**FINAL REPORT**

# Exploring the use of Optium Xceed™ as a cow-side test to effectively diagnose Subclinical Ketosis in early lactating dairy cows

This report summarises the concordance and correlation among concentrations of β-hydroxybutyrate (BHB) in whole blood, plasma and milk using two cow-side kits and laboratory methods in postpartum lactating dairy cows. This project formed the basis of an Animal Science Honours project, generously funded by Western Dairy, 2014.

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# Executive Summary

The objective of this project was to compare the cow-side commercial kits available with laboratory methods for the diagnosis of subclinical ketosis in lactating dairy cows. Cross-validation of the laboratory assays and commercial diagnostic cow-side kits will assist to avoid the misdiagnosis and misclassification of cattle and to reduce associated intervention costs, improving dairy cow performance, health and welfare.

- Subclinical ketosis (SCK) is a condition that is defined with an elevation of circulating ketone bodies (eg. β-hydroxybutyrate (BOHB), acetoacetate) without the presence of the clinical signs of ketosis, with the exception of a decline in milk production. The reported prevalence or incidence of SCK varies between studies and herds, which may be due to the use of different diagnostic tests and different thresholds for each test.
- The blood concentration of BOHB has been used as "gold standard" test for the diagnosis of SCK. There are also several cow-side tests using blood, milk and urine samples for SCK diagnosis. However, the detection limits of the milk ketone body tests do not describe their epidemiologic test characteristics (Duffield, 2000) for the diagnosis of SCK in dairy herds. The sensitivity and specificity of these tests vary between kits.

- This study was completed in 2 stages to:

    - Conduct a pilot study to explore the differences between plasma concentrations of BOHB using laboratory methods (colorimetric and kinetic methods), whole blood (using the cow-side kit Optium Xceed™) and milk concentrations of BOHB using the cow side test PortaBHB.

    - Establish a relationship within and between laboratory methods and cow-side kits and develop a matrix to be able to compare the BOHB data using these methods

    - Conduct a field study to measure the concentration BOHB of cows commercial dairy herds during the early lactation (DIM: 7 ± 3) using the Optium Xceed™ meter, and compare the herd production performance in cows with high and low BOHB. An initial estimate of prevalence of subclinical ketosis was used to determine sample size for the future studies.

**Pilot study (Stage 1):**

- Blood and milk samples were collected from 40 multiparous lactating cows during early lactation for Stage 1. Plasma samples were analysed for BOHB using colorimetric and kinetic laboratory methods. Whole blood and milk samples were analysed for BOHB using Optium Xceed™ cow-side kits and PortaBHB™ ketolac strips, respectively.

- Whole blood concentrations of BOHB measured using the Optium Xceed™ meter and plasma concentrations of BOHB measured using the kinetic and colorimetric laboratory methods were highly correlated (r=0.953 and r = 0.972 respectively). The correlation between milk concentration of BOHB using PortaBHB™ ketolac strips and plasma BOHB using kinetic assays was 0.71.

- Concordance (the level of agreement between two variables) between plasma and whole BOHB concentrations measured using kinetic laboratory method and the cow-side kit Optium Xceed™, respectively, was high (ccc=0.92). Concordance between the colorimetric methods and Optium Xceed™ or the kinetic assay was poor (ccc=0.82 and ccc=0.76 respectively).

- The cut-point of 1.4mM that is suggested to identify cows with subclinical ketosis (SCK) is based on the kinetic laboratory method. The high concordance between the kinetic assay and Optium Xceed indicates that the cut-point of 1.4mM can be considered as reliable threshold for the diagnosis of SCK in lactating dairy cows.

**Field study:**

- Blood samples were collected from 174 multiparous lactating cows during early lactation from 5 farms in south west of Western Australia for Stage 2 and analysed for BOHB concentration using the cow-side kit Optium Xceed™. A multivariate linear regression was performed on log BOHB data using the 2 cut-off points for BOHB concentration (1.1mM and 1.4mM).

- The prevalence of cows with blood BOHB concentration of greater than or equal to 1.4mM was 6.4%. The prevalence of cows with blood concentrations of BOHB greater than or equal to 1.1mM was 14.5%.

- A mixed linear regression model was performed to determine if there was a difference among the herds. This showed that herds didn't significantly contribute to the variations in whole blood BOHB concentrations and production performance. However this should be interpreted with caution, because of small number of herds in this study (n=5) and also small number of samples obtained from each herd.

These results indicate that there is a need for further studies to determine the prevalence of subclinical ketosis in Western Australian dairy farms, based on BOHB concentrations measured using Optium Xceed meter and production performance of lactating dairy cows. The ability to accurately diagnose subclinical ketosis in dairy herds using cow-side tests will improve dairy cow performance, health and welfare.

# Contents


1. Background ..... 6
2. Relevance to the Australian Dairy Industry ..... 7
3. Objectives ..... 7
    3.1 Stage 1 ..... 7
    3.2 Stage 2 ..... 7
4. Methods ..... 8
    4.1 Stage 1 ..... 8
        4.1.1 Sample size calculation ..... 8
        4.1.2 Animals and diet ..... 8
        4.1.3 Sampling procedures ..... 11
            4.1.3.1 Blood collection ..... 11
            4.1.3.2 Milk collection ..... 11
        4.1.4 Laboratory methods ..... 11
            4.1.4.1 Kinetic assay ..... 11
            4.1.4.2 Colorimetric assay ..... 12
    4.2 Stage 2 ..... 12
        4.2.1 Sample size calculation ..... 12
        4.2.2 Animal selection ..... 12
        4.2.3 Sampling procedures ..... 12
            4.2.3.1 Blood collection ..... 12
    4.3 Statistical analyses ..... 13
5. Results ..... 13
    5.1 Stage 1 ..... 13
    5.2 Stage 2 ..... 17
6. Discussion and conclusions ..... 20
7. References ..... 22

## 1. Background

Bovine ketosis is a metabolic disease of ruminants characterised by hypoglycaemia, elevated concentrations of serum free fatty acids and ketone bodies, and a depressed appetite (Foster 1988). It has been recognised as a disease of substantial economic significance in dairy management systems (Lean, *et al*., 1991). Lactating dairy cows are most susceptible to ketosis at three weeks post-partum, with a variation of one to six weeks (Foster 1988). Subclinical ketosis is characterised by increased concentrations of circulating ketones, such as β-hydroxybutyrate (BOHB) and acetoacetate (AcAc), in the absence of clinical signs (Anderson, 1988). It is not simply the presence but the abnormally high concentration of circulating ketone bodies that determines subclinical ketosis. BOHB is the most stable ketone, and is therefore the most widely used in ketosis assessment (Oetzel, 2004). Animals that develop subclinical ketosis are at a threefold risk of developing clinical ketosis (Duffield, 2000), increased risk of metritis, cystic ovarian disease and reproductive problems (Duffield *et al.*, 1998). The reported prevalence or incidence of subclinical ketosis varies between studies and herds, which may be due to the use of different diagnostic tests and different thresholds for each test. There are several reviews that have documented the prevalence and lactational incidence of ketosis and subclinical ketosis in lactating dairy cows (Lean, 2002; Lean *et al*., 1991; Lean *et al.*, 1992; Duffield, 2000; Anderson, 1988). The incidence of SCK in some herds in Australia is estimated to be around 58.6% (ranges from 0 to 94%) (Rabiee and Lean 2007, unpublished).

Diagnosis of subclinical ketosis is primarily done through measurement of blood β - hydroxybutyrate concentrations. This ketone body is more stable in blood than acetone or acetoacetate (Työppönen and Kauppinen, 1980). The normal range for blood BOHB is considered no higher than 1.2-1.4 mM. The most commonly used cut point for subclinical ketosis is generally accepted at ≥ 1.4mM of blood BOHB concentration using a kinetic laboratory assay (Oetzel, 2004, Gordon, *et al.* 2013, Carrier, *et al*. 2004 and Iwersen, *et al.,* 2009). Early lactation cows with blood BOHB concentrations above this cut point are at threefold greater risk to develop clinical ketosis, and cows with BOHB concentrations above 2mM are at risk for reduced milk production (Duffield, 1997).

In order to diagnose subclinical ketosis, the concentration of BOHB must be measured quantitatively, and a reliably defined threshold must be used to separate normal cows from those with subclinical ketosis. The two most quantitative laboratory diagnostic tests that are commonly used for the measurement of blood concentrations of BOHB in lactating dairy herds are the colorimetric and kinetic methods. The disadvantage of these laboratory methods for diagnosis of ketosis is that they are time consuming to perform, costly and not normally available commercially.

The dairy industry needs a rapid, reliable, repeatable and convenient on farm diagnostic tool for subclinical and clinical ketosis. As a result, over the years, a number of cow-side tests using blood, milk and urine samples have been developed for diagnosis of subclinical ketosis (Oetzel 2004). However, the sensitivity of the tests used for measuring ketones in milk is too low to be useful for epidemiologic interpretation (Duffield, 2000). Moreover, the sensitivity and specificity of these milk tests vary between kits (Oetzel 2004).

Although not as convenient as milk samples, whole blood BOHB concentration is recognised as the accepted standard for determining subclinical and clinical ketosis (Gordon, *et al.,* 2013). Current meters for measuring blood ketones on farm as distinct from in the laboratory are limited to Precision Xtra and Optium Xceed. These meters are commonly used for human diabetes tests and ketone test systems. The range of BOHB concentrations in diabetic humans is lower than that observed in ruminants where ketones can reach a concentration of 12mM and are often greater than 3mM (Pethick and Lindsay, 1982). Therefore, the Optium Xceed meter may be standardised on the lower range of BOHB concentrations observed in humans. The current sensitivity and specificity measured for the Precision Xtra meter is approximately 95% (Oetzel, 2004), but it is not available in Australia.

## 2. Relevance to the Australian Dairy Industry

There are a number of published studies on SCK including prevalence of SCK in dairy herds. Many of these studies used different diagnostic methods for SCK. Rabiee et al (pers. comm., June 2014) found significant difference between BOHB concentrations when different assays are conducted on the same sample. Currently, the majority of research scientists, veterinarians and farm advisors are using the cut-point of 1.4mM, however, there are a number of some studies that used cut-point of 1.0 or 1.2 for the diagnosis of SCK, regardless of type of laboratory methods or diagnostic cow-side kits. This has led to misclassification of cows which subsequently led to overestimation or underestimation of SCK within and among herds. It is vital for researchers who are working in the dairy industry and veterinarians in the field to be able to validate and compare laboratory methods and cow-side kits used for reporting the prevalence of SCK in dairy cattle. In this study we attempted to determine if Optium Xceed meter is an accurate method for measuring ketones in lactating dairy cows. Therefore, the Optium Xceed meter will have the potential of ensuring herd monitoring for ketosis is much simpler, faster and less costly for Australian dairy farmers resulting in earlier and more reliable detection of SCK and improved health and welfare of dairy cows.

## 3. Objectives

### 3.1. Stage 1

The objectives of first stage were:

- To measure plasma concentrations of BOHB using laboratory (colorimetric and kinetic) methods
- To measure whole blood concentrations of BOHB using the cow-side kit Optium Xceed™
- To measure milk concentrations of BOHB using the cow side milk test PortaBHB™
- To compare the results of blood concentrations of BOHB collected from the same lactating dairy cows using laboratory methods and cow-side kits, validating the Optium Xceed meter, a whole blood glucose and ketone meter for use in the diagnosis of subclinical ketosis in Australian dairy herds.

### 3.2. Stage 2

We proposed that if the validation of the Optium Xceed meter was successful, we would collect blood samples from 400 early lactating dairy cows in the Harvey region of WA and measure the concentration of blood BOHB using the Optium Xceed meter. The results would be compared with herd production data collected from the cows sampled. The concentrations of BOHB measured using the Optium Xceed meter should provide an initial estimation of the prevalence of subclinical ketosis in West Australian herds. This can then be used for estimation of required sample size for the future studies. Unfortunately, due to difficulty sourcing enough early lactating cows in the area that met the conditions required (for example, herds were required to be regularly herd testing, heifers could not be sampled), only 174 cows were sampled.

## 4. Methods

### 4.1. Stage 1

#### 4.1.1 Sample size calculations

This was a pilot study with the aim of validating the Optium Xceed ketone meter for use in diagnosing subclinical ketosis in dairy cattle. A sample size of 40 dairy cows was adequate to achieve this objective.

#### 4.1.2 Animals and Diet

Samples were collected from 40 multiparous lactating Holstein-Friesian (HF) dairy cows during early lactation (DIM: 7 ± 3) from a dairy farm in Mundijong, Western Australia in February-May 2014, with regular herd recording tests. Cows were selected based on their parity and days in milk when the sampling days were scheduled. The selected cows were drafted following the morning milking and sampled 2 hours after feeding a total mixed ration (TMR). Cows were kept in the same herd and received the same diet as other cows on the farm during the trial period. Cows with ill-health during the postpartum period, as was diagnosed by farm manager were excluded from the study. The diet of the cows at the time of sampling is presented in Table 1.

**Table 1**. Ingredients of diet for lactating cows during the experiment

| Diet ingredients | Amount fed (kg/cow/day) | |
|---|---|---|
| | As fed | Dry matter (DM) |
| Potato peelings | 10 | 2.46 |
| Lupins | 2 | 1.88 |
| Canola meal | 1 | 0.9 |
| Bread crumbs | 2 | 1.24 |
| Triticale | 2.25 | 2.00 |
| Brewers grain | 6 | 1.44 |
| Malt skimmings | 5 | 1.2 |
| Fruit salad | 2 | 0.23 |
| Rolled oats | 3 | 2.74 |
| Ryegrass/clover silage | 10 | 5.77 |
| Vegetable waste | 2 | 0.12 |
| Lactating cow premix | 0.3 | 0.3 |

The diet ingredients were entered in CPM Dairy program (Cornell*Penn*Miner, 2003, version 3.0.4.a) to estimate the chemical components of the diet. Feed values of the ingredients were either obtained from the feedbank values of CPM Dairy or from previous feed samples tested for chemical compositions. The output of CPM Dairy of the diet provided the estimated chemical compositions of the diet. This diet included 17.3% crude protein (CP) of DM, 68.1% rumen degradable protein (RDP) of CP, 31.73% neutral detergent fibre (NDF) of DM, 43.13% non-fibrous carbohydrates (NFC) of DM, and 11.82 MJ/kg metabolisable energy (ME). The calving dates, age, herd tests and milk production and compositions during the trial period (2014) are presented in Table 2. The herd test data (Table 2) was the first herd test for each animal after calving.

**Table 2**. Calving date, age, milk production and composition of cows sampled for BOHB

| Cow ID | Calving date | Age | Date first herd test 2014 | Milk fat% | Milk protein % | Litres/cow/day |
|---|---|---|---|---|---|---|
| X41 | 29/1/14 | 5 | 3/3/14 | 2.8 | 2.9 | 28.8 |
| Z5 | 28/1/14 | 3 | 3/3/14 | 3.2 | 3.2 | 22.4 |
| S28 | 3/2/14 | 9 | 3/3/14 | 2.8 | 3.0 | 31.6 |
| Y29 | 1/2/14 | 4 | 3/3/14 | 3.4 | 3.4 | 25.8 |
| Y4 | 28/1/14 | 4 | 3/3/14 | 3.3 | 3.0 | 29.6 |
| X60 | 30/1/14 | 5 | 3/3/14 | 3.0 | 2.6 | 34.8 |
| S16 | 2/2/14 | 9 | 3/3/14 | 3.2 | 2.6 | 41.1 |
| Y51 | 6/2/14 | 4 | 5/5/14 | 2.8 | 3.1 | 24.4 |
| V24A | 8/2/14 | 7 | 31/3/14 | 3.2 | 2.8 | 41.1 |
| Y42 | 10/2/14 | 4 | 3/3/14 | 4.3 | 3.0 | 38.9 |
| S14 | 26/3/14 | 9 | 5/5/14 | 4.6 | 3.3 | 38.2 |
| X21 | 26/3/14 | 5 | 5/5/14 | 2.9 | 2.7 | 43 |
| P52 | 25/3/14 | 11 | 5/5/14 | 3.8 | 3.2 | 41.2 |
| Y83 | 25/3/14 | 4 | 5/5/14 | 4.1 | 3.4 | 28.1 |
| S66 | 24/3/14 | 9 | 5/5/14 | 2.9 | 2.5 | 48.4 |
| T39 | 22/4/14 | 8 | 5/5/14 | 3.0 | 3.2 | 41.7 |
| X54 | 23/4/14 | 5 | 5/5/14 | 3.9 | 2.9 | 32.3 |
| Y46 | 22/4/14 | 4 | 5/5/14 | 4.3 | 3.0 | 38.2 |
| Z6 | 9/5/14 | 3 | 2/6/14 | 2.0 | 2.6 | 37.8 |
| V22 | 6/5/14 | 7 | 2/6/14 | 3.8 | 3.1 | 36.3 |
| Y21 | 9/5/14 | 4 | 2/6/14 | 3.9 | 3.9 | 27 |
| Y18 | 7/5/14 | 4 | 2/6/14 | 3.9 | 3.0 | 38.8 |
| X68 | 6/5/14 | 5 | 2/6/14 | 3.9 | 3.0 | 41.9 |
| Y80 | 6/5/14 | 4 | 2/6/14 | 3.1 | 3.0 | 51.5 |
| S52 | 16/5/14 | 9 | 2/6/14 | 2.8 | 2.8 | 33 |
| Y24 | 13/5/14 | 4 | 2/6/14 | 3.4 | 3.2 | 31.6 |
| X49 | 13/5/14 | 5 | 2/6/14 | 4.3 | 3.2 | 45.4 |
| Y13 | 15/5/14 | 4 | 2/6/14 | 2.2 | 2.8 | 40.1 |
| W36 | 14/5/14 | 6 | 2/6/14 | 3.0 | 2.9 | 37.9 |
| W58 | 13/5/14 | 6 | 2/6/14 | 2.9 | 2.8 | 34.7 |
| X61 | 16/5/14 | 5 | 2/6/14 | 3.2 | 2.9 | 29.4 |
| W39 | 17/5/14 | 6 | 2/6/14 | 3.5 | 2.9 | 45 |
| W39 | 17/5/14 | 6 | 2/6/14 | 2.8 | 3.0 | 45 |
| Y28 | 19/5/14 | 4 | 2/6/14 | 3.3 | 2.7 | 46.3 |
| W73 | 20/5/14 | 6 | 2/6/14 | 2.7 | 3.3 | 47.4 |
| X25 | 18/5/14 | 5 | 2/6/14 | 3.2 | 2.9 | 39.7 |
| X16 | 22/5/14 | 5 | 2/6/14 | 3.2 | 3.5 | 33.6 |
| V38 | 26/5/14 | 7 | 2/6/14 | 4.6 | 3.3 | 22.1 |
| 150 | 26/5/14 | 7 | 2/6/14 | 4.2 | 4.0 | 28.9 |
| W71 | 26/5/14 | 6 | 2/6/14 | 4.2 | 3.9 | 25 |

### 4.1.3 Sampling procedures

*4.1.3.1 Blood collection*

Blood samples were collected on day 7 ± 3 postpartum, at the same of time of the day (2 hours after morning feeding). Blood sampling was performed by venepuncture of the coccygeal vein using an 18 gauge, 32cm needle. Blood samples were collected into 2 heparinised (anticoagulant) glass tubes (green: 2 x 10ml). Blood samples were centrifuged at 3,000 rpm for 15 minutes. The plasma samples were then decanted into labelled 5ml polypropylene tubes, and frozen at -20$^{o}$C for later analysis of BOHB.

A sample of whole blood (0.1mL) was also used for the determination of the concentrations of BOHB at the time of sampling using the Optium Xceed$^{TM}$ ketone meter (Abbott Diabetes Care Inc., Alemeda, CA 94502, USA).

*4.1.3.2 Milk collection*

Milk samples were collected at the same time that the cows were sampled for blood. Individual milk samples were aseptically collected from all 4 quarters (composite sample) into a sterile container. The milk samples were analysed at the time of sampling for BHB using the cow-side test PortaBHB$^{TM}$ milk ketone test (PortaCheck Inc., Moorestown, NJ, USA). A milk sample was placed on the stick and the change of colour was matched with the colour codes on the side of the bottle to estimate the concentration of BOHB in milk.

### 4.1.4 Laboratory Methods

*4.1.4.1 Kinetic assay*

Plasma samples were analysed for BOHB using the kinetic laboratory assay. The kinetic method was based on that described by the Stormont Veterinary Laboratory, Belfast procedure (McMurray *et al*, 1984). Plasma samples were first defrosted at room temperature, then 0.5mL of plasma was mixed with 0.5mL of 5% perchloric acid in a 2 mL Eppendorf tube and centrifuged for 10 minutes at 5000 rpm. A 0.5 mL of acid supernatant was collected and then a 100μL subsample of the acid supernatant was adjusted to a pH level of 8 by adding 100μL of 3M $KHCO_3$. The neutralised samples were then centrifuged at 3000rpm for 2 minutes. The neutralised supernatant was then collected in an Eppendorf tube. The following solutions were pipetted into a semi-micro cuvette: 500μL of 200mM tris-HCl pH 8.5 buffer, 50μL of 40mM $NAD^+$, 250μL of neutralised supernatant and 195uL of distilled water. A water blank was prepared by adding 445μL of distilled water and no supernatant. The contents of the cuvettes were mixed using parafilm and incubated for 5 minutes at room temperature and the starting optical density at 340nm recorded. Five μL of D-3-hydroxybutyrate dehydrogenase was then added to start each reaction and the increase in optical density measured at 340nm using a Shimadzu spectrophotometer (model UVmini 1240) at times 0, 15, 30, and 60 minutes. The D-3-OH butyrate concentrations were calculated using nanomolar extinction coefficient (0.00622) for $NAD^+$/NADH.

*4.1.4.2 Colorimetric assay*

Plasma samples were analysed for BOHB using the colorimetric laboratory assay. This assay was based upon the oxidation of BOHB to acetoacetate and the concomitant reduction of $NAD^+$ to NADH by the enzyme D-3-hydroxybutyrate dehydrogenase. The NADH stoichiometrically reduced the developer dye WST-1, via diaphorase to produce a yellow colour. The colorimetric assay used a kit supplied by Cayman Chemical Company, Ann Arbor, MI, USA, (cat. No.700190). Eight standard solutions were made up of BOHB concentrations; 0mM (blank), 0.025mM, 0.05mM, 0.1mM, 0.2mM, 0.3mM, 0.4mM and 0.5mM. Samples were diluted 1:5 with assay buffer (100mM tris-HCl, pH 8.5). A Gilson pipette was used to pipette 50μL of standard (or diluted sample) and 50μL of developer solution into each well of a 96 well plate. The plate was then incubated at 25°C in the dark for 30 minutes. The absorbance was then read at 450nm using a plate reader. All standards and samples were performed in duplicate.

## 4.2. Stage 2

### 4.2.1 Sample size calculations

The number of dairy herds and cows that were considered for Stage 2 (field study) of this study was based on accessibility and agreement of dairy farmers to participate in this trial. A total of 5 dairy farmers participated in this study. A total of 174 cows were sampled for determination of whole blood BOHB concentration using the Optium Xceed cow-side kit. The number of dairy cows sampled from each herd ranged from 5 to 25, with an average DIM of 7 ± 3.

### 4.2.2 Animal selection

Samples were collected from 174 multiparous lactating Holstein-Friesian (HF) dairy cows during early lactation from 5 dairy farms in the Harvey area, Western Australia in March - October 2014. Herds were only enlisted in the project if they were regularly herd testing in the 2014 season. Cows were identified following the morning milking and sampled 2 hours after feeding concentrates in the bail. Cows were kept in the same herd and received the same diet as other cows on the farm before sampling. Cows were selected based on their parity and days in milk when the sampling days were scheduled. Cows suffering from a debilitating illness from the day of calving up until the day of sampling will be excluded from the study.

### 4.2.3 Sampling procedures

*4.2.3.1 Blood collection*

Blood samples were collected on day 7 ± 3 postpartum, at the same of time of the day (2 hours after morning feeding). Blood sampling was conducted by venepuncture of the coccygeal vein using an 18 gauge, 32cm needle. Blood samples were collected into a heparinised (anticoagulant) glass tube (green: 10ml). Blood samples were centrifuged at 3,000 rpm for 15 minutes. The plasma samples were kept at -20°C for further future analysis. However, due to limited time and budget, we were not able to analyse these samples for this report. A sample of whole blood (0.1mL) was decanted for the determination of the concentrations of BOHB at

the time of sampling using the Optium Xceed™ ketone meter (Abbott Diabetes Care Inc., Alemeda, CA 94502, USA).

### 4.3 Statistical analyses

Pearson correlation among different cow-side kits and analytical laboratory methods and sample types (whole blood, plasma and milk) were estimated using R program (R Core Team, 2014).

The level of agreement (concordance correlation coefficient; ccc) among different cow-side kits and analytical laboratory methods and sample types (urine, milk, plasma and serum) were measured using the methods of Lin's (1989, 2000) using R program (R Core Team, 2014) with epiR package (R package version 0.9-58). The concordance correlation coefficient combines measures of both precision and accuracy to determine how far the observed data deviate from the line of perfect concordance (i.e., the line at 45 degrees on a square scatter plot). Lin's coefficient increases in value as a function of the nearness of the data's reduced major axis to the line of perfect concordance (the accuracy of the data) and of the tightness of the data about its reduced major axis (the precision of the data).

## 5. Results

### 5.1 Stage 1 (Pilot study)

Samples collected from 40 early postpartum lactating dairy cows were analysed for BOHB on farm using an Optium Xceed™ meter (whole blood) and PortaBHB™ ketolac strips (milk). Plasma samples collected on farm were also analysed for BOHB using the kinetic and colorimetric laboratory assay methods. The blood and plasma BOHB concentrations (mM) as measured by Optium Xceed meter, kinetic and colorimetric laboratory assays are presented in Table 3. Milk BOHB concentrations were not included in the statistical analysis because of low sensitivity of PortaBHB™ ketolac strips for measuring BOHB concentrations in milk.

**Table 3**. Concentration of BHB (mM) in Stage 1 using Optium Xceed™, Kinetic and Colorimetric methods and PortaBHB™

| Cow ID | BOHB concentration (mM) | | | |
|---|---|---|---|---|
| | **Optium Xceed™** | **Kinetic assay** | **Colorimetric assay** | **PortaBHB** |
| | Blood | Plasma | Plasma | Milk |
| X41 | 0.2 | 0.29 | 0.56 | 0.05 |
| Z5 | 0.3 | 0.37 | 0.62 | 0 |
| S28 | 0.4 | 0.40 | 0.66 | 0.05 |
| Y29 | 0.3 | 0.32 | 0.60 | 0.05 |
| Y4 | 0.4 | 0.34 | 0.61 | 0 |
| X60 | 0.3 | 0.34 | 0.62 | 0 |
| S16 | 3.4 | 2.59 | 3.12 | 0.2 |
| Y51 | 0.3 | 0.29 | 0.63 | 0 |
| V24A | 0.8 | 0.69 | 0.91 | 0 |
| Y42 | 0.6 | 0.62 | 0.85 | 0 |
| S14 | 0.5 | 0.53 | 0.75 | 0.05 |
| X21 | 0.4 | 0.37 | 0.75 | 0 |
| P52 | 0.3 | 0.20 | 0.64 | 0 |
| Y83 | 0.5 | 0.41 | 0.77 | 0 |
| S66 | 0.6 | 0.44 | 0.74 | 0.05 |
| T39 | 0.4 | 0.31 | 0.63 | 0.1 |
| X54 | 0.6 | 0.48 | 0.74 | 0.05 |
| Y46 | 0.3 | 0.52 | 0.66 | 0 |
| Z6 | 0.6 | 0.49 | 0.78 | 0 |
| V22 | 0.4 | 0.64 | 0.74 | 0 |
| Y21 | 0.4 | 0.51 | 0.65 | 0 |
| Y18 | 0.6 | 0.81 | 0.83 | 0 |
| X68 | 0.4 | 0.39 | 0.78 | 0 |
| Y80 | 0.6 | 0.95 | 1.03 | 0.05 |
| S52 | 0.4 | 0.56 | 0.63 | 0.05 |
| Y24 | 0.3 | 0.22 | 0.58 | 0 |
| X49 | 0.3 | 0.35 | 0.70 | 0 |
| Y13 | 0.3 | 0.38 | 0.84 | 0 |
| W36 | 1.1 | 0.84 | 1.09 | 0.1 |
| W58 | 0.4 | 0.43 | 0.81 | 0 |
| X61 | 0.3 | 0.27 | 0.69 | 0 |
| W39 | 0.8 | 0.62 | 1.05 | 0.05 |
| W39 | 0.4 | 0.32 | 0.63 | 0 |
| Y28 | 0.6 | 0.65 | 0.95 | 0 |
| W73 | 0.3 | 0.19 | 0.52 | 0 |
| X25 | 0.4 | 0.31 | 0.63 | 0 |
| X16 | 0.5 | 0.53 | 0.82 | 0 |
| V38 | 0.7 | 0.51 | 0.81 | 0 |
| 150 | 0.7 | 0.53 | 0.87 | 0 |
| W71 | 0.6 | 0.49 | 0.54 | 0 |

The correlation and concordance correlation coefficients (ccc) of plasma and blood concentrations of BOHB measured using Optium Xceed™, kinetic and colorimetric laboratory methods are presented in Table 4. Whole blood concentrations of BOHB measured using the Optium Xceed™ test were highly correlated with kinetic and colorimetric laboratory methods (Table 4).

**Table 4.** Correlation (and 95% CI) for the concentrations of BOHB measured using Optium Xceed™ test (blood), colorimetric and kinetic (plasma) methods.

| Assay procedures | Correlation & 95% CI (N= 40) | P value |
|---|---|---|
| | ***Whole blood (Optium Xceed™ Meter)*** | |
| Kinetic- Plasma | 0.953 (0.920 – 1.00) | <0.001 |
| Colorimetric- Plasma | 0.972 (0.952 – 1.00) | <0.001 |
| | ***Kinetic - Plasma*** | |
| Colorimetric-Plasma | 0.959 (0.931 – 1.00) | <0.001 |

The concordance correlation coefficient (ccc) is indicative of the level of agreement between two variables. McBride (2005) suggests that a concordance coefficient of less than 0.9 indicates a poor level of agreement between variables, a concordance coefficient of 0.9 – 0.95 indicates a moderate level of agreement and a concordance coefficient of 0.95 – 0.99 a substantial level of agreement. The ccc and line of agreement (LOA) between the laboratory assays and the Optium Xceed were estimated (Table 5, Figures 1 and 2). Interestingly, the level of agreement between Optium Xceed™ and the kinetic assay was greater (ccc=0.92) than the concordance between Optium Xceed™ and the colorimetric assay (ccc=0.82) (Table 5). With the exception of two outliers, the data for the Optium Xceed and kinetic laboratory assay were within the 2 standard deviations from the line of perfect concordance (Figure 1). The data comparing Optium Xceed and the colorimetric assay were also within the 2 standard deviations) with greater variation (Figure 2).

**Table 5.** Concordance (and 95% CI) for the concentrations of BOHB measured using Optium Xceed™ test (blood), colorimetric and kinetic (plasma) methods

| Assay procedures | Concordance & 95% CI (N= 40) | P value |
|---|---|---|
| | ***Whole blood (Optium Xceed™ Meter)*** | |
| Kinetic- Plasma | 0.916 (0.877-0.954) | <0.001 |
| Colorimetric- Plasma | 0.819 (0.745-0.893) | <0.001 |
| | ***Kinetic – Plasma*** | |
| Colorimetric-Plasma | 0.755 (0.660-0.849) | <0.001 |

**Figure 1.** Concordance Correlation Coefficient and Line of Agreement between Optium Xceed™ test and kinetic (plasma) method for BOHB concentrations

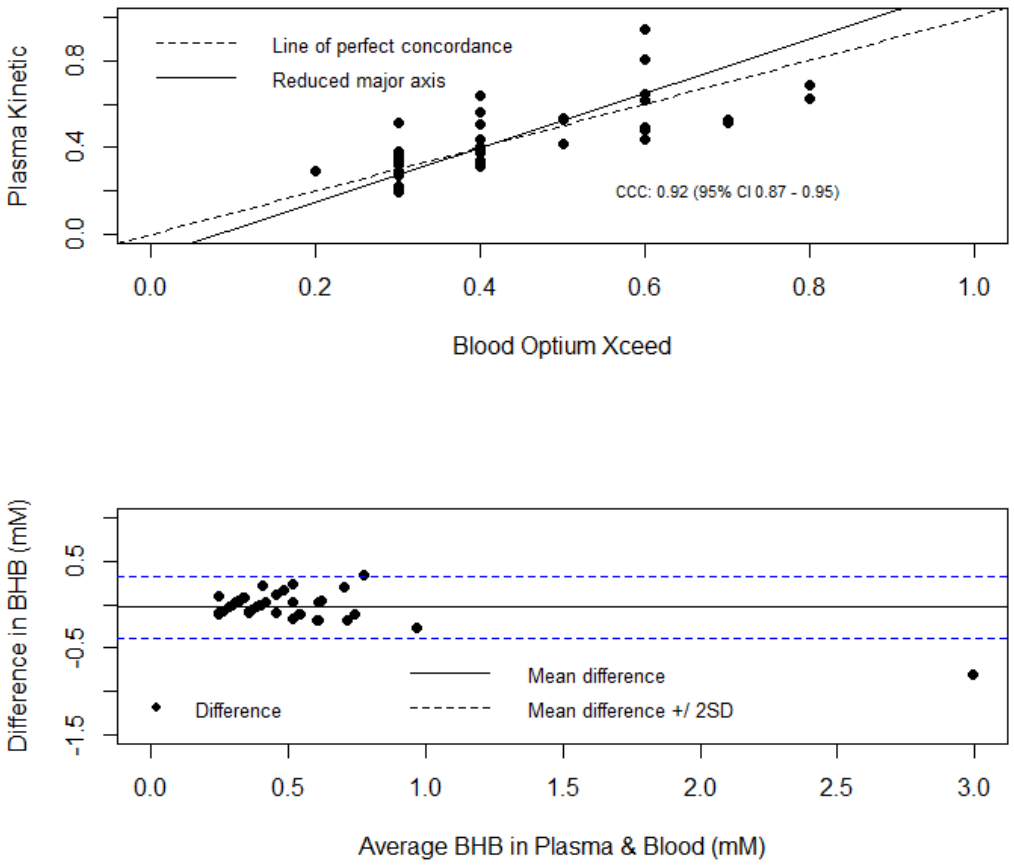
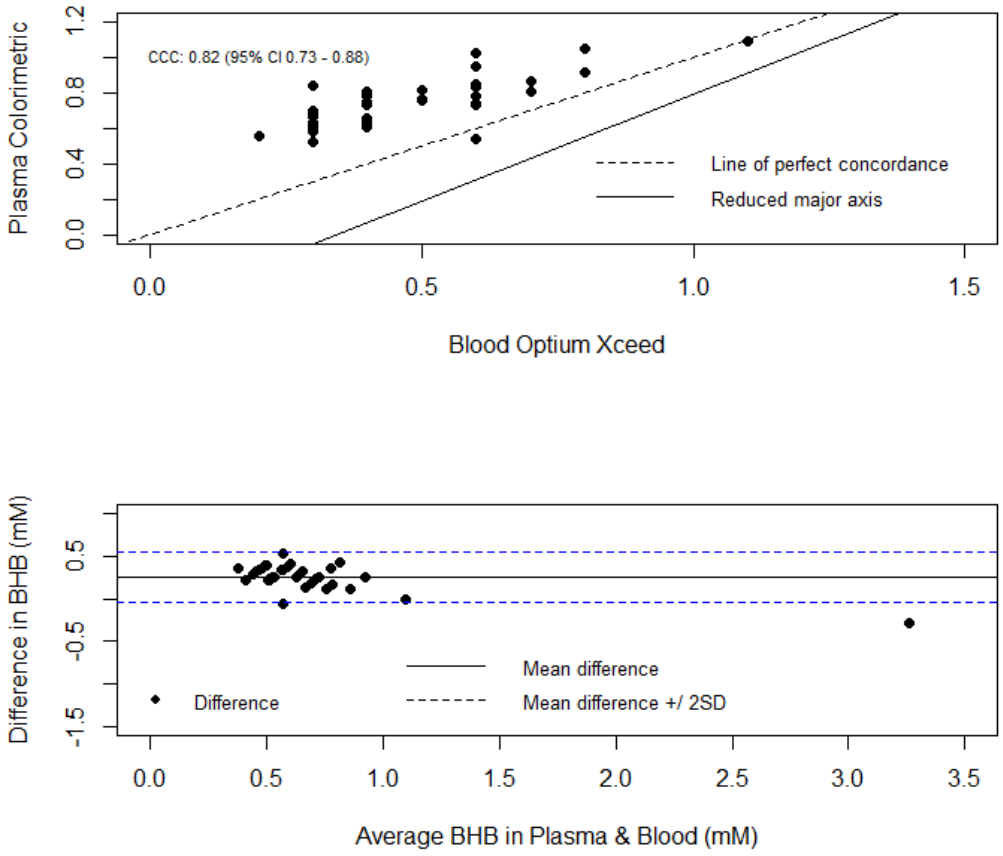


**Figure 2.** Concordance Correlation Coefficient and Line of Agreement between Optium Xceed™ test and colorimetric (plasma) method for BOHB concentrations.




## 5.2 Stage 2 (Field Study)

Blood samples collected from 174 early postpartum lactating dairy cows on 5 dairy farms in South-West Western Australia were analysed for BOHB on farm using an Optium Xceed™ meter. The production data (e.g. milk yield) of cows were compared for cows with high and low BOHB concentrations using two thresholds of 1.4mM and 1.1mM, respectively. Initial descriptive statistical analysis showed that BOHB concentration data that were measured using the Optium Xceed™ method were not normally distributed, therefore a log transformation of the data was used for the statistical analysis (Figures 3 and 4).

**Figure 3.** Frequency distribution of whole blood BOHB concentrations of lactating dairy cows measured using the Optium Xceed meter (SW of WA - sampled in Stage 2).

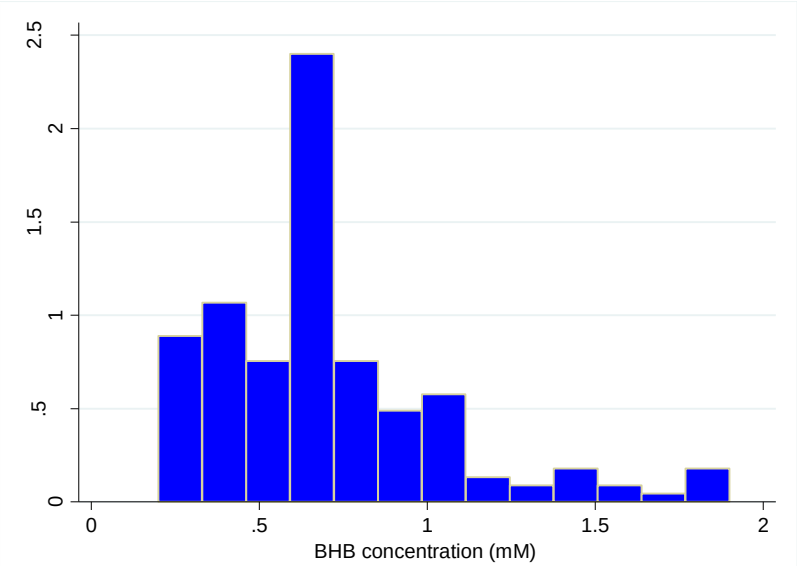
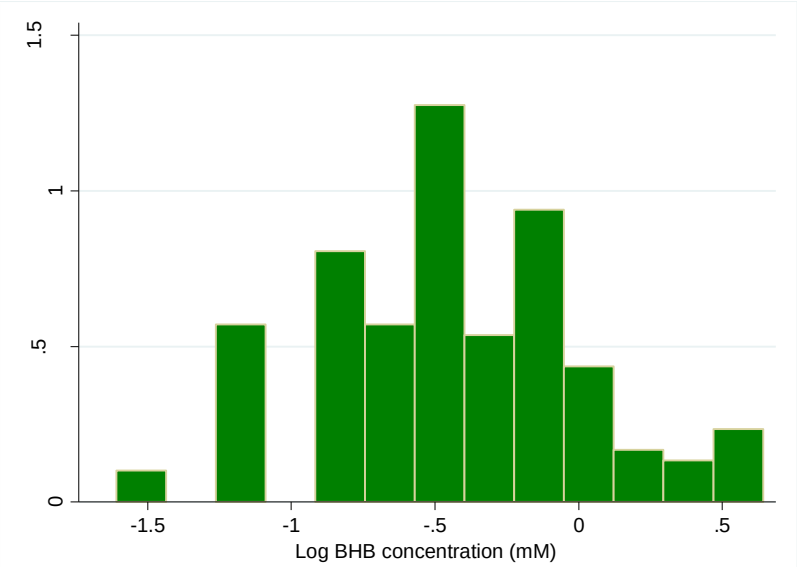



**Figure 4.** Frequency distribution of whole blood BOHB concentrations (log transformed) of lactation dairy cows measured using the Optium Xceed meter (SW of WA - in stage 2).




Whole blood concentrations of BOHB measured using the Optium Xceed meter were analysed using a cut-off of 1.4mM of BOHB. The prevalence of cows with blood BOHB concentration of greater than or equal to 1.4mM was 6.4%. Table 6 summarises the descriptive statistics of the two groups of cows; those with a blood BOHB concentration of < 1.4mM and those with a blood BOHB concentration of ≥ 1.4mM.

**Table 6**. Summary of production data (±SD) for cows below and above the cut-point of 1.4mM of blood BOHB. Numbers in brackets indicate the range.

| **Production data** | **Cows with blood BOHB Mean ± SD (range)** | |
|---|---|---|
| | **<1.4mM** | **≥1.4mM** |
| Litres/cow/day | 34.29 ± 8.35 (12.2 – 53.8) | 37.87 ± 8.59 (21 – 51.3) |
| Milk fat % | 3.81 ± 0.62 (2.19 – 5.3) | 3.99 ± 0.64 (2.8 – 5.0) |
| Milk protein % | 3.12 ± 0.28 (2.50 – 3.86) | 2.99 ± 0.13 (2.80 – 3.20) |
| Days in milk (DIM) | 7.02 ± 2.43 (2.0 – 13.0) | 6.73 ± 3.41 (4.0 – 16.0) |
| Age | 5.05 ± 1.91 (2.0 – 12.0) | 6.45 ± 2.98 (3.0 – 13.0) |
| Optium Xceed BOHB concentration | 0.63 ± 0.41 (0.20 – 1.30) | 1.63 ± 0.20 (1.40 – 1.90) |
| Log transformed BOHB concentration | -0.54 ± 0.41 (-1.61 – 0.26) | 0.49 ± 0.12 (0.34 – 0.64) |

The data were also analysed using a cut-off point of 1.1mM of whole blood concentrations of BOHB measured using the Optium Xceed meter for the diagnosis of sub-clinical ketosis. There are some studies that have used 1.1mM as a cut-off point to define sub-clinical ketosis. The prevalence of cows with blood concentrations of BOHB greater than or equal to 1.1mM was 14.5%. Table 7 summarises the descriptive statistics of the two groups of cows; those with a blood BOHB concentration < 1.1mM and those with a blood BOHB concentration ≥ 1.1mM.

**Table 7**. Summary of production data (±SD) for cows below and above the cut-point of 1.1mM of blood BOHB. Numbers in brackets indicate the range.

| **Production data** | **Cows with blood BOHB Mean ± SD (range)** | |
|---|---|---|
| | **<1.1mM** | **≥1.1mM** |
| Litres/cow/day | 34.0 ± 8.49 (12.2 – 53.8) | 37.8 ± 7.07 (21.0 – 51.3) |
| Milk fat % | 3.8 ± 0.6 (2.2 – 5.2) | 4.1 ± 0.64 (2.8 – 5.3) |
| Milk protein % | 3.1 ± 0.29 (2.5 – 3.9) | 3.0 ± 0.14 (2.8 – 3.4) |
| Days in milk (DIM) | 7.1 ± 2.37 (2 – 13) | 7.1 ± 2.37 (3 – 16) |
| Age (years) | 5.0 ± 1.9 (2 – 12) | 5.9 ± 2.29 (3 – 13) |
| Optium Xceed BOHB concentration (mM) | 0.58 ± 0.2 (0.2 – 1.0) | 1.4 ± 0.28 (1.1 – 1.9) |

A multivariate linear regression was performed on log BOHB data using the 2 cut-off points for BOHB concentration (1.1mM and 1.4mM). The production performance of cows were estimated for both 1.1mM and 1.4mM cows, after controlling for DIM and lactation number

(age). A mixed linear regression model was also performed to determine if there was a difference among the herds. This showed that herds didn't significantly contributed to the variations in whole blood BOHB concentrations and production performance. However this should be interpreted with caution, because of small number of herds in this study (n=5) and also small number of samples obtained from each herd.

## 6. Discussion and Conclusions

In this study three methods that are used to measure BOHB concentration in lactating dairy cows were compared. This included i) a cow side test Optium Xceed and ii) two laboratory methods (kinetic and colorimetric). The Optium Xceed meter was strongly correlated to both the colorimetric assay (r = 0.953) and kinetic assay (r = 0.972). The ccc shows the strength of a relationship between two variables, but not the agreement between the two variables (Bland and Altman, 1986). There is perfect agreement only if the points lie along the line of equality, and we have perfect correlation if the points lie along any straight line (Bland and Altman, 1986). The kinetic assay is classified as the "gold standard" laboratory assay in the industry. The concordance between the concentrations of BOHB that were measured using Optium Xceed meter and kinetic laboratory method was moderately high (ccc = 0.92). The level of agreement between Optium Xceed meter and colorimetric laboratory method was low (ccc = 0.82). The moderately high level of agreement between the Optium Xceed and kinetic laboratory assay is encouraging, and can provided confidence in the use of this method as a reasonably reliable cow-side test in the field. The repeatability of this cow-side test was also established over a range of BOHB concentrations, proving it may be a reliable indicator of BOHB concentrations consistent with values determining diagnosis of subclinical ketosis, i.e. greater or equal to 1.4mM. Given the accuracy of the data, the conductivity measures from the Optium Xceed meter did not appear to be confounded by the presence of other organic acids interfering with the determination.

The consistently higher readings for the colorimetric laboratory assay were also found by Rabiee and Lean (2007, unpublished). The colorimetric assay is based on the measurement of reduction of coloured tetrazolium salts (a reducing dye) by NADH in the presence of diaphorase. Any non-specific reducing agents present in the samples could react with the dye to consistently increase the final absorbance reading. This outcome will give a falsely high reading for BOHB in the samples. Therefore studies that use the colorimetric method will produce greater estimates of the incidence and prevalence of ketosis if not corrected for the interference of these non-specific reducing agents.

The Optium Xceed meter was used to measure blood BOHB concentrations in 174 multiparous cows in early lactation on five farms in SW Western Australia after it was validated for use in Stage 1 of the project. One of the objectives of the study was to measure BOHB concentrations in 400 multiparous cows in early lactation and compare with production data obtained from the sampled cows. This would give an initial estimate of prevalence of SCK in West Australian dairy herds, enabling a sample size estimation to be determined for a larger study. The reported prevalence or incidence of SCK varies between studies and herds; the incidence of SCK in some herds in Australia is estimated to be around 58.6%, ranging from 0 to 94% (Rabiee and Lean 2007, unpublished). The difference may be due to the use of different diagnostic tests and different thresholds for each test. In this project, if 1.4mM of

BOHB was used as a cut point for SCK, the prevalence was 6.4%. If however the cut point was moved to 1.1mM of BOHB, the prevalence was significantly higher at 14.5%. Early diagnosis of even 6.4% of the herd with SCK (if a cut point of 1.4mM is assumed) would significantly decrease the intervention costs with treating clinical cases as well as reducing the increased risk of these cows developing metritis, ovarian cysts and reproductive problems. It is important to note, however, that there were only a small number of herds in this study (n=5) and also small number of samples obtained from each herd. The results of stage 2 of the study should therefore be interpreted with caution. The mixed linear regression model showed that herds didn't significantly contribute to the variations in whole blood BOHB concentrations and production performance. Again, this may have been as a result of the small number of cows and herds enrolled in the study. In some of the herds there was a lag time between the time of herd test and when BOHB concentrations were measured, which could also be a potential confounder. Ideally, it would be better to determine the production data on the same day as the whole blood BOHB concentration are measured, with serial samplings over a period of time during the early lactation. However, most commercial dairies do not have the milk meter equipment in place, and as a result it would be difficult to obtain the yield data to establish the association between production performance and whole blood BOHB concentrations.

In conclusion, we have been able to validate the use of the Optium Xceed meter for use in diagnosing SCK in Australian dairy herds. This meter is available in most pharmacies across Australia. The cut-point of 1.4mM that is suggested in the literature to identify cows with subclinical ketosis (SCK) is based on the kinetic laboratory method. The high concordance between the kinetic assay and Optium Xceed indicates that the cut-point of 1.4mM can be considered as reliable threshold for the diagnosis of SCK in lactating dairy cows using the Optium Xceed meter. Using the cut-point of 1.4mM, an initial estimate of the prevalence of SCK in West Australian dairy herds is 6.4%. This should however be interpreted with caution given the small number of samples (N = 174) in the study. There is a need for further studies to determine more accurately the prevalence of SCK in Western Australian dairy herds, as defined by both BOHB concentrations and production parameters, particularly as we strive for higher producing cows and increase the risk of negative energy balance in early lactation.

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