-250 cows), or large (more than 250 cows). Dry period lengths were provided by farmers as a target number of days in a free-text response, and these were subsequently reclassified into weeks, with the number of days being rounded to the closest full week in either direction. Housing arrangements for dry cows were stated by farmers for both the early and late dry period for both summer and winter. The responses to this question were then grouped together to provide total

numbers of farmers using each overall approach, incorporating the season and both the early and late aspects of the dry period.

The number of years a vet had spent in practice was given as a free-text response. These were retrospectively classified into groups: 1-2 years, 3-5 years, 6-10 years, 11-19 years, and 20+ years.

Both farmers and veterinarians were asked to list their top three dairy cow health concerns in an open text box. Prior to data analysis, these answers were categorised in accordance to Table 2.2, with eight main categories, plus a category for 'none' and a category for 'other'.

Table 2.2. Categorisation of free-text responses to questions regarding dairy cow health concerns reclassified for the purposes of data analysis

Category name	**Key terms included in category**
Calving and calf health	Abortions, stillbirths, calf health, calving, metritis, retained foetal membranes
Infectious disease	TB, leptospirosis, Johne's, salmonella, IBR, BVD
Transition cow health	Transition/fresh/dry cow health
Locomotion	Lameness, foot health, dermatitis, arthritis
Mastitis, cell counts and udder health	Mastitis, cell counts, udders
Infertility	Infertility
Parasites	Parasites, liver fluke, neospora, coccidiosis
Nutrition and metabolic disorders	Nutrition, digestive disorders, maintaining body condition, deficiencies, iodine deficiency, white muscle disease, ketosis, metabolic diseases, milk fever, sub-acute ruminal acidosis, negative energy balance
Other	Comfort
None	None

Thematic analysis was carried out on question 22 in the farmer survey: “what health/production issues are you aware of that are influenced by subclinical hypocalcaemia?”. These were initially classified into the categories shown in Table 2.3. Blank responses were omitted but any responses stating “none” or otherwise actively indicating that they did not have an answer were grouped together.

Table 2.3. Initial categories used to classify responses to question 22 of the farmer questionnaire

What health/production issues are you aware of that are influenced by subclinical hypocalcaemia?
Reduced yield
Fertility problems
Loss of appetite
Retained foetal membranes
"None"/"do not know"
Displaced abomasum
Mastitis/high somatic cell count
Ketosis
Uterus health (including metritis and endometritis)
Sluggish/wobbly
Know it is detrimental but not aware of specifics
Loss of body condition
Slip under the radar
Prevention better than cure
Dull coat
Low temperature
Laminitis
Energy balance
Immune system
Fatty liver
Dystocia

2.2.7. Statistical analysis

Statistical analysis was carried out using chi square tests or two-tailed Fisher's exact tests where appropriate, and used a level of significance of $p<0.05$.

2.2.8. Calculation of weighted averages

Weighted averages were used in the analysis of question 16 (appendix A) of the veterinarian survey. Veterinarians were asked to rank answers from 1 to 6 in terms of importance, with 1 being the most important. During analysis, the number of responses to each question in each number category (1-6) were totalled individually (e.g. total number of vets scoring 'ease of use' as 1, total number of vets scoring 'ease of use' as 2, etc.). These totals were then individually multiplied by the score that related to them (e.g. number of vets scoring 'ease of use' as 5 was multiplied by 5). This gave six scores for each possible response outcome and these were then combined for each outcome. Each total was then divided by the total number of respondents to give a 'weighted average', with the lowest number representing the factor considered to be 'most important' by the overall group of vets who responded to the question, and the highest number representing the 'least important' factor. Veterinarians who did not respond to this question were excluded from this analysis.

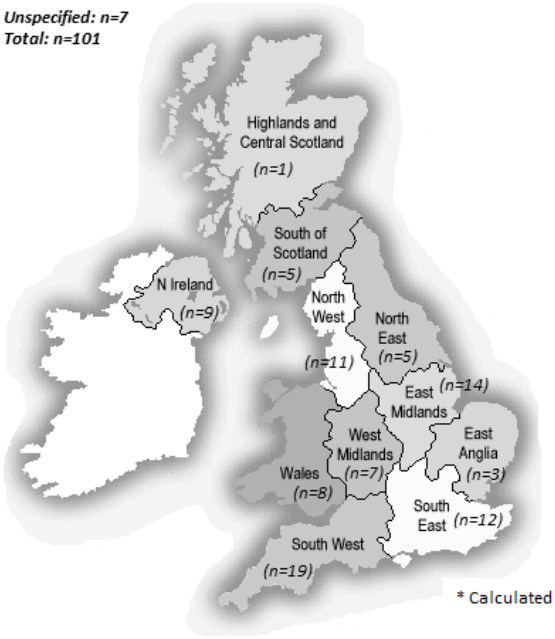
2.3. Results

2.3.1. Response rates

There were 108 respondents to the farmer survey. Six respondents were excluded from data analysis, using the eligibility criteria detailed in Section 2.2.3. Of the 102 responses remaining for analysis, 19 (18.6%) did not complete every section of the questionnaire. Data from these incomplete responses were still used in the analysis where relevant.

Due to the nature of the open distribution of this questionnaire, a response rate cannot be calculated. However, farmers supplied geographical information with regard to the county in which they were located. The

geographical regional distribution and number of farmer respondents are shown in Figure 2.1, along with the percentage of farmers within each region of England and Wales represented by this survey data.



Region	Proportion of region's producers represented by survey respondents *
Wales	0.42%
North west England	0.49%
North east England	0.63%
West Midlands	0.46%
East Midlands	2.12%
East Anglia	3.13%
South west England	0.63%
South east England	2.47%

* Calculated from data from Agriculture & Horticulture Development Board (2015)

Figure 2.1. The number of farmer respondents within each region of the UK, and the proportion of dairy farmers represented by the survey within regions of England and Wales in 2012 (AHDB, 2015)

The veterinarian survey was sent to 191 individual vets or practices and 109 responses were received, of which 108 met the inclusion criteria. As with the farmer survey, incomplete responses were used within analysis where relevant, leading to the varying sample sizes in different aspects of the data, as noted. Geographical data was not obtained from the participating veterinarians.

2.3.2. Background information: farmers

- #### 2.3.2.1. Herd composition

Within the farmer survey, 'black and white' herds accounted for 47.1% (n=48) of the survey population, with 26.5% (n=27) of herds being in the 'Channel Island' category, and 26.5% (n=27) of the herds were classified as 'other'. There was a mean herd size of 191, with a standard deviation of 115.5 cows.

The majority of herds fell into the 'medium' category (55.9%, n=57), with 21.6% (n=22) of herds being categorised as 'small' and 22.6% (n=23) as 'large'.

- 2.3.2.2. Dry cow management and husbandry

The mean (± s.d.) dry period length given by respondents was 54 (±6.9) days. The most common dry period length was 6 weeks (n=20), followed by 7 weeks (n=16) and 8 weeks (n=16). The majority of respondents used a year-round calving pattern (77.5%, n=79).

Housing arrangements used by farmers for both early and late dry periods in both summer and winter are shown in Table 2.4.

Table 2.4. Housing of dry cows reported by farmer survey respondents (n=91)

Survey question: Where are dry cows kept?	Number of respondents	Percentage of respondents (%)
All year at grass	1	1.1
All year at grass for early dry period, all year housed for late dry period	1	1.1
All year at grass for early dry period, winter indoors or summer at grass in late dry period	2	2.2
All year housed - full dry period	15	16.5
Winter indoors and summer at grass for early dry period, all year housed for late dry period	22	24.2
Winter indoors, summer at grass - full dry period	50	54.9

- 2.3.2.3. Farmer perceptions of milk fever problem within herd

Farmers were asked whether they considered milk fever to be a problem within their herd, selecting from a list of options: 'no problem', 'yes - slight problem', 'yes - moderate problem' or 'yes - major problem'. Around a quarter of respondents (24.7%, n=24) reported having no problem with milk fever within their herd. Approximately three-quarters of respondents reported some level of milk fever problem: 53.6% (n=52) reported a 'slight' problem, with 18.6% (n=18) and 3.1% (n=3) reporting 'moderate' or 'major'

problems respectively. The number of cases of clinical milk fever within each herd was reported as a number (number of responses = 97) and then converted into a percentage using the herd size data also provided by respondents. The calculated mean incidence (±s.d.) within a herd was 7% (±6.3). When the farmers' views of their level of milk fever problem were compared to the actual percentage incidence within those herds, those who reported having 'no problem' with the condition had a mean incidence within their herd of 2% (±2.0), compared to a mean incidence of 10.4% (±11.0) in those reporting experiencing a 'major' problem (n=3). Those experiencing a 'moderate' problem reported a mean percentage of cows affected by milk fever as 11% (±6.0).

2.3.3. Background information: veterinarians

Of the veterinarians participating in the survey, the mean number of years spent in practice was 9.9 (s.d. 8.4) years. The majority worked in a farm-only practice (60.2%, n=65), with the remainder working in mixed practices. The majority of vets (62.0%, n=67) spent more than 60% of their work time working with dairy herds, with 31.5% (n=34) spending 61-80% of their time with dairy herds, and 30.6% (n=33) falling into the 81-100% category. The majority of vets questioned had no postgraduate qualifications (65.7%, n=71), but 10.2% (n=11) of the study population held a Diploma in Bovine Reproduction (DBR) and 10.2% (n=11) held a Certificate in Cattle Health and Production (CertCHP).

2.3.4. Herd health and production concerns of farmers and veterinarians

Both farmers and vets were asked to list their top three dairy cow health concerns in an open text box. Vets were also asked which health concerns they believed were the most important to UK dairy farmers. Concerns were then reclassified into categories, as described in Table 2.2, and the results are shown in Figure 2.2.

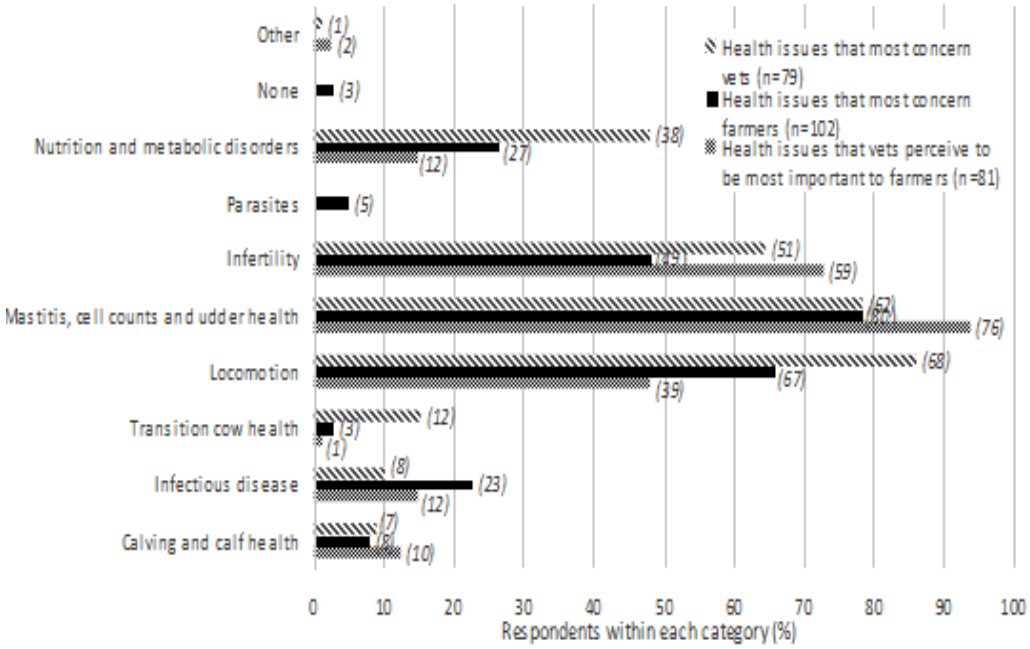


Figure 2.2. Comparison of the top three herd health issues in the opinions of both vets and farmers, along with vets' perceptions of farmers' opinions (number of respondents noted in brackets).

In the opinion of both vets and farmers, the same three issues were of concern: locomotion, infertility, and mastitis/cell counts/udder health. However, the most popular answer differed between vets and farmers, whose most popular answers were locomotion and mastitis/cell counts/udder health respectively. When compared to farmers, vets demonstrated greater concern for transition cow health (chi squared, p=0.003), and nutrition and metabolic disorders (chi squared, p=0.003). Compared to vets, farmers were more likely to express concern regarding infectious disease (chi squared, p=0.028). Concern regarding parasites was expressed by five farmers and was not mentioned by any vets, although this was not significant (Fisher's exact test, p=0.069). Three farmers reported having no health concerns/issues within their herds.

The most popular answer from vets was that they perceived that mastitis/cell counts/udder health would be the most important cow health concern of farmers (n=76), with infertility (n=59) second and locomotion (n=39) third. Only 12% of vets gave the answer that they perceived infectious disease to be

a ‘top three’ health concern to farmers, and 23% of farmers actually reported this to be the case. Locomotion was perceived to be a ‘top three’ health concern to farmers by 39% of vets, with 67% of farmers reporting this concern. This difference was statistically significant (chi-squared, p=0.022).

2.3.5. Farmer survey: choices of milk fever prevention strategies

The milk fever prevention strategies used by farmers (n=96) are shown in Figure 2.3. For the purposes of data analysis, the three calcium supplementation approaches were combined into one group. Of these targeted calcium approaches, intravenous/subcutaneous calcium was the most popular (n=11), followed by calcium boluses (n=8) and drenches (n=4). A number of respondents stated that they used ‘other / combination’ of approaches to milk fever prevention and some provided a free-text response to outline their approach. Of these, 11 reported using targeted calcium approaches but stated that these were only being used for older cows or those otherwise deemed to be higher risk, and in some cases this approach was used in conjunction with another strategy. There were 8 respondents in this category who specifically mentioned magnesium supplementation, and 1 respondent mentioned the use of vitamin D.

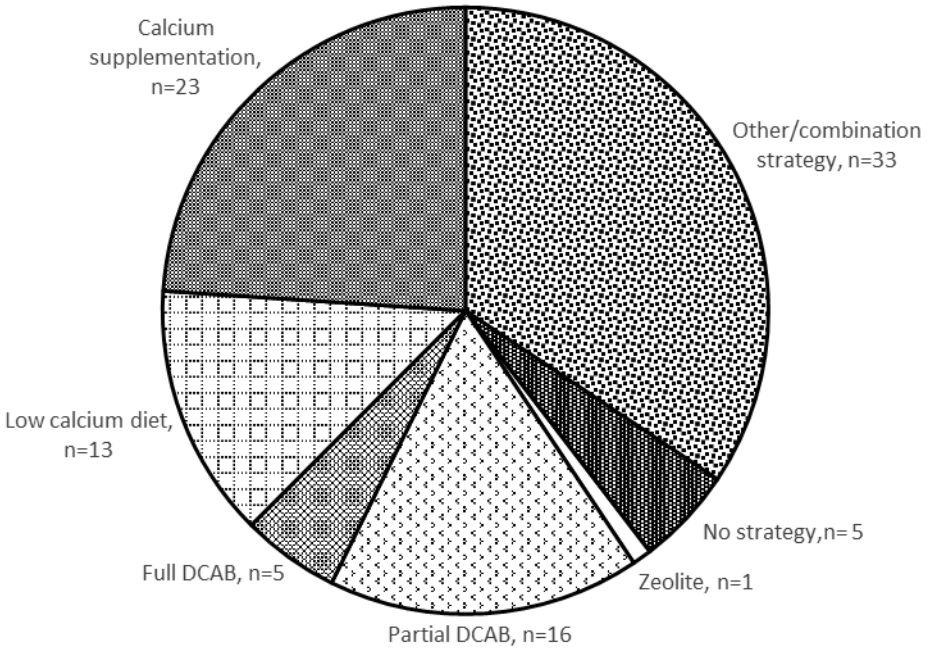


Figure 2.3. Milk fever prevention strategies used by questionnaire's farmer respondents (n=96)

The milk fever prevention strategy choices by farmers were combined with the relevant breed classification data in Figure 2.4. 'Other/combination' strategies were most popular with both black & white and Channel Island herds (n=17 and n=20 respectively), with the next most popular strategy being administration of calcium prophylactically around calving for both of these breed classifications. Feeding a low calcium diet in the dry period was only used by 13 herds in total and over half of these herds fell into the 'other/mixed' bred category (n=7). Partial DCAB diets were more popular than full DCAB for all dairy breed groups.

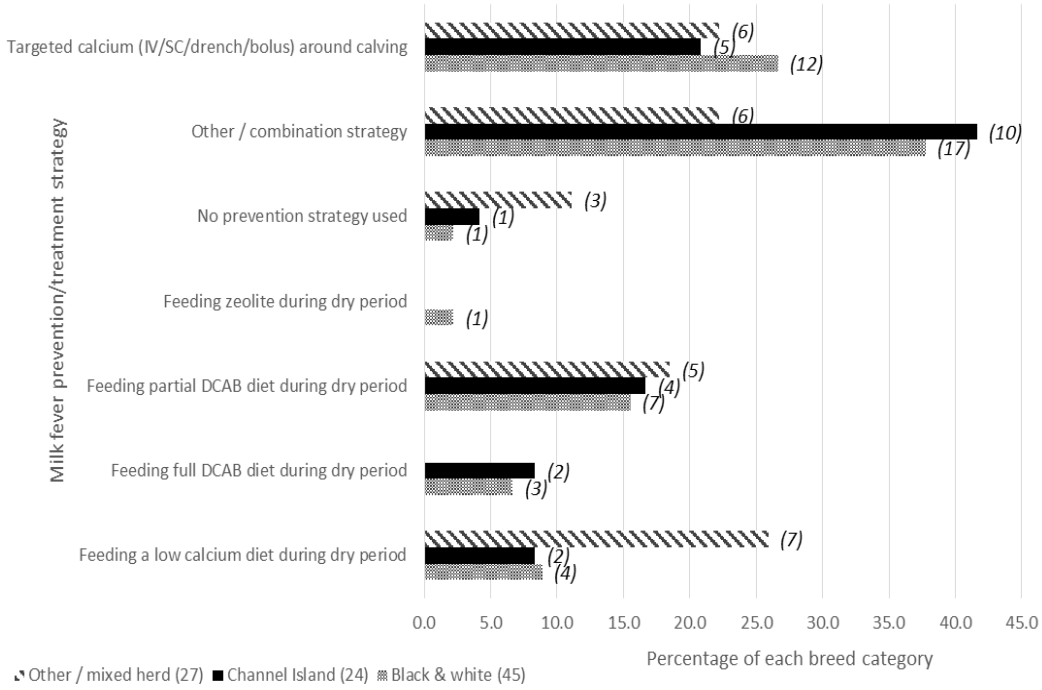


Figure 2.4. Milk fever prevention/treatment strategies used within different breed herds, displayed as a percentage of each breed category (number of respondents displayed in brackets)

Farmers with small herds most commonly used targeted calcium supplementation around calving (n=9). Farmers with medium or large herds most commonly used 'other/combination' strategies (n=19 and n=7 respectively). The second most popular choice for farmers of medium herds

was joint between targeted calcium supplementation (n=9) and feeding a partial DCAB diet (n=9). A partial DCAB diet was also the second most popular choice for farmers with large herds (n=6). All of the results for comparing herd size with choice of milk fever prevention strategy are shown in Figure 2.5. As can be seen in this figure, 27.2% of large herds use a partial DCAB diet, compared to 17.0% and 4.8% of medium and small herds respectively. This difference was not statistically significant (Fisher's exact test, p=0.13). Targeted calcium supplementation around calving was used by 42.9% of small herds, compared with 17.0% of medium herds and 22.7% of large herds. This difference was not statistically significant (chi-squared test, p=0.06).

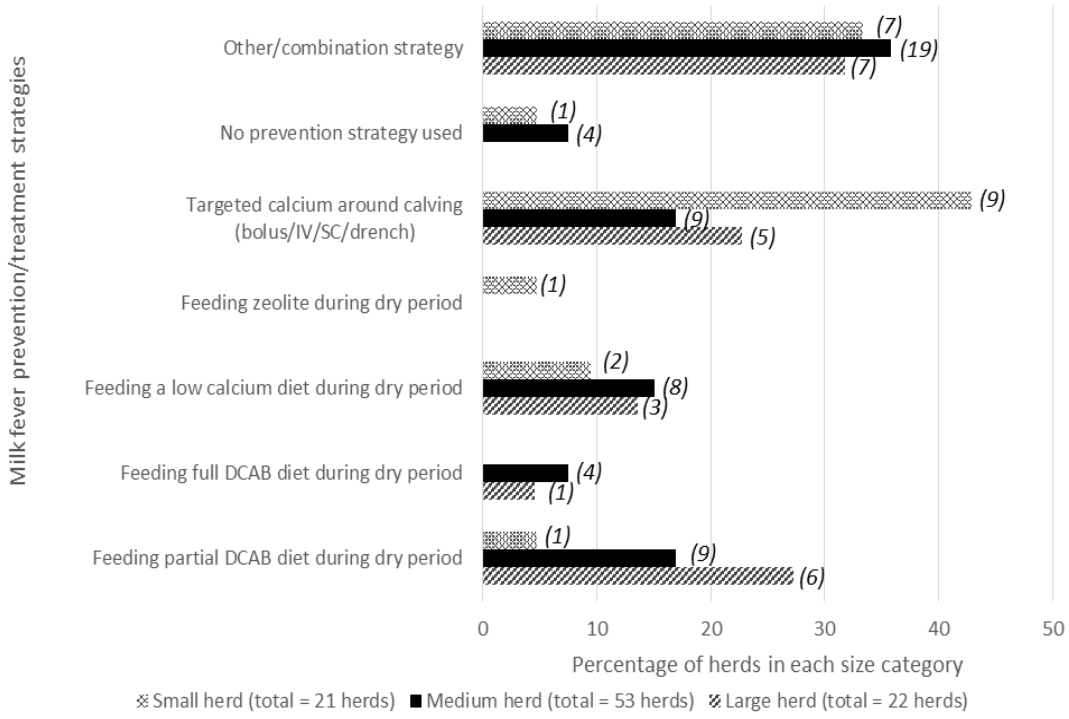


Figure 2.5. Milk fever prevention strategies used by different herd sizes, plotted as a percentage of each herd size category (number of respondents displayed in brackets)

Farmers also provided data on milk fever prevention strategies they had previously tried but were no longer using (n=90). Of these respondents, 30% (n=27) had never tried any alternative milk fever prevention strategies. Full DCAB diets had been tried and discontinued by 12.2% (n=11) of respondents. In some cases, reasons had been provided for the discontinuation of use of this method, in the form of free-text. The most common reasons given for

discontinuing the full DCAB approach was that it was considered to be ineffective (n=4), complicated (n=3) and expensive (n=2). A low calcium diet approach had previously been used by 18.9% (n=17) of respondents. As before, free-text explanations were optionally provided. The most common reason provided for discontinuing using a low calcium approach was poor efficacy (n=7).

The number of farmers using each type of milk fever prevention strategy within each type of housing set-up is shown in Table 2.5. Partial and full DCAB diets were combined due to low numbers of respondents. Only one farmer stated zeolite and so this was moved into 'other/combination strategy'. Of the most popular dry cow housing approach – keeping dry cows indoors in winter and at grass in summer – the most popular milk fever prevention strategy was to use calcium supplementation around calving, with 36% (n=18) of this housing type using this approach to milk fever prevention. Of these eighteen respondents, half (n=9) preferred to use calcium injections (SC/IV), with the other half choosing calcium boluses and calcium drenches (n=5 and n=4 respectively).

Table 2.5. The number of farmers using different milk fever prevention strategies in different types of housing set-ups (n=89)

	Approaches to dry cow housing			
Milk fever prevention strategy	Other	Winter indoors and summer at grass for early dry period, all year housed for late dry period	Winter indoors, summer at grass - full dry period	TOTAL
Targeted calcium supplementation around calving	2	2	18	22
Feeding a low calcium diet during dry period	0	4	8	12
Feeding DCAB diet (full or partial) during dry period	11	4	5	20
Other / combination strategy	6	10	16	32
No prevention strategy used	0	0	3	3
TOTAL	19	20	50	89

2.3.6. Farmer survey: motivators and reasons for choice of milk fever prevention strategy

Farmers were asked to select all the factors involved in their decision about a milk fever prevention strategy from a multiple choice list and the results of this are shown in Figure 2.6. The expected efficacy of a strategy and the ease of its use were both the joint highest scoring answers, each being selected by 50.9% (n=55) of respondents. Only 1.9% (n=2) of respondents report being influenced by a product manufacturer's recommendations or marketing. Veterinary recommendations were considered by 30 respondents, compared to 20 respondents considering the opinion of a consultant or feed advisor. When farmers were asked for their main motivation for choosing a milk fever prevention strategy (Figure 2.7), the most popular reason given was the expected efficacy of a strategy (n=38) and the second most popular reason was ease of use (n=22). This difference was statistically significant (p=0.023). A category for 'experience' was retrospectively added to these results, as this was stated in free text by 5 of the 6 respondents who selected 'other'.

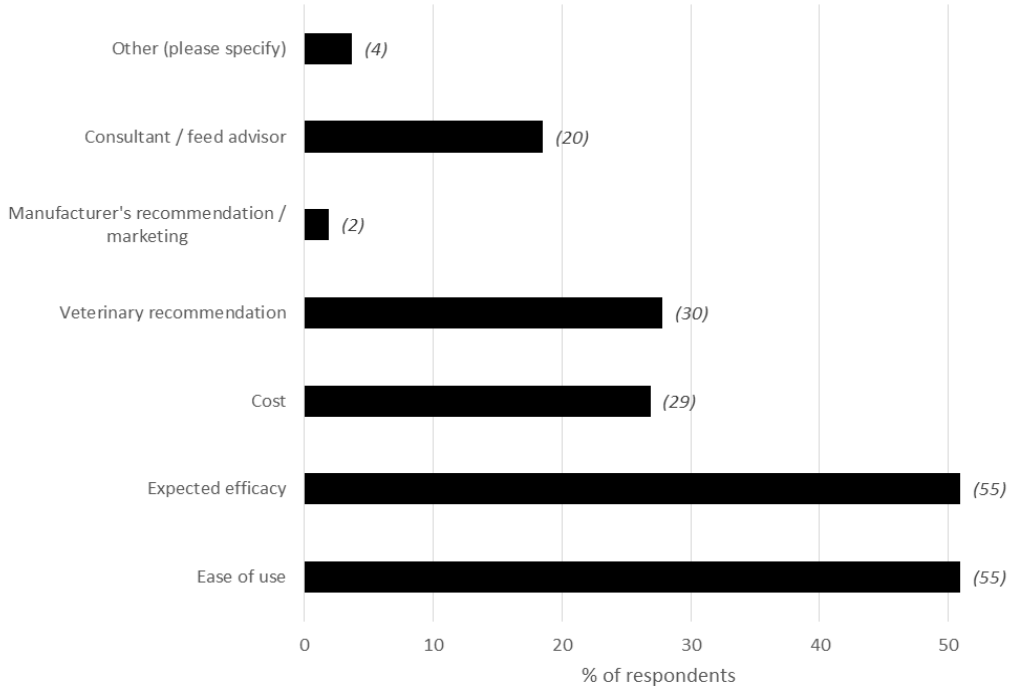


Figure 2.6. Factors influencing farmers' choice of milk fever prevention strategy (number of respondents displayed in brackets, total number of respondents = 108)

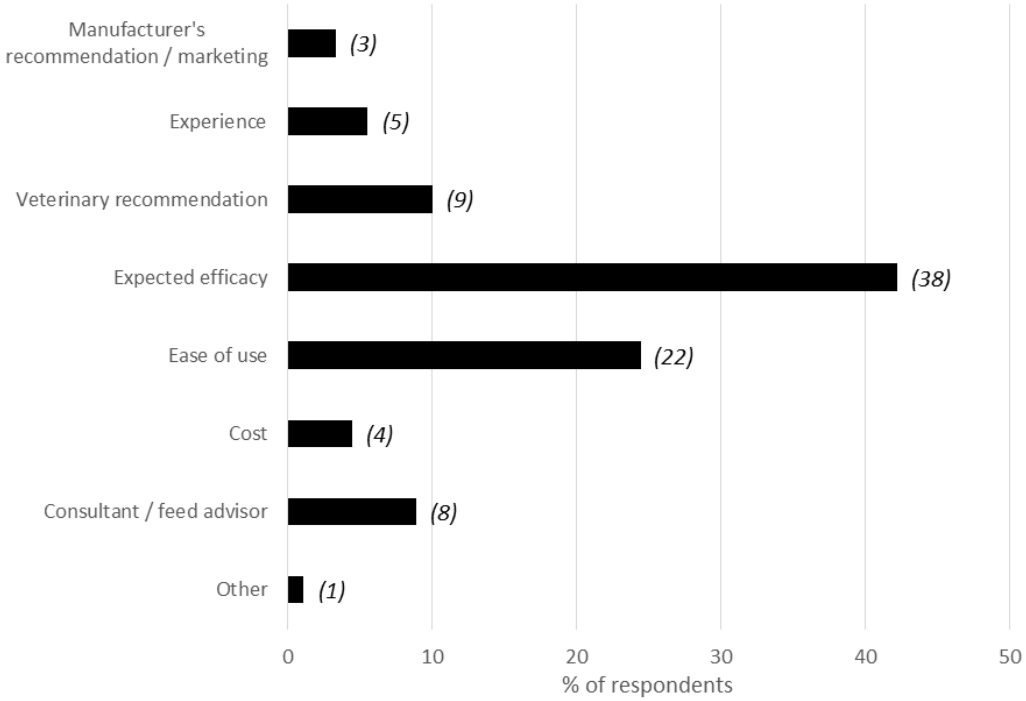


Figure 2.7. Farmers' main reasons for choosing a milk fever prevention strategy (number of respondents displayed in brackets, total number of respondents = 90)

The data collected from farmers who provided both a main reason for their choice of milk fever prevention strategy and also their actual chosen strategy are compared in Table 2.6. Farmers who were most interested in the expected efficacy of a strategy most commonly used either an 'other/combination' strategy (n=10) or a partial DCAB diet (n=10). Those choosing a strategy based on its ease of use most commonly used calcium supplementation around calving (n=10). Partial and full DCAB diets were combined due to low numbers of respondents. Only one farmer stated zeolite and so this was moved into 'other/combination strategy'.

Table 2.6. Farmers' main reasons for choice of milk fever prevention strategy compared to methods of milk fever prevention actually used (n=83)

	Main reason for choice of milk fever prevention strategy						
Method of milk fever prevention	Consultant / feed advisor	Cost	Ease of use	Expected efficacy	Manufacturer's recommendation / marketing	Veterinary recommendation	TOTAL
Feeding a low calcium diet during dry period (n=12)	2	1	1	5	0	3	12
Feeding DCAB diet (full or partial) during dry period (n=21)	2	0	3	12	1	3	21
Calcium supplementation around calving(n=22)	0	2	10	9	0	1	22
Other / combination strategy (n=28)	4	1	8	11	2	2	28
TOTAL	8	4	22	37	3	9	83

2.3.7. Farmer survey: farmers' understanding of the effects of subclinical hypocalcaemia

Farmers were asked to list, in the form of free-text, any problems that they were aware of that could occur as an effect of subclinical hypocalcaemia.

Farmers were able to provide more than one response to this question. There were 89 farmers who actively provided a response to this question, although that includes 14 who stated either that there were no associated problems or that they did not know any associated issues. Thematic analysis was carried out on these responses into the categories shown in Table 2.3 in Section 2.2.6. The initial analysis indicated that over two-thirds of the categories used had ≤7 respondents. The responses were then reclassified into broader categories and these results are shown in Table 2.7.

Table 2.7. Abridged results to thematic analysis of farmer responses to the question "what health/production issues are you aware of that are influenced by subclinical hypocalcaemia?" (total number of respondents to question = 89)

Final category	Original categories included	Number of respondents
Reduced yield	Reduced yield	45
Fertility problems	Fertility problems	24
Uterine problems	Retained foetal membranes, uterus health (including metritis and endometritis)	23
Loss of appetite	Loss of appetite	21
Displaced abomasum	Displaced abomasum	14
Mastitis	Mastitis and high somatic cell count	12

Some respondents provided answers in more than one category. There were 6 respondents who gave answers that fell into both the 'fertility problems' and 'uterine problems' categories.

2.3.8. Veterinarian survey: opinions on milk fever prevention strategies

Veterinarians were presented with a list of milk fever prevention strategies and, with a multiple-choice option, were asked to select which of the list they a) had heard about, b) had previously recommended, c) would recommend, and d) would not recommend. The results of this question are represented as percentages in Figure 2.8.

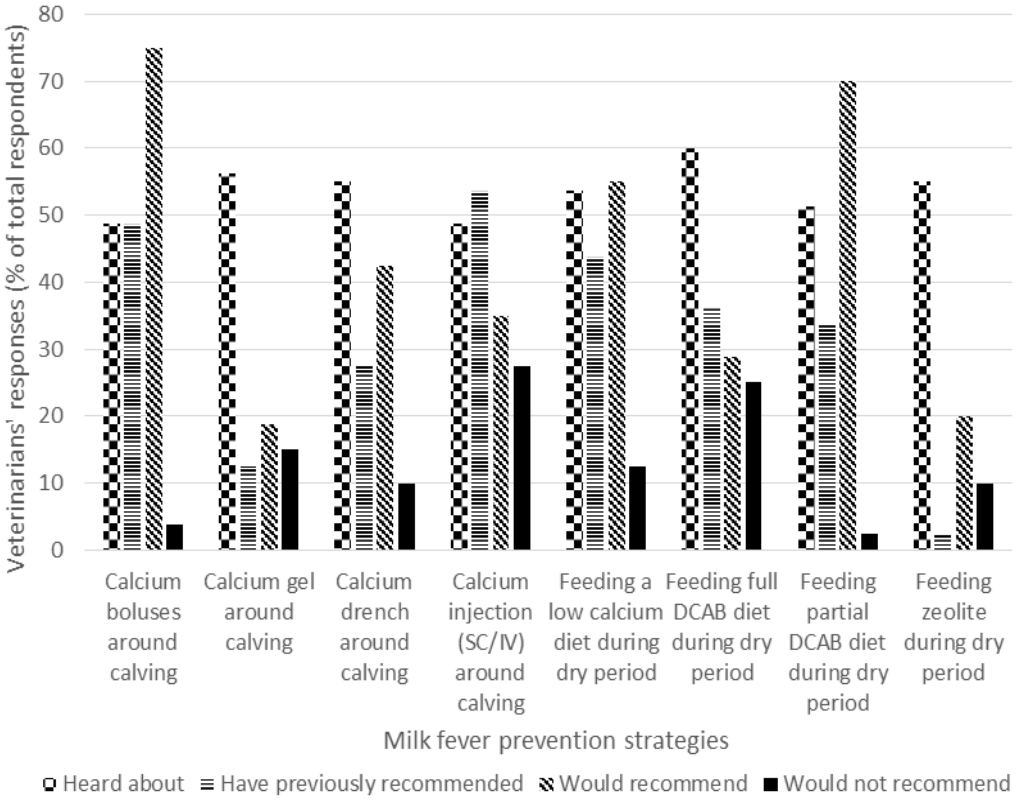


Figure 2.8. Veterinarians' opinions on recommending a selection of milk fever prevention strategies (n=80).

The most commonly recommended milk fever prevention approach by veterinarians was the use of calcium boluses (75%, n=60), followed by partial DCAB diets (70%, n=56). From the veterinarians questioned, calcium injections was the strategy most commonly 'not recommended' (27.5% of vets, n=22), followed by full DCAB diets (25%, n=20). Zeolite use in the dry period had been heard about by 55% (n=44) of vets questioned but had only been previously recommended by two vets within the sample (2.5% of respondents). However, 20% (n=16) said they would recommend it as a strategy, with 10% (n=8) actively saying they would not recommend its use.

2.3.9. Veterinarian survey: perceptions of farmer motivations when choosing a milk fever prevention strategy

Vets were asked to rank six potential reasons for a farmer's choice of milk fever prevention strategy from the most important (1) to least important (6).

The results are shown in Table 2.8. A weighted average was calculated for each option to determine its importance within the population of respondents (n=78). 'Ease of use' was the most important option, with a weighted average of 2.65. Overall, vets believed that manufacturer's recommendations/marketing were the least important decision-making factor for farmers, as this had a weighted average of 5.6, and 74.4% (n=58) of respondents believed this was the least important factor.

Table 2.8. Veterinarians' perceptions of the importance of a selection of potential reasons for farmers choosing a milk fever prevention strategy

	Answer ranking						Total	Weighted average
	1	2	3	4	5	6		
Ease of use	30.8% (n=24)	20.5% (n=16)	19.2% (n=15)	12.8% (n=10)	15.4% (n=12)	1.3% (n=1)	78	2.65
Cost	15.4% (n=12)	33.3% (n=26)	20.5% (n=16)	14.1% (n=11)	11.5% (n=9)	5.1% (n=4)	78	2.88
Expected efficacy	26.9% (n=21)	14.1% (n=11)	20.5% (n=16)	15.4% (n=12)	16.7% (n=13)	6.4% (n=5)	78	3.00
Veterinary recommendation	14.1% (n=11)	14.1% (n=11)	23.1% (n=18)	21.8% (n=17)	24.4% (n=19)	2.6% (n=2)	78	3.36
Consultant / feed advisor	12.8% (n=10)	17.9% (n=14)	14.1% (n=11)	29.5% (n=23)	15.4% (n=12)	10.3% (n=8)	78	3.47
Manufacturer's recommendation / marketing	0.0% (n=0)	0.0% (n=0)	2.6% (n=2)	6.4% (n=5)	16.7% (n=13)	74.4% (n=58)	78	5.63

2.3.10. Veterinarian survey: opinions on farmer accuracy when recording milk fever incidence

Vets were asked about their beliefs in relation to the reliability of farmers' data recording of milk fever cases. Only 2.6% (n=2) of vets believed that farmers accurately record the number of clinical cases of milk fever, compared with 64.1% (n=50) of vets who believed that farmers under-report milk fever, as shown in Figure 2.9.

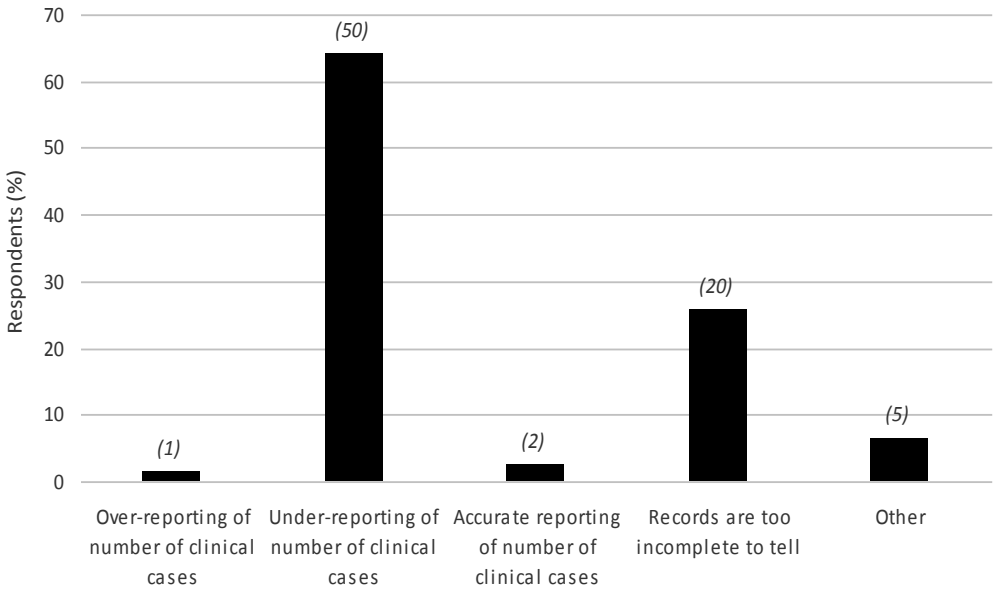


Figure 2.9. Veterinarians' opinions on the accuracy of farmers' data recording in relation to incidence of milk fever (displayed as a percentage of respondents, actual number of respondents displayed in brackets, total number of respondents = 78)

In the opinion of the veterinarians questioned (n=77), 96.1% (n=74) believed that farmers consult their vets for advice on prevention of milk fever, and 94.8% (n=73) believed that they seek advice from consultants/feed advisors. Fewer vets believed that farmers consult product manufacturers' marketing information and this scored the same as 'other farmers' in this question, with both being selected by 68.8% (n=53) of respondents. The farming press was believed to be a source of farmers' information on milk fever prevention by 67.5% (n=52) of vets.

Vets were also asked which source of information they believed was most important to farmers when making decisions about milk fever prevention and the results of this are shown in Figure 2.10. No vets believed that the farming press was the primary source of information to farmers and so this option is omitted from the figure. Consultants/feed advisors were selected by the most vets and represent 43.4% (n=33) of the population of respondents, with 'vets' being the second most popular option selected by 36.8% (n=28).

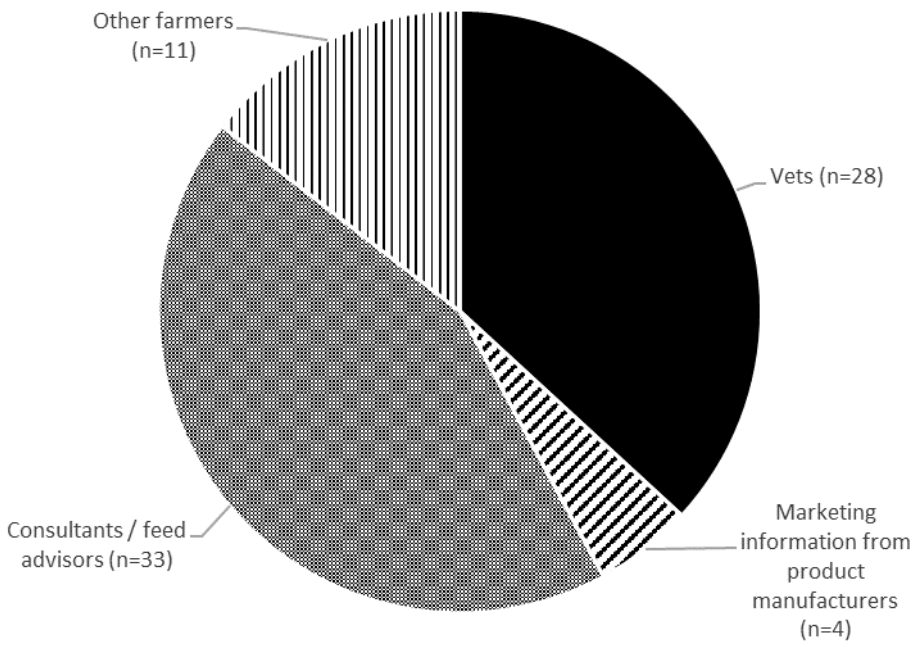


Figure 2.10. Veterinarians' opinions on the source of information most important to farmers when making decisions regarding the prevention of milk fever (n=76)

2.4. Discussion

2.4.1. Response rates, background information and selection bias

Due to the nature of the distribution of the questionnaires, it is not possible to calculate a response rate for either the farmer or veterinarian survey. This may have led to bias amongst the respondents, as it is impossible to know what proportion of potential respondents these views represent. The farmer survey was distributed on social media and so the participants obtained in this way may represent a limited demographic, and although the publication of the questionnaire details in British Dairying may have improved this, there is no way of knowing how many people viewed either or which responses came from which source. In August 2015, there were 13,815 dairy farmers in the UK (AHDB, 2015), and so the 108 respondents who began completing the survey represent 0.78% of dairy farmers at the present time (August 2015), although a number of these respondents failed to complete the full questionnaire. From the farmer respondents, the mean incidence of milk fever within a herd

was calculated at 7%, which is within the typical incidence ranges of 5-10% commonly reported in the literature (Sorensen *et al*., 2002; Mulligan *et al*., 2006a; Hayes *et al*., 2012).

Channel Island breed herds are over-represented in this dataset. Within the UK, 'black and white' dairy breeds considerably outnumber 'Channel Island' breeds, with around twenty-four times the number of 'black and whites' in the UK herd than Jerseys and Guernseys (DEFRA, 2008). However, in this study, only 47.1% (n=48) of respondents had predominantly 'black and white' herds, with over a quarter (26.5%, n=27) of respondents having predominantly Channel Island breeds. This may skew the data from the reality across the entire UK dairy herd. This bias may have occurred due to selection biases coming from the unknown distribution of the survey, as the survey may have unintentionally reached more of these farmers, or potentially is from selection bias of the owners of Jersey/Guernsey herds having a greater interest in milk fever due to their breeds' increased susceptibility to the condition (Lean *et al*., 2006).

The veterinarian survey was sent to a specific number of e-mail addresses from a mailing list but these belonged to both individuals and practices, and were sometimes then distributed around the same practice to different clinicians, which may lead to issues of selection bias. The mailing list of veterinarians was a list of users of a commercial laboratory that carries out nutritional analysis for veterinary practices. This may also affect the selection of participants as they may be practices or individuals who are particularly focused on nutrition and diagnostic testing for metabolic disease, and so the sample population may be skewed.

2.4.2. Herd health concerns of farmers and veterinarians

The three most popular answers for herd health issues were the same for both farmers and veterinarians: locomotion, infertility and mastitis. These findings are supported by other studies that have found the same thing: Pothmann *et al*. (2014) conducted a survey of dairy farmers in Austria and

found that fertility, mastitis and high cell counts, and issues of lameness were of the greatest concern. Boersema *et al*. (2013) found that mastitis was the issue of most concern for both vets and farmers in the Netherlands, and Caraviello *et al*. (2006) found that mastitis was the greatest health concern to dairy farmers in the USA, with lameness the third biggest issue. In the present study, 'nutrition and metabolic disorders' was the fourth most popular concern mentioned by farmers and vets. This may be due to the nature of this survey, which specifically aimed to investigate milk fever and associated issues, and so this may have been over-represented in the results due to it being forefront in participants' minds. However, Boersema *et al*. (2013) found that long-lasting milk fever in first-lactation heifers was the fourth highest concern to veterinarians in the Netherlands but was only the sixth most important issue to farmers.

Vets were more likely than farmers to be concerned about nutrition and metabolic disease ($p=0.003$), and vets were also more likely to give the answer of transition cow health ($p=0.003$). Potentially these were overstated in the results due to the nature of the questionnaire, with its potential leading title and aim. However, it could be the case that vets have a greater understanding of these issues and see them as priorities in terms of preventing associated diseases (e.g. nutrition to reduce the risk of NEB and its knock-on effects), whereas farmers may not form the same associations and so may focus on issues they can actually see (e.g. cases of mastitis). Farmers were more likely than vets to mention infectious disease ($p=0.028$). This may be because certain individual farmers were experiencing issues with infectious disease and so they could have been mentioned due to personal relevance, whereas vets are less likely to consider them a problem overall across all of the herds they see. This may be especially true for issues such as the threat of bovine tuberculosis, where a positive test and subsequent cull will have a financial impact on farmers, as replacement heifers are considered to be one of the largest costs to dairy farmers (Hadley *et al*., 2006), as well as there

being a psychological impact when animals are culled (Olff *et al*., 2005; Farm Crisis Network, 2009).

Of the veterinarians who responded to the question, 48.1% (n=39) expected locomotion to be in the farmers' 'top three' health concerns, when in reality 65.7% (n=67) of the farmers who responded gave this answer, and this difference was statistically significant (p=0.021). Whilst no information was collected on why this discrepancy occurred, it may be that farmers are more likely to seek advice or express concerns about issues surrounding locomotion and foot health with other professionals, such as foot trimmers. This theory is supported by Pothmann *et al*. (2014), who found that vets were considered to be 'not important' by 52.6% of their farmer survey respondents (n=911), and were only considered to be 'important' by 13.9%, with regard to advice relating to foot-trimming, which may mean that vets are not fully aware of farmers' concerns relating to foot-health.

2.4.3. Farmers' choices of milk fever prevention strategies

The most popular answers for farmers' choices of milk fever prevention strategies were 'other/combination' strategies, targeted calcium supplementation around calving, and partial DCAB diets. No statistically significant differences were found between herd size and choices of milk fever prevention strategy. The sample size in the present study was relatively small. Herd size has previously been reported to alter decision-making around issues such as this. Pothmann *et al*. (2014) conducted a survey of 1018 dairy farmers in Austria and found that those with high-yielding herds were more likely to involve feed consultants than those with low-yielding herds.

2.4.4. Farmers' motivations when choosing a milk fever prevention strategy

The most popular reasons given by farmers when asked to list all of the factors influencing their choice of milk fever prevention strategy were the expected efficacy of a strategy and its ease of use, with both of these reasons being selected by 50.9% (n=55) of respondents.

Veterinary recommendations were reported to be considered by 27.8% (n=30) of respondents and feed consultants were considered by 18.5% (n=20) when participants were asked to list all reasons involved in their choice of milk fever prevention strategy. These numbers were closer when farmers were asked to give their main reason for choice (n=9 and n=8 respectively). In Austria, Pothmann *et al*. (2014) found that on issues related to feeding, feed consultants were the most common source of information to farmers, with vets the second most common. This is potentially relevant but possibly depends on the way in which hypocalcaemia is being thought about, and whether it is something being prevented through diet (e.g. DCAB approaches) or whether it is being treated when clinical cases are seen.

When farmers were asked for the main reason behind their choice of milk fever prevention strategy, the most popular reason provided was its expected efficacy (n=38), followed by its ease of use (n=22), and this was statistically significant. Whilst it may seem obvious that most people would be concerned about a strategy actually working, this was not the most popular answer given by vets when they were asked what they perceived to be most important to farmers. However, it is important to consider the possibility of bias in the responses of farmers to this question. Social desirability bias may be involved, where research participants can have a tendency to choose responses that they believe may be more socially acceptable or desirable than their true thoughts, feelings or beliefs (Grimm, 2010). This can alter the answers provided by research subjects, potentially leading to the over-reporting of factors that are considered to be socially desirable, along with the risk of the under-reporting of less desirable or socially undesirable responses. The cost of a milk fever prevention strategy is an example of an answer that may be influenced by social desirability bias, as farmers may not wish to appear to be most concerned with the financial aspects of a strategy and so it may play a bigger role than stated. Cost is a factor that may be particularly susceptible to issues of social desirability bias and issues such as those relating to money and income can be considered to be ‘sensitive’ issues within survey-based

research (Tourangeau & Yan, 2007). It may be that cost is a greater issue than these findings would suggest, although it is also possible that there is little difference between the costs of the options that farmers are choosing between, and so it may be that it genuinely is not considered to be an important factor on this occasion.

Only 1.9% (n=2) of respondents mention the influence of product manufacturer's recommendations or marketing when making a choice of milk fever prevention strategy. It is impossible to tell from this present study but is important to consider that advertising potentially has a bigger impact than this on farmers, either without their full awareness or due to under-reporting associated with the idea of being influenced by marketing potentially being seen as a socially undesirable trait (Kaid & Holtz-Bacha, 2006). Despite the risks of potential social desirability bias, the fact that this questionnaire was conducted online, instead of face-to-face, should reduce the effects of this (Grimm, 2010), as should the fact that the results were anonymised and this was stated at the beginning of the questionnaire.

2.4.5. Farmers' awareness of subclinical hypocalcaemia and its potential effects

Subclinical hypocalcaemia has been associated with raised NEFA levels pre-calving and raised BHB levels post-calving (Martinez *et al*., 2012; Chamberlin *et al*., 2013). This poorer energy status has been associated with reductions in milk yields, and increased odds of displaced abomasum and other disorders during the lactation (Ribeiro *et al*., 2013; Sweeney *et al*., 2014). In the present study, 15.7% (n=14) of respondents to the question regarding issues related to subclinical hypocalcaemia were unable to name any associated conditions. Reduced yield was the most commonly stated answer, noted by 50.6% (n=45) of respondents to this question. A reduction in milk yield may occur due to a reduced DMI during periods of hypocalcaemia (Wilde, 2006). This problem was the most widely recognised amongst the respondents but was only suggested by half of them, and it may be that further education of farmers as to the potential impact of subclinical hypocalcaemia, as well as clinical milk

fever, may have benefits to the productivity, health and welfare of their animals. Pothmann *et al*. (2014) found that only 14.4% of their survey respondents (n=911) in Austria considered metabolic disorders to be a 'large' or 'moderate' problem, with 53.4% reporting them to be an 'occasional problem' and the remaining 32.2% considering them to be 'no problem'. However, in that study, as with this survey, mastitis and issues of fertility were considered to be much higher priorities. Whilst those issues are likely to be multifactorial, subclinical hypocalcaemia may be an underlying issue that is being overlooked if there is a lack of awareness and understanding of the potential links between these conditions.

2.4.6. 'Prevention' strategies versus 'treatment'

The incidence of subclinical hypocalcaemia in dairy cows in the UK has not been quantified but Reinhardt *et al*. (2011) suggests an estimated incidence of 50% in second lactation or greater cows in the USA. As discussed in Chapter 1, subclinical hypocalcaemia can lead to a reduction in smooth muscle function (Houe et al., 2001; Roche & Berry, 2006). Preventing hypocalcaemia, rather than just treating clinical cases, is widely believed to be beneficial, and subclinical cases are thought to have a cost four times greater than clinical milk fever to individual dairy farmers (Oetzel, 2013). Some of the milk fever prevention strategies, such as low calcium diets, can be considered to be true preventatives, as they aim to improve calcium mobilisation around calving, whereas supplementing with boluses merely masks the underlying issue during the delay in adaptation of the calcium homeostatic regulation mechanisms, and can in fact exacerbate the problem (Agger, 2003). Of the farmers questioned, almost a quarter (24.0%, n=23) were using targeted calcium approaches only, suggesting their approach is more treatment-based than actually preventative. Farmers considered mastitis and infertility to be 'top three' health concerns but in the thematic analysis only 13.5% (n=12) and 27.0% (n=24) stated a belief in an association between these conditions and subclinical hypocalcaemia. If these potential links were more clearly

understood by farmers then it may alter the way they think about hypocalcaemia and its prevention, as opposed to treatment.

2.4.7. Veterinarians' awareness of milk fever prevention strategies and their recommendations

The most commonly recommended milk fever prevention approach by veterinarians was the use of calcium boluses (75%, n=60), and this was followed by partial DCAB diets (70%, n=56). However, there do appear to be some discrepancies within the answers given to this question, and so the results should be viewed with caution. It would appear that a number of vets have interpreted the question differently, and it is suggested that some vets only selected one answer per prevention strategy, instead of all that applied. For example, only 41 people report having heard about the use of calcium boluses, yet 60 respondents would recommend this approach. In retrospect, this question could have been presented in a different multiple-choice format to obtain potentially more accurate data.

2.4.8. Veterinarians' perceptions of farmers' views on milk fever and its prevention

The majority of the vets questioned (64.1%, n=50) believed that farmers under-report their incidence of milk fever and only 2.6% (n=2) of vets questioned believe that farmers keep accurate records of their milk fever cases. Espetvedt *et al*. (2012) examined the 'completeness' of records of milk fever on Nordic dairy farms and their findings also suggest an under-reporting of cases of metabolic disease.

When vets were asked to list sources of information that they believe farmers consult for advice on milk fever, 96.1% (n=74) of veterinarians stated that they believe that vets are consulted. However, it is not clear what proportion of farmers they believe are approaching vets for this advice, or taking on board advice that they are offered, as this was not covered by this question. When vets were asked to give the main source of advice they believed farmers relied upon, 36.8% (n=28) believed that veterinarians were the main

source of information. In the farmer survey, 10% (n=9) of farmers stated that veterinary recommendations were the main factor that they considered when choosing a milk fever prevention strategy.

When asked what they perceived to be the most important factors for farmers when choosing a milk fever prevention strategy, the most popular answers given by vets was ease of use, followed by cost, followed by the expected efficacy of a strategy, although the differences between these weighted averages were small. This finding differs from the farmer respondents in the present study. However, the number of participants is small and responses, particularly to the farmer survey, may be subject to social desirability bias and issues relating to the study population itself, as previously discussed.

2.4.9. Limitations of the study and recommendations for future work

Whilst this study aims to investigate current practices with regard to milk fever prevention in the UK dairy industry, the data only represents a small percentage of UK farmers and veterinarians. This study also only presents what those respondents *say* they believe or actions they *say* they undertake, and it cannot be confirmed that this is actually true in practice.

As the questionnaires were intended to focus on the understanding of and attitudes towards milk fever and its prevention, this may have skewed the results. It may be that farmers and veterinarians with a greater than average interest in milk fever and its prevention may have completed the questionnaire. Similarly, when asked about herd health concerns, milk fever and metabolic disease may have appeared more frequently than would have been the case had the influence of a potentially leading survey title and aim not been present.

A number of free-text box style questions were present in the questionnaires. Whilst these were designed to reduce the risk of asking leading questions as

much as possible, they potentially caused problems with the analysis of the results. Where free-text answers are retrospectively categorised or thematic analysis was carried out, this does rely on accurate interpretation of the meanings of the free-text responses, and this may not be the most reliable method to deal with this data. The survey also had a high drop-out rate: for example, with the farmer survey, there were 108 respondents to the initial questions, but this dropped to as low as 83 for some of the questions analysed. This may have been due to the nature of the survey, as it could be considered to be quite long and potentially time-consuming, as thought would be required to fill out the numerous open text boxes. Pennings *et al*. (2002) investigated farmers' willingness to partake in survey-based research and found that the mean amount of time a farmer was willing to spend on a questionnaire was 13.45 minutes. They also found that in surveys where compensation was not being paid, over a third of respondents would be unwilling to spend more than five minutes completing a survey, which may have affected the number of complete responses in this study.

There was a time delay of over two years between the distribution of the farmer questionnaire and that of the veterinarians. This may have influenced the responses, although there are no major developments in milk fever prevention that are likely to have changed the responses. Thomsen *et al*. (2012) conducted a survey on farmers and veterinarians with a similar time gap between the distributions of the two and did not consider this to be an issue with their data. In retrospect, it may have been more appropriate to distribute both questionnaires at the same time, or at least to have received the farmer results and analysed those prior to designing the veterinarian questionnaire, as some issues (e.g. large numbers of text boxes being difficult to analyse effectively) may have been reduced.

This study may act as a useful pilot study for future questionnaire-based research on the subject of milk fever and its prevention. The answers provided in the free-text boxes may be useful in formulating more specific multiple-choice style questions in future work for more effective quantitative

analysis. It would also be recommended that the study population of farmers be balanced in terms of reflecting the UK herd, or that different breed herds were considered separately, in order to reduce the risk of the results being skewed, such as due to the considerable overrepresentation of Channel Island herds in the present study.

2.5. Conclusion

A variety of approaches to milk fever prevention are being used by UK farmers and recommended by UK vets. A large proportion of farmers are choosing to use combination strategies to deal with this condition, and farmers are most concerned with choosing a strategy that actually works but also require it to be easy to use. Cost appears to be a less important motivator, although caution should be taken with this data due to the inherent risks of social desirability bias when conducting questionnaire-based research.

Whilst vets and farmers do seem to share some herd health priorities, such as the importance of mastitis and issues of infertility, there were also some differences between the two groups. It appears that farmers are not fully aware of the importance of subclinical hypocalcaemia and many are concerned with issues such as mastitis and infertility but many are lacking the understanding that these issues could be connected. Vets place a higher degree of importance on metabolic disease than farmers appear to and, whilst it is impossible to say from the present study to what extent this knowledge is being transferred, it is clear that more could be done with respect to educating farmers on this subject. Veterinary recommendations are taken on board by over a quarter (27.8%, n=30) of farmer respondents with regard to milk fever prevention, and it may be that those who do not consider veterinary recommendations are simply not being offered sufficient information. Further research could be carried out in this area. Co-operation is needed between farmers, their vets and feed consultants, and any other individuals or organisations involved in dairy farming in order to most

effectively manage herd health (Jansen *et al*., 2010; Derks *et al*., 2013; Pothmann *et al*., 2014).

This survey has provided some information on the current state of milk fever prevention and attitudes towards the condition in the UK dairy industry, which was previously lacking within the literature. However, this chapter has a variety of limitations and, as such, there is scope for further questionnaire-based research in this area, and this study may be suitable for use as a pilot for future work.

CHAPTER 3: A RETROSPECTIVE STUDY OF FACTORS ASSOCIATED WITH RECORDED MILK FEVER EVENTS IN UK DAIRY HERDS

3.1. Introduction

Milk fever in dairy cows is a multifactorial disease, and is widely regarded as being related to factors that include increasing parity (Curtis *et al*., 1984; Reinhardt *et al*., 2011) and high milk yields (Horst *et al*., 1997). As discussed in Chapter 1, it has been associated with an increased risk of conditions such as dystocia (Curtis *et al*., 1983) and retained foetal membranes (RFM) (Klerx & Smolders, 1997), and has also been linked to an increased risk of culling (Hayes *et al*., 2012). However, many of the outcomes that can be associated with milk fever can also be risk factors for each other, and these relationships are often complex and can be bidirectional.

This chapter focuses on both risk factors for milk fever and on other outcomes that may be affected by its occurrence. Due to the confounding nature of some of the variables previously discussed, this chapter will use regression modelling in order to take into account some other main predictors when investigating the association between milk fever and a particular outcome.

There are two main approaches to this type of work: prospective and retrospective studies. A prospective study would involve approaching farmers to collect future data, agreeing disease definitions and following herds for a set number of lactations. Whilst this can enable accurate data recording, studies of this nature are sometimes limited in terms of sample size. For example, the work of Hayes *et al*. (2012) examined data from 8 herds across around 2000 lactations. As the outcomes examined in the present study are considered to be multifactorial, it is possible that the relationships between these outcomes and milk fever may differ between herds with different management strategies and levels of disease, and so studies that use small datasets may be difficult to generalise across the wider population of dairy cows in the UK. This chapter is a retrospective study, utilising data that has already been collected. Whilst this does have the drawback of having the risk

of varying data accuracy and the potential for differing diagnostic criteria to be used, it provides a relatively large dataset for analysis from a number of herds.

This study uses a relatively large dataset of over 59,000 lactations from 78 UK herds. Other studies have been carried out to investigate associations between diseases in dairy cattle, including milk fever, but this work appears to be very limited within the UK. The data used is relatively recent, obtained from herds between 2000 and 2008, and there appears to be limited UK data available in the literature regarding milk fever incidence and its association with other diseases from this time.

This chapter aims to:

- Investigate potential predictors for milk fever risk, including parity, previous yield and incidence of clinical disease, whilst accounting for any measured variables that may confound these relationships
- Investigate the potential associations of milk fever with the occurrence of other outcomes, including dystocia, RFM, and death, whilst accounting appropriately for any measured variables which may confound these relationships

3.2. Materials & methods

3.2.1. Data collection, initial data handling, and data quality assessment

The data used in this chapter was collected by Hudson *et al*. (2012) from a group of 20 vets across England and Wales, who provided datasets from herds which were considered to have high standards of data recording, and as such represents a convenience sample. The initial data handling was described by Hudson *et al*. (2012) and involved the data being anonymised and converted into a consistent format for ease of analysis. This data was then subject to further data quality assessment for use in this study.

- *Stage 1: herd year quality thresholds*

This dataset was initially made up of 468 herds. Data used in this chapter was subject to data quality criteria applied at a herd-year level, where herds were only included for the years in which their data met the criteria stated. Herd years were considered to be recording milk fever if ≥1% of lactations contained a milk fever event, and herd years that were not considered to be recording milk fever were removed from the dataset. Herd years were also assessed at this stage in terms of herd size and average 305 day yield, as described in Table 3.1. Herd years that did not meet these thresholds were removed from the dataset, but a herd still contributed data from any *herd year* where the quality thresholds were met.

Table 3.1. Herd-year quality thresholds required to be met for inclusion in the dataset

	Outcome	**Criteria required for inclusion of herd year**	
Criteria required by a herd-year for inclusion in the dataset as a whole	Milk fever	Milk fever event in ≥1% of lactations	
	Herd size	≥50 lactations within the herd year	
	Average 305 day yield	>3000 litres in 305 days	
Criteria required by a herd-year for inclusion at a specific disease level (i.e. from models where that factor was included)	Dead calf	Dead calf in ≥1% of lactations	
	Dystocia	Dystocia in ≥2.5% of lactations	
	Retained foetal membranes	RFM in ≥1% of lactations	
	Twins	Twins born in ≥0.5% of lactations	

The data finally used in this chapter was obtained from 78 herds from across England and Wales and the vast majority of herds included were Holstein or Holstein-Friesian. The main reason for herds being excluded was failure to meet the recording threshold for milk fever, with 378 herds removed from the dataset for this reason. Failure to meet the herd size threshold excluded 8 herds, and 12 herds were removed due to not meeting the threshold for average 305 day yields. The sum of these numbers exceeds that of the actual number of herds that were excluded (390 herds from the original 468 were removed), as some herds failed to meet the inclusion criteria in more than one category.

- *Stage 2: incidence rate criteria*

Each individual herd year was assessed for its recorded incidence of a number of different outcomes, such as retained foetal membranes and dystocia. The thresholds used for this assessment are shown in Table 3.1. Herd years that failed to meet these thresholds were classified as 'not recording' for that particular variable and lactations from such herd years were given a blank value for that particular outcome. Herd years that met the stated thresholds were classified as 'recording' for that particular variable.

Herd years that were classified as 'not recording' remained in the dataset in order to maximise the sample size. However, lactations where an outcome was blank were then omitted from any analysis that involved that particular variable, and so the number of lactations included within each multivariate model varies. This dataset used in this chapter was initially made up of 59,043 lactations from a total of 29,643 cows. The numbers of lactations included are noted with each model.

- *Stage 3: binary categorisation of variables*

The data used in this chapter was organised into lactations, with each of these representing a line of data. Lactations in herd years that were classified as 'recording' for a given variable were then given a binary classification for that particular variable. This is detailed in Table 3.2.

Table 3.2. Information recorded by herds within the dataset

Data category	Description of data category	Additional criteria / missing values
Parity ID	Identifying number for each individual parity (i.e. each individual lactation within the dataset)	
Cow ID	Identifying number for each individual animal	
Herd ID	Identifying number for each individual herd	
Calving month	Categorical by month	
Calving year	Categorical by year (2000 to 2008)	
Parity	Categorical (parity of current lactation described by number, parity ≥5 were grouped as a single category due to small numbers)	
Cull	Binary (0 no cull, 1 cull)	Missing values represent herd-years where the event was not recorded at all, or was recorded below the threshold stated
Death	Binary (0 no death, 1 death)	Missing values represent herd-years where the event was not recorded at all, or was recorded below the threshold stated
Exit herd by day 60	Binary (0 remains in herd after day 60, 1 exits herd by day 60)	
Exit herd by day 30	Binary (0 remains in herd after day 30, 1 exits herd by day 30)	
305 day yield (in current lactation) (in thousand litres)	Continuous	
Previous 305 day yield (in thousand litres)	Continuous	
Milk fever	Binary (0 no milk fever, 1 milk fever)	'1' represents a case of milk fever within 10 days of calving date
Twins	Binary (0 single calf, 1 twins)	Missing values represent herd-years where the event was not recorded at all, or was recorded below the threshold stated

Dead calf	Binary (0 live calf, 1 dead calf)	Missing values represent herd-years where the event was not recorded at all, or was recorded below the threshold stated
Dystocia	Binary (0 no dystocia, 1 dystocia)	'1' represents a case of dystocia within 1 day of calving date Missing values represent herd-years where the event was not recorded at all, or was recorded below the threshold stated
Retained foetal membranes	Binary (0 no RFM, 1 RFM)	'1' represents a case of RFM within 14 days of calving date Missing values represent herd-years where the event was not recorded at all, or was recorded below the threshold stated

The categorisation of a number of health events into binary outcomes during a specific time period post-partum reduces the risk of the results being influenced by different styles of recording on different farms. Some farms do record health events multiple times, as was reported by Wenz & Giebel (2012) when they evaluated on-farm data recording. Whilst some farmers may record the reoccurrence of a condition, the present study is concerned with whether or not the event occurs at all, regardless of how many times it may happen.

3.2.2. Restructuring data for analysis

As milk fever is the main focus of this chapter, parity 1 animals were excluded from the dataset before regression models were constructed, due to the low milk fever risk expected within this group (Koeck *et al*., 2012). This was also seen within the dataset, as only 0.19% of parity 1 animals were recorded as having milk fever.

3.2.3. Univariate analysis

The proportion of lactations affected by a case of milk fever was calculated against a number of potential predictors, and the differences were tested using a chi-square test. The potential predictor variables were death during

the lactation, exiting the herd by day 30 of the lactation, delivery of twins, occurrence of RFM, dystocia, and delivery of a dead calf.

3.2.4. Multivariate analysis: methods for regression modelling and model fit checking

The variables tested in each of the models are shown in Table 3.3.

Table 3.3. Predictors tested during model building for each outcome

Outcome	**Predictors tested during model building**
Milk fever	Twins, previous 305 day yield, parity, calving month, calving year, dystocia, dead calf, RFM
RFM	Milk fever, twins, dead calf, dystocia, parity, previous 305 day yield, calving month, calving year
Dystocia	Milk fever, twins, dead calf, previous 305 day yield, parity, calving month, calving year
Dead calf	Milk fever, dystocia, twins, previous 305 day yield, parity, calving month, calving year
Death	Milk fever, parity, RFM, dystocia, dead calf, calving month, calving year, twins, previous 305 day yield
Exit by day 30	Milk fever, RFM, dead calf, dystocia, twins, parity, previous 305 day yield, month, calving year
Exit by day 60	Milk fever, RFM, dead calf, dystocia, twins, parity, previous 305 day yield, calving month, calving year
Subsequent 305 day yield	Milk fever, parity, previous 305 day yield, twins, calving month, calving year, RFM

As the regression models were constructed to look at the relationship between milk fever and other factors, outcomes were not reported where

milk fever was not significant in the final model. Other predictors remained in the models where they had a statistically significant ($p<0.05$) association with the outcome in their own right.

Regression modelling was carried out in MLwiN version 2.36 (Rasbash *et al*., 2016). The individual models used are described below. Separate models were built for each outcome, and these are listed in Table 3.3. Yield in the subsequent lactation was the only continuous outcome and so here a linear regression model was built. However, no significant association with milk fever was found and so the results from this model are not presented in this thesis. All other outcomes were binary and so logistic regression models were used. The general form taken by the logistic regression models was $\mathbf{=\beta_0 cons + \beta_1 X_1 + e}$, where β_0 represents the intercept, β_1 the vector of predictor variables, and e is an error term for each level.

The model specification took the form

$$\mathbf{Outcome_{ij} \sim Bernoulli\ (mean = \mu_{ij})}$$

$$\mathbf{\ln(\mu_{ij}/(1- \mu_{ij})) = \alpha + \beta_1 x_1 + \beta_2 x_2 + ... + \beta_n x_n + u_j}$$

$$\mathbf{u_j \sim N(0, \sigma^2_u)}$$

where μ_j is the fitted probability for the outcome in lactation i within herd j, α is the regression intercept, B_m represents the m^{th} regression coefficient, X_m represents the m^{th} predictor variable, n represents the number of predictor variables in the model, u_j represents the random effect representing variation in risk of the outcome between herds, and σ^2_u represents the variance of the normal distribution of the herd-level random effects.

Models were built by forward selection, where potential predictor variables were added one at a time. Where the 95% confidence interval (CI) of their coefficient did not cover zero they were retained in the model, as their association with the outcome was significant ($p<0.05$). Variables where the 95% CI of their coefficient covered zero were removed from the model.

Initially the models were set up with multilevel structures, where lactations were nested within herds, and using a random effect at each level. For all models where the outcome could occur several times during a cow's lifetime, random effects structures to account for repeated contributions from the same cow were evaluated. Models built with these structures produced substantively the same results as those reported here, with variables retained and directions of association the same, and effect sizes extremely similar. A high proportion of cows only contributed a single lactation to the dataset and so it was considered more appropriate to present the results from single-level models.

These models were checked for fit through visual assessment, comparing predicted versus observed incidence of disease across subsets of the data, and then the Hosmer-Lemeshow goodness of fit test (Hosmer & Lemeshow, 1980) was used. However, some of the models were found to have an unsatisfactory fit. Further exploration of this showed that the fit was good for the majority of herds but was poor for a small number in each case. Therefore, these models were changed to single level models with individual binary indicator variables for the herds where poor fit was demonstrated. This used fixed effects to represent the herds where predictions had been shown to be poor. All final models reported in the results were demonstrated to have satisfactory fit according to this method of model checking.

3.2.5. Limitations of farm records

The records used to compile this dataset were obtained from routine farm records. Whilst these individual sets of records were collated via veterinary practices, the diseases recorded were done so on-farm and are not veterinary diagnoses. Any associations reported within this chapter are therefore associations between the *recording* of one of more milk fever events and other recorded outcomes. No further information regarding the criteria used by individual farms to identify a case of milk fever or any other disease event is available.

3.3. Results

3.3.1. Univariate analysis

- 3.3.1.1. Milk fever incidence by herd and parity

The proportion of lactations with ≥1 milk fever event recorded by herd within the dataset is shown in Figure 3.1. This includes data from the 78 herds within the dataset, which were all included due to their recording of a milk fever incidence rate of >1%, as previously noted. The mean number of lactations reported by a herd is 757.9, with a range of 60 to 4928. The mean proportion of lactations reported to include ≥1 milk fever event is 4.26% and the median proportion of lactations reported to include ≥1 milk fever is 3.57% (lower quartile = 2.64%, upper quartile = 5.41%). This data includes cows of parities 1 to ≥5, as most studies reporting incidence data appear to do so across all parities, although the heifers were removed prior to multivariate analysis.

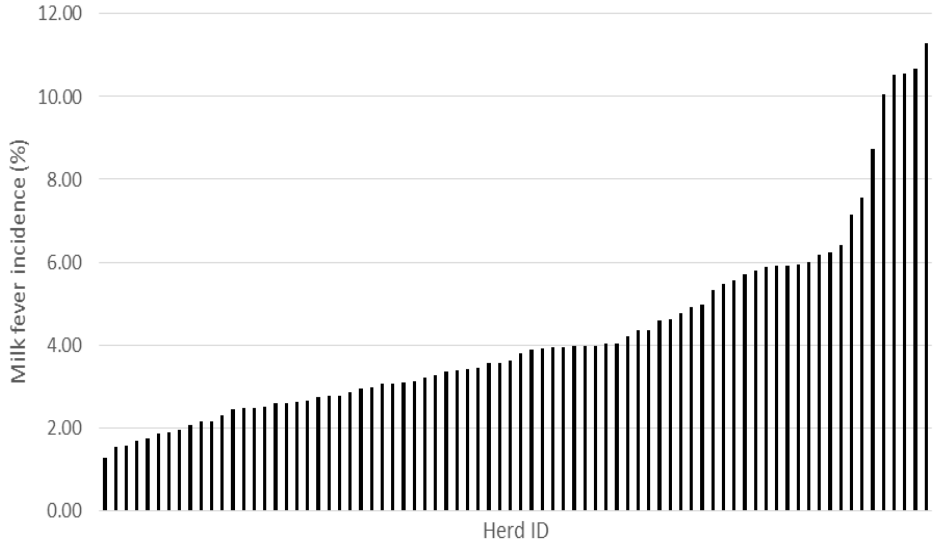


Figure 3.1. Incidence of milk fever within each herd used in the final dataset (n=78), with each line representing an individual herd

The proportion of lactations with a case of milk fever recorded by parity is shown in Table 3.4. These differences in the proportion of lactations recording a milk fever event by parity were analysed using a chi-square test and were significant ($p<0.0001$).

Table 3.4. Proportion of lactations with ≥1 milk fever event recorded by parity (total n=59,052)

Parity	Number of lactations	Proportion of lactations with ≥1 milk fever event recorded
1	15566	0.19%
2	12596	0.78%
3	10168	3.30%
4	7378	7.71%
≥5	13344	14.80%

- 3.3.1.2. Univariate analysis between milk fever and the occurrence of potential predictors

This section includes the univariate analysis between the occurrence of milk fever and a number of different potential predictors. For each individual analysis, the total number of lactations used is the total number of lactations that recorded the presence or absence of the predictor in question, and so this total number varies depending on the factor being discussed. Heifers were removed from the dataset prior to this analysis and so this is for cows of parity 2 or greater, in order to be in line with the multivariate analysis also carried out.

There was a significant association between the occurrence of a case of milk fever and the lactation ending in the death of the animal. Of the lactations with no milk fever recorded (n=40812), 2.68% resulted in death. Of the lactations with a recorded milk fever case (n=2668), 4.50% resulted in the death of that animal. This difference was statistically significant (chi-squared test, $p<0.01$).

Of the deaths in animals with milk fever (deaths n=120), 67.5% of deaths had occurred by day 30 of the lactation, compared with 39.7% of the deaths in animals without milk fever (deaths n=1094). This difference was statistically significant (chi-squared, $p<0.0001$). When deaths by day 60 were considered,

74.2% of the deaths in milk fever cases had occurred by this time point, compared to 49.5% of the deaths in the group that did not have milk fever, and again this difference was statistically significant (chi-squared, $p<0.0001$).

There was a significant association between the occurrence of a case of milk fever and the occurrence of dystocia in the same lactation. Of the lactations where no milk fever was recorded (n=9033), 8.28% experienced dystocia. Of the lactations where milk fever did occur (n=615), 18.05% experienced dystocia. This difference was statistically significant (chi-squared test, $p<0.01$).

There was a significant association between the occurrence of a case of milk fever and the delivery of a dead calf. A dead calf was recorded in 5.16% of lactations where milk fever occurred (n=1647), compared to in 4.52% in lactations where milk fever did not occur (n=24009). This difference was statistically significant (chi-squared test, $p<0.05$).

There was a significant association between the occurrence of milk fever and a cow exiting the herd within the first 30 days of the lactation. Of the lactations where milk fever was recorded (n=2668), 7.76% left the herd within the first 30 days. Of the lactations that did not record a case of milk fever (n=40812), 2.74% of cows left the herd within the first 30 days. This difference was statistically significant (chi squared test, $p<0.01$).

No significant associations were found in univariate analysis between the occurrence of a case of milk fever and the birth of twins (chi squared test, $p=0.335$), or between the occurrence of a case of milk fever and RFM (chi squared test, $p=0.198$).

3.3.2. Multivariate analysis

- #### 3.3.2.1. Association between the occurrence of milk fever and potential predictors

The results for the model representing factors associated with milk fever risk used a dataset of 33,732 lactations. This number only represents a proportion of the overall dataset as some herds do not record for all predictors and so

animals with missing values for an included predictor are omitted. The results are shown in Table 3.5. The category of 'autumn' represents months August to October, as these were the only months significantly associated with milk fever occurrence in the model.

Table 3.5. Odds ratios for the single level regression model representing factors associated with milk fever risk (binary indicator variables for three herds were also included in the model in order to improve fit, as described in section 3.2.4, but coefficient estimates for these terms are not reported) (n=33,732)

Model term	Odds ratio	95% confidence interval
Intercept	0.003	0.003-0.005
Parity 2	Reference	
Parity 3	3.747	2.904-4.835
Parity 4	8.998	7.043-11.496
Parity ≥5	18.672	14.845-23.484
Single calf	Reference	
Twins	0.712	0.547-0.926
Calving: not autumn	Reference	
Calving: autumn	1.262	1.147-1.390
Previous 305 day yield ('000 litres)	1.076	1.053-1.099

The odds of a case of milk fever being recorded were 18.7 times higher for a parity ≥5 cow compared to a parity 2, and this is not dissimilar to the findings in the univariate analysis (Table 3.4). The odds of a cow experiencing milk fever were reduced by 30% if the cow delivered twins compared to a single calf. Each additional 1000 litres of milk production in the previous lactation was associated with a 7.6% increase in the odds of milk fever occurring.

No significant associations were found between milk fever and calving year, dystocia, dead calf or RFM, and so these are not included in the final model.

- 3.3.2.2. Association between the occurrence of death during a lactation and potential predictors

The results for the outcome of the cow dying during the lactation used a dataset of 4,104 lactations. This number only represents a proportion of the overall dataset as some herds did not record for all predictors and so animals with missing values for an included predictor are omitted. The results are shown in Table 3.6.

Table 3.6. Odds ratios for the multilevel logistic regression model, representing the lactation within the herd, and showing the outcome of the cow dying during the lactation (n=4,104)

Model term	Odds ratio	95% confidence interval
Intercept	0.035	0.017-0.069
Parity 2	Reference	
Parity 3	1.009	0.583-1.747
Parity 4	2.028	1.221-3.369
Parity ≥5	1.385	0.842-2.279
No milk fever	Reference	
Milk fever	1.737	1.007-2.995
No dystocia	Reference	
Dystocia	1.616	1.008-2.592
Live calf	Reference	
Dead calf	2.250	1.230-4.115
Calving: January	Reference	
Calving: February	0.495	0.206-1.191
Calving: March	0.304	0.113-0.816
Calving: April	0.847	0.405-1.773
Calving: May	0.429	0.176-1.049
Calving: June	0.904	0.440-1.856

Calving: July	0.485	0.220-1.068
Calving: August	0.369	0.161-0.846
Calving: September	0.464	0.211-1.020
Calving: October	0.758	0.382-1.505
Calving: November	0.650	0.312-1.355
Calving: December	0.675	0.326-1.397

The odds of a cow dying during the lactation was 1.7 times higher where a case of milk fever was recorded compared to where a cow did not experience milk fever. The odds of a cow dying in a lactation following delivery of a dead calf were 2.3 times higher than if the calf was born live. Dystocia increased the odds of a cow dying during the subsequent lactation by 1.6 times compared to a cow that did not experience dystocia. Variations were seen occurrence according to month but no distinct seasonal pattern was noted.

No significant associations were found between death during the lactation and RFM, twins or previous 305 day yield, and so these are not included in the model.

- 3.3.2.3. Association between an animal exiting the herd during the first 30 days of the lactation and potential predictors

The results for the outcome of a cow exiting the herd by day 30 used a dataset of 25,654 lactations. This number only represents a proportion of the overall dataset as some herds do not record for all predictors and so animals with missing values for an included predictor are omitted. The results are shown in Table 3.7.

Table 3.7. Odds ratios for the single level logistic regression model representing the outcome of a cow exiting the herd by day 30 (binary indicator variables for four herds were also included in the model in order to improve fit, as described in section 3.2.4., but coefficient estimates for these terms are not reported) (n=25,654)

Model term	Odds ratio	95% confidence interval
Intercept	0.008	0.006-0.012
Parity 2	Reference	
Parity 3	1.293	1.008-1.659
Parity 4	2.173	1.711-2.760
Parity ≥5	2.743	2.220-3.389
No milk fever	Reference	
Milk fever	2.243	1.833-2.745
Live calf	Reference	
Dead calf	3.248	2.618-4.029
Calving year: 2000	Reference	
Calving year: 2001	0.879	0.560-1.380
Calving year: 2002	1.840	1.248-2.713
Calving year: 2003	1.309	0.840-2.038
Calving year: 2004	1.556	1.055-2.294
Calving year: 2005	1.921	1.306-2.827
Calving year: 2006	1.716	1.176-2.505
Calving year: 2007	1.627	1.117-2.371
Calving year: 2008	1.908	1.320-2.758

The odds of a cow exiting the herd during the first thirty days were 2.2 times higher in a cow that experienced milk fever around calving. Delivering a dead calf was associated with increased odds of a cow leaving the herd within the first thirty days 3.2 times compared to delivering a live calf. The odds of leaving the herd within the first thirty days increased with increasing parity.

No significant associations were found between a cow leaving the herd in the first thirty days and RFM, dystocia, twins, previous 305 day yield or calving month, and so these are not reported in the results.

Cows that exited the herd in the first 30 days of the lactation did so through either culling or death. No difference was found in the 'exit by day 30' group between the animals that did or did not experience milk fever with regard to the manner in which they left the herd (within the animals that exited by day 30, 60.8% of milk fever cases were culled, compared with 61.2% of non-milk fever cases).

- 3.3.2.4. Association between the occurrence of dystocia and potential predictors

The results for the outcome of dystocia used a dataset of 4,763 lactations. This number only represents a proportion of the overall dataset as some herds do not record for all predictors and so animals with missing values for an included predictor are omitted. The results are shown in Table 3.8.

Table 3.8. Odds ratios for the single level logistic regression model representing the outcome of dystocia (binary indicator variables for four herds were also included in the model in order to improve fit, as described in section 3.2.4., but coefficient estimates for these terms are not reported) (n=4,763)

Model term	Odds ratio	95% confidence interval
Intercept	0.179	0.113-0.284
No milk fever	Reference	
Milk fever	2.312	1.747-3.060
Live calf	Reference	
Dead calf	2.423	1.679-3.496
Previous 305 day yield ('000 litres)	0.892	0.846-0.941

The odds of dystocia occurring were 2.3 times higher in a cow with milk fever compared to one without milk fever. The odds of dystocia being associated

with a dead calf were 2.4 times higher than dystocia being associated with a live calf.

No significant associations were found between dystocia and twins, parity, calving month or calving year.

- 3.3.2.5. Association between the occurrence of milk fever and other outcomes

Models were tested for other outcomes including culling, RFM and previous 305 day yield. However, the occurrence of milk fever had no statistically significant association with any of these listed outcomes and so these models have not been reported.

3.4. Discussion

3.4.1. Incidence of milk fever

The mean incidence rate of milk fever found within the current dataset of lactations obtained from 78 herds, with the inclusion of heifers, was 4.26%. Typically the incidence of milk fever has been reported within the literature as being 5-10% per lactation (Sorensen *et al*., 2002; Mulligan *et al*., 2006a; DeGaris & Lean, 2008; Hayes *et al*., 2012), and so the incidence reported in this present study is on the low side of what is often regarded as typical. The majority of papers reporting figures for milk fever incidence appear to be from data obtained from outside the UK, and this may differ due to factors such as husbandry practices. When focusing specifically on UK incidence of milk fever, Husband (2005) reported an estimation of 4-9% incidence within UK dairy herds, although the source of data, if any, on which this is based is unclear. Whitaker *et al*. (2004b) reported milk fever incidence of between 4.7% and 5.3% in British dairy herds from 1998 to 2002 (number of herds ranged from 244 to 434 for each year studied). Contrary to the present study, the data used by Whitaker *et al*. (2004b) included herds that did not have any recorded milk fever cases. Whitaker *et al*. (2004b) reported the highest annual

incidence rate for a single farm as being 41%. The present study did not consider incidence by year but instead incidence was reported by herd overall for the total of all lactations submitted by that herd, and the highest recorded incidence for a single farm was 11.3%. Hayes et al. (2012) also looked at incidence of milk fever in UK herds and reported an overall mean incidence of 5.0% (range of 0.8% to 15.7%), but this was a small study (herds n=8, lactations n=2105).

The mean incidence rate found in the present study is again on the low side of the widely accepted 5-10% incidence estimate, but it is worth considering the fact that this dataset excluded herds with a <1% incidence rate, in an attempt to control for herds that were not routinely recording milk fever events. It is also only obtained through routine farm records. This may bias the sample population towards farms with better management practices. The perceived importance of a condition to farmers or the purpose of record completion may also affect how accurately it is recorded.

The pattern of increasing milk fever risk by increasing parity, shown in Table 3.4, is well reported within the literature (Curtis *et al*., 1984; Horst *et al*., 1997; Bigras-Poulin & Tremblay, 1998; Fleischer *et al*., 2001). The pattern reported in the present study is not dissimilar to that found by Parker-Gaddis *et al*. (2012) from the analysis of records from 100 farms in the USA. They also found a very small incidence of milk fever in heifers (0.23%) and also chose to exclude these from further analysis. Koeck *et al*. (2012) also report a heifer milk fever incidence rate of 0.2%, suggesting that this present study does appear to fit well with the existing literature in this respect. The low incidence of milk fever amongst heifers is considered to be due to the existing upregulation of calcium homeostatic mechanisms in the growing animal (Martin-Tereso *et al*., 2011).

3.4.2. Previous 305 day yield

Each additional 1000 litres of milk produced in the previous lactation was associated with a 7.6% increase in the odds of milk fever occurring. This may be because a higher milk yield increases the demand for calcium (Horst *et al*., 1997) and so animals producing more milk experience a greater challenge to calcium homeostasis around calving. An association between previous yield and milk fever risk has not always been found in other studies. Grohn *et al*. (1995) carried out logistic regression on a dataset of 25 herds of Holsteins in the USA and found no association between previous yield and milk fever. Grohn *et al*. (1995) did attempt to account for the potential confounders of parity, season and herd. However, Fourichon *et al*. (1999) conducted a review of papers that investigated relationships between disease events, and they reported that only half of the studies in their review attempted to control for previous yield as a confounder when investigating milk fever. The present study does suggest an increased risk of milk fever with a higher milk yield and did take into account parity, which can sometimes act as a confounder, as milk yield tends to increase with increasing parity, as does the risk of milk fever. These effects are therefore additive, with the model suggesting that a later parity cow with a higher yield would have her risk for milk fever increased by both of these factors. However, the effect of yield on the increase in odds of milk fever demonstrated by this model is smaller than that of parity.

3.4.3. Twins

There appears to be limited data within the literature regarding the potential effects of twin pregnancies on incidence of milk fever. This may be due to the fact that both events are relatively rare in themselves, and even more unusual in combination, and so the data available for study may be relatively limited in itself. In the present study, 36,555 lactations recorded a binary outcome for the occurrence of twins, and of these only 70 lactations reported a twin birth and milk fever in combination (0.19%). A large number of lactations is therefore required within a dataset in order to investigate this association,

and a prospective study would be unlikely to obtain sufficient data for this. The univariate analysis conducted in this study did not find any significant relationship between twin births and milk fever. However, the multivariate analysis indicated a significant association, with cows that gave birth to twins having their odds of experiencing milk fever reduced by 30%. The reasons for this are unclear and do not appear to be reported within the literature. Causality in the present study cannot be determined due to the nature of the study design. However, it could be speculated that a cow carrying twins may have already upregulated her hormonal mechanisms of calcium homeostasis owing to the greater losses of calcium pre-calving into two foetuses. The dietary restriction of calcium pre-calving is widely regarded as an effective means of preventing milk fever (Belyea *et al*., 1974; Green *et al*., 1981; Mulligan *et al*., 2006a). Cows with reduced blood calcium pre-calving have been demonstrated to have increased levels of parathyroid hormone circulating and thus have increased calcium resorption from bone (Goff, 2008). The increased loss of calcium into multiple foetuses pre-calving could therefore be suggested as a theory to explain the association and apparent protective nature of twin pregnancy against milk fever reported in this present study.

3.4.4. Dystocia

This study found an association between dystocia and the occurrence of milk fever, with the odds of a cow experiencing dystocia being 2.3 times higher if she also experiences milk fever. This association was found in the regression model but is not dissimilar to the findings of the univariate analysis. Milk fever has previously been reported as being associated with dystocia, and Curtis *et al*. (1983) reported that cows with milk fever were 3 times more likely to develop dystocia compared to normocalcaemic animals. This association cannot be identified as a cause here due to the present study, but within the literature this association has previously been described being a consequence of reduced uterine motility (Mulligan *et al*., 2006b), owing to reduced smooth muscle function associated with hypocalcaemia (Hanesen *et al*., 2003).

Cows that delivered dead calves were 2.4 times more likely to experience dystocia. The present study does not allow for an indication of causality. Schuenmann *et al*. (2011) reported an increased risk of stillbirth following dystocia, with 22.1% of cows with dystocia (n=32) delivering dead calves compared to 1.5% of cows without dystocia (n=60). This is a much smaller sample size than the present study. It also does not take into account other factors that the present study incorporated into the regression models. In univariate analysis of all lactations (including heifers) in the present study, 11.68% of cows that experienced dystocia delivered stillborn calves, compared to 4.40% of cows that did not experience dystocia, and this difference was statistically significant (chi-squared test, $p<0.0001$). The difference in the size of this effect to that found by Schuenmann *et al*. (2011) may be a reflection of the differing parity profile of the study populations. Schuenmann *et al*. (2011) had a small study population (n=92), of which 63.04% were heifers (n=58), compared to the present dataset that included only 26.36% heifers (n=15565). Dystocia has previously been reported to be associated with age. Heifers are considered to be at the greatest risk of dystocia, and reasons suggested for this include the calf size relative to the mother, and the fact that heifers are not fully developed (Mee *et al*., 2011). For example, Funnell & Hilton (2016) reported that in a study of 270 herds in Canada (lactations n=29,970), 17.3% of calving heifers experienced dystocia compared to 2.9 to 4.7% of multiparous cows. The present study found less disparity between the two groups but found that 14.5% of heifers (total n=3336) experienced dystocia, compared to 8.91% of multiparous animals (total n=9646), and this difference was statistically significant (chi-squared, $p<0.0001$). This may explain why the multivariate analysis in the present study did not find a significant association with parity. This is likely to be due to the removal of heifers from the dataset prior to multivariate analysis, as they were deemed to be of less interest due to the focus of this present study on milk fever, as previously explained.

3.4.5. Death during the lactation

Cows experiencing milk fever were 1.7 times more likely to die during the subsequent lactation compared to cows that did not have a recorded case of milk fever. This describes natural deaths only and does not include culling.

Of the deaths in animals with milk fever, 67.5% had occurred by day 30 of the lactation, compared with 39.7% of the deaths in animals without milk fever, and this difference was statistically significant ($p<0.01$). In deaths by day 60 of the lactation, 74.2% of the deaths in animals with milk fever events recorded had occurred, compared to only 49.5% of the deaths in animals without milk fever, and again this was statistically significant ($p<0.01$). Both of these points demonstrate that deaths in animals with milk fever were more likely to happen at the earlier available time points of the lactation than for cows that died but which did not experience milk fever. Severe milk fever in itself can result in death post-partum and Kossaibati & Esslemont (1997) estimated that around 8% of cases are fatal. Deaths may also occur further into the lactation from other factors. However, this may include diseases that have previously been shown to be associated with the occurrence of milk fever, including mastitis (Curtis *et al*., 1983; Goff & Horst, 1997; Berry *et al*., 2007) and displaced abomasum (Correa *et al*., 1990), and it is not possible to investigate these associations with the dataset used in this chapter.

3.4.6. Exiting the herd by day 30 of the lactation

Cows leaving the herd by day 30 of the lactation did so by either death or culling, and these occurred in the same proportion regardless of whether or not the animals experienced milk fever. Of the animals that exited the herd by day 30 of the lactation, 60.8% of milk fever cases were culled, compared to 61.2% of non-milk fever cases. The overall proportional split of deaths and culls is not dissimilar to the findings of Thomsen *et al*. (2004), who reported that out of the overall mortality rate of dairy cows studied (herds n=196), 58% were euthanised.

Milk fever was shown to be associated with the risk of a cow leaving the herd within the first 30 days, as the odds of this happening were 2.2 times higher when a cow experienced milk fever, through either death or culling. Hayes *et al*. (2012) studied 2105 lactations across 8 herds and reported that 16.2% of cows with milk fever were culled within the first 100 days of the lactation (OR=4.0), compared to 5.3% that did not experience milk fever. This present study showed that having a dead calf increased a cow's likelihood of exiting the herd during the first 30 days of the lactation by 3.2 times. Hayes *et al*. (2012) reported an increased risk for cows that gave birth to a dead calf in the first 100 days of the lactation, with an odds ratio of 2.4.

3.4.7. Limitations of this study

Whilst this retrospective cross-sectional study has been able to make a number of associations between milk fever and other variables, there is no opportunity to demonstrate causality of these relationships. This study has aimed to focus on relationships in which the chronology of the events implies the causal direction of the relationship. For example, a case of milk fever will not influence whether or not a cow has delivered twins. However, the possibility of confounding variables confusing the apparent relationships between factors cannot be ruled out.

There may be additional confounding variables that were not recorded within the current dataset and the associations found in this study may have been altered were additional information available. For example, breed information was not available in this current study and whilst the vast majority of animals were Holstein/Holstein-Friesian, different results may have been found if the study population had consisted of solely Holsteins, as breed differences are widely recognised within the literature (Bendixen *et al*., 1987; Schnier *et al*., 2004; Lean *et al*., 2006). Body condition could potentially also be a hidden factor here, as over-conditioned cows have been shown to have a higher risk of milk fever (Heuer *et al*., 1999), and as body condition scores were not recorded within the dataset these were unable to be incorporated into the analysis.

As a number of herds did not record all of the predictors used, the numbers for some of the models were considerably smaller than others and so did not represent the entire dataset. For example, the final model for factors associated with death during the lactation only included data for 4104 lactations. However, these are still relatively large numbers of lactations and are comparable with some other studies reported within the literature.

No information is available regarding the criteria used to diagnose milk fever, or any of the other potential predictors, such as RFM and dystocia. Whilst the dataset was obtained through veterinary practices, it is made up of on-farm records and does not represent veterinary diagnosis of any of the disease events.

There is also no background information on the husbandry and milk fever prevention used within the herds included in this study, or whether these represent 'typical' herds in terms of their milk fever control. A relatively large sample size was used, however, and the incidence of milk fever found in this trial appears to fit within the range reported within the literature, as previously discussed.

Herds that are keeping records of lactations with the details used in this study may represent herds that are better than average at record-keeping. This study sample represented a convenience sample of farms using the veterinary practices that were originally approached for data collection (Hudson *et al*., 2012). It is entirely possible that these herds may also implement other good management practices, for which they may be above average when compared with other UK dairy farms, and this may affect how these results fit with the true picture of the UK dairy industry.

3.5. Conclusion

This study provides some more up to date data on milk fever incidence within the UK than was currently available within the literature. Within the current study, 4.26% of lactations (from heifers to parity 5+) recorded at least one case of milk fever. This figure is on the low side of UK milk fever incidence

ranges reported by other studies, such as Whitaker *et al*. (2004b) and Husband (2005). It is not clear whether this is potentially due to the nature of this current study, which relied on retrospectively collated data from existing farm records, as criteria for recording milk fever may vary between farms, the records may not be entirely complete and accurate, and farmers who record herd health data such as this may also implement other good management practices to reduce disease events. The finding of milk fever risk increasing by parity is widely reported in the existing literature (Horst *et al*., 1997; Bigras-Poulin & Tremblay, 1998; Koeck *et al*., 2012), and the present study appears to confirm that this is still relevant within UK dairy herds in recent years.

Previous yield was associated with a 7.6% increase in the odds of milk fever occurring per 1000 litres. This finding took into account parity, which can act as a confounding variable, as cows of increasing parity are also reported to have increasing yields.

Retained foetal membranes have previously been reported to be more likely in cows that experienced milk fever (Klerx & Smolders, 1997) or dystocia (Melendez *et al*., 2003). However, no such associations were found in the multivariate analysis in the present study.

The present study found that the split of cows exiting the herd by day 30 of the lactation into either deaths or culls was very similar to the existing findings of Thomsen *et al*. (2004). Leaving the herd by day 30 was 2.2 times more likely in cows that experienced milk fever than those that did not, and death was 1.7 times more likely in the cows with milk fever. Again, causality cannot be explicitly stated in this study. However, increased mortality rates with milk fever have previously been reported in the literature (Hayes *et al*., 2012) and the present study adds evidence in support of this issue, which has a detrimental financial impact on farmers, in recent UK conditions.

There was a 2.3 times higher risk of dystocia in cows that experienced milk fever compared to those that did not. Curtis *et al*. (1983) report an increased risk of 3 times, and so this finding is not dissimilar to existing literature.

The association between twin pregnancy and a 30% reduction in milk fever risk appears to be a finding previously unreported within the literature. The present study cannot prove causation, but it could be postulated that an increasing demand for calcium placed on the body pre-partum by two developing foetuses may initiate the upregulation of calcium homeostatic mechanisms sooner than in animals with a single calf.

CHAPTER 4: INVESTIGATING THE EFFECTS OF FEEDING RUMEN-PROTECTED RICE BRAN DURING THE DRY PERIOD ON COWS IN A COMMERCIAL UK DAIRY HERD

4.1. Introduction

The use of dietary calcium binders is a relatively new approach to the prevention of milk fever, as detailed in Chapter 1. The binding of dietary calcium means that it is not available for absorption by the animal and passes out in the faeces (Goff, 2008), thus mimicking the low calcium diet approach. This works on the basis of activating the mechanisms of calcium homeostasis and increasing the production of parathyroid hormone, stimulating the resorption of calcium from bone. Upregulating the calcium homeostatic system using low calcium diets during the late dry period has been shown to enable cows to have a more rapid return to normal blood calcium concentrations after calving (Belyea *et al*., 1974; Green *et al*., 1981), reducing the risk of milk fever and associated problems. Rice bran has the capacity to bind with dietary calcium, due to its high phytate content, but ordinarily would be unsuitable for use in dairy cows in this way due to rumen degradation. However, in recent years, a method of treating rice bran with formaldehyde has enabled the development of a 'rumen-protected' rice bran product, capable of binding a reported 7g of calcium per kilogram of product (Martin-Tereso *et al*., 2009). This rumen-protected rice bran (RPRB) as a feed product has to date only been tested in small trials on experimental farms. This chapter aims to trial RPRB on a larger scale in a commercial UK dairy herd and to investigate its effects on both clinical milk fever and subclinical hypocalcaemia, along with considering the potential effects of its use on magnesium and phosphorous status, energy status, protein parameters, and milk yields. This trial also aims to provide a greater insight into the practicalities of using such a strategy on a commercial farm.

The terms 'treatment feed', 'rumen-protected rice bran' and 'RPRB' will be used interchangeably within this chapter.

4.2. Materials & methods

4.2.1. Farm background

The trial was carried out on a commercial dairy farm in Derbyshire during 2013. At this time, the milking herd consisted of around 280 pedigree Holstein cows. The milking herd were grazed during spring and summer months but were housed in cubicle sheds throughout autumn and winter. All freshly-calved cows used on the trial were kept in the same shed, alongside other non-trial cows. The farm targeted a 50 day dry period.

4.2.2. Trial animals and dry cow husbandry

The trial aimed to recruit 100 multiparous pedigree Holstein cows. Heifers were excluded from the trial on the basis that first-calvers are at a lower risk of hypocalcaemia (Horst *et al*., 1997; Koeck *et al*., 2012) and so are not typically targeted by milk fever prevention strategies. The trial animals were recruited onto the trial at drying-off, when they were moved from the milking shed into a dry cow shed, where the animals were loose-housed and bedded on straw. This large shed was divided into two sections: approximately three-quarters of it formed the main dry cow yard, with the remaining quarter being treated as a 'close up' yard, where the farmer moved cows when calving was considered to be imminent. The number of days that individual cows spent in the smaller section of this shed varied but animals in both sections of the shed had unrestricted access to identical forage and the water supply was shared between the two pens. Identical out-of-parlour feeders were installed in each section of the dry cow shed. These were installed back-to-back and the layout of the shed can be seen in Figure 4.1.

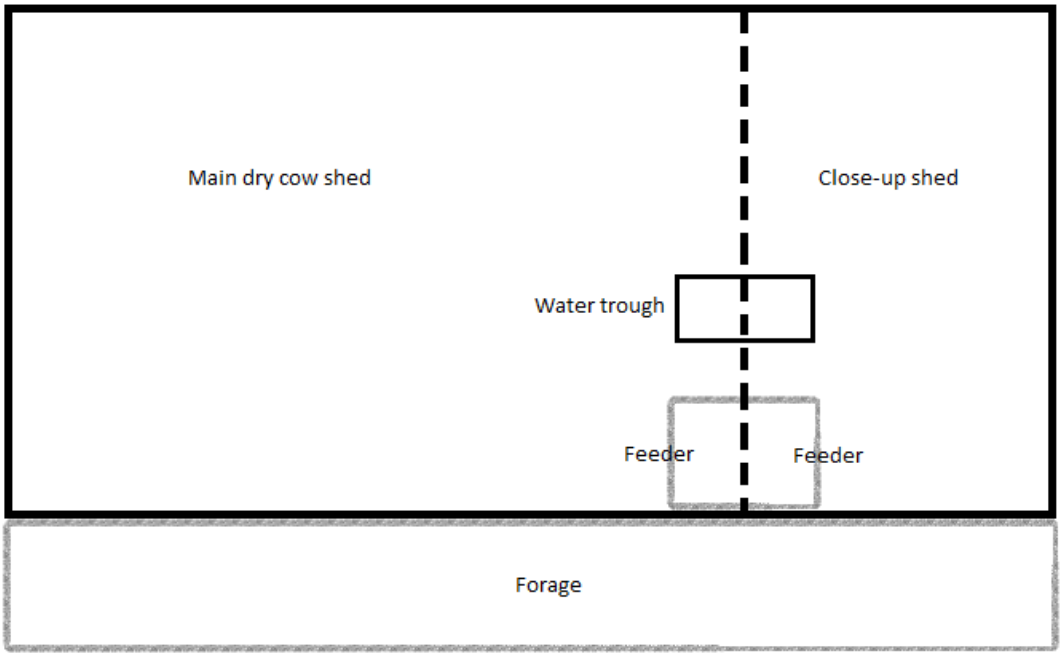


Figure 4.1. Diagram of dry cow shed used during feed trial

The trial took place on a commercial dairy farm with the agreement that it would not be too disruptive to normal farm activities and with restrictions on how much the diet could be altered from the farmer's usual dry cow feeding regime.

4.2.3. Trial allocation and design

The cows were assigned to one of two groups. Half of the cows were allocated to receive a control diet, with the other allocated to receive a treatment diet. In order to balance the treatment and control groups as effectively as possible, cows were blocked according to parity and yield. Dry-off dates were determined by the farmer and cows were typically dried off in groups of between six and twelve at a time. These cows were divided up into matched pairs, based on both parity and previous 305 day yield. Each pair was randomly split to assign one cow to the rumen-protected rice bran (RPRB) treatment group and one to the control diet. This block randomisation design should negate any effects of the time of year, such as variations in forage or weather effects, as a matching cow was assigned to each feed group at the same time. The farmer and all farm staff were blind to the treatment groups throughout the trial.

All parities noted in the dataset from this trial are recorded at the time of recruitment, at the point of drying-off (i.e. a 'parity 1' cow reported in this chapter entered her second lactation following on from her dry-period on the trial diet).

4.2.4. Trial changes

Initially the trial sampling began on 29th January 2013. At the beginning of April there were some issues with both the RPRB and control diets and these were subsequently altered. In light of this, the trial effectively was restarted on 5th April 2013.

The issues arose following complaints from the farmer regarding an unexpectedly high number of clinical milk fever cases. This was primarily a concern regarding animal welfare, but also risked the farmer withdrawing from participation in the trial.

For approximately the first two months of the trial, the cows receiving RPRB had been doing so at a rate of 4kg/day from their dry-off date up until calving. Due to the problems that were occurring, it was decided that the time on RPRB would be reduced to three weeks before the expected calving date. Any cows that were due to calve imminently that had been on RPRB for longer than three weeks were monitored closely around calving and treated if clinical signs were observed, as outlined in section 4.2.10. Any cows that were on RPRB but had more than three weeks left until expected calving were changed onto the control feed up until 21 days before their expected calving date, at which point they were switched back onto RPRB. From 5th April 2013 onwards, all trial cows were put onto the control pellet at drying-off and were only changed over to RPRB at three weeks before expected calving. This is represented in Figure 4.2.

At the point where the RPRB feeding regime was altered, additional standard and mineral analyses of the pelleted feeds were requested and were carried out by Frank Wright Ltd (Trouw Nutrition GB, Ashbourne, Derbyshire, UK). This analysis indicated that the control pellet had been incorrectly

manufactured and was oversupplying calcium by 11.6g/head per day and phosphorous by 10.46g/head per day, leading to a total calcium intake of 64.66g/head per day and total phosphorous intake of 53.56g/head per day in the total diet for control cows. As the diet was in a pelleted form and was being fed through an out-of-parlour feeder, additional magnesium chloride in an attempt to counteract this was added to the forage mix at a rate of 20g/head per day. This increase in magnesium chloride was received by both the control and treatment groups, as it was not possible to split the cows into separate housing. The decision to add magnesium chloride was made primarily in order to safeguard animal welfare and attempt to reduce the number of clinical cases of milk fever that were occurring on the original diet. Financial and logistical constraints meant that it was not possible to repeat the manufacture of the diets in order to correct the error that had been noticed, and substantial investments of both time and money had already been put into setting up the trial.

It was subsequently noted that a deviation from the specification also occurred in the RPRB diet. When the analysis was carried out in early April on the RPRB diet, it was found to contain more phosphorous than was formulated, supplying RPRB cows with an additional 19.8g of phosphorous per day. This meant that the total phosphorous intake for RPRB cows was 96.7g/head per day. As previously noted, these cows also received an additional 20g/head per day of magnesium chloride, although this was only as a consequence of supplying this to the control animals at this time.

All cows affected by this early diet change were removed from the dataset prior to the analysis of the results. A comparison between the original trial design and the new trial design, which applied to all animals included in the results data, is shown in Figure 4.2.

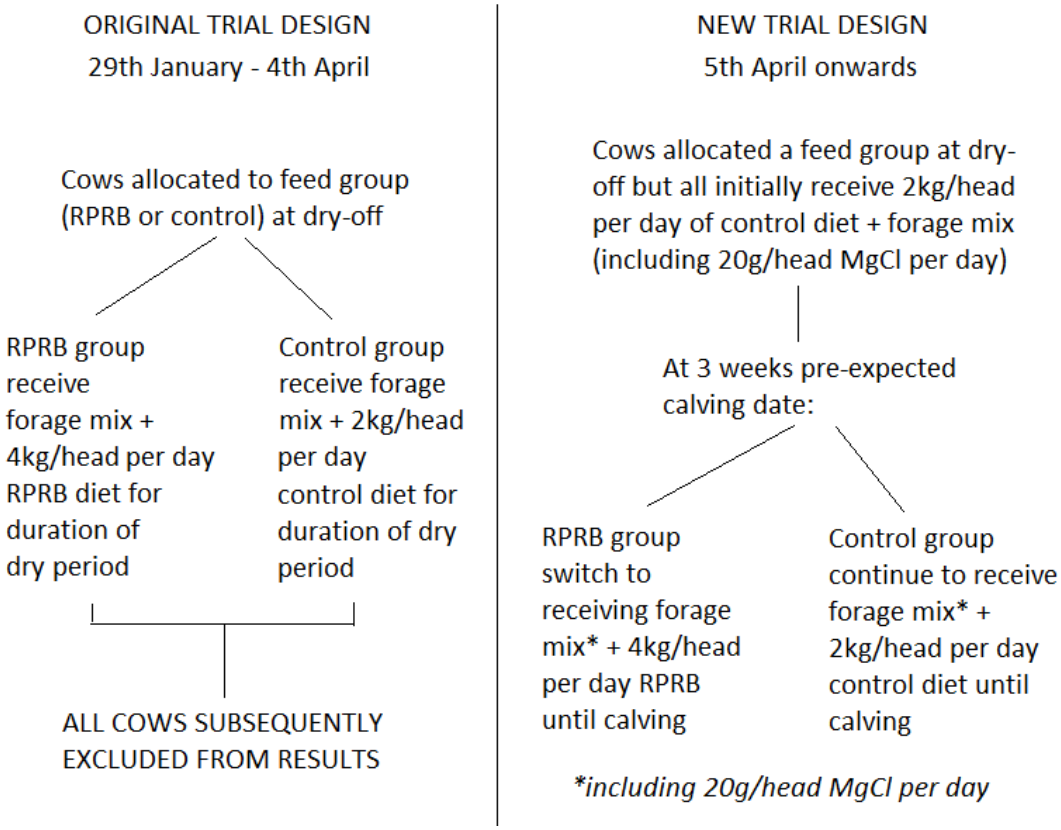


Figure 4.2. Flowchart to represent diet changes occurring during feed trial

4.2.5. Trial diet formulations

RPRB is a feed product made from rice bran. Its high phytate content gives it the capacity to act as a calcium binder, with Martin-Tereso *et al*. (2009) reporting that it may be capable of binding 7g of dietary calcium per kilogram of product. The rice bran is treated with formaldehyde to prevent its degradation in the rumen.

The treatment and control diets differed only by the pelleted feed received by the animals and the forage mix was shared by both groups. The diets were formulated on a 12kg/day DMI basis using commercial ration formulation software (carried out by Rachel Fowers, Nutreco Ruminant Research Centre, Boxmeer, Netherlands) and the control diet was formulated as closely to the original pre-trial dry cow diet as possible, due to restrictions placed by the farm. The control and treatment diets were manufactured by Lloyds Animal Feeds (Oswestry, Shropshire, UK) and Hendrix UTD (Zwolle, Netherlands) respectively.

Unfortunately there was a requirement to change the diet formulation approximately two months into the trial, as previously explained in section 4.2.4. The initial composition is detailed in Table 4.1, with further details on the composition of the pelleted feeds included in appendix B.

Table 4.1. Ration formulations for trial dry cows, using silage mineral analysis carried out prior to commencement of the trial

	Pre-trial ration formulation	**Trial control ration formulation**	**Trial treatment ration formulation (RPRB)**
Forage mix (kg)	22.00	22.00	18.00
Milking blend (kg)	2.00	0.00	0.00
Pre-trial dry cow	0.10	0.00	0.00
Magnesium chloride	0.10	0.00 (up to 5th April 2013) *(0.02 from 5th April 2013 onwards)*	0.00 (up to 5th April 2013) *(0.02 from 5th April 2013 onwards)*
Trial dry cow mineral	0.00	0.10	0.10
Trial control pellets	0.00	2.00	0.00
Trial RPRB pellets	0.00	0.00	4.00
TOTAL	24.20	24.10	22.10
NUTRIENT ANALYSIS	*(nutrient analysis of initial trial formulations, before additional MgCl was added to ration)*		
Dry matter intake	11.90	11.80	11.80
Forage dry matter	9.95	9.95	8.14
Dry matter (%)	49.20	49.10	53.40
M.E. (MJ)	115	114	113
M/D (MJ/kgDM)	9.70	9.61	9.55
CP (%DM)	12.9	12.7	13.7
MPB (g)	320	317	308
MPN (g)	1026	1013	1074
MPE (g)	1002	987	899
Excess MPN (g)	24.9	26.3	174.8
Rumen stability	196	198	152
NDF (%DM)	51.0	50.8	46.4
Oil (%DM)	2.94	2.89	2.65
RF starch (g/kgDM)	32.6	29.7	69.9
RU starch (g/kgDM)	3.46	3.02	18.58
Sugar (g/kgDM)	30.7	34.8	49.1
YieldME	0.000	0.000	0.000
YieldMP	0.000	0.000	0.000
Calcium (g)	54.9	53.3	43.2
Phosphorous (g)	36.3	43.1	76.9
Magnesium (g)	47.0	46.5	62.5
Sodium (g)	33.8	33.5	39.2
Iron (mg)	3972	4215	3511
Copper (mg)	312	319	334
Manganese (mg)	1176	1144	1344
Cobalt (mg)	11.3	11.5	11.6
Zinc (mg)	1137	1118	1266
Iodine (mg)	48.7	53.8	52.2
Selenium (mg)	4.47	4.55	4.50

4.2.6. Feeding regime

Both the control and treatment diets were fed in a pelleted form through an out-of-parlour feeder (Fullwood Ltd., Shropshire, UK). There were two feeders, one for each section of the dry-cow shed (Figure 4.1), and each feeder had two separate hoppers in order to deliver both the control and treatment feed. These were refilled daily and were recalibrated weekly by weighing delivered portions of feed and inputting the weight data back into the feeder computer. At drying-off, all trial cows were fitted with a collar containing a transponder. This was synchronised to the out-of-parlour feeders using the herd management software 'Crystal' (Fullwood Ltd., Shropshire, UK), enabling concentrate intakes to be controlled and recorded.

When the control diet was fed, it was done so at a rate of 2kg/day, split into four four-hourly 'meals' across 24 hours, and each of these 500g 'meals' was delivered in 125g 'portions'. The treatment feed (RPRB) was fed at a rate of 4kg/day, split into four four-hourly 1kg 'meals' each day.

When a cow placed its head over the transponder reader on the edge of the out-of-parlour feeder, a portion of feed was delivered. If the animal remained standing at the feeder then additional portions of feed were delivered until either the cow left the feeder or the entire meal had been dispensed. If a cow returned to the feeder within four hours after receiving an entire meal then no additional feed would be delivered at that time. Feeding times and quantities of feed delivered were automatically recorded through Crystal and could then be downloaded and monitored daily.

Forage was mixed by the herdsman using a mixer-wagon and was intended to comprise of 86.2% grass silage and 13.8% wheat straw, plus 100g/head of a powdered mineral supplement (Table 4.2). The forage feeding routine depended upon the time of year and weather: in hotter weather, the forage was mixed daily, but sometimes was mixed every two of three days during colder months. The forage was distributed evenly along the outside of the building, allowing access to cows in both the far-off and close-up sides of the shed. Forage intakes were neither controlled nor monitored. The forage mix

was altered after 5th April to include additional magnesium chloride, as outlined in section 4.2.4, and this change applied to all cows included in the final dataset.

Table 4.2. Composition of trial powdered mineral supplement

Mineral/vitamin	Form	Inclusion rate
Calcium		Nil
Phosphorous		Nil
Magnesium		15%
Sodium		11%
Selenium	Sodium selenite	30mg/kg
Selenium	CNCM I-3060	5mg/kg
Cobalt	Cobalt carbonate	90mg/kg
Iodine	Calcium iodate	450mg/kg
Manganese	Manganous oxide	5000mg/kg
Zinc	Zinc oxide	8000mg/kg
Copper	Cupric sulphate	2500mg/kg
Vitamin A	Retinyl acetate	400,000IU/kg
Vitamin D	Cholecalciferol	100,000IU/kg
Vitamin E	di-alpha-tocopheryl acetate	6500IU/kg

4.2.7. Mixed forage sampling

Samples of the mixed forage ration were collected every morning. Each daily sample comprised of five handfuls, taken from approximately equally sized intervals along the entire feed-face, across both the close-up and far-off sides of the dry cow shed. These samples were then bagged, dated and frozen at -20°C, and were later defrosted and pooled into monthly samples for analysis.

4.2.8. Sampling of diet components

Fortnightly samples were collected of both the control and RPRB diets, along with samples of the straw, grass silage and mineral that made up the mixed forage ration. The concentrate samples were taken directly from the feeder hoppers. The straw was sampled from the bale currently in use for dry cow feed. Grass silage samples were taken from five different points at different heights along the face of the silage clamp in a 'W' shape – the silage directly on the face of the clamp itself was discarded and the sample was taken from just behind it in order to reduce any effects of exposure to the weather. These samples were bagged, labelled and frozen at -20°C for subsequent analysis.

4.2.9. Access to drinking water

All cows had unrestricted access to water in the dry cow shed. This was supplied through a trough shared between both sections of this shed (Figure 4.1) and the water came from a mains source.

4.2.10. Procedures surrounding calving

Calving dates and number of calves born were recorded by the herdsmen. A record of assisted calvings was also requested. The transponder collars required for the use of the out-of-parlour feeders supplying the trial diets were removed after calving, with cows being moved from the dry cow shed and into the milking herd as soon as possible.

4.2.11. Trial animal health events

Any freshly calved cows considered by the farmer or herdsmen to be displaying signs of clinical milk fever, such as unsteadiness or recumbency, were treated by subcutaneous and/or intravenous injection of a calcium and magnesium solution (Calciject 40CM Solution for Injection, Norbrook Laboratories). A 400ml injection of this solution supplies 11.9g calcium in the forms of calcium gluconate and calcium borogluconate and 1.85g magnesium in the form of magnesium hypophosphite. This method of treatment for cases assumed to be clinical milk fever was standard practice on the farm and was not altered for this research trial, although any such diagnoses and treatments were recorded for the trial.

Cases of retained foetal membranes, either witnessed or as reported by farm staff, were recorded. Retained foetal membranes were defined as foetal membranes not expelled by 24 hours post-partum (Kelton *et al*., 1998).

The occurrence of health events was either recorded at the time or records were obtained retrospectively from the farm diary.

4.2.12. Blood and urine sampling

Blood and urine samples were collected once a week on a Tuesday from all dry cows and from all trial cows for twelve weeks post-calving.

- 4.2.12.1. Blood sampling protocol

Blood samples were taken, Animals (Scientific Procedures) Act 1986 (PPL 40/3586) with local ethical approval, from the tail vein using single-use 1.5" 18G needles (Vacuette® single sample needles, Greiner Bio-One, Gloucestershire, UK) and 10ml vacuum tubes for both serum ('CAT' clot activator tubes, catalogue number REF367896, BD Vacutainer®, Plymouth, UK) and ('LH' lithium heparin tubes, catalogue number REF367880, BD Vacutainer®, Plymouth, UK). Immediately after sampling these were gently inverted several times to ensure thorough mixing.

- 4.2.12.2. Blood processing and storage

All blood samples were processed in the laboratory on the day they were collected. The length of time between collection and processing varied but did not exceed twelve hours.

An aliquot of approximately 1ml of whole blood was removed from the LH tube after mixing and was stored in a 75x12mm 4ml polypropylene tube (Sarstedt, Leicester, UK) at -20°C. The remainder of blood in the LH tube was then centrifuged at 2000*g* for 15 minutes, before the plasma was removed and divided into three 75x12mm 4ml polypropylene tubes (Sarstedt, Leicester, UK), in aliquots of approximately 1ml. Within thirty minutes of centrifugation, these aliquots of plasma were then stored at -20°C prior to subsequent analysis.

Clotted CAT tubes were centrifuged at 2000g for 15 minutes. The serum was then removed and divided into three 75x12mm 4ml polypropylene tubes (Sarstedt, Leicester, UK), in aliquots of approximately 1ml. These were stored at -20°C, within thirty minutes of centrifugation, prior to subsequent analysis. All results obtained from the analysis of blood samples are following a single freeze-thaw cycle.

- 4.2.12.3. Urine sampling protocol

Urine samples were midstream specimens, obtained either as a voluntary free-catch sample or following manual stimulation of the perivulvar area. The pH of these urine samples was measured using a waterproof portable pH meter (Hanna Instruments, Bedfordshire, UK), prior to then being mixed by inversion, pipetted into aliquots and stored in 14ml polypropylene tubes (Sarstedt, Leicester, UK) at -20°C prior to analysis.

4.2.13. Clinical chemistry blood and urine analysis

The analysis of serum, plasma and urine samples was all carried out in single replicates using a Randox RX Imola clinical chemistry analyser (Randox Laboratories Ltd., Belfast, UK). The analytes tested for, kits and sample types used are outlined in Table 4.3. With the exception of non-esterified fatty acids (NEFA), kits were used according to the manufacturer's instructions. Volumes of reagents and samples were reduced for NEFA analysis but the ratios remained in accordance to the kit instructions and so no assay parameters were altered.

Table 4.3. Clinical chemistry analyser kit details

Analyte	**Randox catalogue number**	**Sample types analysed using this kit**
Calcium	CA3871	Serum, plasma, urine
Magnesium	MG3880	Serum, plasma, urine
Phosphorous (inorganic)	PH3820	Serum, plasma, urine
Total protein	TP3869	Serum
Albumin	AB3800	Serum
NEFA	FA115	Serum
BHB	RB1007	Serum
Urea	UR3825	Serum
Creatinine	CR3814	Urine

- 4.2.13.1. Serum analysis

Serum was analysed for calcium, magnesium, phosphorous, BHB, NEFA, urea, total protein and albumin. This analysis was carried out as single replicates

using the Randox RX Imola clinical chemistry analyser (Randox Laboratories Ltd., Belfast, UK), as outlined in section 4.2.13.

- 4.2.13.1.1. NEFA and BHB

NEFA and BHB concentrations were given binary classifications, where '1' represented an 'elevated' value. The threshold for elevated NEFA was 0.8mmol/l and the threshold for an elevated BHB concentration was >1mmol/l.

- 4.2.13.1.2. Albumin and globulin

Serum samples were analysed for total protein (TP) and albumin, using the clinical chemistry kits detailed in Table 4.3.

- 4.2.13.1.3. Serum minerals

The concentrations of calcium, magnesium and inorganic phosphorous within the blood of dairy cows all have relevance to milk fever and hypocalcaemia. Measuring calcium itself is important, as discussed in depth in Chapter 1. Magnesium has an important role in hypocalcaemia, as being in a hypomagnesaemic state has been shown to reduce the body's responsiveness to PTH (Lean *et al*., 2013), thus increasing the risk of milk fever. Serum concentrations of phosphorous are also important as increasing serum phosphorous levels are associated with an increased risk of milk fever (Lean *et al*., 2013). The references ranges for the serum minerals analysed for in this trial are shown in Table 4.4.

Table 4.4. Reference ranges for the relevant selection of serum minerals (Aiello & Moses, 2015)

Analyte	Reference range (mmol/l)
Calcium	2.0 to 2.8
Magnesium	0.6 to 0.9
Phosphorous	1.8 to 2.6

- 4.2.13.2. Urine analysis

Urine samples were analysed in single-replicate using the Randox RX Imola clinical chemistry analyser (Randox Laboratories Ltd., Belfast, UK) and were tested for calcium, magnesium, phosphorous and creatinine, using the method outlined in section 4.2.13. Urine samples underwent a single freeze-thaw cycle prior to analysis and were thoroughly defrosted and mixed on a tube-roller before being loaded onto the analyser.

Urine mineral results were interpreted as ratios to creatinine.

4.2.14. Forage analysis

Standard and mineral analysis was carried out by Frank Wright Ltd. (Trouw Nutrition GB, Ashbourne, Derbyshire, UK) on the monthly pooled mixed forage samples and on the fortnightly grass silage samples. Dry matter was determined by the UKAS-accredited method Chem/6 (Trouw Nutrition GB, Ashbourne, Derbyshire, UK). Crude protein was analysed for using a non-accredited near infrared reflectance spectroscopy (NIR) method (Trouw Nutrition GB, Ashbourne, Derbyshire, UK). Major minerals, trace elements and antagonists were quantified using the UKAS-accredited method TE/1 by inductively coupled plasma optical emission spectrometry (ICP-OES) and microwave digestion (Trouw Nutrition GB, Ashbourne, Derbyshire, UK). Chloride was tested for using a chloride analyser using the UKAS-accredited method Chem/7 (Trouw Nutrition GB, Ashbourne, Derbyshire, UK).

4.2.15. Analysis of results

- 4.2.15.1. Data quality and restructuring

Sampling dates were rounded into the nearest calving week, with sampling point 0 being the sample taken closest to calving. This sample was excluded from the analysis due to the disparity between the actual days of these samples and the potential for the result to be influenced by the number of days from calving. The samples that were actually taken on day 0 were not included in any further data analysis due to the small number of samples this applied to.

- 4.2.15.2. Statistical analysis

The categorical predictor variables were feed group, parity, calving month, dry period length, and incidence of clinical milk fever or RFM. The feed groups used were 'RPRB' (animals consumed >75% of allocated rumen-protected rice bran diet for 3 weeks pre-calving) and 'control' (animals consumed >75% of allocated control diet for 3 weeks pre-calving), and the RPRB group was used as the reference. 'Parity' refers to the parity at drying-off and was split into parity 1, 2, 3 and 4+ (parity 4 and higher were combined due to small numbers within this subset of data). Parity 1 was used as the reference and refers to cows that had previously completed one lactation, entering their second lactation following the trial dry period. Calving month was reclassified to simplify the models where possible. Dry period lengths were allocated into one of three groups: group 1 represents cows with a dry period length of <30 days, group 2 represents cows with a dry period length of 30 to 60 days, and group 3 represents cows with a dry period length of >60 days.

As previously stated, cows that were affected by the early diet changes were excluded from all data analysis.

Lactation data was reported in units of '000 litres. The yield data was centred, with the population mean subtracted from each cow's data.

Only one cow in the final dataset had given birth to twins. As this cow was an exceptional case, it was decided that her data should be removed from the final dataset, leaving a total of 62 animals in the final analysis.

- 4.2.15.2.1. Clinical disease incidence

When clinical event outcomes were analysed, both milk fever and RFM were analysed as binary outcomes (score 0 = no clinical condition, score 1 = clinical condition). These were tabulated and tested for statistical significance using Fisher's exact test. Multivariate analysis was not carried out due to small numbers of these outcomes within the dataset.

- 4.2.15.2.2. Serum mineral (Ca, Mg & P) concentrations

Serum calcium, serum phosphorous and serum magnesium were analysed using multilevel regression models, to account for repeated measures within each cow's dataset. As previously noted, the data was restructured to allocate a sample to each weekly time point relative to calving. Serum minerals were analysed for weeks -3 to -1 pre-calving, as this was where any changes due to the dry period diet were expected to be seen. As previously stated, samples from week 0 were excluded due to their varied nature, as they were from animals ranging between 3 days pre- and 3 days post-calving. It was decided that individual samples that happened to fall on 'day 0' would not be analysed due to the small number of samples that this would involve.

- 4.2.15.2.3. Urine mineral (Ca, Mg & P) ratios to creatinine

Serum and urine minerals (Ca, Mg, P) were analysed for the sample point at -1 week pre-calving. Urine minerals were analysed as ratios to creatinine, using a simple univariate comparison of the three feed groups, as the urine mineral:creatinine ratios were right-skewed, rather than being normally distributed. The urine calcium/magnesium/phosphorous results of the RPRB and control groups at 'week -1' (one week pre-calving) were compared directly against each other using the Wilcoxon rank sum test.

- 4.2.15.2.4. NEFA and BHB

Serum NEFA were analysed at week +1 post-calving, and serum BHB was analysed at weeks +1, +2 and +3 post-calving. Serum NEFA results were treated as binary outcomes and were considered to be 'high' if >0.8mmol/l and serum BHB results were considered to be high if >1mmol/l. Univariate analysis of both the NEFA and BHB results was carried out using Fisher's exact test. Multivariate analysis, using logistic regression, was carried out on serum NEFA and BHB results from 'week 1', treating 'high NEFA' or 'high BHB' as binary outcomes.

An association between serum NEFA and globulin results was analysed for statistical significance using Wilcoxon's rank sum test with continuity correction.

- 4.2.15.2.5. Serum protein (albumin & globulin)

Serum albumin was analysed using a regression model with feed group and time point used as predictors. Globulin was calculated from the difference between total protein and albumin, and the results were analysed using a multilevel model, from week -6 pre-calving to week +6 post-calving, taking into account the repeated measures from each cow and using a cow-level random effect.

- 4.2.15.2.6. Milk yields

Previous 100 day yield, from the lactation before the trial dry period, was only used where trial yield was being measured as an outcome. A univariate relationship was tested for using the Wilcoxon rank sum test, followed by further analysis using simple linear regression.

- 4.2.15.3. Statistical modelling and model-checking

Across all analyses, probability values of $P<0.05$ were considered to represent a significant relationship. All analysis and data restructuring was carried out using R (R Core Team, 2015).

The multivariate models used for NEFA and BHB results were checked using the Hosmer-Lemeshow goodness of fit test. Where a simple linear regression was used, as in the case of the trial 100 day yield results, the residuals were checked as being close to a normal distribution. Multilevel models were checked by ensuring that the residuals at each level (i.e. at both time point and cow level) were normally distributed.

Feed group was always included as a predictor, and other predictors remained in the model if they had a statistically significant association with the outcome in their own right, or if they had a large effect on the association between the outcome and the feed group.

4.2.16. BLOOD SAMPLE COMPARISONS: A DETERMINATION OF APPROPRIATE SAMPLE CHOICE

Serum samples were chosen for analysis for this feed trial chapter. However, whole blood and plasma samples had also been stored and so some experimental work was carried out using these to compare the results from each sample type and this study is included here.

- 4.2.16.1. Blood sample comparisons: introduction

The blood of vertebrates consists of cells suspended in a liquid matrix called plasma, which makes up around 55% of the volume of blood (Campbell *et al*., 2008). The remaining 45% of blood volume consists of cellular elements, including red blood cells, white blood cells and platelets. Plasma consists of around 90% water and also contains ions, plasma proteins (including clotting factors), and substances that are required to be transported by the blood (including respiratory gases, metabolic waste products, hormones and nutrients). Fibrinogen is a glycoprotein found within plasma that aids in blood clotting. Serum is plasma with these clotting factors removed.

The levels of essential inorganic compounds, including some minerals, are constantly maintained within the body through homeostasis in order to avoid cases of either toxicity or deficiency (Harrington *et al*., 2014). This section will focus on measuring the three minerals also discussed in Chapter 4: calcium, phosphorous and magnesium. The importance of calcium is discussed in Chapter 1. Phosphorous has roles in bone and tooth formation (Campbell *et al*., 2008). It is essential for the formation of cell membranes and nucleic acid molecules, as well as having an important role in metabolic activity, including carbohydrate and energy metabolism. Magnesium is required for the activation of enzymes, and for the synthesis of RNA, DNA and protein. It is a co-factor for over three hundred enzyme reactions within the body and also has a role in muscle contraction due to its involvement in the movement of calcium into smooth muscle cells.

Serum samples are obtained from blood that has been allowed to clot (Tuck *et al*., 2009), and can be done so from tubes of blood with no additives or from blood collected in tubes containing clotting activator. Plasma samples are

obtained from tubes containing anticoagulants, such as lithium heparin, that prevent the formation of a clot. Blood in both types of tubes separates on centrifugation, yielding serum or plasma respectively.

A selection of the plasma and whole blood samples taken during the main trial included in this chapter were analysed, with the aim of this smaller study being to investigate the potential agreement between these sample types and the chosen analysis methods for the selected analytes, treating the serum sample as the 'gold standard'. Studies such as that of Mohri *et al*. (2007) have looked at comparing levels of different blood constituents in different types of samples, but did stress a need for further work in this area. If different types of blood samples and different methods of analysis show agreement in terms of the results, then it may make it easier to directly compare different sets of research findings within literature. It may also have practical benefits for veterinarians and farmers with regards to diagnostic testing for toxicities and deficiencies relating to calcium, magnesium and phosphorous, particularly if only one tube of blood is available.

- 4.2.16.2. Blood sample comparisons: materials and methods
    - 4.2.16.2.1. Sample selection

The samples obtained for use in this study were taken from those already collected in the farm trial detailed in this chapter. Ten cows were selected for the multiple sample type analysis. In order to select these animals, the serum phosphorous results were taken and the standard deviations of these were calculated for each animal. The ten cows with the highest standard deviations were then selected, as these were considered likely to have the most distinctive peaks and troughs when displayed on a graph. This was deemed to be important when comparing the serum results with the subsequent plasma and whole blood analysis, as potential time lags between different sample types would be most evident.

- 4.2.16.2.2. Plasma and serum analysis

Plasma samples were analysed as single replicates using a Randox RX Imola clinical chemistry analyser (Randox Laboratories Ltd., Belfast, UK) and were tested for calcium, magnesium and phosphorous, as outlined in section 4.2.13.1. The plasma samples were obtained from centrifuged whole blood in a lithium heparin tube, collected according to the protocol outlined in section 4.2.12. Plasma samples had been stored at -20°C prior to analysis and were thoroughly defrosted and mixed on a tube-roller before being loaded onto the analyser. Serum analysis for calcium, magnesium and phosphorous had already been carried out as part of the farm trial discussed in this chapter. Serum was obtained from blood collected in a clot activator tube following centrifugation, as detailed in section 4.2.12.2. The analysis was completed in the same way as the plasma analysis, the methods of which are described in section 4.2.13.1.

- 4.2.16.2.3. Whole blood ICP method

Whole blood was collected in lithium heparin tubes and an aliquot was stored at -20°C prior to analysis. Whole blood samples were analysed by inductively coupled plasma mass spectrometry (ICP-MS). The samples were defrosted at room temperature, undergoing only a single freeze-thaw cycle prior to ICP-MS analysis, and were mixed thoroughly before being pipetted into 0.5ml aliquots in 14ml polypropylene tubes (Sarstedt, Leicester, UK). These were then diluted with 9.5ml of diluent, which contained 0.1% Triton X-100 non-ionic surfactant and 1% HNO3, before being analysed using a Thermo-Fisher Scientific iCAP-Q (Thermo Fisher Scientific, Bremen, Germany).

- 4.2.16.2.4. Method comparison statistical analysis

The results of the serum analysis are shown in Figures 4.3 to 4.5. These were compared individually to both the whole blood and plasma results for all three analytes. This was carried out using Bland-Altman limits of agreement plots (Bland & Altman, 1986) to visually represent the data.

- 4.2.16.3. Blood sample comparisons: results

Individual graphs for calcium, magnesium and phosphorous for each of the ten cows are shown in Figures 4.3 to 4.5. For each of the three analytes (calcium, phosphorous and magnesium), a Bland-Altman plot (Bland & Altman, 1986) was generated to display the results for comparing serum to plasma, and serum to whole blood. Bland-Altman plots were developed as a graphical means of plotting the difference of two sets of measurements against the mean of the two measurements in order to visually evaluate the potential agreement between the two methods in question (Bland & Altman, 1986). These Bland-Altman limits of agreement plots are displayed in Figures 4.6 to 4.11.

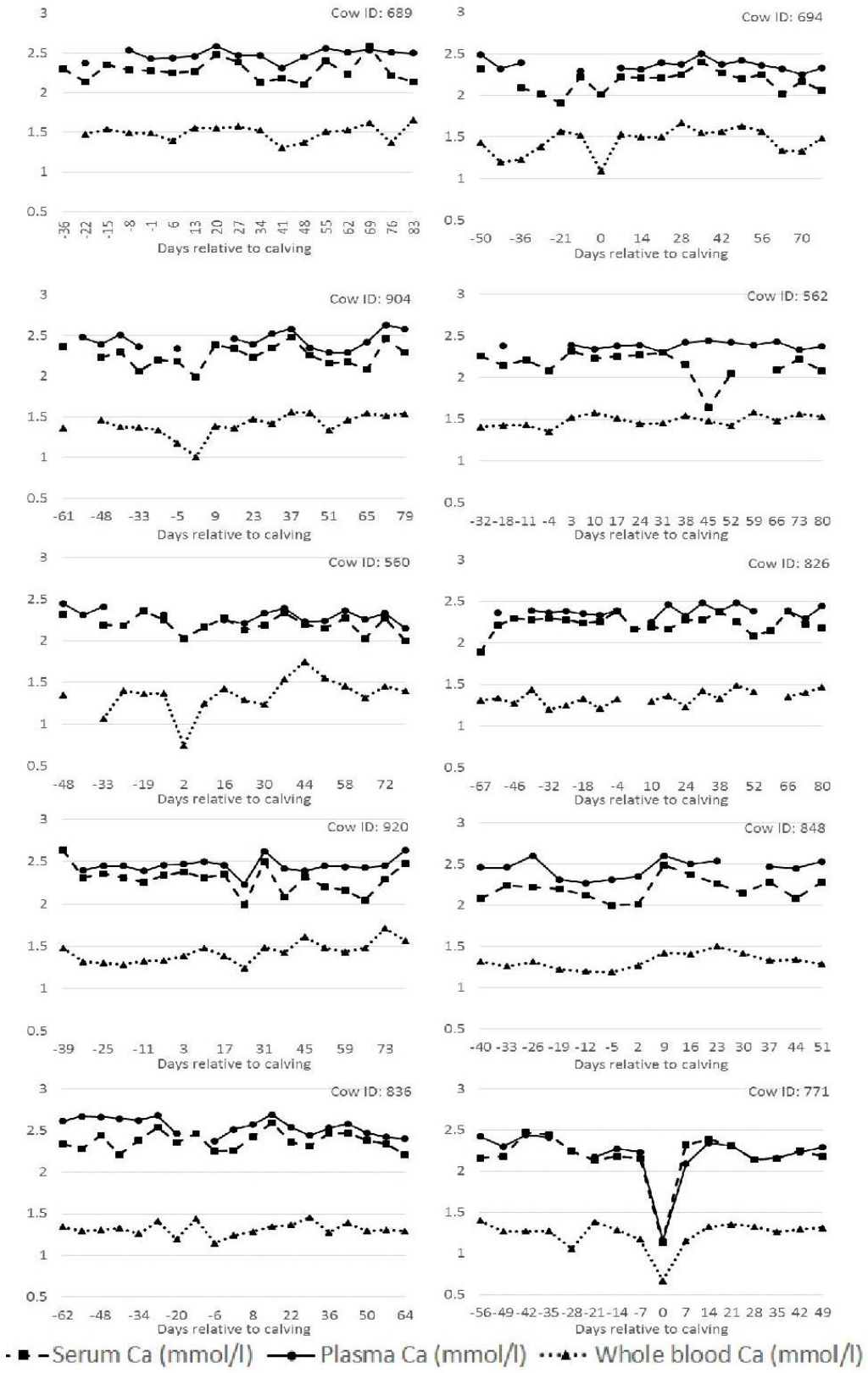


Figure 4.3. Individual cow profiles for calcium reported for serum, plasma and whole blood

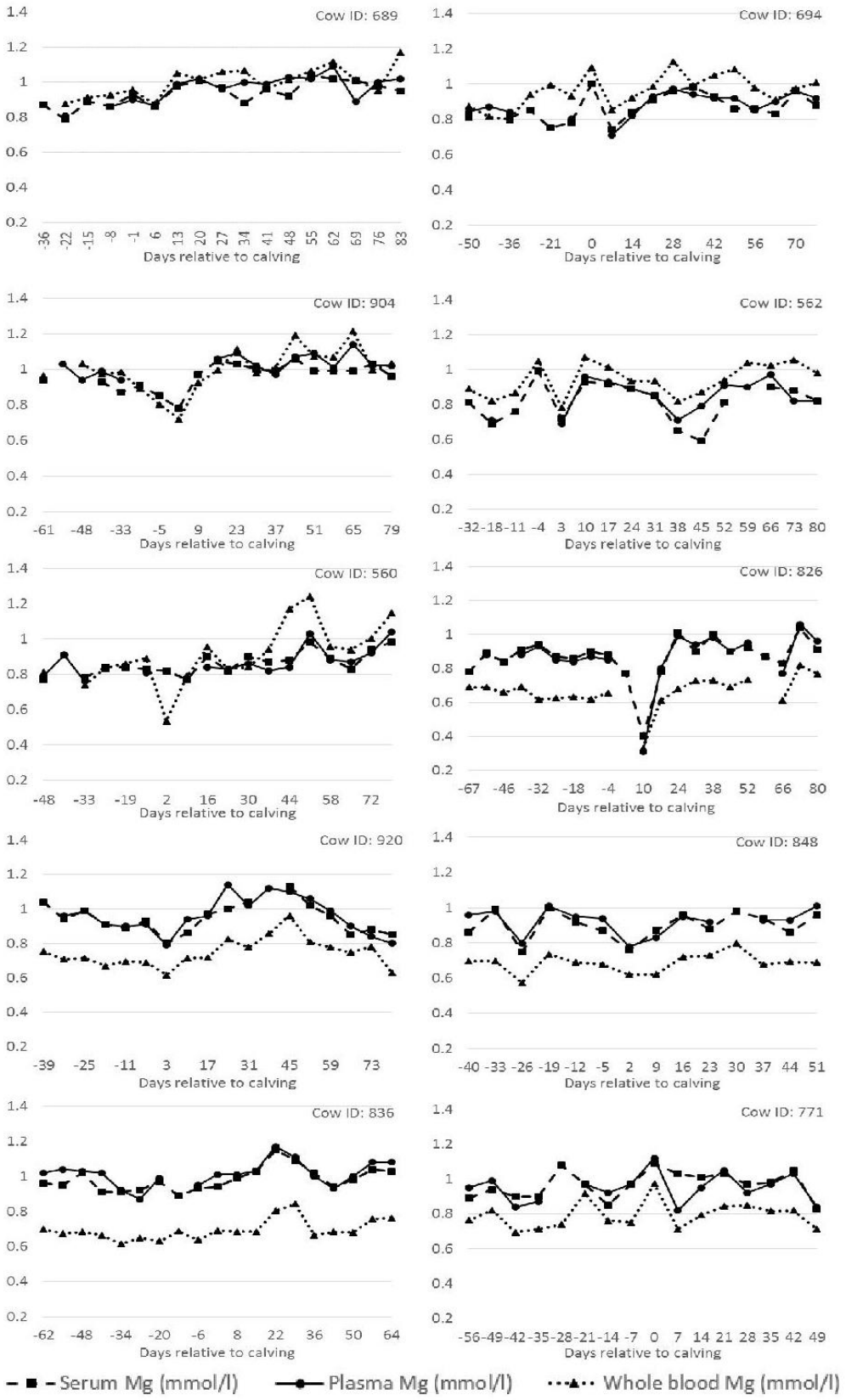


Figure 4.4. Individual cow profiles for magnesium reported for serum, plasma and whole blood

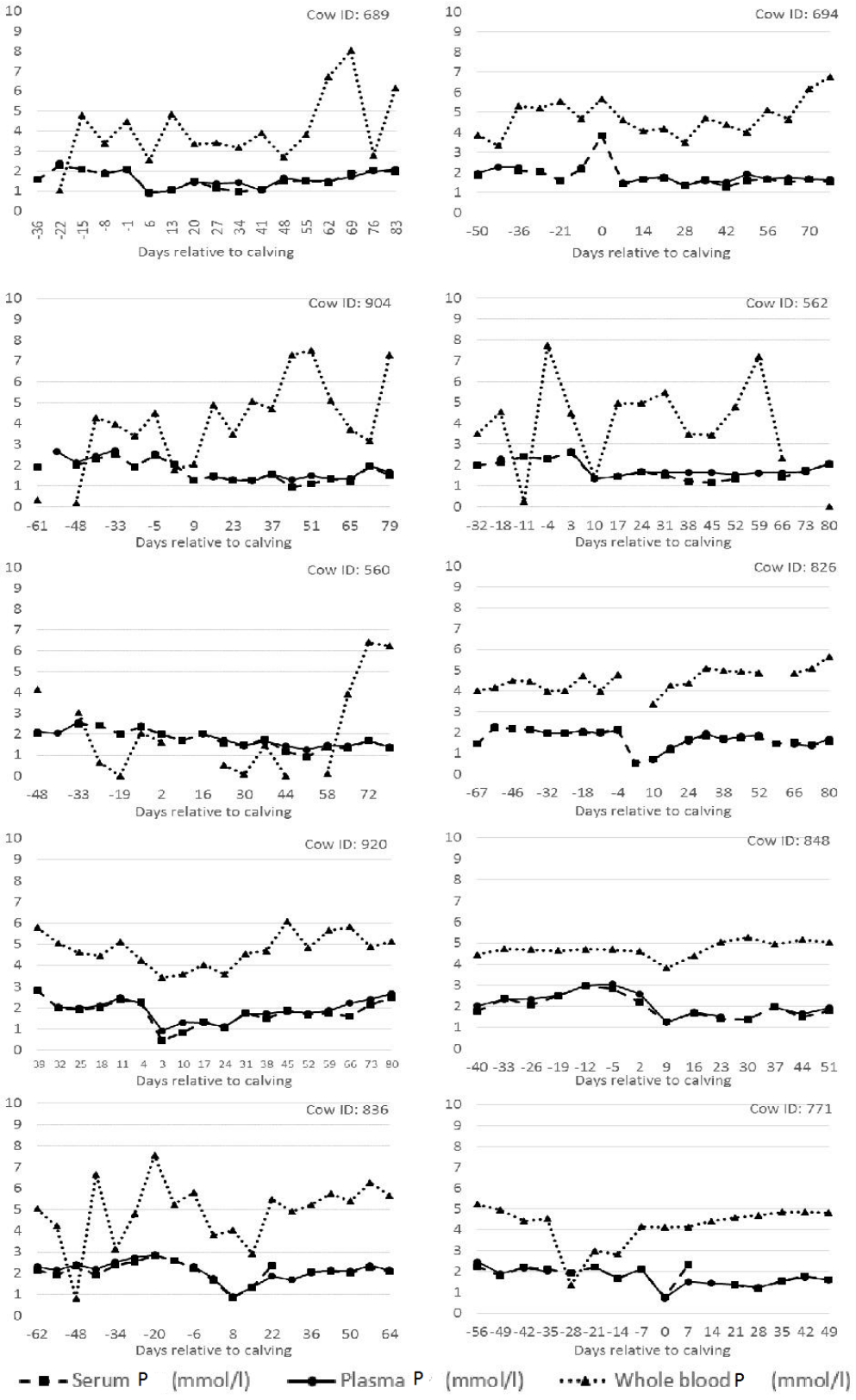


Figure 4.5. Individual cow profiles for phosphorous reported for serum, plasma and whole blood

- 4.2.16.1. Comparing plasma to serum

Figure 4.6 shows the differences plotted against the means for serum and plasma calcium concentrations. The majority of points are clustered around the mean and fall within two standard deviations of the mean, although there are a couple of outliers.

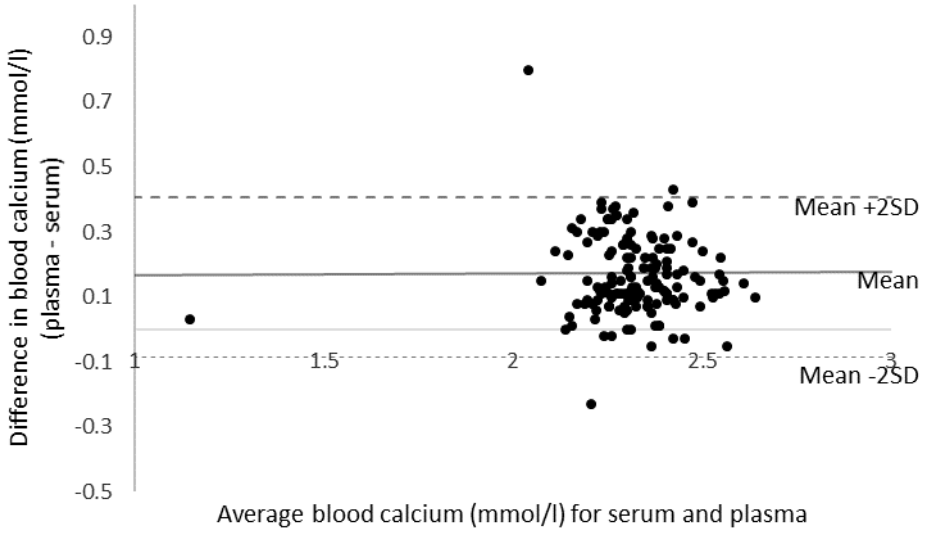


Figure 4.6. Bland-Altman plot showing difference against mean for serum and plasma calcium

Figure 4.7 shows the differences plotted against the mean for serum and plasma inorganic phosphorous concentrations. Most points do fall within two standard deviations of the mean, although there are some that fall outside of these boundaries.

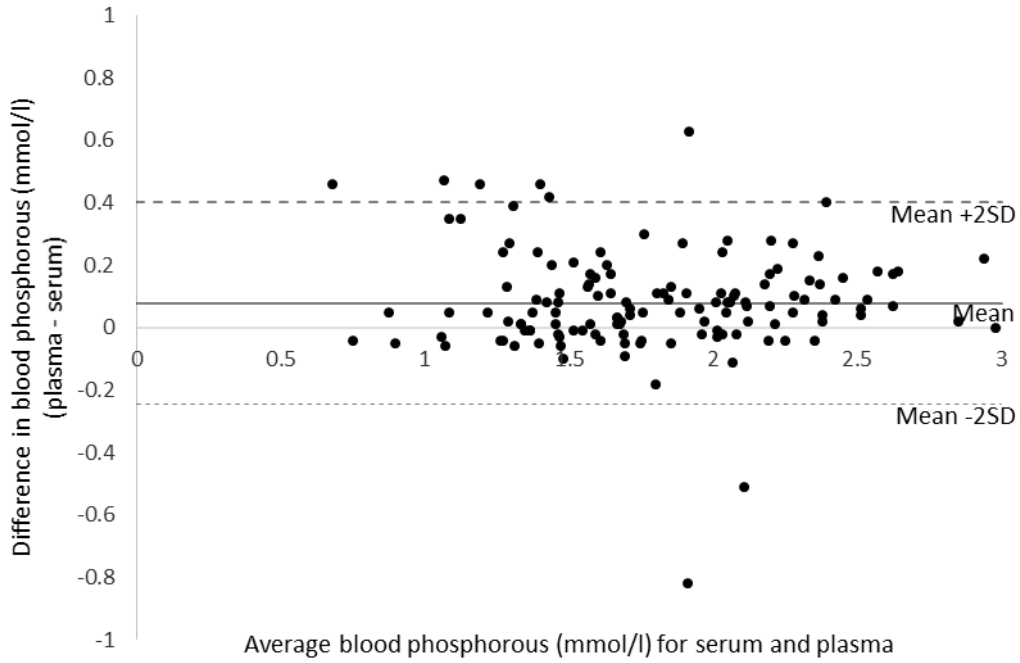


Figure 4.7. Bland-Altman plot showing difference against mean for serum and plasma inorganic phosphorous

Figure 4.8 shows the differences plotted against the mean for serum and plasma magnesium concentrations. The majority of points are clustered around the mean and fall within two standard deviations of the mean, although there are some exceptions that fall outside of these limits.

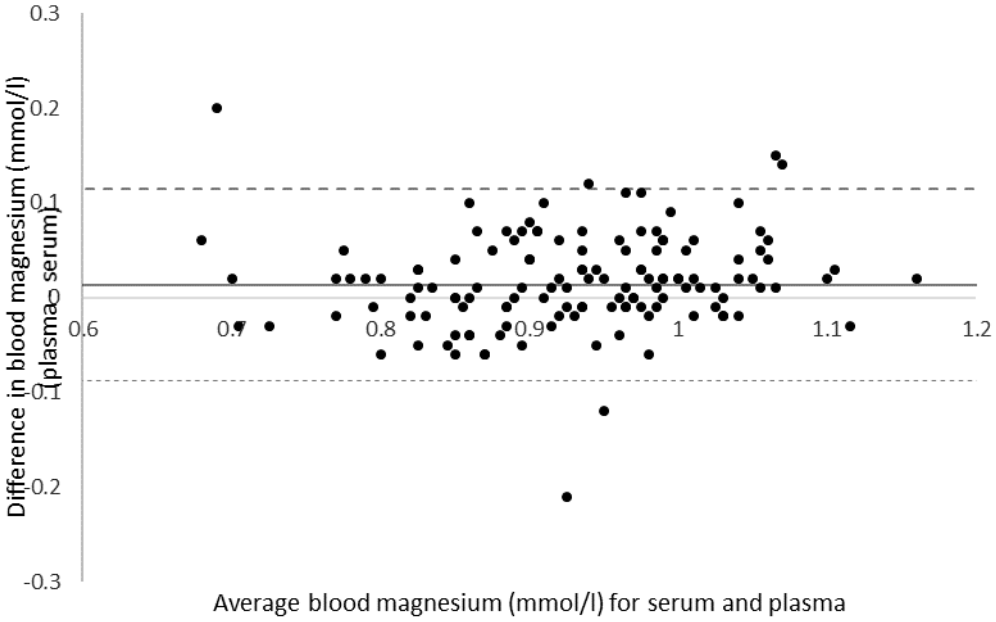


Figure 4.8. Bland-Altman plot showing difference against mean for serum and plasma magnesium

- 4.2.16.3.2. Comparing whole blood to serum

Figure 4.9 shows the differences plotted against the mean for serum and whole blood calcium. There are a few outliers but the majority of samples are clustered around the mean and fall within two standard deviations of the mean.

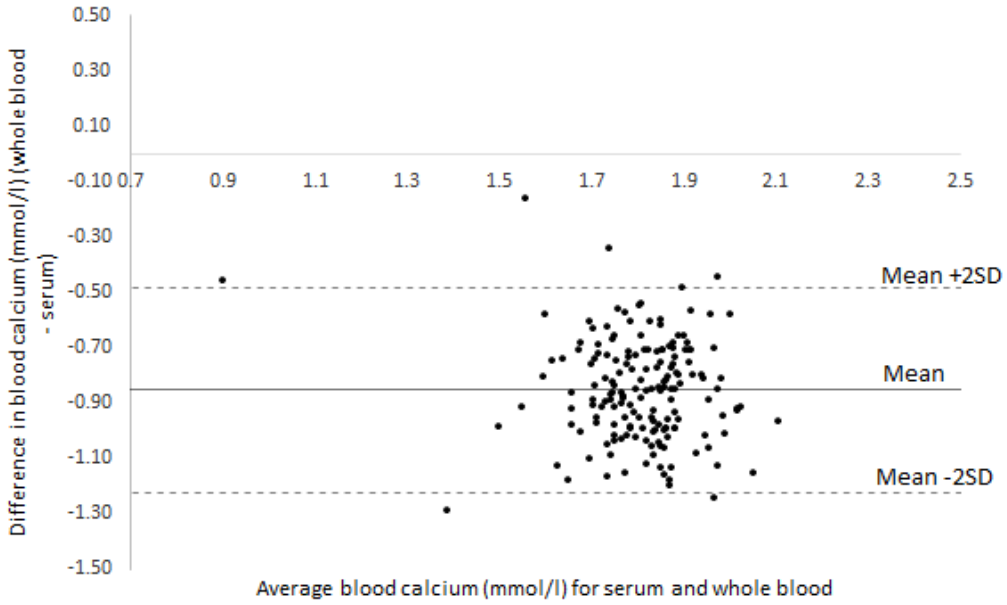


Figure 4.9. Bland-Altman plot showing difference against mean for serum and whole blood calcium

Figure 4.10 shows the differences plotted against the mean for serum inorganic phosphorous and whole blood total phosphorous. A regression line has been plotted to show the relationship between the difference and the average for blood phosphorous for the serum inorganic phosphorous and whole blood total phosphorous. The majority of points are clustered around the mean and most fall within two standard deviations of the mean but there are some outliers.

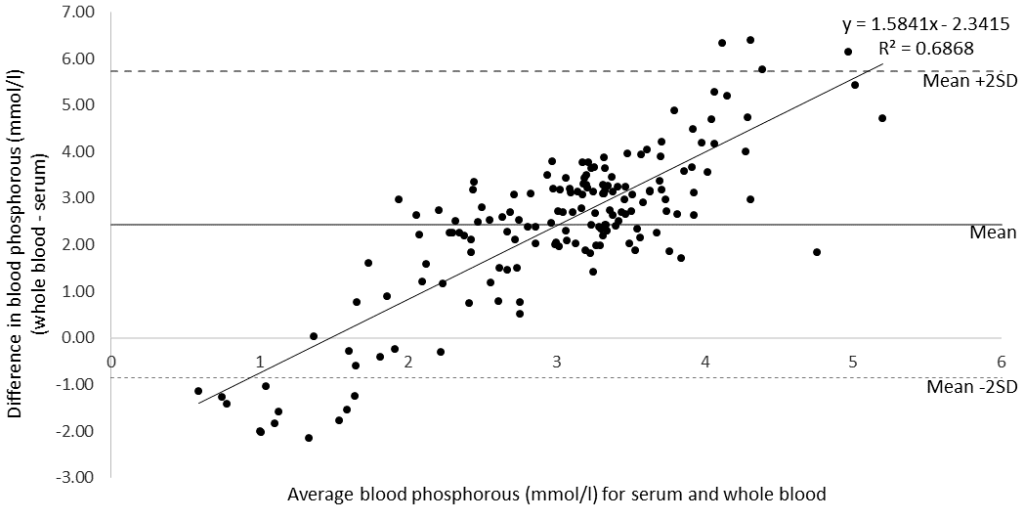


Figure 4.10. Bland-Altman plot showing difference against mean for serum inorganic phosphorous and whole blood total phosphorous, with added trendline

Figure 4.11 shows the differences plotted against the mean for serum and whole blood magnesium. The majority of points fall within two standard deviations of the mean, with a couple of exceptions.

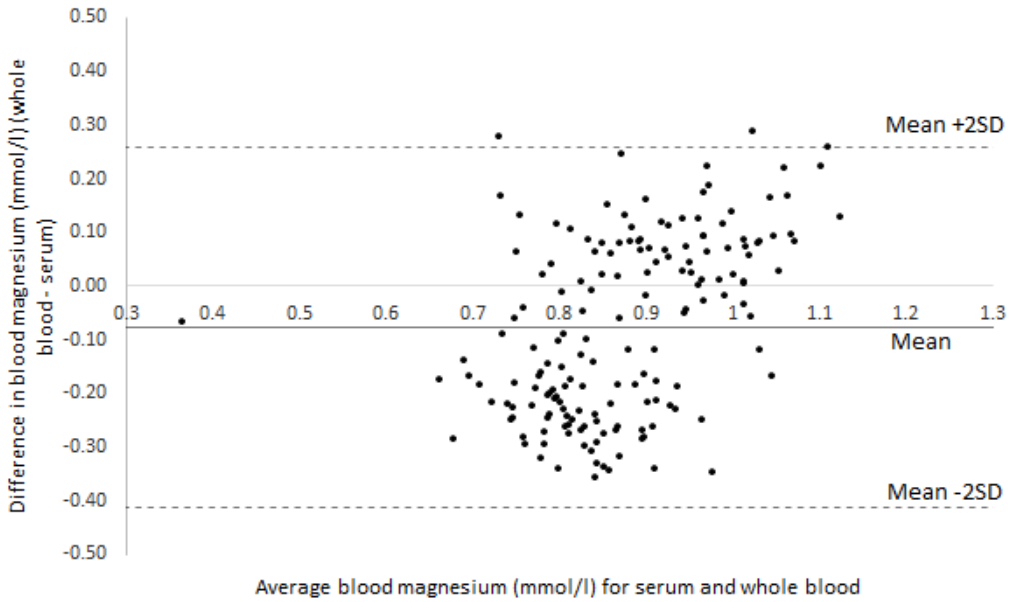


Figure 4.11. Bland-Altman plot showing difference against mean for serum and whole blood magnesium

- 4.2.16.4. Blood sample comparisons: discussion
  - 4.2.16.4.1. Comparing serum and plasma

The individual cow graphs for calcium (Figure 4.3) show that plasma and serum results were both consistently higher than the results for whole blood in all cows. All three lines appear to visually show a broadly similar pattern of corresponding peaks and troughs throughout the weeks of sample points.

The serum and plasma phosphorous results appear visually very similar throughout. Serum and plasma phosphorous have been reported within the literature to differ to the extent of affecting clinical interpretation in human medicine (Miles *et al*., 2004), which will subsequently be discussed further.

The serum and plasma individual cow results for magnesium (Figure 4.4) appear visually very similar throughout, although there are some apparently small differences which may have an important clinical effect due to the relatively tight reference range of 0.6 to 0.9mmol/l for this particular mineral in bovine blood (Aiello & Moses, 2015). It is reported that serum magnesium has a tendency to be higher than plasma (Moodie, 1975). This may be due to the dilution effect of plasma containing fibrinogen, whereas serum does not and may therefore contain a higher concentration of magnesium.

Within the Bland-Altman plots comparing serum and plasma data (Figures 4.6, 4.7 and 4.8), there generally appears to be good levels of agreement between the serum and plasma results across all three minerals tested, with the majority of points falling within two standard deviations of the mean. There are a few outliers within each plot, most notably in the results for calcium (Figure 4.6), with one value for the difference in sample concentration appearing to be much higher than the majority of points in the plot. This appears to be most likely due to a sampling error on the analyser, given the level of agreement seen with the other samples within this dataset.

Miles *et al*. (2004) report that many consider serum and heparinised plasma to be equivalent for many different types of analysis, but draw attention to the fact that for many assays these two sample types are not identical,

including analysis for calcium and phosphorous (Ladenson *et al*., 1974). In some cases, these discrepancies were considered to be large enough to affect the clinical interpretation of the results, which highlights the importance of assessing whether or not two different blood fractions or analysis methods are comparable. Guthold *et al*. (2004) suggest that concentrations of analytes measured in plasma may be seen to be lower than in corresponding serum samples, due to the fibrin clot retaining some water and thus making serum more concentrated. Plasma and whole blood samples used in this study contained the anticoagulant lithium heparin. Whilst heparinised samples are recommended as being suitable for use with the arsenazo III reagent in the calcium analysis kit, the kit instructions for the phosphorous assay do state that serum is the recommended sample, as opposed to plasma.

Despite the apparent visual agreement between serum and plasma phosphorous, the data sheet for the UV method kit used indicates that the recommended sample for testing should be serum, and plasma is not mentioned. Serum phosphorous is considered to be an unreliable indicator of the phosphorous status of an animal (Aiello & Moses, 2015). This is due to the compartmental shifts of phosphorous between the intracellular space (ICS) and the extracellular space (ECS), which includes serum or plasma, and less than 1% of the body's phosphorous reserves are maintained within the ECS. Despite this, serum phosphorous is considered to be the most widely used measurement to assess phosphorous status within the veterinary profession (Aiello & Moses, 2015).

In this study, lithium heparin was used as the anticoagulant for plasma samples, and heparin is considered to be the most widely used anticoagulant in bovine clinical chemistry analysis (Mohri *et al*., 2007). There is no anticoagulant universally suited to all tests (Banfi *et al*., 2007) but there are alternatives that are better suited to some assays (Vaught, 2006). Ethylenediaminetetraacetic acid (EDTA) is frequently recommended for use in haematology, as it allows the best preservation of the morphology of blood cells (Banfi *et al*., 2007). However, it would not have been a suitable

alternative in this present study, due to the fact that it interferes with assays for the quantitative analysis of both calcium and magnesium (Bowen & Remaley, 2014). Acid-citrate-dextrose is another option for the anticoagulation of blood, but again this has the capacity to chelate calcium and so would affect the results of this study. If plasma and serum samples can be used interchangeably then plasma may have advantages in situations where a fast turnaround of sample analysis is required, due to the coagulation time between sampling and centrifugation not being required (Mohri *et al*., 2007).

- 4.2.16.4.2. Comparing serum and whole blood

The individual cow graphs for calcium (Figure 4.3) show that whole blood results were consistently lower than the results for plasma and serum in all cows. Broadly speaking, all three lines appear to visually show a similar pattern of corresponding peaks and troughs throughout the weeks of sample points.

In the individual cow graphs (Figure 4.4), magnesium in serum and plasma visually appear to be very similar. However, whole blood magnesium appears different, sometimes appearing consistently higher across all time points for an individual cow, but mostly appearing consistently lower. Generally the same pattern can be seen in terms of peaks and troughs at corresponding time points but this is not always the case and it is unclear as to why this might be. For calcium and magnesium, the Bland-Altman plots for comparing serum and whole blood (Figures 4.9 and 4.11 respectively) appear to show agreement, with the data points generally falling within two standard deviations of the mean. The difference on the y-axis was consistently low, with mean whole blood concentrations being 0.855mmol/l lower than serum for calcium, and 0.076mmol/l lower than serum for magnesium.

However, the individual cow graphs for phosphorous (Figure 4.5) suggest that the whole blood results are far less consistent with the serum and plasma results than was the case when looking at calcium. In some cases the whole

blood results are lower than plasma/serum, but at other times they are in excess of them, and sometimes the peaks and troughs at different time points do appear to correspond but at other times this does not look to be the case.

In the Bland-Altman plot depicting the agreement between whole blood and serum phosphorous, the difference between the samples appears to increase as the mean value increases, and a trendline has been added to this graph to demonstrate this (R^2=0.6868). This is likely to be due to the different methods involved in the analysis of these samples, and the different result types each method yields. Serum results were analysed using a kit intended for the determination of inorganic phosphorous concentrations in serum and urine, which uses the reaction between inorganic phosphorous with ammonium molybdate in the presence of sulphuric acid to form a phosphomolybdate complex. However, this assay is unsuitable for using with whole blood. Within the instructions for the kits used for the analysis of serum inorganic phosphorous, it is noted that haemolysis can interfere with the assay. Whole blood analysis was carried out using ICP-MS, as previously described. It may have been more appropriate to perform this same analysis in order to obtain the phosphorous concentrations from serum to make the results more directly comparable.

Serum phosphorous represents only a small percentage of the total phosphorous within the body, but is easy to measure and so is frequently used as an indicator of the overall body phosphorous reserves of an animal (Bansal, 1990). Almost all of the phosphorous within the extracellular fluid is in the form of inorganic phosphate (Bansal, 1990). Total blood phosphorous is equally divided into acid-soluble and acid-insoluble fractions, with the acid-soluble fraction being divided equally again between organic and inorganic phosphorous (Moodie, 1975). The total phosphorous concentration of plasma is usually around half that of whole blood, but the concentration of inorganic phosphorous is reported to be higher in plasma than in whole blood (Moodie, 1975), due to low levels of inorganic phosphorous in red blood cells. The results of the whole blood ICP-MS analysis for phosphorous are total

phosphorous concentrations, as opposed to the inorganic phosphorous results obtained from the analysis of serum. This difference may explain the trendline demonstrated in Figure 4.10, as whilst there is an association between total phosphorous and inorganic phosphorous levels, they are two different measurements. As the selected inorganic phosphorous kit was unsuitable for the analysis of whole blood, in retrospect it may have been more appropriate to change the method of serum analysis to one more comparable to the whole blood analysis, such as running the serum samples through ICP-MS analysis also.

- 4.2.16.4.3. Blood sample comparisons: further limitations and considerations

Serum samples must be allowed sufficient time to clot before centrifugation but there are limits on the length of time it can be left to stand, with Tuck *et al*. (2009) recommending a period of 30-60 minutes. Tuck *et al*. (2009) state that samples given less time to clot may mean that the serum retains cellular components, but also state that samples left for longer than sixty minutes may experience lysis of cells within the clot, which would lead to the release of cellular elements into the serum. Both of these issues could affect the subsequent analysis of serum samples. In this present study, all samples were allowed a sufficient period of time to clot in CAT tubes. However, the actual amount of time allowed varied and was always well in excess of this recommended sixty minutes – all samples underwent centrifugation on the same day but the time scale varied and could be upwards of eight hours for some samples at some sampling points. This may have affected the findings and is worth being aware of, but in retrospect it would have been more appropriate to maintain consistency in terms of sampling to centrifugation time in all samples included within this experiment to avoid potential variation owing to this.

The storage time of blood samples may also impact on the results of any subsequent analysis. Lester and Varghese (1977) found that the mean calcium concentration of human serum stored at -20°C remained stable over a storage

period of eight months, but found that plasma calcium levels decreased significantly when stored for between one and six months. It is important to consider that blood sampling and storage equipment, including tubes and stoppers, can impact on the samples over time. Components from the tubes themselves can potentially leach into samples, or components of the blood samples can adsorb to the surfaces they come into contact with (Bowen & Remaley, 2014), which may affect the subsequent analysis of those samples, and this may explain the differences reported by Lester and Varghese (1977). If there is a true difference in stability between serum and plasma samples then this may have an effect on the apparent agreement between these two samples. In the present study, the storage time of samples varied considerably. Some of the serum samples were analysed within one month of collection, having been stored at -20°C, but the analysis of plasma samples took place up to two years after the collection and freezing of samples, and this varied within the data set due to the fact that sample collection itself took place over a period of eleven months. In retrospect, the samples selected for plasma analysis would have been better analysed within a set time frame following collection, possibly in line with the one month of stability recommended by Lester and Varghese (1977), in order to improve the quality of any findings. Cray *et al*. (2009) report that limited work appears to have been done in looking at the stability of blood samples over time in the veterinary field, with most of the data available being from human blood studies, and there is evidence of interspecies differences, which needs to be taken into consideration or further investigated.

It is important to consider whether any differences between samples affect their clinical interpretation with regards to cattle health and disease. Reference ranges for normal serum phosphorous, calcium and magnesium in cows are shown in Table 4.4 and thresholds for a selection of related metabolic diseases are displayed in Table 4.5.

Table 4.5. Thresholds for metabolic disease relating to low blood phosphorous, calcium and magnesium in dairy cows

Mineral	Condition	Mineral concentration representing threshold for disease	Reference
Phosphorous (inorganic)	Hypophosphataemia	<1.4mmol/l	Grunberg *et al*., 2005
Calcium	Hypocalcaemia	<2.0mmol/l	Goff, 2008
Magnesium	Hypomagnesaemia	<0.65mmol/l	Aiello & Moses, 2015

Further work with the current data could consider whether or not serum or plasma measurements for individual cows would alter a diagnosis of a metabolic disease state, as this present study only considered the fairly crude visual analysis of the data through the use of Bland-Altman plots.

- 4.2.16.5. Blood sample comparisons: conclusion

Different types of blood samples are made up of different constituents and this may have an impact on the mineral concentrations within them. It is important to consider whether or not different sample types and different analysis methods are actually comparable in order to determine which sample types are most appropriate for specific analytical tests. This experimental work was undertaken to assess whether or not the analysis of plasma and whole blood samples would be of benefit to the farm trial study detailed in this chapter, as these sample type comparisons do not appear to have been previously reported in the literature for cattle.

This present study suggests that calcium, magnesium and phosphorous levels between serum and plasma samples are relatively similar, as a good level of agreement was seen when Bland-Altman plots were constructed and examined. These samples are often used interchangeably within the literature

(Miles *et al*., 2004) but more work could be done to quantify the actual differences between these for the analytes investigated here and others, as this work is reported to be fairly limited within the veterinary field (Mohri *et al*., 2007).

Whole blood calcium concentrations appear to be lower than in serum, and this may be due to the diluting effect of the cells present in whole blood samples. The relative concentrations of magnesium in whole blood and plasma appear to be less consistent and this may warrant further investigation. It was identified that the whole blood and serum measurements for phosphorous concentration were reporting different measurements, for total phosphorous and inorganic phosphorous respectively. These different measures of phosphorous do appear to be correlated and it may be beneficial to investigate this area further using more comparable methods of blood analysis.

It is clear that there is scope for more research in the area of comparing sample types and methods of analysis to further consider levels of agreement, and this could have practical benefits in both research and for veterinarians working in practice. Future work in this area should take into account issues of sample processing and storage, with attempts being made to ensure consistency between storage conditions for all samples involved, and there may be a need for further focus on the actual clinical significance of any variations elucidated between sample types. Upon reviewing the data collected from this limited plasma and whole blood analysis, it was decided that only serum results would be reported for the main study included in this chapter.

4. 3. Results

4.3.1. Trial feed changes and animals excluded from the trial

The changes that occurred to the diet early in the trial are described in section 4.2.4. These effectively meant that the trial was restarted from that date and all animals recruited to the trial prior to that were removed from the analysis.

A further batch of excluded animals occurred in the case of 'non-eaters'. These were animals that failed to consume the required quantity of their allocated diet for the time frame of 3 weeks prior to calving. The timeline of this and the destinations of dry cows recruited onto the feed trial is shown in Figure 4.12.

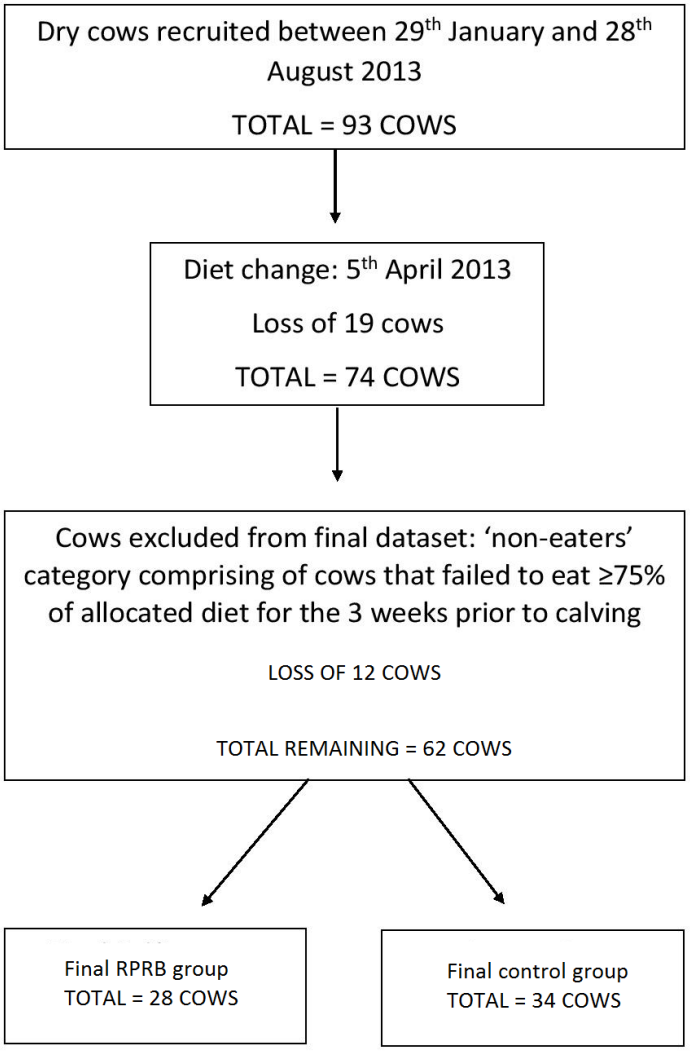


Figure 4.12. Destinations of dry cows recruited onto the feed trial

4.3.2. Mixed forage and grass silage analysis

The cation-anion balance (CAB) analysis of both the pooled monthly mixed forage samples and the fortnightly grass silage samples are shown in Figures 4.13 and 4.14 respectively.

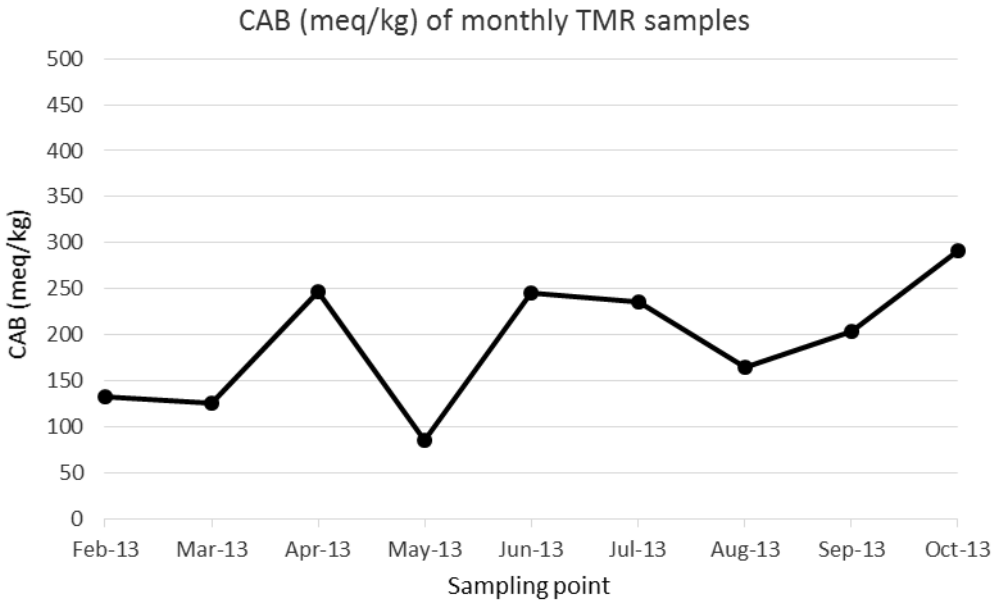


Figure 4.13. CAB of monthly mixed forage samples

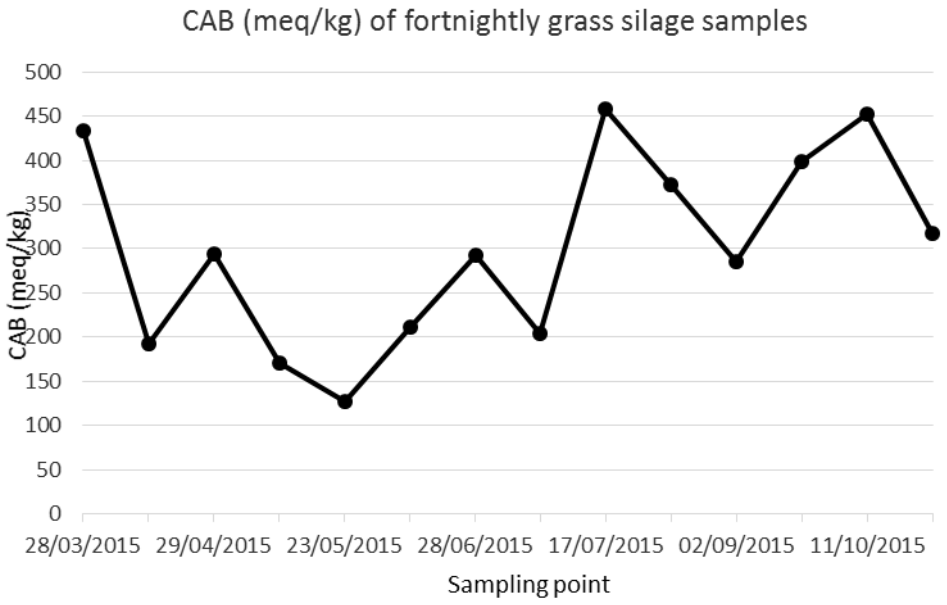


Figure 4.14. CAB of fortnightly grass silage samples

4.3.3. Details of trial animals

- 4.3.3.1. Parities

As explained in section 4.2.3, cows were recruited onto the trial in matched pairs, based on parity and previous yield in order to balance the RPRB and control groups as evenly as possible. The original numbers of cows in each group are shown in Figure 4.15. The numbers of cows in each group in the final analysis, once excluded animals were removed from the dataset, are shown in Figure 4.16. For the purposes of statistical analysis, cows were grouped into parity 1/2/3/4+.

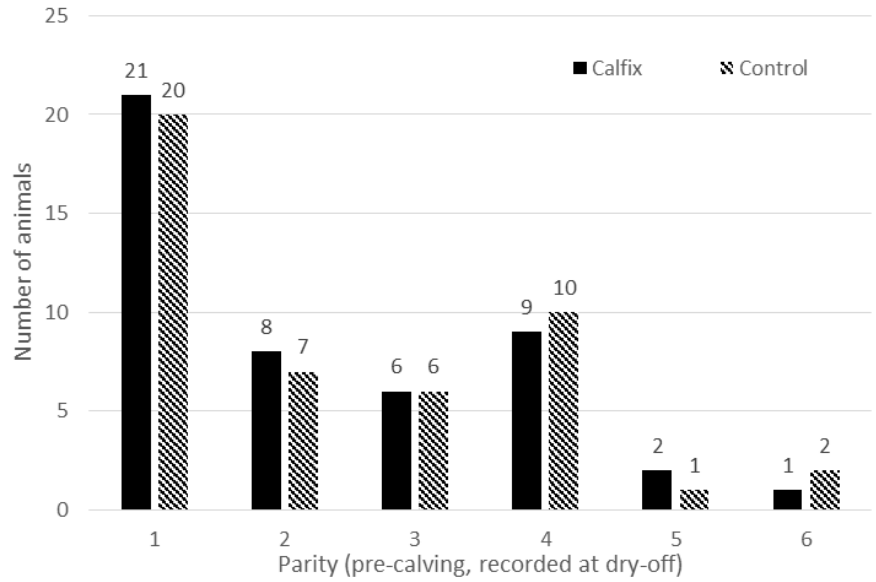


Figure 4.15. Original cow allocations to feed groups based on parity (n=93)

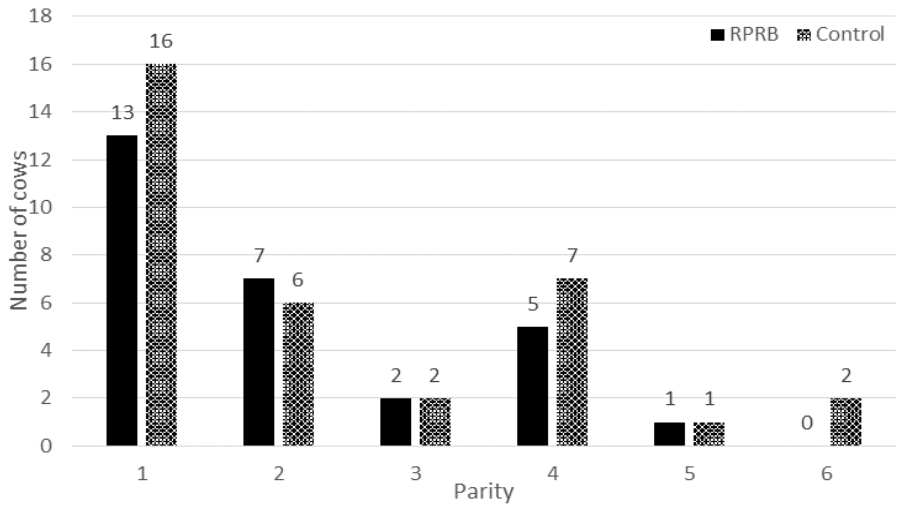


Figure 4.16. Parities of cows in each feed group after exclusions, as used in the final analysis (n=62)

- 4.3.3.2. Milk yields

The previous lactation yields were also taken into consideration when originally forming the matched pairs. The mean 100-day yields from the previous lactation of the cows used in the final analysis are shown in Figure 4.17.

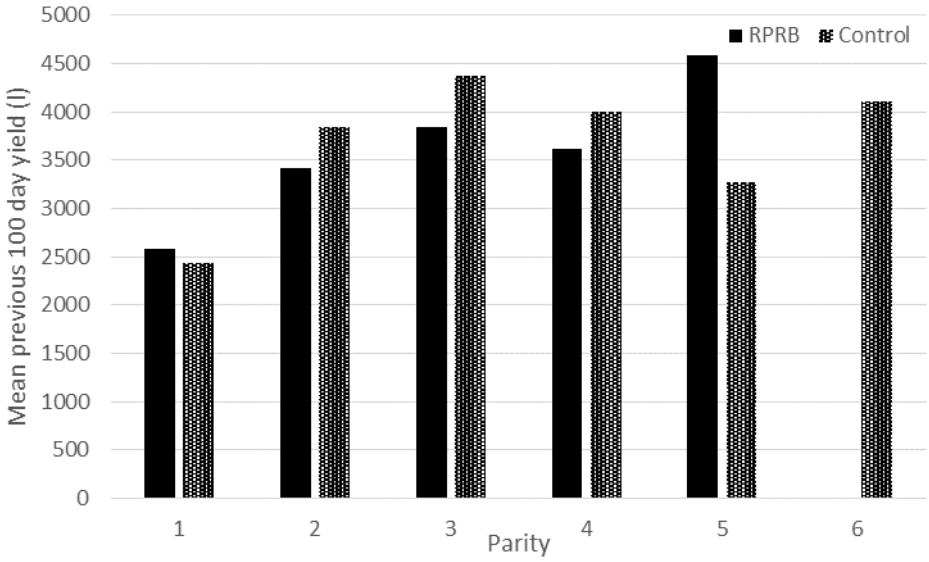


Figure 4.17. Mean pre-trial lactation 100-day yields for each parity and feed group, as used in the final analysis (n=62)

The overall mean pre-trial 100-day yield for cows in the RPRB group was 3141l (s.d.=936), compared to 3245l (s.d.=1024) in the control group.

- 4.3.3.3. Dry period lengths

Of the cows used in the final analysis, the mean dry period length for RPRB cows was 48 days (s.d.=13, range = 21 to 72 days). The mean dry period length for control cows was 46 days (s.d.=19, range = 23 to 93 days).

4.3.4. Concentrate intakes

Meal data from the out-of-parlour feeders was compiled into daily concentrate intakes for each cow's entire dry period. These were then converted into percentages of the allowance eaten. Animals only remained in the final dataset if they had been recorded as having consumed ≥75% of their allocated diet for at least three weeks prior to calving. Animals that did not meet this threshold were excluded as 'non-eaters', as shown in Figure 4.12.

4.3.5. Urine pH

Urine pH measurements were obtained where possible, although at the majority of the sampling points there were some cows that would not produce a specimen, or the sample provided was not of a sufficient volume to test using the portable pH meter. However, these were later deemed to be unreliable and so the results of these tests are not included in this thesis.

4.3.6. Incidence of clinical disease – retained foetal membranes and milk fever

Two cases of RFM were recorded in the final dataset. As one of these occurred in each feed group, no statistical analysis has been carried out on this data.

The frequencies of clinical milk fever cases in each feed group are shown in Table 4.6. The difference between the two feed groups was not statistically significant (Fisher's exact test, p=0.277).

Table 4.6. Frequency of cases of clinical milk fever within each feed group from the final dataset of cows (cows dried-off after 5th April 2013)

	Feed group		
	RPRB	**Control**	**Total**
No milk fever	22 (78.6%)	31 (91.2%)	53
Milk fever	6 (21.4%)	3 (8.8%)	9
Total	28	34	62

4.3.7. Serum mineral concentrations

- 4.3.7.1. Serum calcium concentrations

The mean serum calcium concentrations for each feed group throughout the trial are shown in Figure 4.18.

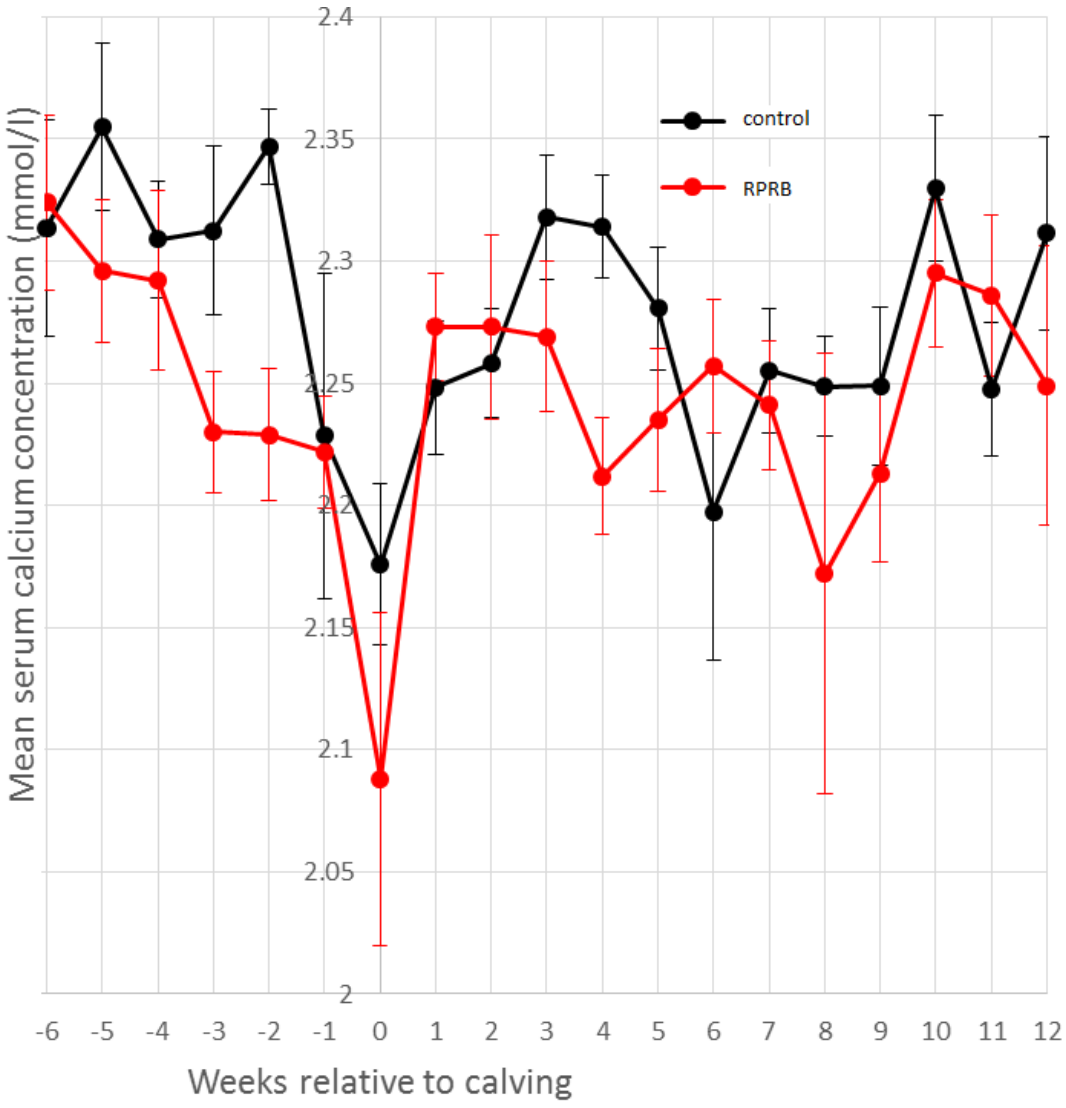


Figure 4.18. Mean serum calcium concentrations for cows in each feed group by week relative to calving (error bars represent +/- standard error of the mean)

Serum mineral results were analysed using multilevel regression models, to account appropriately for repeated measures within individual cows.

Coefficient estimates for a model with the outcome of serum calcium level are shown in Table 4.7.

Table 4.7. Parameter estimates for a multilevel regression model (with a random effect at cow level) with the outcome serum calcium concentration (mmol/l)

	Coefficient	**95% confidence interval**
Intercept	2.248	
Parity 1	Reference	
Parity 2	-0.026	-0.074 to 0.021
Parity 3	-0.048	-0.139 to 0.044
Parity 4+	-0.061	-0.106 to -0.015
Feed group: RPRB	Reference	
Feed group: control	0.109	0.072 to 0.146

The model intercept represents the predicted serum calcium concentration for an average cow in the reference category for each variable (i.e. a parity 1 cow in the trial feed group at time point week -1). The coefficient estimates represent the predicted change from the intercept associated with each category of each variable. For example, serum calcium concentration in a control cow was predicted to be 0.109mmol/l higher than a cow in the RPRB group. Where the 95% confidence interval for a coefficient estimate does not include zero, that category of that variable is considered to have a statistically significant association with the outcome (equivalent to $p<0.05$). A cow of parity 4+ was predicted to have a serum calcium concentration of 0.061mmol/l lower than that of the reference group (a parity 1 cow). No other parity associations were statistically significant.

- 4.3.7.2. Serum inorganic phosphorous concentrations

The mean serum phosphate concentrations for each feed group throughout the trial are shown in Figure 4.19.

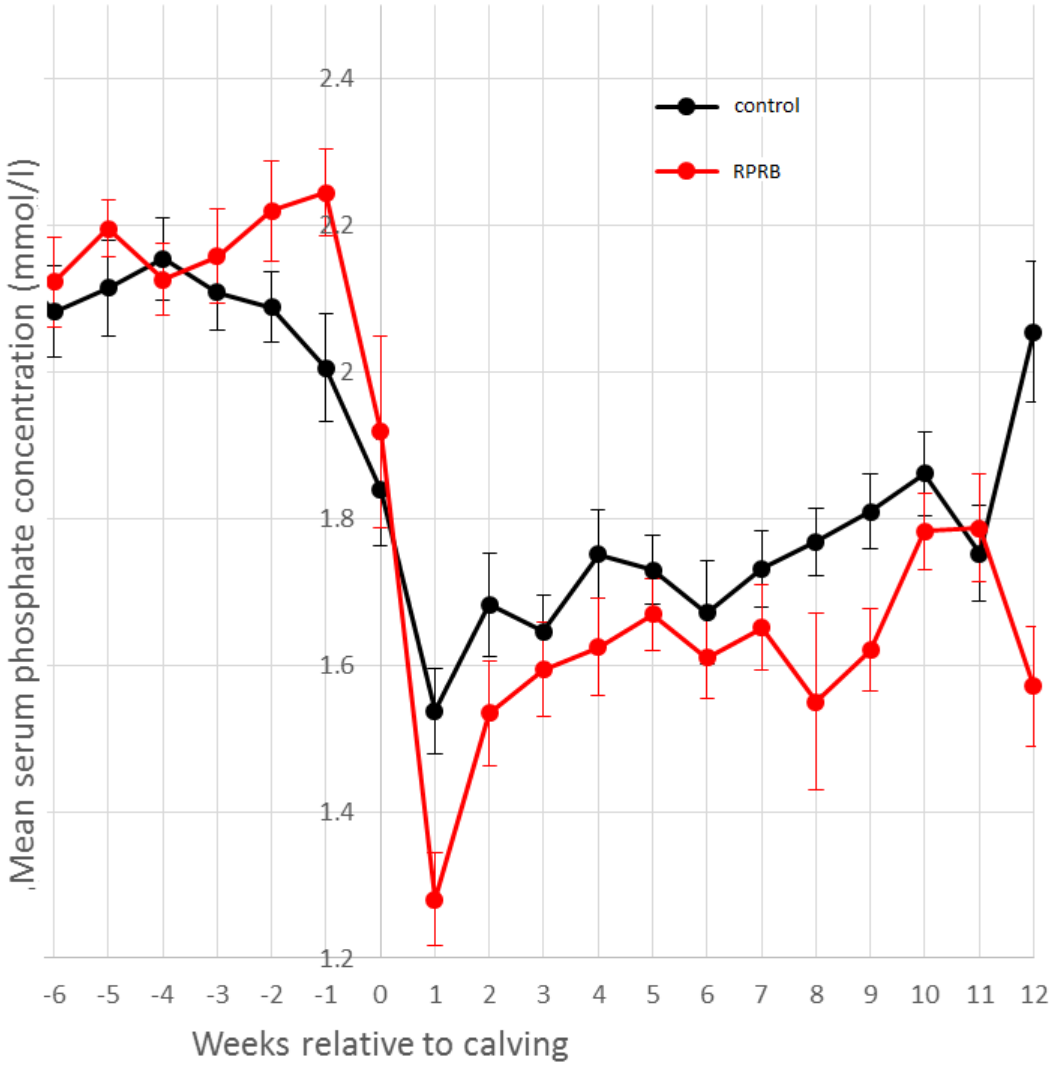


Figure 4.19. Mean serum inorganic phosphorous concentrations for cows in each feed group by week relative to calving (error bars represent +/- standard error of the mean)

Coefficient estimates for a multilevel regression model to predict serum inorganic phosphorous concentration are shown in Table 4.8. Parity 4+ was associated with significantly lower inorganic phosphorous results when compared to parity 1, with parity 4+ cows predicted to have serum inorganic phosphorous concentrations 0.209mmol/l lower than parity 1 animals. The control group was associated with significantly lower serum phosphorous concentrations, as these were predicted to be 0.201mmol/l lower than animals in the RPRB group at week -1 pre-calving.

Table 4.8. Coefficient estimates for multilevel regression model (with a random effect at cow level) with the outcome serum inorganic phosphorous concentration

	Coefficient estimate	**95% confidence interval**
Intercept	2.318	
Parity 1	Reference	
Parity 2	-0.140	-0.281 to 0.001
Parity 3	-0.107	-0.380 to 0.165
Parity 4+	-0.209	-0.345 to -0.075
Feed group: RPRB week -1	Reference	
Feed group: control week -1	-0.201	-0.359 to -0.044
Feed group: RPRB week -2	-0.030	-0.176 to 0.115
Feed group: control week -2	0.071	-0.061 to 0.203
Feed group: RPRB week -3	-0.035	-0.180 to 0.110
Feed group: control week -3	0.134	0.002 to 0.266

- 4.3.7.3. Serum magnesium concentrations

There were no significant associations found between serum magnesium concentrations and any of the potential predictor variables. The mean serum magnesium concentrations for cows in each group across all weeks relative to calving are shown in Figure 4.20.

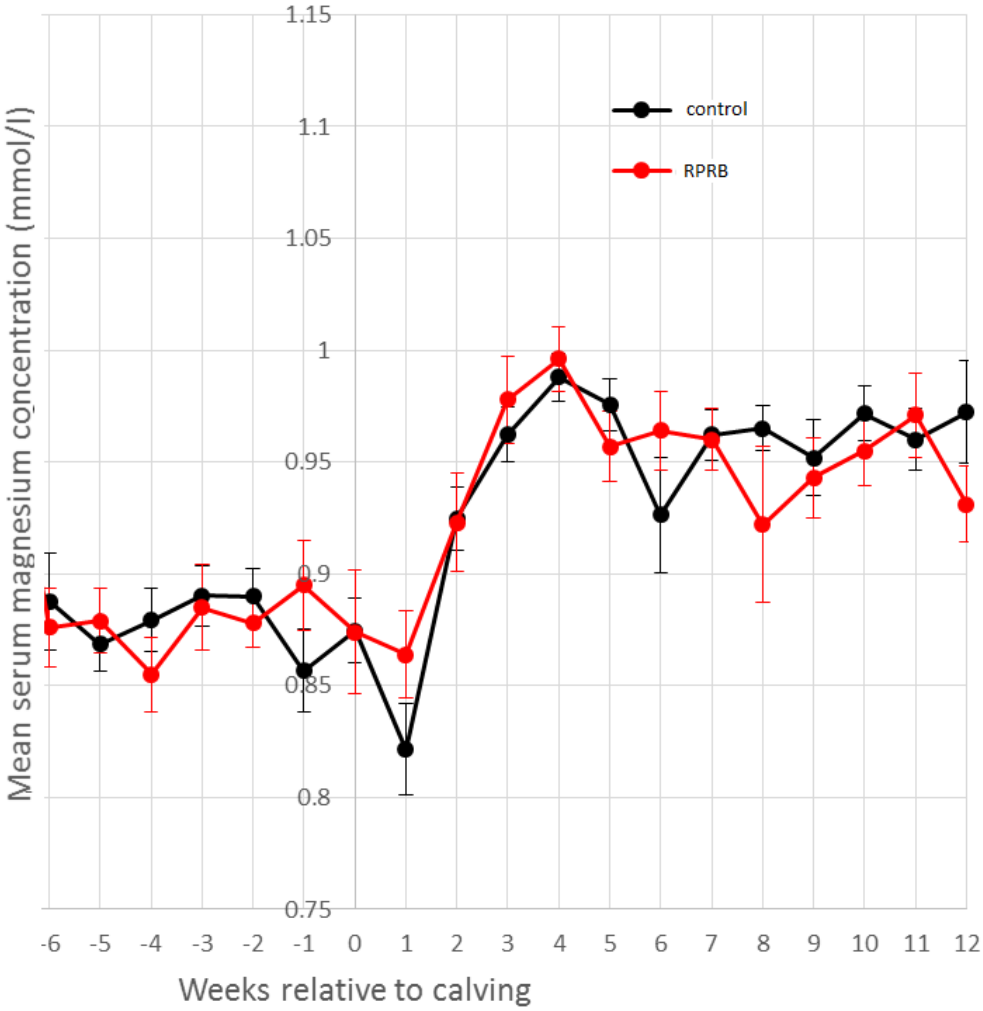


Figure 4.20. Mean serum magnesium concentrations for cows in each feed group by week relative to calving (error bars represent +/- standard error of the mean)

4.3.8. Urine mineral concentrations

The ratios between urine mineral and urine creatinine did not approximate a normal distribution (in general, they were strongly right skewed, but this differed between treatment groups). This made construction of a robust model much more complex; initial exploratory model building work revealed substantively similar results to those from a simple univariate analysis of urine mineral to creatinine ratios at week -1, so the latter is presented below.

- 4.3.8.1. Urine calcium to creatinine ratio

Figure 4.21 shows the distributions of urine calcium to creatinine ratio for each feed group at week -1. There was a 0.0003 difference between the RPRB and control group's calcium to creatinine ratios, and testing the difference between these two distributions using the Wilcoxon rank sum test, revealed a significant difference (p=0.02).

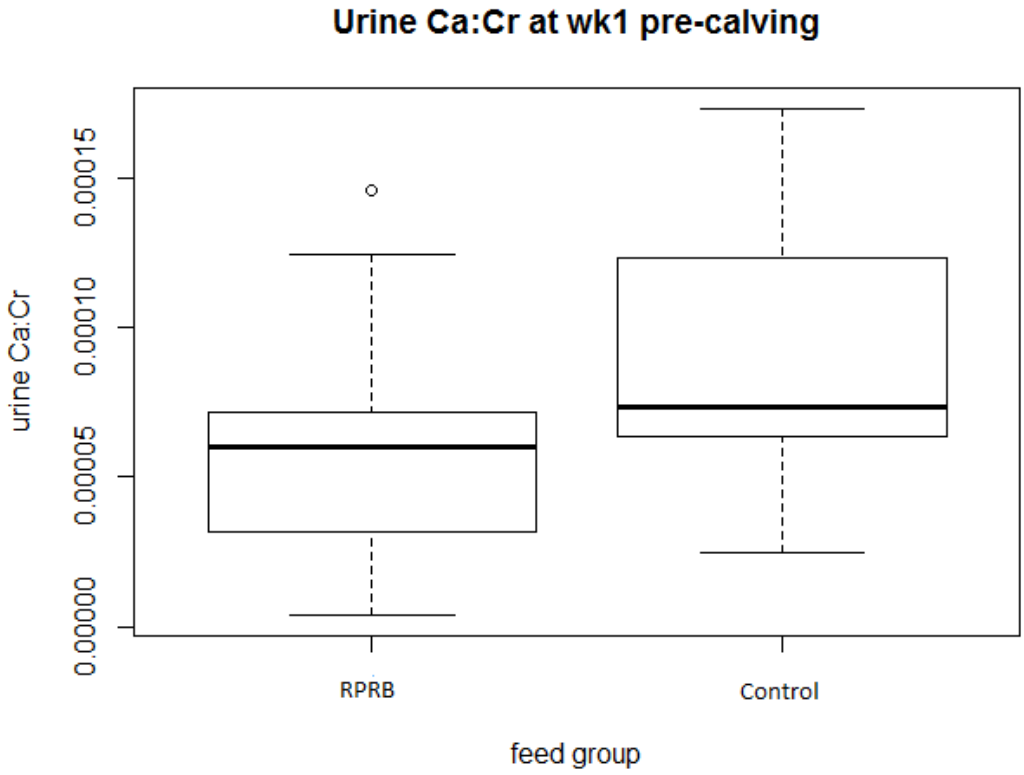


Figure 4.21. Boxplot showing urine calcium to creatinine ratio results for the two different feed groups (box represents lower to upper quartiles, with centre line representing median urine Ca:creatinine, and with whiskers representing data to a maximum value of 1.5 x IQR, with outlier points beyond these limits displayed as dots)

- 4.3.8.2. Urine inorganic phosphorous to creatinine ratio

The urine inorganic phosphorous to creatinine ratio results appear to be slightly higher for the RPRB group (Figure 4.22). This was tested using the Wilcoxon rank sum test and no significant difference between the control and RPRB groups.

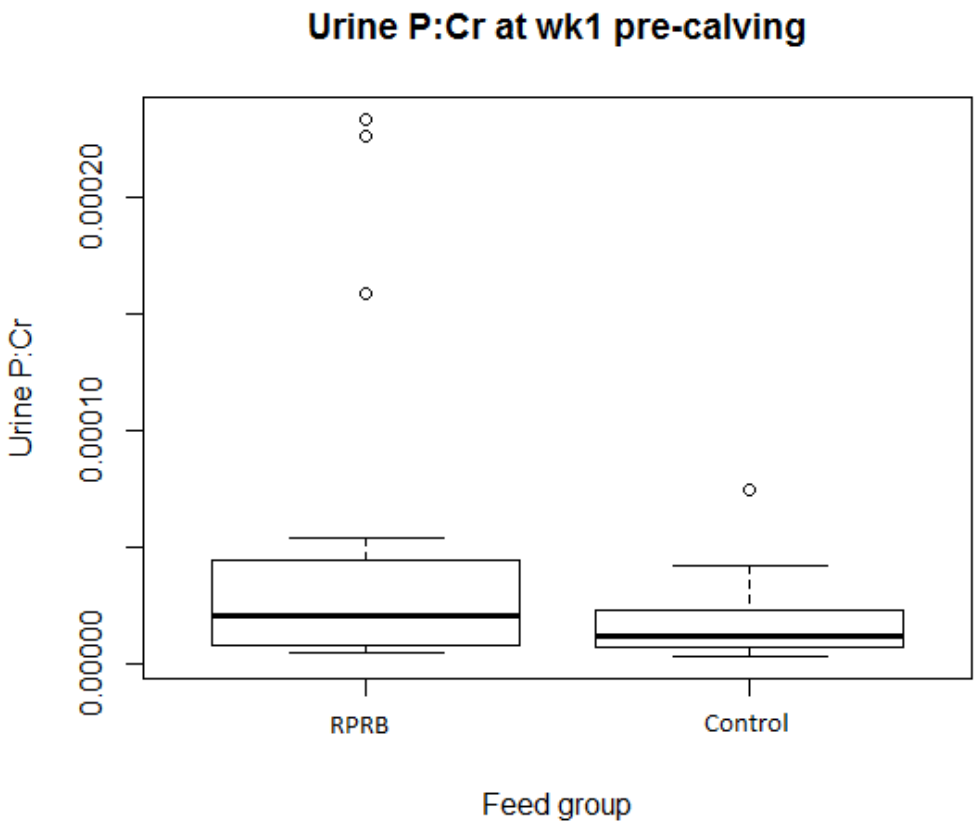


Figure 4.22. Boxplot showing urine phosphorous to creatinine ratio results for the two different feed groups (box represents lower to upper quartiles, with centre line representing median urine P:creatinine, and with whiskers representing data to a maximum value of 1.5 x IQR, with outlier points beyond these limits displayed as dots)

- 4.3.8.3. Urine magnesium to creatinine ratio

The urine magnesium to creatinine ratios for the two feed groups are shown in Figure 4.23. A significant difference was seen between the two feed groups (Wilcoxon rank sum test, p=0.02).

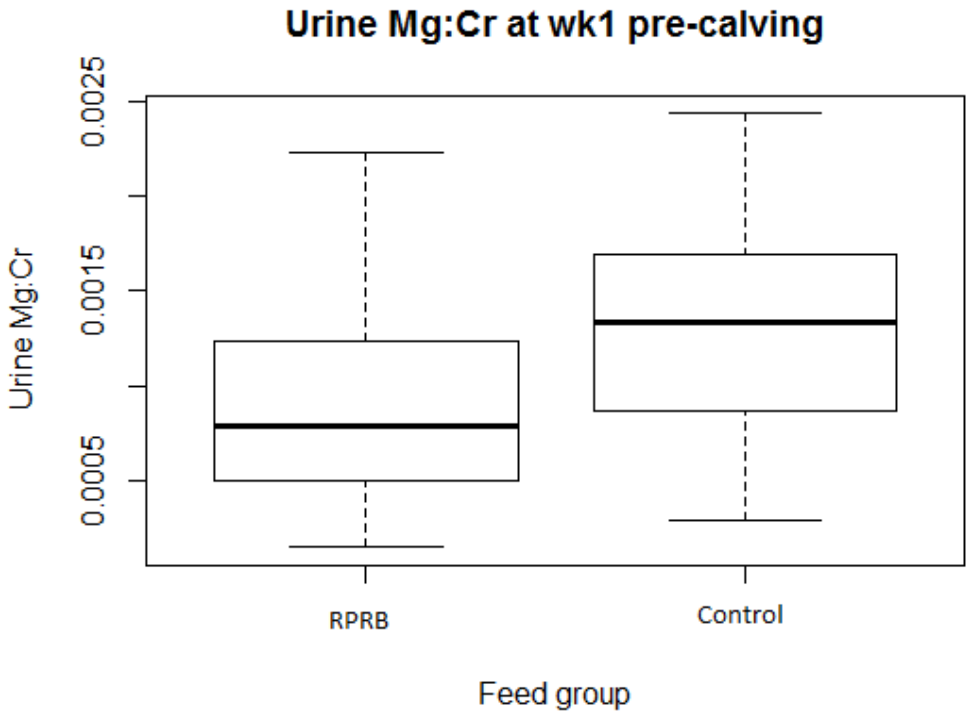


Figure 4.23. Boxplot showing urine magnesium to creatinine ratio results for the two different feed groups (box represents lower to upper quartiles, with centre line representing median urine Mg:creatinine, and with whiskers representing data to a maximum value of 1.5 x IQR, with outlier points beyond these limits displayed as dots

4.3.9. Serum energy parameters

- 4.3.9.1. Serum NEFA

In order to simplify the outcome variable, NEFA results were inspected in the raw data. It was clear that the vast majority of cows that ever experienced a NEFA above 0.8 mmol/l (the threshold value chosen to represent excessive fat mobilisation: Roberts *et al.*, 2012) did so at week 1 post calving. A binary variable was therefore created representing a cow's NEFA value at week 1, where a value of one represented an elevated serum NEFA concentration. The number of high NEFA cows in each feed group is shown in Figure 4.24. There was no significant univariate association between feed group and proportion of cows with elevated NEFA values (Fisher's exact test).

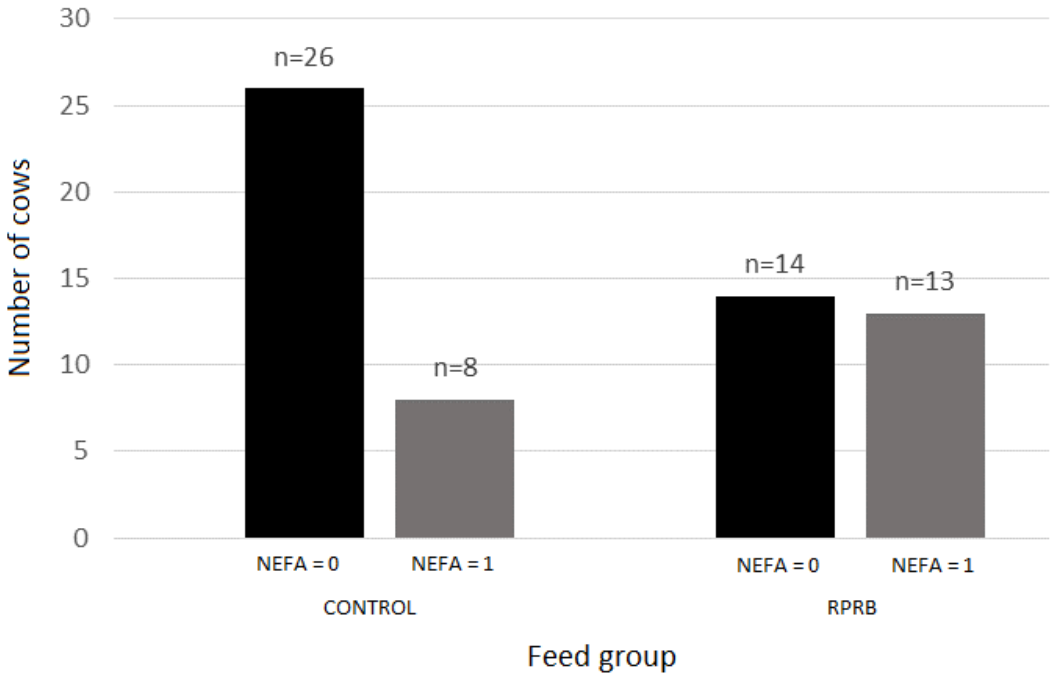


Figure 4.24. Frequency of high NEFA ('NEFA = 1', where NEFA>0.8 mmol/l) results at week 1 post-calving, according to feed group

Table 4.9 shows parameter estimates for a logistic regression model to predict the outcome of a cow having an elevated NEFA concentration at week 1 post-calving. The odds of a cow having an elevated NEFA result at week 1 post-calving was almost 90% lower in the control group compared to the RPRB group (odds ratio 0.119, 95% confidence interval 0.0125 to 0.673, $p<0.05$). The odds of a cow experiencing an elevated NEFA in week 1 post-calving were also significantly higher in cows of parity 4+ compared to cows of parity <4, and in cows with higher 100 day yields in the trial lactation.

Table 4.9. Parameter estimates for a logistic regression model to predict the odds of cows experiencing a high NEFA result (>0.8 mmol/l) at sampling week 1 post-calving

Model term	n	Coefficient estimate (standard error)	Odds ratio (95% confidence interval)
Intercept		-0.900 (0.588)	
Feed group: RPRB	21	*Reference*	
Feed group: control	26	-2.13 (0.980)	0.119 (0.0125 – 0.673)
Parity: <4	36	*Reference*	
Parity: 4+	11	2.57 (1.04)	13.0 (2.01 – 138)
100 day yield (trial lactation, '000 ltrs)	47	1.54 (0.729)	4.65 (1.28 – 24.4)

- 4.3.9.2. Serum BHB

Again, the raw data was examined in order to generate a single binary outcome to represent the occurrence of an elevated serum beta-hydroxybutyrate (BHB) concentration. For BHB, elevated values tended to occur at weeks 1 to 3 post-calving, but there was no consistent pattern in timing of the high BHB event. The outcome chosen was therefore a cow having a high BHB reading (>1 mmol/l) at any of the timepoints from 1 to 3 weeks post-calving. Figure 4.25 shows the frequencies of elevated BHB results in each feed group. The difference in frequencies between feed groups was not significant (Fisher's exact test).

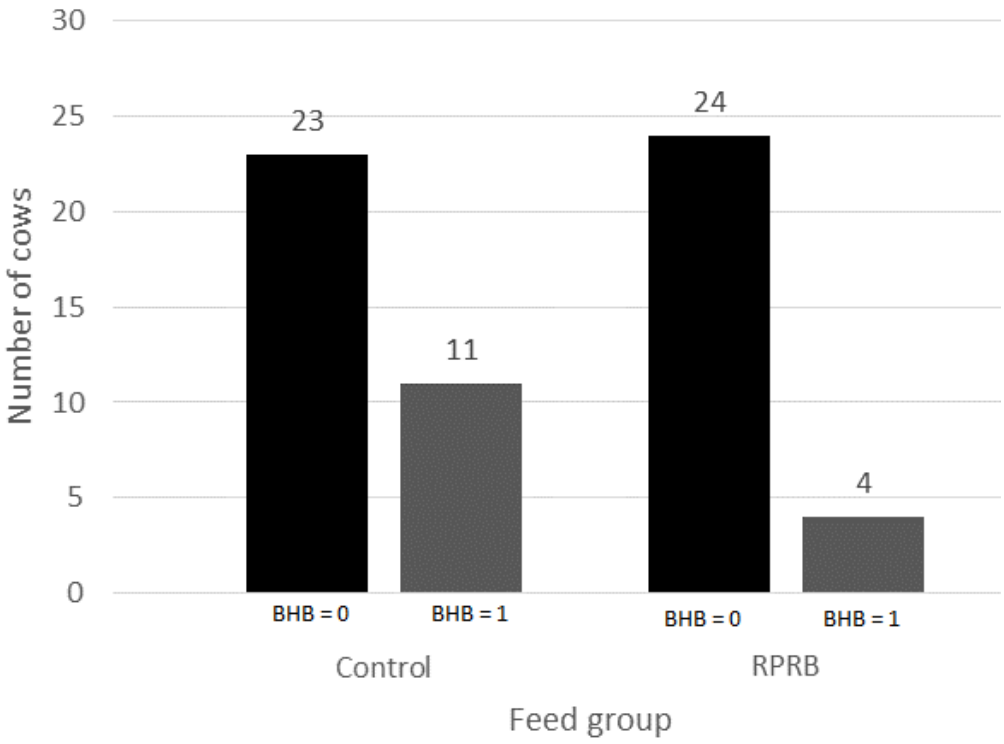


Figure 4.25. Frequency of high BHB (where BHB=1 represents BHB >1mmol/l) results at weeks 1, 2 or 3, according to feed group

The BHB results were also analysed in a multivariate logistic regression model. There was no significant difference between feed group and so this model is not reported.

4.3.10. Protein results

- 4.3.10.1. Serum albumin

Distributions of serum albumin concentrations in each feed group at each timepoint (from week -6 pre-calving to week +6 post-calving) are shown in Figure 4.26. No associations were seen between albumin and fed group, or between albumin and any other predictors.

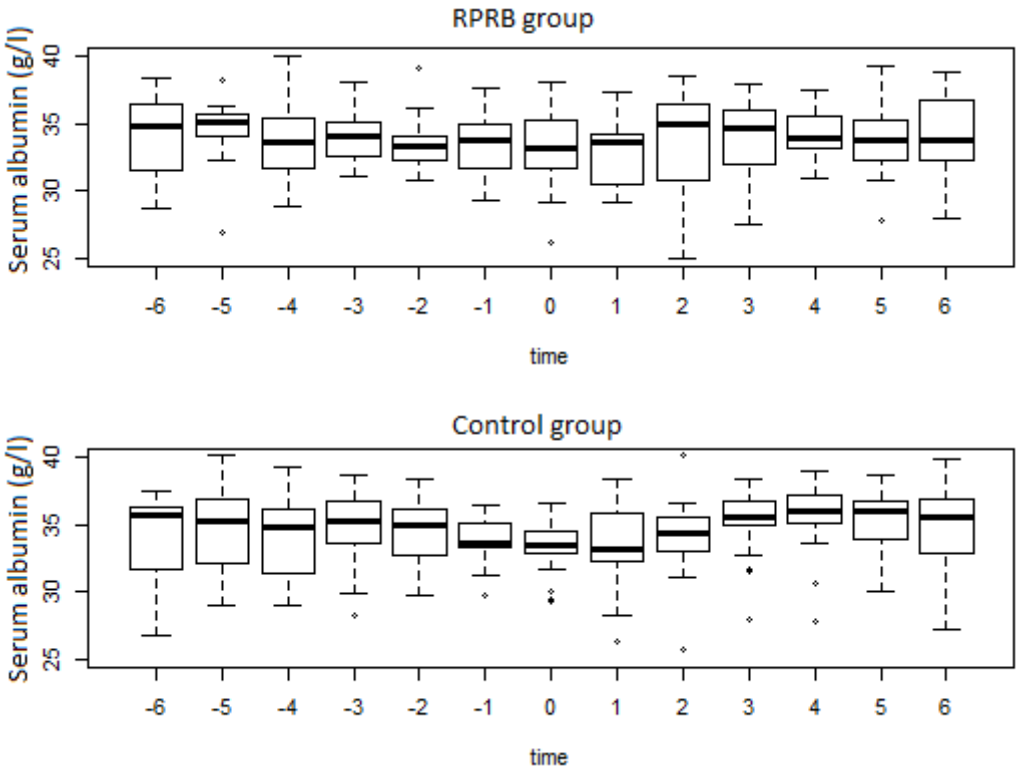


Figure 4.26. Distribution of serum albumin results in each feed group at each time point (number of weeks relative to calving, sample closest to calving represented as time point '0') (box represents lower to upper quartiles of serum albumin values for each time point, with centre lines representing median albumin concentrations at each time point, and with whiskers representing data to a maximum value of 1.5 x IQR, with outlier points beyond these limits displayed as dots)

- 4.3.10.2. Serum globulin

The distributions of serum globulin concentration at each time point and in each feed group are shown in Figure 4.12. Globulin concentrations tend to show a decrease around the time of calving. Globulin concentrations between feed groups appear more similar from week -3 onwards.

Table 4.10 shows parameter estimates for a multilevel model (accounting for repeated measures of globulin within cow) with the outcome serum globulin concentration. This model revealed significant associations between feed group and predicted globulin concentration in <2 weeks pre-calving, with predicted globulin levels 1.569g/l higher in control cows compared to those in the RPRB group <2 weeks pre-calving. The reduction in serum globulin

concentrations between weeks -2 and weeks +2 that were seen in Figure 4.27 were statistically significant.

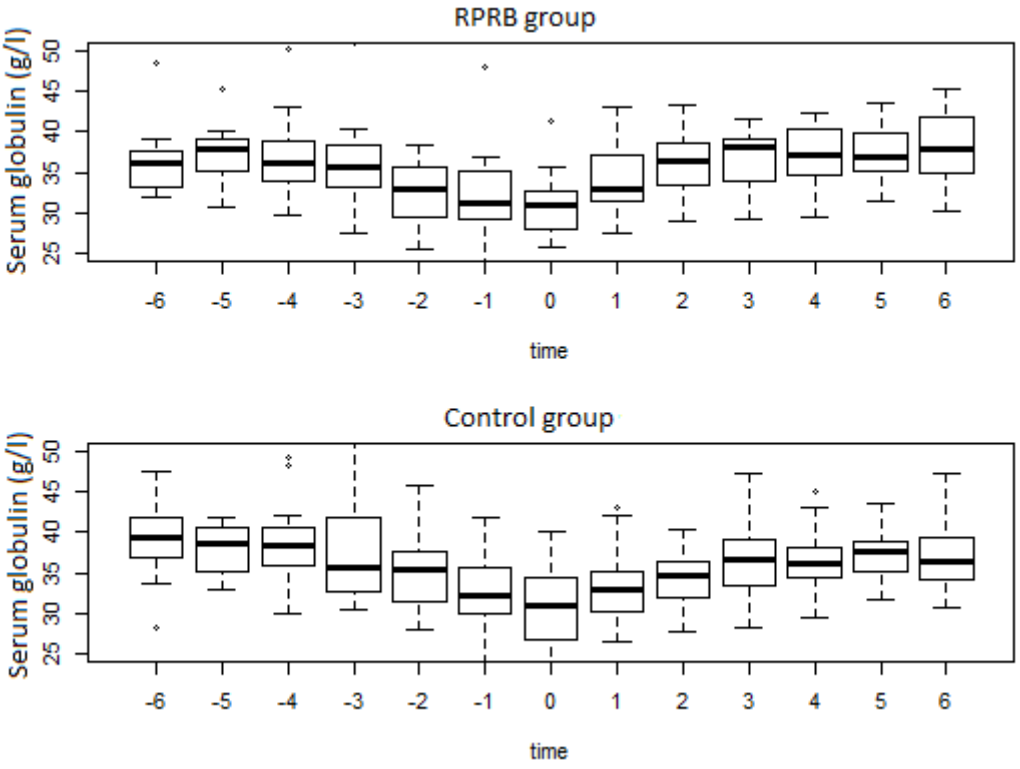


Figure 4.27. Distribution of serum globulin results in each feed group at each time point (number of weeks relative to calving, sample closest to calving represented as time point '0') (box represents lower to upper quartiles of serum globulin values for each time point, with centre lines representing median globulin concentrations at each time point, and with whiskers representing data to a maximum value of 1.5 x IQR, with outlier points beyond these limits displayed as dots)

Table 4.10. Parameter estimates for a multilevel regression model (with a random effect at cow level) to predict serum globulin concentration

	Coefficient estimate	95% confidence interval
Intercept	35.652	
Feed group: RPRB (<2 weeks pre-calving)	Reference	
Feed group: control (<2 weeks pre-calving)	1.569	0.360 to 2.777
Timepoint: week -6	Reference	
Timepoint: week -5	0.305	-1.326 to 1.937
Timepoint: week -4	-0.039	-1.568 to 1.491
Timepoint: week -3	-0.959	-2.457 to 0.538
Timepoint: week -2	-2.606	-4.235 to -0.976
Timepoint: week -1	-5.011	-6.645 to -3.376
Timepoint: week 0	-6.018	-7.656 to -4.380
Timepoint: week +1	-3.156	-4.785 to -1.526
Timepoint: week +2	-1.875	-3.505 to -0.246
Timepoint: week +3	-0.234	-1.879 to 1.411
Timepoint: week +4	-0.304	-1.940 to 1.332
Timepoint: week +5	0.485	-1.152 to 2.121
Timepoint: week +6	0.850	-0.791 to 2.490
Calving month: not April	Reference	
Calving month: April	6.032	1.787 to 10.276
Parity 1	Reference	
Parity 2	1.202	-0.998 to 3.402
Parity 3	2.358	-1.869 to 6.584
Parity 4+	2.558	0.418 to 4.694

4.3.11. Production data – trial 100 day milk yield

No significant univariate relationship between feed group and milk yield was found when the distributions of 100 day yield (in the trial lactation) were compared between feed groups (Wilcoxon rank sum test). This comparison is shown graphically in Figure 4.28.

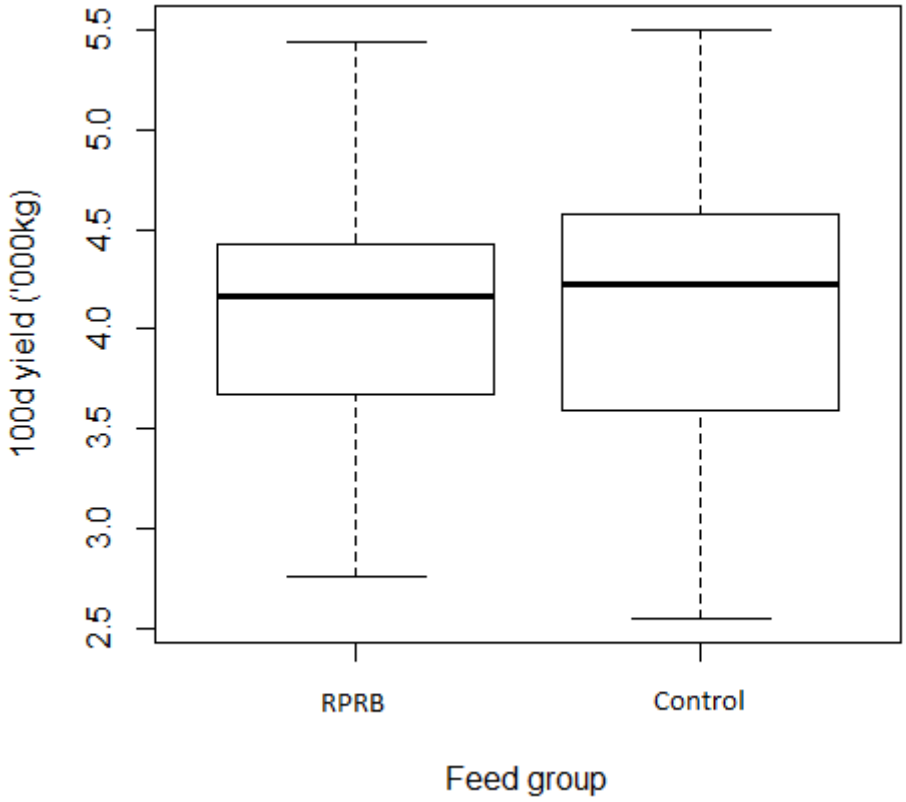


Figure 4.28. Distribution of milk yields in each feed group in the 100 days post-calving in the trial lactation (boxes represent lower to upper quartiles of 100 day milk yield values following the trial, with centre lines representing median milk yields within each feed group, and whiskers representing minimum and maximum values)

A simple linear regression model was constructed to predict trial 100 day yield. However, feed group showed no significant association even when other potential predictors were included and so this model is not reported.

4.4. Discussion

4.4.1. Clinical incidence of retained foetal membranes and milk fever

No significant difference was found between the two feed groups for the clinical incidence of milk fever. This may be due to the relatively small dataset used in this trial and the fact that these health events are already anticipated to be relatively infrequent. Prior to the commencement of the trial, the farmer reported an estimate of around 10 milk fever cases per year in the herd, which equates to around 3.57% of the animals across all parities. If accurate, this estimate would place the herd on the low end of the scale of milk fever incidence within the UK, with 4-9% being suggested as a typical incidence by Husband (2005). RFM were reported in only 2 of the 62 cows included in the final analysis. As these were spread evenly across the two feed groups, statistical analysis was not carried out as no difference was seen. RFM and milk fever recording was carried out when clinical cases were observed, but primarily relied on communication from farm staff and the follow-up of farm records. Whilst this is not considered to be a large problem due to the care taken to accurately record this data, it is possible that some cases may not have been recorded.

4.4.2. Serum mineral concentrations

- #### 4.4.2.1. Serum calcium concentrations

From the multivariate model, a cow in the control group was predicted to have a serum calcium concentration 0.109mmol/l higher than a cow in the RPRB group in weeks -3 to -1 pre-calving, and this difference was significant ($p<0.05$). Blood calcium levels in the adult cow are generally maintained between 2.12 and 2.90mmol/l (Goff, 2004) and so this difference is fairly small, and whilst it is statistically significant, it is unlikely to be of biological significance. The reason for the statistical difference between the serum calcium concentrations of the two feeds groups cannot be determined. It is postulated that this is either due to dietary intake of calcium, or is due to the action of calcium binding. The lower serum calcium concentrations in the RPRB group could be due to the lower dietary calcium content of the RPRB

diet compared to the control ration, as analysis of the diets revealed that the RPRB diet supplied 47.0g/head per day of calcium compared to the 64.7g/head per day of the control ration. Alternatively, RPRB has been reported to have calcium binding properties, with Martin-Tereso *et al*. (2014) finding reduced pre-calving serum calcium concentrations in cows receiving a diet containing RPRB, when compared to a control diet that supplied an identical level of dietary calcium. The difference found by Martin-Tereso *et al*. (2014) was attributed to the calcium binding properties of the rice bran.

A cow that entered the trial at parity 4+ (i.e. a cow about to enter its 5th lactation or greater) had a statistically significantly lower serum calcium concentration in weeks -3 to -1 pre-calving than a cow that was parity 1 at recruitment (i.e. a cow about to enter its 2nd lactation) by 0.061mmol/l. This difference was smaller than the difference seen between feed groups, and again is unlikely to have biological significance. Age-related factors relating to serum calcium concentrations are reported in the literature, with Horst *et al*. (1997) reporting that older cows have a reduced ability to effectively respond to challenges of calcium homeostasis, owing to factors such as their reduced efficiency of intestinal calcium absorption and a reduced ability to resorb calcium from bone. However, as this present study did not look at serum calcium concentrations immediately pre-calving it is not possible to assess potential parity-related differences in calcium status at the onset of lactation.

- 4.4.2.2. Serum inorganic phosphorous concentrations

The multilevel regression modelling showed that serum inorganic phosphorous concentrations were 0.201mmol/l lower at week -1 pre-calving in control cows compared to the RPRB cows, and this difference was significant ($p<0.05$). The significant difference seen between the two groups is likely to be due to the much higher level of phosphorous supplied by the RPRB diet. This would have been the case anyway, as the original formulation specified a daily intake of 76.9g/head on the RPRB diet, compared to the 43.1g/head supplied to the control group. Rice bran is inherently rich in phosphorous, owing to its high phytate content. This is a storage form of

phosphorous in plants and is reported to have calcium-binding properties (Martin-Tereso *et al*., 2009), and so a higher inclusion rate of dietary phosphorous compared to the control group was expected. However, the misformulation of the diets meant that the RPRB diet actually supplied phosphorous at a level of 96.7g/head to the RPRB cows, and 53.6g/head to the control cows, meaning that the phosphorous intake for the RPRB was almost double that of the control animals. This has the potential to be problematic with regard to milk fever, as high levels of dietary phosphorous have been associated with an increase in milk fever risk (Peterson *et al*., 2005; Goff, 2014b). Blood phosphate concentrations that fall within the upper end of normal, around 6mg/dL (1.94mmol/l), can have an inhibitory effect on the renal enzyme required for production of $1,25(OH)_2D$. This is required for intestinal transport of calcium and so, even if PTH was secreted and recognised by the tissues, impaired calcium homeostasis could still occur (Goff, 2014b). The mean serum phosphorous concentrations pre-calving exceed this in the present trial (Figure 4.19). Peterson *et al*. (2005) report that feeding <50g/day of phosphorous appears to be safe with relation to the risks to calcium homeostasis. The initial formulation for the control diet fell below this reportedly 'safe' threshold, although the misformulated diet exceeded it. However, the RPRB original formulation already exceeded it, and this issue relating to milk fever risk does not appear to have been explored in the existing literature on rumen-protected rice bran.

The multivariate model also showed that parity 4+ had a 0.209mmol/l lower serum inorganic phosphorous than parity 1 animals across weeks -3 to -1 pre-calving, and this difference was significant ($p<0.05$). Younger animals tend to have higher blood phosphorous concentrations due to the activity of growth hormone as they are still developing (Cozzi *et al*., 2011), and this may explain the difference seen across all analysed time points (weeks -3 to 1 pre-calving) in this present study.

- 4.4.2.3. Serum magnesium concentrations

No significant associations were found between serum magnesium concentrations and any of the potential predictor variables. This is in line with the findings of Martin-Tereso *et al*. (2014), who compared the feeding of RPRB to a low DCAD diet and found no difference in serum magnesium between the two groups. The magnesium content of the RPRB diet in the present trial was higher than that of the control diet (formulated at 62.5g and 46.5g respectively, prior to the additional inclusion of 20g/head per day of magnesium chloride to both groups), but RPRB may bind to dietary magnesium due to its non-specific action (Lean *et al*., 2013), reducing its availability to those animals.

It would have been interesting to consider the effects on serum magnesium concentrations had the trial been able to continue without the additional inclusion of magnesium chloride into the forage ration, which was provided to all dry cows. Whilst no significant difference was found between the two feed groups, this may have been different had the overall supply of magnesium been lower in the diets. As previously mentioned, RPRB is non-specific in its action and if it is capable of binding magnesium at levels that would risk causing hypomagnesaemia when lower levels of dietary magnesium are supplied then this may have detrimental effects on calcaemic status. Hypomagnesaemia can increase the risk of hypocalcaemia by both reducing tissue sensitivity to PTH and reducing PTH secretion itself (Goff, 2014). It is not possible to determine whether or not this would have been seen in the present trial without the inclusion of magnesium chloride, but this may warrant further investigation and the potential effects of RPRB on magnesium status are worth continuing to consider in future trials of RPRB.

4.4.3. Urine mineral concentrations

Creatinine is a waste product of creatine phosphate in muscle and its excretion, via the kidneys, is relatively constant and therefore is considered to be an appropriate means of 'standardising' the excretion of minerals within a urine sample (Matos *et al*., 1997; Price *et al*., 2005).

- 4.4.3.1. Urine calcium to creatinine ratios

The RPRB diet supplied less dietary calcium than the control diet and also is reported to have the ability to bind with dietary calcium (Martin-Tereso *et al*., 2014). Cows in the control group had a significantly higher (p=0.02) calcium to creatinine ratio than cows in the RPRB group. However, this difference was very small (0.0003) and so is regarded to be of little physiological significance.

- 4.4.3.2. Urine inorganic phosphorous to creatinine ratios

Whilst dietary phosphorous intake was considerably higher in the RPRB group compared to the control group, as previously discussed, no significant difference was found between urine inorganic phosphorous to creatinine ratios between the two feed groups at week -1 pre-calving. In cows, excess phosphorous is excreted through urine, faeces and saliva, and excretion is strongly linked to intake (Knowlton & Herbein, 2002). The present study did not consider these alternative means of phosphorous excretion.

- 4.4.3.3. Urine magnesium to creatinine ratios

The urine magnesium to creatinine ratio in week -1 pre-calving was significantly higher in the control group compared to the RPRB group (p=0.02). Dietary magnesium was anticipated to be higher in the RPRB group, as previously discussed, although forage intakes that contained magnesium chloride cannot be determined. However, despite the fact that excess magnesium can be excreted via the kidneys (Goff, 2014), the group with the higher expected magnesium intake had a significantly lower urine magnesium to creatinine ratio. It can be postulated that this difference could occur due to the non-specific binding action of phytate in rice bran binding to magnesium (Lean *et al*., 2013), meaning that some will be lost in this way through the faeces, although this is not measured in this present study. The difference reported here, whilst statistically significant, is small and therefore is unlikely to have biological or clinical significance.

4.4.4. Serum energy parameters

- #### 4.4.4.1. Serum NEFA concentrations

NEFA concentrations were converted to binary values, with '1' representing an elevated NEFA concentration (>0.8mmol/l) in week 1 post-calving. The time period was chosen as the vast majority of high NEFA events recorded occurred during this time. No significant difference was found between the two feed groups during univariate analysis. The logistic regression model revealed that the odds of a cow having an elevated NEFA result at week 1 post-calving was significantly lower in the control group compared to the RPRB group ($p<0.05$). Increased NEFA concentrations are considered indicative of a negative energy balance (Huzzey *et al*., 2012). A high level of fat mobilisation, and corresponding increase in NEFA concentrations, post-partum can follow on from overfeeding in the dry period (Huzzey *et al*., 2012), but both the RPRB and control diets supplied the same levels of energy, assuming the predicted quantities of forage were also consumed. A significant difference in serum calcium concentrations was also found between the two feed groups. NEFA concentrations have previously been associated with calcaemic status, with Chamberlin *et al*. (2013) reporting higher NEFA concentrations post-partum in hypocalcaemic animals. However, the differences in serum calcium concentrations found between the two feed groups in the present study are small and are not considered to have biological significance, as both groups remained within a normal serum calcium range.

There was a significant association between an elevated NEFA concentration and higher 100 day yields in the subsequent lactation. Cows producing more milk will require more energy to put into that production, and so it is more likely that those animals will be mobilising greater amounts of fat at this time. This is thought to be related to energy partitioning controlled by genetics in high-yielding animals, rather than being due to an inability to increase feed intakes in line with the demand (Veerkamp *et al*., 2003).

- 4.4.4.2. Serum BHB concentrations

BHB concentrations were converted to binary values, with '1' representing an elevated BHB concentration (>1mmol/l) in weeks 1 to 3 post-calving. No significant difference was found between the two feed groups.

The threshold of >1mmol/l for an 'elevated' BHB concentration was chosen in line studies such as those of Kelly (1977) and Kessel *et al*. (2008). However, Geishauser *et al*. (2000) and Jackson *et al*. (2011) use >1.4mmol/l as a threshold for measuring ketosis, and this may have altered the findings. Weeks 1 to 3 post-calving were chosen for the serum BHB analysis, as in the present dataset these were the time points where high BHB values tended to be recorded. However, Duffield *et al*. (1997) report that when clinical ketosis occurs it is typically between weeks 2 to 7 post-calving, and so it may have been appropriate to statistically analyse a wider section of the data.

4.4.5. Serum protein concentrations

- 4.4.5.1. Serum albumin concentrations

Serum albumin concentrations from week -6 pre-calving to week 6 post-calving were not found to be associated with feed group or any other predictors. Overton & Burhans (2013) also report little variation in albumin results across the transition period.

- 4.4.5.2. Serum globulin concentrations

Serum globulin concentrations showed a decrease between week -2 pre-calving and week 2 post-calving and this was statistically significant ($p<0.05$). Grunberg *et al*. (2011) attribute a decrease in globulin levels shortly before calving to the increased uptake of IgG1 by the mammary gland prior to the onset of lactation.

The difference in serum globulin concentrations between feed groups was also significant, with predicted globulin levels 1.6g/l higher in control cows compared to those in the RPRB group at <2 weeks pre-calving. Grunberg *et al*. (2011) also reported a dietary difference in globulin levels, and described a

decrease in globulin levels in cows fed a low DCAD diet, although stated that the clinical relevance of this was not clear.

4.4.6. Limitations of this study and future recommendations

- #### 4.4.6.1. Effects of the errors in diet manufacture

The control diet was not made to the specification provided to the manufacturers and, as a consequence, magnesium chloride was added to the mixed forage supplied to both feed groups. This was carried out in order to safeguard welfare, as the farmer was concerned about suspected cases of milk fever that were occurring at this early stage of the trial. It was not logistically possible at that stage to move the trial to a different farm, but effectively the trial restarted at that point with a differing protocol. The trial diets were already produced in a pelleted form and the only way to supplement the control animals with magnesium chloride was to do so through the mixed forage that was supplied to all of the dry cows. Magnesium chloride is an anionic salt which in itself can act to reduce the risk of milk fever (Aiello & Moses, 2015), and so this was not ideal, as it may have added a further level of protection against hypocalcaemia that may make the results more difficult to interpret.

Following the initial issue with the control diet, subsequent analysis of the RPRB diet revealed it also did not match its intended specification. Perhaps the most notable issue is the RPRB diet containing an even higher level of phosphorous than was already included in its intended formulation. The RPRB diet was always intended to supply a higher level of phosphorous than the control diet as it phytate content is responsible for its reported calcium binding properties. However, the increase in phosphorous content over and above what was expected may have influenced the results.

4.4.6.2. Issues surrounding the use of out-of-parlour feeders

Out-of-parlour feeders were considered to be the only practical way to monitor concentrate intakes in this commercial herd. Their use was particularly effective at eliminating cows from the results analysis that did not

consume enough (or any) of the trial diets prior to calving, as this would not have been detected had the intakes not been recorded and may have had a considerable impact on the validity of the results reported. However, the use of such feeders carries the small risk of cows being able to access the incorrect diet or additional portions of feed that are dispensed for other animals in the shed. Whilst only one animal could fit in the feeder at any one time, this could occur if an animal triggered the dispensation of a meal but then left the feeder before consuming it, allowing another animal access to that meal. In an attempt to combat this, the portion sizes were small, meaning that if animals did trigger the feeder and then left feed behind, it was likely to only be a small proportion of the daily intended intake.

As stated, some animals did not consume adequate quantities of the diet for the stated period of time and so were subsequently excluded from the trial. Attempts were made to physically manoeuvre such cows into the feeders and once in position they did then eat the feed supplied to them, suggesting it was more an issue of entering the feeders, rather than an issue of palatability. It is unclear as to why some animals did not enter the feeders but it may be related to the confined space or potential risk of bullying behaviour by other animals within the shed.

- 4.4.6.3. Practical issues of future use of rumen-protected rice bran

Out-of-parlour feeders were chosen for use in this trial as they allowed for the monitoring and recording of concentrate feed intakes, along with fitting in with the husbandry and management strategies already in place on the farm. Theoretically, if RPRB can be shown to have a positive effect on calcaemic status and post-partum health and productivity then the use of out-of-parlour feeders may be the best approach in terms of practically supplying the diet. It allows for intakes to be monitored, which may highlight at-risk animals if some do not consume their full ration, as was found in this study. The use of the accompanying computer software means that cows can be switched onto the diet at the point deemed most appropriate. The diet may also be suitable to be fed as a top-dressing or as part of a TMR.

4.4.6.4. Further limitations of the study and recommendations for future research

One of the main limitations of this study surrounds the time frame of sample collection. Blood and urine samples were collected every Tuesday, regardless of the calving date of animals. It was not logistically possible to sample all cows immediately after calving, and so samples obtained within the time frame deemed within the literature to be the most relevant to the diagnosis of hypocalcaemia, such as the 12 to 24 hours post-partum proposed by Goff (2008), were only obtained coincidentally. Some calvings did occur on a Tuesday and therefore the samples for those cows truly reflect 'week 0', but due to the relatively small overall sample size these were very limited. Only seven cows calved on their sampling date and these samples occurred at varying times pre- and post-calving on those days, and so the decision was made not to include samples from week 0 in the analysis. Week 0 included cows sampled from between day -3 pre-calving to day 3 post-calving, and the number of cows calving on each of these relative days varied and was not necessarily well-matched between the feed groups.

It could be argued that the control ration formulated for this trial was 'too good', especially after the inclusion of additional magnesium chloride following the early changes to the diets, as this itself can reduce the risk of milk fever (Aiello & Moses, 2015). Due to the trial taking part on a commercial dairy farm, there were limitations on what aspects of herd management could be altered and the trial had to fit around existing farm practices. Complete freedom to change the experimental diets was not possible, as these had to be agreed with the farmer and were required to be based around the farm's existing dry cow ration and available forage. One issue with the farm chosen for the study was the already low incidence of milk fever in previous years, with the farmer reporting approximately 10 cases of milk fever per annum in a herd of approximately 280 milking cows. An estimated incidence percentage of 3.6% for the year prior to the trial is low when compared to reported UK averages (Husband, 2005), although a level of subclinical hypocalcaemia

would still be anticipated within such a herd (Jonsson *et al*., 1999; Roche *et al*., 2002). It may have been more appropriate to trial RPRB on a farm that had more of an existing milk fever concern.

Whilst this trial is the largest commercial trial of RPRB for the prevention of milk fever to date, the dataset was limited in terms of cow numbers. Fewer cows were able to be recruited onto the trial than had been anticipated, and there was a high rate of exclusions, mainly due to issues surrounding feed intakes. However, extending the length of the trial in order to recruit more animals was not possible or practical due to a major change in farm management at the end of 2013, including changing dry cow management and milking practices.

It was not possible to monitor forage intakes during this trial and so these may not have accurately reflected the formulated rations. There may have been considerable variation between the two groups in terms of forage intakes, which would also impact on the intakes of the supplemented magnesium chloride. Similarly, the intakes of the freshly calved cows were not monitored or controlled at all.

Ionised calcium was not analysed for in this trial. It was initially considered and an ionised calcium electrode was purchased (Lazar Research Laboratories Inc., Los Angeles, California, USA). However, this was not considered to be reliable during attempts at calibration, and the decision was made not to use this measure, after consulting the literature, as previously discussed in Chapter 1. Whilst ionised calcium levels would indicate the concentrations of the physically active form of calcium within an animal (Schenck & Chew, 2008), there are a number of problems widely associated with sample handling and analysis (Lyon *et al*., 1995; Pfitzenmeyer *et al*., 2007). The combination of these factors and the apparent unreliability of the ionised calcium electrode available meant that this method of analysis was not pursued further.

Urine pH data was collected during this study but was deemed to be unreliable due to the pH meter used, and so the results of this analysis are not presented in this thesis. Urine pH data can be useful in monitoring dietary acidification in full DCAB diets, but such acidification would not be expected on the diets used in this study.

The collection and analysis of faecal samples may have provided more information about the mineral status of the animals used in the trial. This option was not investigated or considered but may have been of particular use with regard to levels of phosphorous excretion. As previously discussed, no significant difference was found between the RPRB and control feed groups in terms of urinary phosphorous excretion. However, the cows in the RPRB group had a considerably higher phosphorous intake and phosphorous excretion occurs in line with phosphorous intake (Knowlton & Herbein, 2002), via the saliva, urine and faeces (Wu *et al*., 2000). Not only might it be interesting to have information regarding faecal phosphorous excretion from a nutrition perspective, but it might also have environmental relevance. The recommendations for ruminant phosphorous intakes have changed in recent years, in part due to the environmental concerns relating to the role of high levels of faecal phosphorous excretion by livestock in the pollution of surface water (Knowlton *et al*., 2001), leading to eutrophication (Ekelund *et al*., 2006). Whilst it is important that the animals' needs for dietary phosphorous are met, it may be important to consider the possible environmental impact of such a strategy if it is supplying excessive levels of the mineral, potentially leading to high levels of faecal phosphorous excretion, and this would warrant further investigation.

Further work regarding the use of rumen-protected rice bran as a strategy for preventing milk fever would be recommended. Whilst the present study did not look at samples immediately post-partum, as would be recommended (Goff, 2008), it did not find benefits in terms of yield or energy status post-partum for cows fed a RPRB diet. However, due to the increased phosphorous included in the misformulated diet, it is not clear if this may have had an

effect, as this has been reported to increase milk fever risk (Goff, 2014b) and may have cancelled out any potential benefits of RPRB on calcaemic status. The potential detrimental effects of phosphorous on both calcaemic status and the environment warrant further investigation, as does the actual efficacy of the RPRB strategy when used outside of an experimental setting.

4.5. Conclusion

Both pre-calving serum calcium concentration and pre-calving urine calcium to creatinine ratio were significantly higher in animals in the control group compared to those fed RPRB. However, this effect was small and is not considered to be biologically significant. Inorganic phosphorous was significantly higher in cows in the RPRB group and this is likely to be due to the high level of phosphorous included in that diet. No difference was seen in urinary inorganic phosphorous to creatinine ratios between the two groups but this may be due to alternative pathways of excretion of excess phosphorous that were not explored in this study. Significantly higher levels of magnesium were excreted in the control cows compared to the RPRB cows in the final week pre-calving. The forage mix contained supplementary magnesium chloride and intakes of this were not measured but may have had an effect, or this could be due to the non-specific binding action of RPRB that has previously been reported and may warrant further investigation in future research. The risk of a cow experiencing an elevated NEFA post-calving was significantly higher in the RPRB group but no significant differences in the risk of experiencing an elevated BHB concentration were found. Serum globulin was higher in control cows in the two weeks pre-calving than in the RPRB cows, although it is not clear as to why this may have occurred.

This study had a number of issues, including the misformulation of the trial diets and the subsequent alterations made to the feeding protocol. This meant that the trial was effectively restarted at this point and so the data included in this chapter was not affected by the initial problems. However, the diet that was used for the period of data collection was perhaps too good in terms of milk fever prevention, even without the potential effects of RPRB as

a calcium binder. This potential factor, combined with an existing low incidence of milk fever on the farm chosen, may have contributed to the lack of significant findings regarding hypocalcaemia. The trial suffered from a high exclusion rate of animals, largely due to issues surrounding cows not consuming sufficient quantities of the trial diets in order to meet the thresholds for inclusion. The sampling point with relation to calving was not ideal, and although alternatives had been considered, they were not logistically possible.

This study is the largest reported study of RPRB as a potential milk fever preventative on a dairy herd to date. The use of out-of-parlour feeders was beneficial in terms of detecting and eliminating animals that did not consume adequate quantities of the trial diets, and is a strength of this study design.

Further research is needed if RPRB is to be marketed as a potential milk fever preventative. Whilst other research has supported its use for this purpose, evidence for its efficacy was not reported in the present trial. Future studies would be recommended to improve numbers of participating animals, improve the timing of sampling points in relation to calving, and may be best suited to farms where an existing milk fever problem is recognised. Issues surrounding the high levels of phosphorous supplied by RPRB may warrant further investigation, both with regard to potential effects on hypocalcaemia and the potentially detrimental environmental impact.

CHAPTER 5: GENERAL DISCUSSION

Clinical milk fever and subclinical hypocalcaemia are important metabolic diseases that can arise during the transition period (Curtis *et al*., 1983), as cows move from the dry period into their early lactation post-calving. Both conditions can have a number of downstream effects on both cattle health and productivity (Oetzel, 2013), and they can have a considerable economic impact on farmers (Husband, 2005), with even subclinical hypocalcaemia having the potential to lead to both milk losses and an increased risk of clinical disease (Oetzel, 2013). Farmers therefore have a financial incentive to prevent the conditions from occurring, and a number of potential milk fever prevention strategies are available to them, as described in Chapter 1. All of the strategies have potential benefits and potential negative issues, as discussed in Section 1.4. The use of calcium binders within the diet is a relatively new approach to milk fever prevention. One option that works on this principle is zeolite, but this has drawbacks relating to its lack of ion specificity (Thilsing *et al*., 2006), its poor palatability (Grabherr *et al*., 2009), and its potential to reduce dry matter intake (Grabherr *et al*., 2009).

Rice bran also has the potential to bind to dietary calcium (Jahnen *et al*., 1992; Siener *et al*., 2001) and, following on from the development of a rumen-protected form of this feedstuff (Martin-Tereso *et al*., 2009), has been considered as a potential milk fever preventative. To date, the data on its efficacy has largely been obtained from small trials on experimental dairy farms and so this thesis aimed to trial its use within a commercial UK dairy herd. The effects of feeding the diet containing the rumen-protected rice bran were compared against a control ration, which was investigated from a number of perspectives, including calcaemic status, energy parameters and production outcomes. A survey of both UK dairy farmers and UK vets was also carried out to gain more insight into current attitudes to hypocalcaemia and its prevention. A retrospective study was also carried out to investigate factors associated with recorded milk fever events in a relatively large dataset from herds across the UK. Finally, this thesis also aimed to investigate the

potential comparable use of different blood sample types for the diagnosis of subclinical hypocalcaemia and other mineral disorders.

5.1. Main findings

The main findings of this thesis were as follows:

A survey of UK dairy farmers and veterinarians on milk fever and its prevention:

- Farmers who completed a questionnaire on milk fever were most likely to state that when choosing a milk fever prevention strategy they were primarily concerned with its efficacy, followed by its ease of use, with cost appearing to be a less important factor
- Veterinarians were more likely than farmers to report that transition cow health and metabolic diseases featured in their top three herd health concerns ($p<0.05$)
- Veterinary recommendations for milk fever prevention strategies featured in the reported decision-making factors for choosing a strategy by just over a quarter of farmers questioned (27.8%, n=30)

A retrospective study of factors associated with recorded milk fever events across >59,000 lactations in 78 UK dairy herds:

- The overall recorded milk fever incidence across herds within the dataset was 4.26%
- Higher milk yields (taking into account parity through regression modelling) in the previous lactation were associated with a higher risk of a milk fever event being recorded, with a 7.6% increase in milk fever risk per 1,000 litres of milk in the previous lactation
- Cows that had a case of milk fever recorded were 2.2 times more likely to leave the herd by day 30 of that lactation than cows that did not have a recorded milk fever event

- A recorded case of milk fever was associated with a 2.3 times higher risk of dystocia than a cow that did not have a recorded milk fever event
- A twin pregnancy was associated with a 30% reduction in the odds of a cow experiencing milk fever

Trialling a diet containing rumen-protected rice bran against a control diet on a commercial UK dairy herd:

- There appears to be a good level of agreement between serum and plasma samples when choosing a sample type for analysis when diagnosing milk fever or subclinical hypocalcaemia
- Cows fed a diet containing rumen-protected rice bran had a significantly lower serum calcium concentration and a significantly lower urinary calcium to creatinine ratio pre-calving to cows fed a control diet, although this was not considered to have biological significance
- Serum phosphorous was significantly higher pre-calving in cows fed rumen-protected rice bran than in cows fed a control diet
- Urinary magnesium excretion was significantly lower in cows fed rumen-protected rice bran compared to those fed a control diet
- No evidence was found in the farm trial conducted to support the use of rumen-protected rice bran as a milk fever preventative

5.2. Clinical and industrial relevance of the main findings

The importance placed by farmers on the efficacy of a milk fever prevention strategy is particularly relevant when conducting trials of new products or strategies, such as the use of rumen-protected rice bran, as it suggests that the evidence of a product improving the calcaemic status around calving would encourage its use by farmers. This supports the idea that there was and is a need for further work to be carried out on trialling the effects of rumen-protected rice bran, including the trial conducted as part of this thesis.

Veterinarians were significantly more likely than farmers to include metabolic disease and transition cow health in their list of top three health concerns. Whilst some farmers were able to describe some of the potential effects of subclinical hypocalcaemia, this was not found across the board, and it may be that some farmers lack knowledge in this area. There may be room for improvement with regard to the education of farmers by veterinarians on this subject. Only around a quarter of farmers questioned were considering veterinary advice when choosing a milk fever prevention strategy, although it is not clear as to whether this is due to a lack of information being offered to them or due to an unwillingness to take on board the opinions of vets.

The milk fever incidence data reported in the retrospective epidemiology study was 4.26%, which is around the low end of previous estimates of UK milk fever incidence. It cannot be determined whether this is low purely due to the approach taken to data collection, but is still a measure of milk fever incidence from a relatively large number of lactations/herds and in relatively recent years. The recording of a milk fever event was associated with a significantly increased likelihood of a cow exiting the herd early in the lactation, and was also significantly associated with an increased risk of dystocia, which can lead to calf loss. Both of these events can have a considerable financial impact on a farmer. Higher milk yields were associated with a higher risk of a milk fever event being recorded, and this may aid farmers when choosing specific animals to target for milk fever monitoring and prevention. The finding that a twin pregnancy was associated with a reduced risk of the recording of a milk fever event appears to be a novel finding previously unreported in the literature.

Plasma and serum samples appeared to show good levels of agreement with regard to the measurement of calcium, magnesium and phosphorous. This work did not appear to have been previously carried out in cows and appears to confirm that these samples will provide comparable results.

The trial of rumen-protected rice bran on a UK commercial dairy herd did not find evidence to support its use as a milk fever prevention strategy. It would

be recommended that further trials be carried out prior to the commercial production of such a product, especially given the priority of efficacy of a product stated by the questionnaire respondents.

5.3. Limitations of this work

The main limitations of the survey work (Chapter 2) were related to the methods of distribution and the formatting of the questions. As discussed in Chapter 3, the farmer survey was sent out to both collect the data on the attitudes of farmers but also acted as a means to identify farmers who may be interested in becoming involved in subsequent feed trials, as farmer recruitment was anticipated to be a difficult aspect of this thesis. This meant that it was distributed as widely as possible but without any monitoring of response rates. Similar issues occurred with the sample used for the vet survey. Both sample groups have the issue of potential bias, as it may be that farmers and vets with a particular issue or interest in hypocalcaemia felt more inclined to respond. The large number of free-text answers also made the interpretation of the data more difficult. The questionnaires may have been better being treated as pilot studies, with improved versions incorporating questions based on the free-text responses already received being designed and then distributed to a wider audience of potential respondents.

The retrospective study of factors associated with the recording of milk fever events in UK dairy herds (Chapter 3) could only identify associations and could not prove causality with any of the factors involved. The dataset was relatively large but may differ from the wider UK population of dairy cows. Whilst the sample size should help to overcome this limitation, it is possible that herds that record disease events such as milk fever may have better (or worse) management practices than other farms around the country.

One major drawback with the trial of rumen-protected rice bran on a commercial farm (Chapter 4) was that the farm did not have a high level of clinical milk fever cases in the first instance. The limitations of working with a

commercial farm meant that only certain aspects of the diet could be altered and the control ration had to remain as close to the original diet as possible. As the farm already appeared to be managing hypocalcaemia effectively, this meant that the control group was already likely to be good at preventing milk fever. The control ration could also be argued to be 'too good', especially after the addition of extra magnesium chloride fairly early on in the trial, due to the errors made in the manufacture of the control pellets. The second main problem related to the timings of samples collected. It was not possible to always sample cows immediately after calving, although this would have been the time point of greatest interest with relation to the calcaemic status of the animals. Due to the seven day spread of the samples closest to calving, as all samples were collected on the same weekday, it was decided that these were too variable to be included in the regression modelling and so the data collected from 'week 0' was not used in the final analysis.

5.4. Recommendations for future work

The use of a combination strategy was preferred by a large proportion of the farmers questioned. It may be that they feel that this is required in order to best control milk fever within their herds. As previously stated, farmers are mainly looking for a strategy that is effective at preventing milk fever but also value its ease of use. Zeolite was only used by one farmer in the survey population, although the reasons for this are not clear. However, there are issues with this strategy reported in the literature, such as its association with reduced serum magnesium and phosphorous concentrations (Thilsing-Hansen *et al*., 2003), along with its poor palatability and risk of reducing dry matter intakes (Grabherr *et al*., 2009). The rumen-protected rice bran diet used in this thesis did not significantly affect the serum magnesium concentrations of cows when compared to the control animals. This experimental ration also supplied high levels of phosphorous, as serum phosphorous concentrations were significantly higher in the cows consuming rumen-protected rice bran compared to the control ration. The potential for a combined approach,

supplementing a diet with a proportion of both zeolite and rumen-protected rice bran, may be worthy of investigation, as there may be potential to counteract the possible negative effects of each approach. This may include the high levels of phosphorous supplied in the rumen-protected rice bran, which may in itself be detrimental to calcaemic status and increase the risk of milk fever (Peterson *et al*., 2005; Goff, 2014b), helping to balance the phosphorous-binding effects reported with zeolite (Thilsing-Hansen *et al*., 2003; Grabherr *et al*., 2009). This binding of phosphorous with zeolite may also help to reduce the levels of phosphorous excretion, reducing the risk of detrimental environmental effects associated with high levels of phosphorous excretion in livestock (Knowlton *et al*., 2001).

Despite the fact that no clear benefits appear to have been seen in the subsequent lactation following a dry cow diet based on rumen-protected rice bran, that is not to say that the strategy does not warrant further investigation, as lowering calcium intake (or absorption) in the dry period has previously been shown to be an effective means of milk fever prevention (Belyea *et al*., 1974; Green *et al*., 1981; Moreira *et al*., 2009). There are a number of reasons, as previously discussed, that may have impacted on the effects of rumen-protected rice bran and so it cannot be discounted as a potential approach, despite the lack of evidence for its efficacy reported in this thesis. Further work would also be recommended with regard to the levels of phytate required for inclusion in a rumen-protected rice bran-based diet, as the farm trial here involved the use of a treatment feed with a phosphorous content that greatly exceeded recommendations (Goff, 2014b), and this may be associated with negative effects on calcaemic status, as discussed in Chapter 4.

5.5. Final conclusions

Rumen-protected rice bran may have benefits as a milk fever prevention strategy but these were not found in the farm trial conducted on a UK dairy farm. It is unclear if this was due to issues that occurred in the trial itself and so further research on this approach would be required.

The retrospective study carried out on UK herd health records confirmed that a number of conditions were associated with recorded milk fever events. Some of these, such as dystocia and exiting the herd early in the lactation, were already reported within the literature. However, the finding of twin pregnancies being associated with a 30% reduction in recorded milk fever events appears to be a finding that has not previously been reported in the literature.

The UK dairy farmers who took part in the survey were most concerned with finding a milk fever prevention strategy that works, with ease of use being their second highest priority. The highest priority cow health concerns of the farmers and veterinarians who completed the survey were generally similar, although vets placed significantly more importance on issues relating to metabolic disease compared to farmers. It appears that there are differing levels of awareness among farmers as to conditions that can be associated with subclinical hypocalcaemia, and there may be a role for greater veterinary involvement in education of farmers in this area. It is clear from the survey carried out that farmers obtain information on the prevention of milk fever from a variety of sources, including veterinarians and feed consultants, and this highlights the need for co-operation between different individuals and organisations towards reducing the incidence of this economically important metabolic disease.

BIBLIOGRAPHY

Agger, N. (2003). "Less calcium – fewer oral doses": a new approach to milk fever prevention. *Acta Veterinaria Scandanavica Supplements* **98**: 243.

Agriculture & Horticulture Development Board (2015). Age profile of GB dairy herd. [ONLINE] Available at: http://dairy.ahdb.org.uk/market-information/farming-data/cow-numbers/age-profile-of-gb-dairy-herd [Accessed 12 August 15]

Aiello, S.E., and Moses, M.A. (2015). The Merck Veterinary Manual. Available at: http://www.merckvetmanual.com [Accessed 13 September 2015]

Anderson, J.J.B. (1991). Nutritional biochemistry of calcium and phosphorous. *Journal of Nutritional Biochemistry* **2**: 300-307.

Andrews, A.H., Blowey, R.W., Boyd, H., and Eddy, R.G. (2004). Bovine Medicine, Diseases and Husbandry of Cattle. 2nd edition. Chapter 46: Major Metabolic Disorders.

Asl, A.N., Nazifi, S., Ghasrodashti, A.R., and Olyaee, A. (2011). Prevalence of subclinical ketosis in dairy cattle in south-western Iran and detection of cut-off point for NEFA and glucose concentrations for diagnosis of subclinical ketosis. *Preventive Veterinary Medicine* **100**: 38-43.

Baird, D.G. (1982). Primary ketosis in the high-producing dairy cow: clinical and subclinical disorders, treatment, prevention and outlook. *Journal of Dairy Science* **65**: 1-10.

Banfi, G., Salvagno, G.L., and Lippi, G. (2007). The role of ethylenediaminetetraacetic acid (EDTA) as *in vitro* anticoagulant for diganostic purposes. *Clinical Chemistry and Laboratory* Medicine **45**: 656-576.

Bansal, V.K., (1990). Serum inorganic phosphorous. In: Walker, H.K., Hall, W.D., and Hurst, J.W. (eds.) (1990). Clinical Methods: the History, Physical and Laboratory Examinations. 3rd edition.

Becker, J., Reist, M., and Steiner, A. (2014). Factors influencing the attitudes of cattle veterinarians, farmers and claw-trimmers towards the pain associated with the treatment of sole ulcers and the sensitivity to pain of dairy cows. *The Veterinary Journal* **200**: 38-43.

Bell, M.J., and Roberts, D.J. (2007). The impact of uterine infection on a dairy cow's performance. *Theriogenology* **68**: 1074-1079.

Belyea, R.L., Coppock, C.E., and Lake, G.B. (1974). Effects of a low calcium diet on feed intake, milk production, and response to blood calcium challenge in lactating Holstein cows. *Journal of Dairy Science* **59**: 1068-1077.

Bendixen, P.H., Vilson, B., Ekesbo, I., and Astrand, D.B. (1987). Disease frequencies in dairy cows in Sweden III: parturient paresis. *Preventive Veterinary Medicine* **5**: 87-97.

Berry, D.P., Lee, J.M., Macdonald, K.A., Stafford, K., Matthews, L., and Roche, J.R. (2007). Associations between body condition score, body weight, somatic cell count, and clinical mastitis in seasonally calving dairy cattle. *Journal of Dairy Science* **90**: 637-648.

Bertics, S.J., Grummer, R.R., Cadorniga-Valino, C., and Stoddard, E.E. (1992). Effect of prepartum dry matter intake on liver triglyceride concentration and early lactation. *Journal of Dairy Science* **75**: 1914-1922.

Bethard, G., Verbeck, R., and Smith, J.F. (1998). Controlling milk fever and hypocalcaemia in dairy cattle: use of dietary cation-anion difference (DCAD) in formulating dry cow rations. Technical Report 31. Agricultural Experiment Station, Cooperative Extension Service, College of Agriculture and Home Economics, New Mexico State University, 1998. Pages 1-5.

Bertoni, G., Trevisi, E., Han, X., and Bionaz, M. (2008). Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. *Journal of Dairy Science* **91**: 3300-3310.

Bigner, D.R., Goff, J.P., Faust, M.A., Burton, J.L., Tyler, H.D., and Horst, R.L. (1996). Acidosis effects on insulin response during glucose tolerance tests in Jersey cows. *Journal of Dairy Science* **79**: 2192-2188.

Bigras-Poulin, M., and Tremblay, A. (1998). An epidemiological study of calcium metabolism in non-paretic post-parturient Holstein cows. *Preventive Veterinary Medicine* **35**: 195-207.

Björkman, M.P., Sorva, A.J., and Tilvis, R.S. (2009). Calculated serum calcium is an insufficient surrogate for measured ionised calcium. *Archives of Gerontology and Geriatrics* **49**: 348-350.

Bland, J.M., and Altman, D.G. (1986). Statistical methods for assessing agreement between two methods of clinical measurement. *The Lancet*, February 1986, **8**: 307-310.

Block, E. (1994). Manipulation of dietary cation-anion difference on nutritionally related production diseases, productivity and metabolic responses in dairy cows. *Journal of Dairy Science* **77**: 1437-1450.

Boersema, J.S.C., Noordhuizen, J.P.T.M., and Lievaart, J.J. (2013). Hazard perceptions of Dutch farmers and veterinarians related to dairy young stock rearing. *Journal of Dairy Science* **96**: 5027-6276.

Bowen, R.A.R., and Remaley, A.T. (2014). Interferences from blood collection tube components on clinical chemistry assays. *Biochemia Medica* **24**: 31-44.

Bradley, A.J. (2002). Bovine mastitis: an evolving disease. *The Veterinary* Journal **164**: 116-128.

Bremmer, D.R., Trower, S.L., Bertics, S.J., Besong, S.A., Bernabucci, U., and Grummer, R.R. (2000). Etiology of fatty liver in dairy cattle: effects of nutritional and hormonal status on hepatic microsomal triglyceride transfer protein. *Journal of Dairy Science* **83**: 2239-2251.

Brozos, C., Mavrogianni, V.S., and Fthenakis, G.C. (2011). Treatment and control of peri-parturient metabolic diseases: pregnancy toxaemia, hypocalcaemia, hypomagnesaemia. *Veterinary Clinics of North America: Food Animal Practice* **27**: 105-113.

Bruijnis, M., Hogeveen, H.A.N., Garforth, C.B.C., and Stassen, E.D. (2013). Dairy farmers' attitudes and intentions towards improving dairy cow foot health. *Livestock Science* **155**: 103-113.

Burke, C.R., Meier, S., McDougall, S., Compton, C., Mitchell, M., and Roche, J.R. (2010). Relationships between endometritis and metabolic state during the transition period in pasture-grazed dairy cows. *Journal of Dairy Science* **93**: 5363-5373.

Busher, J.T. (1990). Serum albumin and globulin. In: Walker, H.K., Hall, W.D., and Hurst, J.W. (eds.) (1990). Clinical Methods: The History, Physical and Laboratory Examinations. 3rd edition. Butterworths, Boston.

Calavas, D., Faye, B., Bugnard, F., Ducrot, C., and Raymond, F. (1996). Analysis of associations among diseases in French dairy cows in two consecutive lactations. *Preventive Veterinary Medicine* **27**: 43-55.

Campbell, N.A., Reece, J.B., Urry, L.A., Cain, M.L., Wasserman, S.A., Minorsky, P.V., and Jackson, R.B. (2008). Biology. Pearson Education Inc., San Francisco, California, USA. 8th edition.

Cameron, R.E.B., Dyk, P.B., Herdt, T.H., Kaneene, J.B., Miller, R., Bucholtz, H.F., Liesman, J.S., VandeHaar, M.J., and Emery, S. (1998). Dry cow diet, management, and energy balance as risk factors for displaced abomasum in high producing dairy herds. *Journal of Dairy Science* **81**: 132-139.

Caraviello, D.Z., Weigel, K.A., Fricke, P.M., Wiltbank, M.C., Florent, M.J., Cook, N.B., Nordlund, K.V., Zwald, N.R., and Rawson, C.L. (2006). Survey of management practices on reproductive performance of dairy cattle on large US commercial farms. *Journal of Dairy Science* **89**: 4723-4735.

Chamberlin, W.G., Middleton, J.R., Spain, J.N., Johnson, G.C., Ellersieck, M.R., and Pithau, P. (2013). Subclinical hypocalcaemia, plasma biochemical parameters, lipid metabolism, postpartum disease, and fertility in postparturient dairy cows. *Journal of Dairy Science* **96**: 7001-7013.

Chan, P.S., West, J.W., and Bernard, J.K. (2006). Effect of prepartum dietary calcium on intake and serum and urinary mineral concentrations of cows. *Journal of Dairy Science* **89**: 704-713.

Cheong, S.H., Nydam, D.V., Galvao, K.N., Crosier, B.M., and Gilbert, R.O. (2011). Cow-level and herd-level risk factors for subclinical endometritis in lactating Holstein cows. *Journal of Dairy Science* **94**: 762-770.

Chorfi, Y., Lanevschi-Pietersma, A., Girard, V., and Tremblay, A. (2004). Evaluation of variation in serum globulin concentrations in dairy cattle. *Veterinary Clinical Pathology* **33**: 122-127.

Clark, W.D., Wohlt, J.E., Gilbreath, R.L., and Zajac, P. (1986). Phytate phosphorous intake and disappearance in the gastrointestinal tract of high producing dairy cows. *Journal of Dairy Science* **69**: 3151-3155.

Contreras, L.L., Ryan, C.M., and Overton, T.R. (2004). Effects of dry cow grouping strategy and prepartum body condition score on performance and health of transition dairy cows. *Journal of Dairy Science* **87**: 517-523.

Cook, T.E., Cilley, W.A., Savltsky, A.C., and Wlers, B.H. (1982). Zeolite A hydrolysis and degradation. *Environmental Science and Technology* **16**: 344-350.

Cook, J.D., Strauss, K.A., Caplan, Y.H., LoDico, C.P., and Bush, D.M. (2007). Urine pH: the effects of time and temperature after collection. *Journal of Analytical Toxicology* **31**: 486-496.

Coppock, C.E., Everett, R.W., Natzke, R.P., and Ainslie, H.R. (1974). Effect of dry period length on Holstein milk production and selected disorders at parturition. *Journal of Dairy Science* **57**: 712-718.

Cozzi, G., Ravarotto, L., Gotardo, F., Stefani, A.L., Contiero, B., Moro, L., Brscic, M., and Dalvit, P. (2011). Short communication: reference values for blood parameters in Holstein dairy cows: effects of parity, stage of lactation, and season of production. *Journal of Dairy Science* **94**: 3895-3901.

Cray, C., Rodriguez, M., Zaias, J., and Altman, N.H. (2009). Effects of storage temperature and time on clinical biochemical parameters from rat serum. *Journal of the American Association for Laboratory Animal Science* **48**: 202-204.

Curtis, C.R., Erb, H.N., Sniffen, C.J., Smith, R.D., Powers, P.A., Smith, M.C., White, M.E., Hillman, R.B., and Pearson, E.J. (1983). Association of parturient hypocalcaemia with eight periparturient disorders in Holstein cows. *Journal of the American Veterinary Medical Association* **183**: 559-561.

Curtis, C.R., Erb, H.N., Sniffen, C.J., and Smith, R.D. (1984). Epidemiology of parturient paresis: predisposing factors with emphasis on dry cow feeding and management. *Journal of Dairy Science* **67**: 817.

Curtis, C.R., Erb, H.N., Sniffen, C.J., Smith, R.D., and Kronfield, D.S. (1985). Path analysis of dry period nutrition, postpartum metabolic and reproductive disorders, and mastitis in Holstein cows. *Journal of Dairy Science* **68**: 2347-2360.

Daniel, R.C.W. (1983). Motility of the rumen and abomasum during hypocalcaemia. *Canadian Journal of Comparative Medicine* **47**: 276.

Derks, M., van Werven, T., Hogeveen, H., and Kremer, W.D.J. (2013). Veterinary herd health management programs on dairy farms in the Netherlands: use, execution and relations to farmer characteristics. *Journal of Dairy Science* **96**: 1623-1637.

DeGaris, P.J., and Lean, I.J. (2008). Milk fever in dairy cows: a review of pathophysiology and control principles. *The Veterinary Journal* **176**: 58-69.

DeGroot, M.A., Block, E., and French, P.D. (2010). Effect of prepartum anionic supplementation on periparturient feed intake, health and milk production. *Journal of Dairy Science* **93**: 5268-5279.

Department for Environment, Food and Rural Affairs (2008). The Cattle Book 2008: Descriptive Statistics of Cattle Numbers in Great Britain on 1st June

2008. [ONLINE] Available at: https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/69220/pb13572-cattlebook-2008-090804.pdf. [Accessed 20 August 15].

Doreau, M., Ollier, A., and Michaelet-Doreau, B. (2001). An atypical case of ruminal fermentations leading to ketosis in early lactating cows. *Revue de Médecine Vétérinaire* **4**: 301-306.

Douglas, G.N., Overton, T.R., Bateman, H.G., and Drackley, J.K. (2004). Peripartal metabolism and production of Holstein cows fed diets supplemented with fat during the dry period. *Journal of Dairy Science* 87: 4210-4220.

Duffield, T.F., Kelton, D.F., Leslie, K.E., Lissemore, K.D., and Lumsden, J.H. (1997). Use of test day milk fat and milk protein to detect subclinical ketosis in dairy cattle in Ontario. *Canadian Veterinary Journal* **38**: 713-718.

Duffield, T.F., Sandals, D., Leslie, K.E., Lissemore, K.D., McBride, B.W., Lumsden, J.H., Dick, P., and Bagg, R. (1998). Efficacy of monensin for the prevention of subclinical ketosis in lactating dairy cows. *Journal of Dairy* Science **81**: 2866-2873.

Duffield, T.F., Lissemore, K.D., McBride, B.W., Leslie, K.E. (2009). Impact of hyperketonemia in early lactation dairy cows on health and production. *Journal of Dairy Science* **92**: 571-580.

Dugdale, A.H.A., Grove-White, D., Curtis, G.C., Harris, P.A., and Argo, C.M. (2012). Body condition scoring as a predictor of body fat in horses and ponies. *The Veterinary Journal* **194**: 173-178.

Ekelund, A., Sporndly, R., and Holtenius, K. (2006). Influence of low phosphorous intake during early lactation on apparent digestibility of phosphorous and bone metabolism in dairy cows. *Livestock Science* **99**: 227-236

El-Samad, H., Goff, J.P., and Khammash, M. (2002). Calcium homeostasis and parturient hypocalcaemia: an integral feedback perspective. *Journal of Theoretical Biology* **214**: 17-29.

Escobosa, A., Coppock, C.E., Rowe, L.D., Jenkins, W.L., and Gates, C.E. (1984). Effects of dietary sodium bicarbonate and calcium chloride on physiological responses of lactating dairy cows in hot weather. *Journal of Dairy Science* **67**: 574-584.

Espetvedt, M.N., Wolff, C., Rintakoski, S., Lind, A., and Østeras, O. (2012). Completeness of metabolic disease recordings in Nordic national databases for dairy cows. *Preventive Veterinary Medicine* **105**: 25-37.

European Food Safety Authority (EFSA) (2004). Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed on the request from the Commission on the use of synthetic sodium aluminium silicate (zeolite) for the reduction of risk of milk fever in dairy cows. *The EFSA Journal* **160**: 1-11.

Farm Crisis Network (2009). Stress and loss: a report on the impact of bovine TB on farming families. [ONLINE] Available at: http://www.tbfreeengland.co.uk/assets/4200. [Accessed 20 August 15].

Fischer, A., Luginbuhl, T., Delattre, L., Delouard, J.M., and Faverdin, P. (2015). Rear shape in 3 dimensions summarised by principal component analysis is a good predictor of body condition score in Holstein dairy cows. *Journal of Dairy* Science **98**: 4465-4476.

Fleischer, P., Metzner, M., Beyerbach, M., Hoedemaker, M., and Klee, W. (2001). The relationship between milk yield and the incidence of some diseases in dairy cows. *Journal of Dairy Science* **84**: 2025-2035.

Fourichon, C., Seegers, H., and Malher, X. (1999). Effect of disease on reproduction in the dairy cow: a meta-analysis. *Theriogenology* **53**: 1729-1759.

Funnell, B.J., and Hilton, W.M. (2016). Management and prevention of dystocia. *Veterinary Clinics of North America: Food Animal Practice* **32**: 511-522.

Gant, R.G., Sanchez, W., and Kincaid, R.L. (1998). Effect of anionic salts on selenium metabolism in nonlactating pregnant dairy cows. *Journal of Dairy Science* **81**: 1637-1642.

Gartner, J.W., Murphy, G.M., and Hoey, W.A. (1982). Effects of induced subclinical phosphorous deficiency on feed intake and growth of beef heifers. *Journal of Agricultural Science* **98**: 23-29.

Geishauser, T., Leslie, K., Tenhag, J., and Bashiri, A. (2000). Evaluation of eight cow-side ketone tests in milk for detection of subclinical ketosis in dairy cows. *Journal of Dairy Science* **83**: 296-299

Gilbert, R.O., Shin, S.T., Guard, C.L., Erb, H.N., and Frajblat, M. (2005). Prevalence of endometritis and its effects on reproductive performance of dairy cows. *Theriogenology* **64**: 1879-1888.

Giuliodori, M.J., Delavaud, C., Chilliard, Y., Becú-Villalobos, D., Lacau-Mengido, I., and de la Sota, R.L. (2011). High NEFA concentrations around parturition are associated with delayed ovulations in grazing dairy cows. *Livestock Science* **141**: 123-128

Goff, J. P. (2014a). Diet-induced pseudohypoparathyroidism: a calcium and milk fever risk factor. *Journal of Dairy Science* **97**: 1520-1528.

Goff, J. P. (2014b). Calcium and magnesium disorders. *Veterinary Clinics: Food Animal Practice* **30**: 359-381.

Goff, J.P. (2000). Pathophysiology of calcium and phosphorous disorders. *Veterinary Clinics of North America: Food Animal Practice* **16**: 319-337.

Goff, J.P. (2004). Macromineral disorders of the transition cow. *Veterinary Clinics of North America: Food Animal Practice* **20**: 471-494.

Goff, J.P. (2008). The monitoring, prevention and treatment of milk fever and subclinical hypocalcaemia in dairy cows. *The Veterinary Journal* **176**: 50-57.

Goff, J.P., and Horst, R.L. (1993). Oral administration of calcium salts for treatment of hypocalcaemia in cattle. *Journal of Dairy Science* **76**: 101-108.

Goff, J.P., and Horst, R.L. (1994). Calcium salts for treating hypocalcaemia: carrier effects, acid-base balance, and oral versus rectal administration. *Journal of Dairy Science* **77**: 1451-1456.

Goff, J.P., Horst, R.L., Jardon, P.W., Borelli, C., and Wedam, J. (1996). Field trials of an oral calcium propionate paste as an aid to prevent milk fever in periparturient dairy cows. *Journal of Dairy Science* **79**: 378-383.

Goff, J.P., and Horst, R.L. (1997). Effects of the addition of potassium or sodium, but not calcium, to prepartum rations on milk fever in dairy cows. *Journal of Dairy Science* **80**: 176-186.

Goff, J.P., and Horst, R.L. (1998). Factors to concentrate on to prevent periparturient disease in the dairy cow with special emphasis on milk fever. *Ruminant Nutrition Workshop: Implications of Health Problems in the Early Lactation Dairy Cow*: 15-34.

Goff, J.P., Reinhardt, T.A., Beitz, D.B., and Horst, R.L. (1995). Breed affects tissue vitamin D receptor concentration in periparturient dairy cows: a milk fever risk factor? *Journal of Dairy Science* **78** (supplement 1): 184 (abstract).

Grabherr, H., Spolders, M., Furll, M., and Flachowsky, G. (2009). Effect of several doses of zeolite A on feed intake, energy metabolism and on mineral metabolism in dairy cows around calving. *Journal of Animal Physiology and Animal Nutrition* **93**: 221-236.

Green, H.B., Horst, R.L., Beitz, D.C., and Littledike, E.T. (1981). Vitamin D metabolites in plasma of cows fed a prepartum low-calcium diet for prevention of parturient hypocalcaemia. *Journal of Dairy Science* **64**: 217-226.

Grimm, P. (2010). Social Desirability Bias. Wiley International Encyclopaedia of Marketing.

Grohn, Y.T., Erb, H.N., McCulloch, C.E., and Saloniemi, H.S. (1989). Epidemiology of metabolic disorders in dairy cattle: associations among host characteristics, disease and production. *Journal of Dairy Science* **72**: 1876-1885.

Grohn, Y.T., Erb, H.N., McCulloch, C.E., and Saloniemi, H.S. (1990). Epidemiology of reproductive disorders in dairy cattle: associations among host characteristics, disease and production. *Preventive Veterinary Medicine* **8**: 25-39.

Grohn, Y.T., Eicker, S.W., and Hertl, J.A. (1995). The association between previous 305-day milk yield and disease in New York State dairy cows. Journal of Dairy Science 78: 1693-1702.

Grummer, R.R. (1995). Impact of changes in organic nutrient metabolism on feeding the transition dairy cows. *Journal of Animal Science* **73**: 2820-2833.

Grunberg, W., Donkin, S.S., and Constable, P.D. (2011). Periparturient effects of feeding a low dietary cation-anion difference diet on acid-base, calcium and phosphorous homeostasis and on intravenous glucose tolerance test in high-producing dairy cows. *Journal of Dairy Science* **94**: 727-745.

Grunberg, W., Constable, P., Schroder, U., Staufenbiel, R., Morin, D., and Rohn, M. (2005). Phosphorous homeostasis in dairy cows with abomasal displacement or abomasal vovulus. *Journal of Veterinary Internal Medicine* **19**: 894-898

Guthold, M., Liu, W., Stephens, B., Lord, S.T., Hantgan, R.R., Erie, D.A., Taylor, R.M., and Superfine, R. (2004). Visualisation and mechanical manipulations of

individual fibrin fibres suggest that fibre cross-section has fractal dimension 1.3. *Biophysical Journal* **87**: 4226-4236.

Hadley, G.L., Wolf, C.A. and Harsh, S.B. (2006). Dairy cattle culling patterns, explanations and implications. *Journal of Dairy Science* **86**: 2286-2296.

Hammon, D.S., Evjen, I.M., Dhiman, T.R., Goff, J.P., and Walters, J.L. (2006). Neutrophil function and energy status in Holstein cows with uterine health disorders. *Veterinary Immunology and Immunopathology* **113**: 21-29.

Han, I.K., and Kim, I.H. (2005). Risk factors for retained placenta and the effect of retained placenta on the occurrence of postpartum diseases and subsequent reproductive performance in dairy cows. *Journal of Veterinary Science* **6**: 53-59.

Hansen, S.S., Nørgaard, P., Pederson, C., Jørgensen, R.J., Mellau, L.S.B., and Enemark, J.D. (2003). The effect of subclinical hypocalcaemia induced by Na_2EDTA on the feed intake and chewing activity of dairy cows. *Veterinary Research Communications* **27**: 193-205.

Hansen, S.S., Ersbøll, A.K., Blom, J.Y., and Jørgensen, R.J. (2007). Preventive strategies and risk factors for milk fever in Danish dairy herds: a questionnaire survey. *Preventive Veterinary Medicine* **80**: 271-286.

Harrington, M.E., Flynn, A., and Cashman, K.D. (2001). Effects of dietary fibre extracts on calcium absorption in the rat. *Food Chemistry* **73**: 263-239.

Harrington, J.M., Young, D.J., Essader, A.S., Sumner, S.J., and Levine, K.E. (2014). Analysis of human serum and whole blood for mineral content by ICP-MS and ICP-OES: development of a mineralomics method. *Biological Trace Element Research* **160**: 132-142.

Hayes, E.P.B., Christley, R.M., and Dobson, H. (2012). Effects of periparturient events on subsequent culling and fertility in eight UK dairy herds. *Veterinary Record* **170**: 540.

Hayirli, A., Grummer, R.R., Nordheim, E.V., and Crump, P.M. (2002). Animal and dietary factors affecting feed intake during the prefresh transition period in Holsteins. *Journal of Dairy Science* **85**: 3430-3443.

Hayton, A., Husband, J., and Vecqueray, R. (2012). Nutritional Management of Herd Health. In: Green, M., Bradley, A., Breen, J., Green, L., Hayton, A., Higgins, H., Hudson, C., Huxley, J., and Statham, J. (eds.). *Dairy Herd Health*. CAB International, pp 268-269.

Heuer, C., Schukken, Y.H., and Dobbelaar, P. (1999). Postpartum body condition score and results from the first test day milk as predictors of disease, fertility, yield and culling in commercial dairy herds. *Journal of Dairy Science* **82**: 295-304.

Horst, R.L., Goff, J.P., Reinhardt, A., and Buxton, D.R. (1997). Strategies for preventing milk fever in dairy cattle. *Journal of Dairy Science* **80**: 1269-1280.

Horst, R.L., and Jorgensen, N.A. (1982). Elevated plasma cortisol during induced and spontaneous hypocalcaemia in ruminants. *Journal of Dairy Science* **65**: 2332-2337.

Hosmer, D.W., and Lemeshow, S. (1980). A goodness-of-fit test for the multiple logistic regression model. *Communications in Statistics* **A10**: 1043-1069.

Houe, H., Østergaard, S., Thilsing-Hansen, T., Jorgensen, R.J., Larsen, T., Sørensen, J.T., Agger, J.F., and Blom, J.Y. (2001). Milk fever and subclinical hypocalcaemia – an evaluation of parameters on incidence risk, diagnosis, risk factors and biological effects as input for a decision support system for disease control. *Acta Veterinaria Scandinavica* **42**: 1-29.

Hu, W., Kung Jr, L., Murphy, M.R. (2007). Relationships between dry matter intake and acid-base status of lactating dairy cows as manipulated by dietary cation-anion difference. *Animal Feed Science and Technology* **136**: 216-225.

Huber, T.L. (1976). Physiological effects of acidosis on feedlot cattle. *Journal of Animal Science* **43**: 902-909.

Hudson, C.D., Bradley, A.J., Breen, J.E., and Green, M.J. (2012). Associations between udder health and reproductive performance in United Kingdom dairy cows. *Journal of Dairy Science* **95**: 3683-3697.

Husband, J. (2005). Strategies for the control of milk fever. *In Practice* **27**: 88-92.

Hutjens, M.F., and Aalseth, E.P. (2005). Caring for Transition Cows. W.D. Hoards & Sons Company, 3rd edition.

Huzzey, J.M., Nydam, D.V., Ospina, P.A., Overton, T.R. (2012) Predicting transition cow health and performance – use of blood and fecal biomarkers for herd-level evaluation and diagnostics. In: *'Proceedings of North-East Dairy Producers Association Conference. Cornell University'*. Available at: https://dspace.library.cornell.edu/bitstream/1813/36568/1/3.3.Overton.Manuscript.pdf [accessed 15th July 2015]

Jackson, R.A., Wills, J.R., Kendall, N.R., Green, M.J., Murray, R.D., and Dobson, H. (2011). Energy metabolites in pre- and postpartum dairy cattle as predictors of reproductive disorders. *Veterinary Record* **168**: 562.

Jahnen, A., Heynck, H., Gertz, B., Claßen, A., and Hesse, A. (1992). Dietary fibre: the effectiveness of a high bran intake in reducing renal calcium excretion. *Urological Research* **20**: 3-6.

Jansen, J., Steuten, C.D.M., Renes, R.J., Aarts, N., and Lam, T.J.G.M. (2010). Debunking the myth of the hard-to-reach farmer: effective communication on udder health. *Journal of Dairy Science* **93**: 1296-1306.

Jonsson, N.N., and Daniel, R.C.W. (1997). Effects of hypocalcaemia to the blood flow to the ovaries of the sheep. *Journal of Veterinary Medicine Series A* **44**: 281-287.

Jordan, E.R., and Fourdraine, R.H. (1993). Characterisation of the management practices of the top milk producing herds in the country. *Journal of Dairy Science* **76**: 3247-3256.

Jorritsma, R., Wensing, T., and Kruip, T.A.M. (2003). Metabolic changes in early lactation and impaired reproductive performance in dairy cows. *Veterinary Research* **34**: 11-26.

Kaid, L.L., and Holtz-Bacha, C. (2006). *The SAGE Handbook of Political Advertising.* Thousand Oaks, California, USA. Print.

Kamgarpour, R., Daniel, R.C., Fenwick, D.C., McGuigan, K., and Murphy, G. (1999). Postpartum subclinical hypocalcaemia and effects on ovarian function and uterine involution in a dairy herd. *Veterinary Journal* **158**: 59-67.

Karatzia, M.A., Pourliotis, K., Katsoulos, P.D., and Karatzias, H. (2011). Effects of in-feed inclusion of clinoptilolite on blood serum concentrations of aluminium and inorganic phosphorous and on ruminal pH and volatile fatty acid concentrations in dairy cows. *Biological Trace Element Research* **142**: 159-166.

Katsoulos, P.D., Roubies, N., Panousis, N., Arsenos, G., Christaki, E., and Karatzias, H. (2005). Effects of long-term dietary supplementation with clinoptilolite on incidence of parturient paresis and serum concentrations of total calcium, phosphate, magnesium, potassium, and sodium in dairy cows. *American Journal of Veterinary Research* **66**: 2081-2085.

Kelly, J.M. (1977). Changes in serum beta-hydroxybutyrate concentrations in dairy cows kept under commercial farm conditions. *Veterinary Record* **101**: 499-502.

Kelton, D. F., Lissemore, K.D., and Martin, R.E. (1998). Recommendations for recording and calculating the incidence of selected clinical diseases of dairy cattle. *Journal of Dairy Science* **81**: 2502-2509.

Kessel, S., Stroehl, M., Meyer, H.H., Hiss, S., Sauerwein, H., Schwarz, F.J., and Bruckmaier, R.M. (2008). Individual variability in physiological adaptation to metabolic stress during early lactation in dairy cows kept under equal conditions. *Journal of Animal Science* **86**: 2903-2912.

Kim, I-H., and Suh, G.H. (2003). Effect of the amount of body condition loss from the dry to near calving periods on the subsequent body condition change, occurrence of post-partum diseases, metabolic parameters and reproductive performance in Holstein dairy cows. *Theriogenology* **60**: 1445-1456.

Kimura, K., Goff, J.P., Kehrli Jr, M.E., and Reinhardt, T.A. (2002). Decreased PMN function as a cause of retained placenta in dairy cattle. *Journal of Dairy Science* **85**: 544-550.

Kimura, K., Reinhardt, T.A, and Goff, J.P. (2006). Parturition and hypocalcaemia blunts calcium signals in immune cells of dairy cattle. *Journal of Dairy Science* **89**: 2588-2595.

Klerx, H.J., and Smolders, E.A.A. (1997). Herd and cow random variation in models of interrelationships between metabolic and reproductive disorders in high yielding multiparous Holstein dairy cattle in the Netherlands. *Livestock Production Science* **52**: 21-29.

Knowlton, K.F., Herbein, J.H., Meister-Weisbarth, M.A., and Wark, W.A. (2001). Nitrogen and phosphorous partitioning in lactating Holstein cows fed different sources of dietary protein and phosphorous. *Journal of Dairy Science* **84**: 1210-1217.

Knowlton, K.F., and Herbein, J.H. (2002). Phosphorous partitioning during early lactation in dairy cows fed diets varying in phosphorous content. *Journal of Dairy Science* **85**: 1227-1236.

Koeck, A., Miglior, F., Kelton, D.F., and Schenkel, F.S. (2012). Health recording in Canadian Holsteins: data and genetic parameters. *Journal of Dairy Science* **95**: 4099-4108.

Kossaibati, M.A., and Esslemont, R.J. (1997). The costs of production diseases in dairy herds in England. *Veterinary Journal* **154**: 41-51.

Kronqvist, C., Emanuelson, U., Sporndly, R., and Holtenius, K. (2011). Effects of prepartum dietary calcium level on calcium and magnesium metabolism in periparturient dairy cows. *Journal of Dairy Science* **94**: 1365-1373.

Ladenson, J.H., Tsai, L.M.B., Michael, J.M., Kessler, G., and Joist, J.H. (1974). Serum versus heparinised plasma for eighteen common chemistry tests. American Journal of Clinical Pathology 62: 545-552. Cited in: Miles, R.R., Roberts, R.F., Putnam, A.R., and Roberts, W.L. (2004). Comparisons of serum and heparinised plasma samples for measurement of chemistry analytes. *Clinical Chemistry* **50**: 1704-1705.

Larsen, Møller, G., and Bellio, R. (2001). Evaluation of clinical and clinical chemical parameters in periparturient cows. *Journal of Dairy Science* **84**: 1749-1758.

Lean, I.J., DeGaris, P.J., McNeil, D.M., and Block, E. (2006). Hypocalcaemia in dairy cows: meta-analysis and dietary cation anion difference theory revisited. *Journal of Dairy Science* **89**: 669-684.

Lean, I.J., van Saun, R., and DeGaris, P. (2013). Mineral and antioxidant management of transition dairy cows. *Veterinary Clinics of North America: Food Animal Practice* **29**: 367-386.

Lester, E., and Varghese, Z. (1977). Differences in the calcium concentration of serum and plasma initially and after storage. *Annals of Clinical Biochemistry* **14**: 39-44.

Liboreiro, D.N., Machado, K.S., Silva, P.R.B., Maturana, M.M., Nishimura, T.K., Brandao, A.P., Endres, M.I., and Chebel, R.C. (2015). Characterisation of peripartum rumination and activity of cows diagnosed with metabolic and uterine disease. *Journal of Dairy Science* **98**: 6812-6827

Littledike, E.T., and Goff, J. (1987). Interactions of calcium, phosphorous, magnesium and vitamin D that influence their status in domestic meat animals. *Journal of Animal Science* **65**: 1727-1743.

Lovelock, J.E., and Porterfield, B.M. (1952). Blood clotting: the function of electrolytes and of calcium. *Biochemical Journal* **50**: 415-420.

Lyon, M.E., Bremner, D., Laha, T., Malik, S., Henderson, P.J., and Kenny, M.A. (1995). Specific heparin preparations interfere with the simultaneous

measurement of ionised magnesium and ionised calcium. *Clinical Biochemistry* **28**: 79-84.

March, M.D., Haskell, M.J., Chagunda, M.G.G., Langford, F.M., and Roberts, D.J. (2014). Current trends in British dairy management regimens. *Journal of Dairy Science* **97**: 7985-7994.

Markusfeld, O. (1987). Periparturient traits in seven high dairy herds: incidence rates, association with parity, and interrelationships among traits. *Journal of Dairy Science* **70**: 158-166.

Marquardt, J.P., Horst, R.L., and Jorgensen, N.A. (1977). Effect of parity on dry matter intake at parturition in dairy cattle. *Journal of Dairy Science* **60**: 929-934.

Martinez, N., Sinedino, L.D.P., Bisinotto, R.S., Ribeiro, E.S., Gomes, G.C., Lima, F.S., Greco, L.F., Risco, C.A., Galvao, K.N., Taylor-Rodriguez, D., Driver, J.P., Thatcher, W.W., and Santos, J.E.P. (2014). Effect of induced subclinical hypocalcaemia on physiological responses and neutrophil function in dairy cows. *Journal of Dairy Science* **97**: 874-887.

Martin-Tereso, J. (2010) Rumen-protected rice bran to induce the adaption of calcium metabolism in dairy cows. PhD Thesis, Wageningen University, the Netherlands.

Martin-Tereso, J., Gonzalez, A., Van Laar, H., Burbano, C., Pedrosa, M.M., Mulder, K., den Hartog, L.A., and Verstegen, M.W.A. (2009). *In situ* ruminal degradation of phytic acid in formaldehyde-treated rice bran. *Animal Feed Science and Technology* **152**: 286-297.

Martín-Tereso, J., Derks, M., van Laar, H., Mulder, K., den Hartog, L.A., and Verstegen, M.W.A. (2010). Urinary calcium excretion in non-lactating dairy cows in relation to intake of fat-coated rice bran. *Journal of Animal Physiology and Animal Nutrition* **94**: 129-136.

Martín-Tereso, J., Distefano, C., van Laar, H., Mulder, K., den Hartog, L.A., and Verstegen, M.W.A. (2011). Effect of feeding rumen-protected rice bran on mineral status of non-lactating dairy heifers. *Journal of Animal Physiology and Animal Nutrition* **95**: 73-80.

Martin-Tereso, J., ter Wijlen, H., van Laar, H., and Verstegen, M.W.A. (2014). Peripartal calcium homeostasis of multiparous dairy cows fed rumen-protected rice bran or a lowered dietary cation/anion balance diet before calving. *Journal of Animal Physiology and Animal Nutrition* **98**: 775-784.

Massey, C.D., Wang, C., Donovan, G.A., and Beede, D.K. (1993). Hypocalcaemia at parturition as a risk factor for left displaced abomasum in dairy cows. *Journal of the American Veterinary Medical Association* **203**: 852.

Matos, V., van Melle, G., Boulat, O., Markert, M., Bachmann, C., and Guignard, J.P. (1997). Urinary phosphate/creatinine, calcium/creatinine, and magnesium/creatinine ratios in a health paediatric population. *The Journal of Pediatrics* **131**: 252-257.

Mee, J.F., Berry, D.P., and Cromie, A.R. (2011). Risk factors for calving assistance and dystocia in pasture-based Holstein-Friesian heifers and cows in Ireland. *The Veterinary Journal* **187**: 189-194.

Melendez, P., Donovan, G.A., Risco, C.A., Littell, R., and Goff, J.P. (2003). Effect of calcium-energy supplements on calving-related disorders, fertility and milk yield during the transition period in cows fed anionic diets. *Theriogenology* **60**: 843-854.

Met Office (2015). 2013 weather summaries. Available at: http://www.metoffice.gov.uk/climate/uk/summaries/2013/july [Accessed 20th September 2015]

Miles, R.R., Roberts, R.F., Putnam, A.R., and Roberts, W.L. (2004). Comparisons of serum and heparinised plasma samples for measurement of chemistry analytes. *Clinical Chemistry* **50**: 1704-1705.

Mohri, M., Shakeri, H., and Zadeh, S.L. (2007). Effects of common anticoagulants (heparin, citrate and EDTA) on routine plasma biochemistry of cattle. *Comparative Clinical Pathology* **16**: 207-209.

Moodie, E.W. (1975). Mineral metabolism. In: Blunt, M.H. (1975) (ed.). The Blood of Sheep: Composition and Function.

Moore, S.J., VandeHaar, M.J., Sharma, B.K., Pilbeam, T.E., Beede, D.K., Bucholtz, J.S., Liesman, J.S., Horst, R.L., and Goff, J.P. (1997). Varying dietary cation-anion difference (DCAD) for dairy cattle before calving. *Journal of Dairy Science (supplement 1)*: 170.

Moore, S.J., VandeHaar, M.J., Sharma, B.K., Pilbeam, T.E., Beede, D.K., Bucholtz, H.F., Liesman, J.S., Horst, R.L., and Goff, J.P. (2000). Effects of altering dietary cation-anion difference on calcium and energy metabolism in peripartum cows. *Journal of Dairy Science* **83**: 2095-2104.

Moreira, V.R., Zeringue, L.K., Williams, C.C., Leonardi, C., and McCormick, M.E. (2009). Influences of calcium and phosphorous feeding on markers of bone metabolism in transition cows. *Journal of Dairy Science* **92**: 5189-5198.

Morrill, K.M., Marston, S.P., Whitehouse, N.L., Van Amburgh, M.E., Schwab, C.G., Haines, D.M., and Erickson, P.S. (2010). Anionic salts in the prepartum diet and addition of sodium bicarbonate to colostrum replacer, and their effects on immunoglobulin G absorption in the neonate. *Journal of Dairy Science* **93**: 2067-2075.

Moyes, K.M., Larsen, T., and Ingvartsen, K.L. (2013). Generation of an index for physiological imbalance and its use as a predictor of primary disease in dairy cows during early lactation. *Journal of Dairy Science* **96**: 2161-2170

Mulligan, F.J., O'Grady, L., Rice, D.A., and Doherty, M.L. (2006a). A herd health approach to dairy cow nutrition and production diseases of the transition cow. *Animal Reproduction Science* **96**: 331-353.

Mulligan, F.J., O'Grady, L., Rice, D.A., and Doherty, M.L. (2006b). Production diseases of the transition cow: milk fever and subclinical hypocalcaemia. *Irish Veterinary Journal* **59**: 697-702.

Mulligan, F.J., and Doherty, M.L. (2008). Production diseases of the transition cow. *The Veterinary Journal* **176**: 3-9.

National Research Council (1989). Nutrient Requirements of Dairy Cattle, 6th edition. National Academic Press, Washington, D.C.

Nobalo Silva, J., and Noakes, D.E. (1984). The effect of experimentally induced hypocalcaemia on uterine activity at parturition in the ewe. *Theriogenology* **21**: 607-623.

Nyamushamba, G.B., Chikwanda, D., Matondi, G.H.M., Marandure, T., Mamutse, J., Tavirimirwa, B., Banana, N.Y.D., and Dhiwayo, M. (2014). The effect of non-genetic factors on milk yield and composition of Red Dane cattle in Zimbabwe. *Livestock Research for Rural Development* **26**

Oba, M., Oakley, A.E., and Tremblay, G.F. (2011). Dietary Ca concentration to minimise the risk of hypocalcaemia in dairy cows is affected by the dietary cation-anion difference. *Animal Feed Science and Technology* **164**: 147-153.

Oetzel, G.R., Fettman, M.J., Hamar, D.W., and Olson, J.D. (1991). Screening of anionic salts for palatability, effects on acid-base status, and urinary calcium excretion in dairy cows. *Journal of Dairy Science* **74**: 965-971.

Oetzel, G.R. (1996). Improving reproductive performance in dairy cattle via milk fever prevention. *The Bovine Proceedings* **28**: 52-59.

Oetzel, G.R. (2013). Oral calcium supplementation in peripartum dairy cows. *Veterinary Clinics: Food Animal Practice* **29**: 447-455.

Ogunsanmi, A.O., and Taiwo, V.O. (2004). Comparative studies on erythrocyte calcium, potassium, haemoglobin concentration, osmotic resistance and sedimentation rates in grey duiker (*Sylvicapra grimmia*), sheep and goats experimentally infected with *Trypanosoma congolese*. *Veterinarski Arhiv* **24**: 201-216

Olff, M., Koeter, M.W.J., van Haaften, E.H., Kersten, P.H., and Gersons, B.P.R. (2005). Impact of a foot and mouth disease crisis on post-traumatic stress symptoms in farmers. *British Journal of Psychiatry* **186**: 165-166.

Overton, T.R. (2004). Optimizing the Transition Cow Management System on Commercial Dairy Farms. In: Florida Ruminant Nutrition Symposium 2004.

Overton, T.R., and Burhans, W.S. (2013). Protein metabolism of the transition cow. In: Proc Cornell Nutr Conf. Syracuse, NY. p. 91–100.

Parker-Gaddis, K.L., Cole, J.B., Clay, J.S., and Maltecca, C. (2012). Incidence validation and relationship analysis of producer-recorded health event data from on-farm computer systems in the United States. *Journal of Dairy Science* **95**: *5422-5435*.

Pallesen, A., Pallesen, F., Jørgensen, R.J., and Thilsing, T. (2008). Effect of pre-calving zeolite, magnesium and phosphorous supplementation on periparturient serum mineral concentrations. *The Veterinary Journal* **175**: 234-239.

Palmquist, D.L., Jenkins, T.C., and Joyner, A.E. (1986). Effect of dietary fat and calcium source on insoluble soap formation in the rumen. *Journal of Dairy Science* **69**: 1020-1025.

Parker-Gaddis, K.L., Cole, J.B., Clay, J.S., and Maltecca, C. (2012). Incidence validation and relationship analysis of producer-recorded health event data from on-farm computer systems in the United States. *Journal of Dairy Science* **95**: 5422-5435.

Pehrson, B., Svensson, C., and Jonsson, M. (1998). A comparative study of the effectiveness of calcium propionate and calcium chloride for the prevention of parturient paresis in dairy cows. *Journal of Dairy Science* **81**: 2011-2016.

Pennings, J.M.E., Irwin, S.H., and Good, D.L. (2002). Surveying farmers: a case study. *Review of Agricultural Economics* **24**: 266-277.

Peterson, A.B., Orth, A.W., Goff, J.P., and Beede, D.K. (2005). Periparturient responses of multiparous Holstein cows fed different dietary phosphorous concentrations prepartum. *Journal of Dairy Science* **88**: 3582-3594.

Pezeshki, A., Mehrzad, J., Ghorbani, G.R., Rahmani, H.R., Collier, R.J., and Burvenich, C. (2007). Effects of short dry periods on performance and metabolic status in Holstein dairy cows. *Journal of Dairy Science* **90**: 5531-5541.

Pfitzenmeyer, P., Martin, I., d'Athis, P., Grumbach, Y., Delmestre, M.C., Blondé-Cynober, F., Derycke, B., Brondel, L., and the Club Francophone de Gériatre et Nutrition (2007). A new formula for correction of total calcium level into ionised serum calcium values in very elderly hospitalised patients. *Archives of Gerontology and Geriatrics* **45**: 151-157.

Piccinini, R., Binda, E., Belotti, M., Casirani, G., and Zecconi, A. (2004). The evaluation of non-specific immune status of heifers in field conditions during the periparturient period. *Veterinary Research* **35**: 539-550.

Pond, W.G., Church, D.C., Pond, K.R., and Schoknecht, P.A. (2005). Basic Animal Nutrition and Feeding. John Wiley & Sons, Inc. 5th edition.

Pothmann, H., Nechanitzky, K., Sturmlechner, F., and Drillich, M. (2014). Consultancy to dairy farmers relating to animal health and herd management on small- and medium-sized farms. *Journal of Dairy Science* **97**: 851-860.

Potter, T.J., Guitian, J., Fishwick, J., Gordon, P.J., and Sheldon, I.M. (2010). Risk factors for clinical endometritis in postpartum dairy cattle. *Theriogenology* **74**: 127-134.

Price, C.P., Newall, R.G., and Boyd, J.C. (2005). Use of protein:creatinine ratio measurements on random urine samples for prediction of significant proteinuria: a systematic review. *Clinical Chemistry* **51**: 1577-1586.

R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/)

Raboisson, D., Mounié, M., and Maigné, E. (2014). Disease, reproductive performance, and changes in milk production associated with subclinical ketosis in dairy cows: a meta-analysis and review. *Journal of Dairy Science* **97**: 7547-7563

Rajala, P.J., and Gröhn, Y.T. (1998). Effects of dystocia, retained placenta and metritis on milk yield in dairy cows. *Journal of Dairy Science* **81**: 3172-3181.

Rajala-Schultz, P.J., Gröhn, Y.T., and McCulloch, C.E. (1999). Effects of milk fever, ketosis and lameness on milk yield in dairy cows. *Journal of Dairy Science* **82**: 288-294.

Rasbash, J., Charlton, C., Browne, W.J., Healy, M., and Cameron, B. (2016). *MLwiN version 2.36*. Centre for Multilevel Modelling, University of Bristol.

Reinhardt, T.A., Horst, R.L., and Goff, J.P. (1988). Calcium, phosphorous and magnesium homeostasis in ruminants. *The Veterinary Clinics of North America: Food Animal Practice* **4**: 331-349.

Reinhardt, T.A.. Lippolis, J.D., McCluskey, B.J., Goff, J.P., and Horst, R.L. (2011). Prevalence of subclinical hypocalcaemia in dairy herds. *The Veterinary Journal* **188**: 122-124.

Ribeiro, E.S., Lima, F.S., Greco, L.F., Bisinotto, R.S., Monteiro, A.P.A., Favoreto, M., Ayres, H., Marsola, R.S., Martinez, N., Thatcher, W.W., and Santos, J.E.P., (2013). Prevalence of periparturient diseases and impacts on fertility of seasonally calving grazing dairy cows supplemented with concentrates. *Journal of Dairy Science* **96**: 5682-5697.

Ringsrud, K.M., and Linne, J.J. (1995). Urinalysis and Body Fluids: A Colour Text and Atlas. Mosby, 1995, page 45.

Risco, C.A., Reynolds, J.P., and Hird, D. (1984). Uterine prolapse and hypocalcaemia in dairy cows. *Journal of the American Veterinary Medical Association* **185**: 1517-1519.

Risco, C.A., Drost, M., Thatcher, W.W., Savio, J., and Thatcher, M.J. (1994). Effects of calving-related disorders on prostaglandin, calcium, ovarian activity and uterine involution in postpartum dairy cows. *Theriogenology* **42**: 183-203.

Roberts, T., Chapinal, N., LeBlanc, S.J., Kelton, D.F., Dubuc, J., and Duffield, T.F. (2012). Metabolic parameters in transition cows as indicators for early-lactation culling risk. *Journal of Dairy Science* **95**: 3057-3063.

Roche, J.R., Dalley, D., Moate, P., Grainger, C., Hannah, M., O'Mara, F., and Rath, M. (2002). Variations in dietary cation-anion difference and the acid-base balance of dairy cows on a pasture-based diet in south-eastern Australia. *Grass Forage Science* **55**: 26-36.

Roche, J.R., Kolver, E.S., and Kay, J.K. (2005a). Influence of pre-calving feed allowance on periparturial metabolic and hormonal responses and milk production in grazing dairy cows. *Journal of Dairy Science* **88**: 677-689.

Roche, J.R., Petch, S., and Kay, J.K. (2005b). Manipulating the dietary cation-anion difference via drenching to early-lactation dairy cows grazing pasture. *Journal of Dairy Science* **88**: 264-276.

Roche, J.R., and Berry, D.P. (2006). Periparturient climatic, animal and management factors influencing the incidence of milk fever in grazing systems. *Journal of Dairy Science* **89**: 2775-2783.

Roche, J.R., Friggens, N.C., Kay, J.K., Fisher, M.W., Stafford, K.J., and Berry, D.P. (2009). Invited review: Body condition score and its association with dairy cow productivity, health, and welfare. *Journal of Dairy Science* **92**: 5769-5801.

Russell, K.E., and Roussel, A.J. (2007). Evaluation of the ruminant serum chemistry profile. *Veterinary Clinics Food Animal Practice* **23**: 403-426.

Schenck, P.A., and Chew, D.J. (2008). Calcium: total or ionised? *Veterinary Clinics of North America: Small Animal Practice* **38**: 497-502.

Schenck, P.A., Chew, D.J., Nagode, L.A., and Rosol, T.J. (2011). Disorders of calcium: hypercalcaemia and hypocalcaemia. In: DiBartola, S.P., editor. Fluid, Electrolyte, and Acid-Base Disorders in Small Animal Practice. W.B. Saunders, 5rd edition. P. 120-194.

Schnier, C., Hielm, S., and Saloniemi, H.S. (2004). Comparison of the disease incidences of Finnish Ayrshire and Finnish Black and White dairy cows. *Preventive Veterinary Medicine* **62**: 285-298.

Schuenemann, G.M., Nieto, I., Bas, S., Galvao, K.N., and Workman, J. (2011). Assessment of calving progress and reference times for obstetric intervention during dystocia in Holstein dairy cows. *Journal of Dairy Science* **94**: 5494-5501.

Schonewille, J.Th., Klooster, ATh van't, and Beynen, A.C. (1994). The addition of extra calcium to a chloride-rich ration does not affect the absolute amount of calcium absorbed by non-pregnant dry cows. *Journal of Animal Physiology A: Animal Nutrition* **72**: 272-280.

Selle, P.H., Cowieson, A.J., and Ravindran, V. (2009). Consequences of calcium interactions with phytate and phytase for poultry and pigs. *Livestock Science* **124**: 126-141.

Shaver, R.D. (1997). Nutritional risk factors for the aetiology of left displaced abomasum in dairy cows: a review. *Journal of Dairy Science* **80**: 2449-2453.

Sheldon, I.M., and Dobson, H. (2004). Postpartum uterine health in cattle. *Animal Reproduction Science* **82-83**: 295-305.

Sheldon, I.M., Williams, E.J., Miller, A.N.A., Nash, D.M., and Herath, S. (2008). Uterine diseases in cattle after parturition. *Veterinary Journal* **176**: 115-121.

Siener, R., Heynck, H., and Hesse, A. (2001). Calcium-binding capacities of different brans under simulated gastrointestinal pH conditions: in vitro study with $_{45}$Ca. *Journal of Agricultural and Food Chemistry* **49**: 4397-4401.

Sørensen, J.T., Østergaard, S., Houe, H., and Hindhede, J. (2002). Expert opinions of strategies for milk fever control. *Preventative Veterinary Medicine* **55**: 69-78.

Steele, W. (1984). Lipid supplementation of dairy cow diets. *Journal of Dairy Science* **67**: 1716-1724.

Sterner, K.E., Grymer, J., Bartlett, P.C., and Miekstyn, M.J. (2008). Factors influencing the survival of dairy cows after correction of left displaced abomasum. *Journal of the American Veterinary Medical Association* **15**: 1521-1529.

Stokol, T., and Nydam, D.V. (2005). Effect of anticoagulant and storage conditions on bovine nonesterified fatty acid and β-hydroxybutyrate concentrations in blood. *Journal of Dairy Science* **88**: 3139-3144.

STROBE (2007). Strengthening the reporting of observational studies in epidemiology. STROBE checklist for cross-sectional studies. http://www.strobe-statement.org/fileadmin/Strobe/uploads/checklists/STROBE_checklist_v4_case-control.pdf [accessed 12th October 2016]

Sweeney, B.M., Martens, E.M., Felippe, M.J., and Overton, T.R. (2014). Impacts and evaluation of subclinical hypocalcaemia in dairy cattle. In: '*Proceedings of 2014 Cornell Nutrition Conference for Feed Manufacturers*'. Available at: https://ecommons.cornell.edu/bitstream/handle/1813/37973/CNC2014_8_Sweeney.pdf [accessed 21st December 2016]

Thilsing, T., Jørgensen, R.J., and Poulsen, H.D. (2006). In vitro binding capacity of zeolite A to calcium, phosphorous and magnesium in rumen fluid as influenced by changes in pH. *Journal of Veterinary Medicine A* **53**: 57-64.

Thilsing-Hansen, T., and Jørgensen, R.J. (2001). Prevention of parturient paresis and subclinical hypocalcaemia in dairy cows by zeolite A administration in the dry period. *Journal of Dairy Science* **84**: 691-693.

Thilsing-Hansen, T., Jørgensen, R.J., Enemark, J.M.D, and Larsen, T. (2002a). The effect of zeolite A supplementation in the dry period on periparturient calcium, phosphorous and magnesium homeostasis. *Journal of Dairy Science* **85**: 1855-1862.

Thilsing-Hansen, T., Jørgensen, R.J., and Østergaard, S. (2002b). Milk fever control principles: a review. *Acta Veterinaria Scandinavica* **43**: 1-19.

Thilsing-Hansen, T., Jørgensen, R.J., Enemark, J.M.D, Zelvyte, R., and Sederevicius, A. (2003). The effect of zeolite A supplementation in the dry period on blood mineral status around calving. *Acta Veterinaria Scandinavica* **97**: 87-95.

Thilsing, T., Larsen, T., Jørgensen, R.J., and Houe, H. (2007). The effect of dietary calcium and phosphorus supplementation in zeolite A treated dry cows on periparturient calcium and phosphorus homeostasis. *Journal of Veterinary Medicine A* **54**: 82-91.

Thomsen, P.T., Kjeldsen, A.M., Sorensen, J.T., and Houe, H. (2004). Mortality (including euthanasia) among Danish dairy cows (1990-2001). *Preventive Veterinary Medicine* **62**: 19-33.

Thomsen, P.T., Anneberg, I., and Herskin, M.S. (2012). Differences in attitudes of farmers and veterinarians towards pain in dairy cows. *The Veterinary Journal* **194**: 94-97.

Tourangeau, R., and Yan, T. (2007). Sensitive questions in surveys. *Psychological Bulletin* **133**: 859-883.

Trevisi, E., Bertoni, G., Lombardelli, R., and Minuti, A. (2013). Relation of inflammation and liver function with the plasma cortisol response to adrenocorticotropin in early lactating dairy cows. *Journal of Dairy Science* **96**: 5712-5722.

Tuck, M.K., Chan, D.W., Chia, D., Godwin, A.K., Grizzle, W.E., Krueger, K.E., Rom, W., Sapda M., Sorbara, L., Stass, S., Wang, W., and Brenner, D.E. (2009). Standard operating procedures for serum and plasma collection: early detection research network consensus statement. *Journal of Proteome Research* **8**: 113-117.

Tucker, W.B., Harrison, G.A., and Hemken, R.W. (1988). Influence of dietary cation-anion balance on milk, blood, urine and rumen fluid in lactating dairy cattle. *Journal of Dairy Science* **71**: 346-354.

Vagnoni, D.B., and Oetzel, G.R. (1998). Effects of dietary cation-anion difference on the acid-base status of dry cows. *Journal of Dairy Science* **81**: 1643–1652

VandeHaar, M.J., Yousif, G., Sharma, B.K., Herdt, T.H., Emery, R.S., Allen, M.S., and Liesman, J.S. (1999). Effect of energy and protein density of prepartum diets on fat and protein metabolism of dairy cattle in the periparturient period. *Journal of Dairy Science* **82**: 1282-1295.

Vanholder, T., Papen, J., Bemers, R., Vertenten, G., and Berge, A.C.B. (2015). Risk factors for subclinical and clinical ketosis and association with production parameters in dairy cows in the Netherlands. *Journal of Dairy Science* **98**: 880-888.

Van Knegsel, A.T.M., Remmelink, G.J., Jorjong, S., Fievez, V., and Kemp, B. (2014). Effect of dry period length and dietary energy source on energy balance, milk yield, and milk composition of dairy cows. *Journal of Dairy Science* **97**: 1499-1512.

Vasseur, E., Gibbons, J., Rushen, J., and de Passillé, A.M. (2013). Development and implementation of a training program to ensure high repeatability of body condition scoring of dairy cows. *Journal of Dairy Science* **96**: 4725-4737.

Vaught, J.B. (2006). Blood collection, shipment, processing and storage. *Cancer Epidemiology, Biomarkers & Prevention* **15**: 1582-1584.

Veerkamp, R.F., Beerda, B., and van der Lende, T. (2003). Effects of genetic selection for milk yield on energy balance, levels of hormones, and metabolites in lactating cattle, and possible links to reduced fertility. *Livestock Production Science* **83**: 257-275.

Verdaris, J.N., and Evans, J.L. (1976). Diet calcium and pH versus mineral balance in Holstein cows 84 days pre- to 2 days postpartum. *Journal of Dairy Science* **59**: 1271-1272.

Wathes, D.C., Fenwick, M., Cheng, Z. Bourne, Z., Llwellyn, S., Morris, D.G., Kenny, D., Murphy, J., and Fitzpatrick, R. (2007). Influence of negative energy balance on cyclicity and fertility in the high producing dairy cow. *Theriogenology* **S232-2241**.

Weaver, C.M., Heaney, R.P., Martin, B.R., and Fitzsimmons, M.L. (1991). Human calcium absorption from whole-wheat products. *The Journal of Nutrition* **121**: 1769-1775.

Wentink, G.H., and van den Ingh, T.S. (1992). Oral administration of calcium chloride-containing products: testing for deleterious side-effects. *The Veterinary Quarterly* **14**: 76-79.

Wenz, J.R., and Giebel, S.K. (2012). Retrospective evaluation of health event data recording on 50 dairies using Dairy Comp 305. *Journal of Dairy Science* **95**: 4699-4706.

Whitaker, D.A. (2004). Metabolic Profiles. In: Andrews, A.H., Blowey, R.W., Boyd, H., and Eddy, R.G. (eds.) (2004). *Bovine Medicine: Diseases and Husbandry of Cattle*. 2nd edition. Blackwell Science, Oxford.

Whitaker, D.A., Macrae, and Burrough, E. (2004b). Disposal and disease rates in British dairy herds between April 1998 and March 2002. *The Veterinary Record* **155**: 43-47.

Whiteford, L.C., and Sheldon, I.M. (2005). Association between clinical hypocalcaemia and postpartum endometritis. *Veterinary Record* **157**: 202-204.

Wiggers, K.D., Nelson, D.K., and Jacobson, N.L. (1975). Prevention of parturient paresis by a low calcium diet prepartum: a field study. *Journal of Dairy Science* **58**: 430-431.

Wilde, D. (2006). Influence of macro and micro minerals in the peri-parturient period on fertility in dairy cattle. *Animal Reproduction Science* **96**: 240-249.

Wilson, G.F. (2003). A new practical method to prevent hypocalcaemia in dairy cows at pasture. *Acta Veterinaria Scandinavica* Suppl. **98**.

Wu, Z., Satter, L.D., and Sojo, R. (2000). Milk production, reproductive performance, and faecal excretion of phosphorous by dairy cows fed three amounts of phosphorous. *Journal of Dairy Science* **83**: 1028-1041.

APPENDIX A: FARMER AND VETERINARIAN SURVEY QUESTIONS

Milk Fever and Calcium Management Survey

The management of milk fever and subclinical hypocalcaemia

This survey has been designed to gather information on milk fever prevention strategies currently used on commercial UK dairy farms.

The data collected will form part of a masters research project at the University of Nottingham. All responses will be anonymised and individuals will not be identified.

The survey should take no more than ten minutes to complete. Your time to complete this survey is very much appreciated.

At the end of the questionnaire you will be given the option to enter a prize draw to win a £50 M&S voucher (closing date: 30th June 2012).

If you have any questions, please do not hesitate to get in touch.

Eleanor Garnett,
MRes student
svxeg@nottingham.ac.uk

Supervised by Dr Nigel Kendall,
Lecturer in Nutrition
nigel.kendall@nottingham.ac.uk
0115 9516447

School of Veterinary Medicine & Science
The University of Nottingham
Sutton Bonington Campus
Sutton Bonington
Leicestershire
LE12 5RD

Your herd

***1. What is the typical size of your milking herd?**

Number of adult dairy cows

***2. Which of the following options best describes your calving pattern?**

- ◯ All Year Round
- ◯ Seasonal - Spring
- ◯ Seasonal - Summer
- ◯ Seasonal - Autumn
- ◯ Seasonal - Winter
- ◯ Other (please specify)

Milk Fever and Calcium Management Survey

***3. What breed(s) of cows are found within your herd (with approximate % of each)?**

4. In which county is your herd?

***5. What do you consider to be the top three health concerns/issues within your herd?**

Your dry cows

***6. Within your herd, what is the target dry period length?**

Number of days

7. Dry period length comments (if applicable).

***8. Where are your dry cows kept?**

	Early dry period	Late dry period
All year housed	☐	☐
All year at grass	☐	☐
Winter indoors, summer at grass	☐	☐

Other (please specify)

Your herd and milk fever

Milk fever is a metabolic disease that most commonly occurs around calving, particularly in high-yielding dairy cows, and is caused by low blood calcium levels.

Clinical signs of milk fever include low body temperature, cold extremities, poor appetite, unsteadiness and an inability to stand.

***9. Do you consider milk fever to be a problem within your herd?**

- ◯ No
- ◯ Yes - slight problem
- ◯ Yes - moderate problem
- ◯ Yes - major problem

Milk Fever and Calcium Management Survey

***10. How many cases of clinical milk fever did you experience within your herd in 2011?**

Number of cases

***11. How did the incidence level of milk fever within your herd in 2011 compare to the previous year?**

- ◯ More cases than in 2010
- ◯ Fewer cases than in 2010
- ◯ Approximately the same / no change

What factors may have influenced any change in incidence during this time?

Milk fever prevention strategies

***12. What is your main strategy for the prevention of milk fever?**

- ◯ No prevention strategy used
- ◯ Calcium bolus(es) around calving
- ◯ Calcium gel around calving
- ◯ Calcium drench around calving
- ◯ Calcium injection (SC/IV) around calving
- ◯ Feeding a low calcium diet during dry period
- ◯ Feeding full DCAB diet during dry period
- ◯ Feeding partial DCAB diet during dry period
- ◯ Feeding zeolite during dry period
- ◯ Other or Combination of strategies (please describe)

Milk fever prevention strategies

Milk Fever and Calcium Management Survey

***13. What is your main reason for not using a milk fever prevention strategy?**

- ◯ Time consuming
- ◯ Expensive
- ◯ Lack of information about options available
- ◯ No success with prevention options tried previously
- ◯ Do not consider milk fever to be a problem
- ◯ Other (please specify)

***14. Have you previously used any milk fever prevention strategies?**

- ☐ I have never tried using a milk fever prevention strategy
- ☐ Calcium bolus(es) around calving
- ☐ Calcium gel around calving
- ☐ Calcium drench around calving
- ☐ Calcium injection (SC/IV) around calving
- ☐ Feeding a low calcium diet during dry period
- ☐ Feeding full DCAB diet during dry period
- ☐ Feeding partial DCAB diet during dry period
- ☐ Feeding zeolite during dry period
- ☐ Other (please specify)

15. Why did you stop using this milk fever prevention strategy? (if applicable)

Milk fever prevention strategies

Milk Fever and Calcium Management Survey

***16. What factors influence your choice of milk fever prevention strategy? (select all that apply)**

- [ ] Ease of use
- [ ] Expected efficacy
- [ ] Cost
- [ ] Veterinary recommendation
- [ ] Manufacturer's recommendation / marketing
- [ ] Consultant / feed advisor
- [ ] Other (please specify)

***17. What is the MAIN reason for your choice of milk fever prevention strategy?**

- ◯ Ease of use
- ◯ Expected efficacy
- ◯ Cost
- ◯ Veterinary recommendation
- ◯ Manufacturer's recommendation / marketing
- ◯ Consultant / feed advisor
- ◯ Other (please specify)

***18. What other milk fever prevention strategies have you previously tried?**

- [ ] Calcium bolus(es) around calving
- [ ] Calcium gel around calving
- [ ] Calcium drench around calving
- [ ] Calcium injection (SC/IV) around calving
- [ ] Feeding a low calcium diet during dry period
- [ ] Feeding full DCAB diet during dry period
- [ ] Feeding partial DCAB diet during dry period
- [ ] Feeding zeolite during dry period
- [ ] None
- [ ] Other(s) (please specify)

Milk Fever and Calcium Management Survey

19. Why did you stop using this prevention strategy? (if applicable)

Herd health/production issues

***20. What do you consider to be the main health/production problems associated with clinical cases of milk fever?**

21. Is the risk of milk fever preventing you from keeping your dry cows at grass?

- ◯ Yes
- ◯ No

Comments (if applicable)

Subclinical hypocalcaemia

Subclinical hypocalcaemia is a reduction in calcium but is not severe enough to cause visible signs of milk fever.

***22. What health/production issues are you aware of that are influenced by subclinical hypocalcaemia? (please list)**

Contact details and future research

One strategy to prevent milk fever is through the feeding of calcium binders in the final weeks of the dry period. These bind to dietary calcium, preventing it from being absorbed within the cow. This enables the cow's hormone system to adapt to low calcium conditions, preparing it for the high calcium demands at calving. Further research into the feeding of calcium binders to prevent milk fever is due to be carried out as part of this masters programme at the University of Nottingham.

It may be useful for us to contact you regarding future milk fever/hypocalcaemia research trials. If this is something you may be interested in receiving further information about, please provide contact details.

Milk Fever and Calcium Management Survey

Two prize draws will take place, each to win a £50 M&S voucher. The winners will be drawn at random from all entrants on 30th April 2012 and 30th June 2012. If you wish to be entered into the prize draw(s), please enter your contact details into the box below.

If you would be interested to receive information regarding the results of the survey, please provide an e-mail address.

Contact details will only be used for the purpose submitted. All results will be anonymised.

***23. Please provide contact details if you answer yes to any of the following:**

	yes	no
To allow us to contact you for survey clarification/follow-up	○	○
To enter the prize draw	○	○
To receive further information regarding potential participation in research trials	○	○
To receive information regarding the results of the survey	○	○

Please enter contact details (e-mail/contact number/address).

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Milk fever and calcium management - a questionnaire for vets

The management of milk fever and subclinical hypocalcaemia

This survey has been designed to gather information from UK vets on attitudes to milk fever and its prevention on UK dairy farms.

The data collected will form part of a PhD research project at the University of Nottingham. All responses will be anonymised and individuals will not be identified.

The survey should take no more than 10 minutes to complete. Your time to complete this survey is very much appreciated.

If you have any questions, please do not hesitate to get in touch.

Eleanor Garnett,
PhD student
svxeg@nottingham.ac.uk

Supervised by Dr Nigel Kendall,
Lecturer in Nutrition
nigel.kendall@nottingham.ac.uk
0115 9516447

School of Veterinary Medicine & Science
The University of Nottingham
Sutton Bonington Campus
Sutton Bonington
Leicestershire
LE12 5RD

*** 1. What type of practice do you work in?**

- ◯ Farm only
- ◯ Mixed farm and equine
- ◯ Mixed farm and small animal
- ◯ Farm, equine and small animal
- ◯ Other (please specify)

*** 2. For how many years have you been in practice?**

Milk fever and calcium management - a questionnaire for vets

***3. Do you have any postgraduate qualifications?**

- [ ] Certificate in cattle health & production (CertCHP)
- [ ] Diploma in cattle health & production (DCHP)
- [ ] Dip. ECBHM
- [ ] DBR
- [ ] Other(s) (please list)

***4. Approximately what proportion of your working hours are spent working with dairy cows?**

- ◯ None
- ◯ 1-20%
- ◯ 21-40%
- ◯ 41-60%
- ◯ 61-80%
- ◯ 81-100%

Comments (if applicable)

Milk fever prevention strategies

***5. In your opinion, what are the three most important health/production issues within dairy herds?**

1.
2.
3.

***6. What do you believe are the top three herd health/production issues that dairy farmers are most concerned about?**

1.
2.
3.

Approaches to milk fever prevention will vary considerably between herds and may depend on a number of different factors. However, for the purposes of this survey we are interested in finding out which strategies you would personally recommend (or have recommended) in an appropriate farm situation.

Milk fever and calcium management - a questionnaire for vets

***7. Which of the following milk fever prevention strategies have you heard about? Which have you recommended? (please select all answers that apply)**

	Heard about	Have previously recommended	Would recommend	Would not recommend
Calcium bolus(es) around calving	☐	☐	☐	☐
Calcium gel around calving	☐	☐	☐	☐
Calcium drench around calving	☐	☐	☐	☐
Calcium injection (SC/IV) around calving	☐	☐	☐	☐
Feeding a low calcium diet during dry period	☐	☐	☐	☐
Feeding full DCAB diet during dry period	☐	☐	☐	☐
Feeding partial DCAB diet during dry period	☐	☐	☐	☐
Feeding zeolite during dry period	☐	☐	☐	☐

Other/comments

Approaches to milk fever prevention will vary considerably between herds and may depend on a number of different factors. However, for the purposes of this survey we are interested in finding out which strategies you would be most likely to personally recommend in the following basic scenarios.

***8. What milk fever prevention strategy would you be most likely to recommend for a spring-calving herd at grass?**

***9. What milk fever prevention strategy would you be most likely to recommend in a dairy herd housed all year round?**

Page 3

Milk fever and calcium management - a questionnaire for vets

***10. What diagnostic techniques do you use for identifying subclinical levels of hypocalcaemia?**

- [ ] None
- [ ] Total blood calcium
- [ ] Ionised calcium
- [ ] Urine pH
- [ ] Urine macrominerals
- [ ] Ration analysis
- [ ] Other (please specify)

11. How often (and on approximately what proportion of dairy farms) do you carry out diagnostic tests for clinical milk fever or subclinical hypocalcaemia? What tests do you use in what situation?

***12. In an ideal scenario, what would your gold standard approach to milk fever prevention be?**

Milk fever and calcium management - a questionnaire for vets

***13. Farmers often do not meet this gold standard approach. What do you believe the reasons for this to be?**

Farmer choice of milk fever prevention strategy

14. What sources do you believe that farmers use to obtain information regarding milk fever prevention?

- [ ] Vets
- [ ] Marketing information from product manufacturers
- [ ] Consultants / feed advisors
- [ ] Farming press
- [ ] Other farmers
- [ ] Other (please specify)

***15. Where do you think that farmers are MOST likely to seek information regarding milk fever prevention?**

- ◯ Vets
- ◯ Marketing information from product manufacturers
- ◯ Consultants / feed advisors
- ◯ Farming press
- ◯ Other farmers
- ◯ Other (please specify)

Milk fever and calcium management - a questionnaire for vets

***16. In your experience, what factors do you believe most strongly influence farmer choice of milk fever prevention? Please RANK the following answers in terms of importance to farmers.**

	1 - Most important	2	3	4	5	6 - Least important
Cost	○	○	○	○	○	○
Ease of use	○	○	○	○	○	○
Consultant / feed advisor	○	○	○	○	○	○
Manufacturer's recommendation / marketing	○	○	○	○	○	○
Veterinary recommendation	○	○	○	○	○	○
Expected efficacy	○	○	○	○	○	○

***17. How accurately do you believe that the majority of farmers record their incidence of milk fever?**

- ○ Over-reporting of number of clinical cases
- ○ Accurate reporting number of clinical cases
- ○ Under-reporting of number of clinical cases
- ○ Records are too incomplete to tell
- ○ Other (please specify)

***18. To what extent do you believe that farmers are aware of the potential detrimental effects of subclinical hypocalcaemia?**

Page 6

Milk fever and calcium management - a questionnaire for vets

19. Do you have any final comments to include on milk fever/subclinical hypocalcaemia and its prevention?

Contact details

Many thanks for completing the survey. If you would be interested to receive information regarding the results of the survey, please provide contact details below.

Contact details will only be used for the purpose submitted. All results will be anonymised.

20. Please provide contact details if you answer yes to any of the following:

	Yes	No
To allow us to contact you for survey clarification/follow-up	○	○
To receive information regarding the results of the survey	○	○

Please enter contact details (e-mail / contact number / address).

APPENDIX B: INTENDED FORMULATION OF RPRB AND CONTROL PELLETS

RUMEN-PROTECTED RICE BRAN DIET

Summary Report

Client:

Adviser:
Rachel Fowers
Nutreco RRC
Veerstraat 38
5831JN Boxmeer
the Netherlands

December 3, 2012

Calfix 2.1

Raw Material	kg	kg DM
D Rice Bran	721.80	648.90
Wheat 11.5%	85.00	73.10
Molasses - Beet	78.60	61.54
Dr. Mol. S.B.P.	50.00	45.40
Linseed	25.00	23.00
Soya Hipro	16.00	14.03
Urea	12.00	11.88
Salt	4.90	4.88
MgOx	4.20	4.20
Fat	2.50	2.48
Total	1000.00	889.41

Nutrient		Amount	kg DM
DMI	(kg)	8.89	1.00
Forage DM	(kg)	0.00	0.00
Dry Matter	(%)	88.94	88.94
M.E.	(MJ)	93.68	10.53
M/D	(MJ/kgDM)	10.53	10.53
CP	(%DM)	21.38	21.38
MPB	(g)	521.32	58.61
MPN	(g)	1282.29	144.17
MPE	(g)	811.82	91.28
Excess MPN	(g)	470.48	52.90
Rumen Stability		-70.36	-70.36
NDF	(%DM)	24.53	24.53
Oil	(%DM)	2.29	2.29
RF Starch	(g/kgDM)	208.26	208.26
RU Starch	(g/kgDM)	61.60	61.60
Sugar	(g/kgDM)	108.59	108.59
YieldME		3.38	3.38
YieldMP		9.49	9.49
Calcium	(g)	12.20	1.37
Phosphorus	(g)	148.34	16.68
Magnesium	(g)	88.01	9.90
Sodium	(g)	29.85	3.36
Iron	(mg)	1693.34	190.39
Copper	(mg)	111.55	12.54
Manganese	(mg)	912.10	102.55
Cobalt	(mg)	2.20	0.25
Zinc	(mg)	671.08	75.45
Iodine	(mg)	1.53	0.17
Selenium	(mg)	0.38	0.04

These feeding recommendations are intended only a guide and depend on the accuracy of the information suppied.

APPENDIX B: INTENDED FORMULATION OF RPRB AND CONTROL PELLETS

CONTROL DIET

Summary Report

Client:

Adviser:
Rachel Fowers
Nutreco RRC
Veerstraat 38
5831JN Boxmeer
the Netherlands

December 3, 2012

Trial Blend

Raw Material	kg	kg DM
Wheat 10%	200.00	173.60
DDG Wheat	200.00	182.00
Soya Hipro	195.00	170.82
Dr. Mol. S.B.P.	100.00	91.80
Soya Hulls (Toasted)	100.00	90.00
Molasses - Cane	60.00	44.46
Rapeseed (00) Ext	50.00	44.00
MgCl2.6H2O	50.00	49.95
M.A.P.	25.00	24.98
Prairie Meal	20.00	18.00
Total	1000.00	889.61

Nutrient		Amount	kg DM
DMI	(kg)	889.61	1.00
Forage DM	(kg)	0.00	0.00
Dry Matter	(%)	88.96	88.96
M.E.	(MJ)	10923.26	12.28
M/D	(MJ/kgDM)	12.28	12.28
CP	(%DM)	25.64	25.64
MPB	(g)	72495.84	81.49
MPN	(g)	156511.52	175.93
MPE	(g)	120730.19	135.71
Excess MPN	(g)	35781.33	40.22
Rumen Stability		-83.20	-83.20
NDF	(%DM)	21.75	21.75
Oil	(%DM)	3.29	3.29
RF Starch	(g/kgDM)	140.08	140.08
RU Starch	(g/kgDM)	20.06	20.06
Sugar	(g/kgDM)	99.07	99.07
YieldME		1943.99	1943.99
YieldMP		2125.99	2125.99
Calcium	(g)	3411.90	3.84
Phosphorus	(g)	10815.20	12.16
Magnesium	(g)	8206.50	9.22
Sodium	(g)	1389.10	1.56
Iron	(mg)	376058.36	422.73
Copper	(mg)	10545.62	11.85
Manganese	(mg)	29366.28	33.01
Cobalt	(mg)	213.89	0.24
Zinc	(mg)	38285.01	43.04
Iodine	(mg)	339.06	0.38
Selenium	(mg)	8.58	0.01

These feeding recommendations are intended only a guide and depend on the accuracy of the information suppied.