Aus der
Tierklinik für Fortpflanzung
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

On-farm diagnosis and effects of subclinical ketosis on milk production and reproductive performance in dairy cows

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Julia Ruoff
Tierärztin aus Freudenstadt

Berlin 2018
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Für meine Eltern

TABLE OF CONTENTS

TABLE OF CONTENTS

1 INTRODUCTION

The management of the transition period of dairy cows has increasingly gained in importance by dairy researchers and producers during the last years. Most dairy cows experience a phase of negative energy balance during the transition period (Herdt, 2000). The sudden changes from the pregnant, non-lactating status to the non-pregnant, lactating status is a big challenge for the adaptational ability of the metabolism of a dairy cow (Goff und Horst 1997). As a consequence of negative energy balance, non-esterified fatty acids (NEFA) and ketone bodies (e.g., ß-hydroxybutyrate) are produced (Herdt, 2000). Excessive production of NEFA and BHBA are indicative of a poor adaptive response to negative energy balance (McArt et al., 2015). Both health and economic aspects of this disease have been evaluated. Several studies have reported the effects of elevated NEFA and BHBA concentrations on reproduction (Walsh et al., 2007; Ospina et al., 2010; McArt et al., 2012), milk production (Duffield et al., 2009; Ospina et al., 2010a; Chapinal et al., 2012) and subsequent negative health events (Ospina et al., 2010b; Chapinal et al., 2011; Seifi et al., 2011, McArt et al., 2012). Besides its impact on health, the disease causes financial losses due to a lower milk production, an increased risk for herd removal and an increased risk for diseases associated with subclinical ketosis (McArt et al., 2015).

To evaluate the metabolic status of dairy herds and to detect affected cows, laboratory analysis of different metabolic parameters was shown to be a useful tool for diagnosis. It has been assumed that diagnosis of individual cases of subclinical ketosis at an earlier point in the course of the disease would permit earlier treatment and help mitigate further losses (Enjalbert et al., 2001; McArt et al., 2014). Furthermore, hyperketonemia screening programs on a herd basis can serve to evaluate and monitor the success of current management and feeding protocols (LeBlanc, 2010).

Circulating concentrations of NEFA and BHBA measure aspects of the success in adaptation to a state of negative energy balance (Herdt, 2000; LeBlanc 2010). The concentration of serum NEFA reflects the magnitude of fat mobilization, whereas the concentration of BHBA reflects the completeness of oxidation of fat in the liver (LeBlanc, 2010). Critical thresholds for NEFA and BHBA have been determined for the prepartum (i.e., NEFA = 0.3 mEq/L to 0.5 mEq/L, BHBA = 0.8 mmol/L) and postpartum (i.e., NEFA = 0.7 mEq/L to 1.0 mEq/L, BHBA = 1.2 mmol/L to 1.4 mmol/L) periods (McArt et al. 2013), respectively. Blood concentrations can be determined by laboratory determination. However, this can be cost intensive as well as time consuming. On-farm methods for the diagnosis of

subclinical ketosis were shown to be an effective alternative to laboratory analysis in order to provide quick results and early treatment as well as keeping the costs low. As measurement of BHBA in laboratories is associated with a delay in diagnosis and treatment of hyperketonemia, cow side ketone body tests can provide practical benefits (McArt et al., 2013). Hand-held devices have been evaluated for the measurement of BHBA on farm (Bach et al., 2016). Unfortunately, there is no electronic meter for on farm testing for NEFA available so far. Therefore, NEFA concentrations are used less commonly for on-farm monitoring, although it actually offers more information on odds of negative health outcomes than BHBA, especially prepartum (Chapinal et al. 2011).

Therefore, the objective of the first study was to evaluate the association between different parameters related to lipid metabolism and to find out if those parameters could be used to predict the concentration of NEFA. Specifically, we set out to determine the relationship of BHBA, cholesterol, TAG and HDL to NEFA and to study, if those parameters could serve as surrogates for NEFA both before and after calving.

To optimize hyperketonemia testing protocols it should be considered that different types of ketosis may exist. Two different pathways potentially leading to hyperketonemia were described depending on their time of occurrence and pathophysiology (Holtenius and Holtenius, 1996; Herdt, 2000). According to this classification scheme, type I ketosis occurs between week 3 and 6 postpartum near the time of peak lactation due to a high demand for glucose to support milk production (Holtenius and Holtenius, 1996). It was proposed that gluconeogenic pathways are maximally stimulated but the supply of glucose precursors is insufficient to permit maximal glucose production (Herdt, 2000). This type of ketosis is characterized by low blood glucose and insulin concentrations (Herdt, 2000). Type II ketosis occurs earlier in lactation between week 1 and 2 postpartum when large amounts of NEFA are delivered to the liver but gluconeogenesis and ketogenesis are not maximally stimulated (Herdt, 2000). This can lead to the development of fatty liver, especially in overfed cows. According to this theory, type II ketosis is associated with hyperglycemia and hyperinsulinemia (Herdt, 2000).

The first 2 weeks after calving have been described as the main risk period for hyperketonemia (Duffield et al., 1998; McArt et al., 2012) and effects on health and production outcomes have been evaluated primarily for this period. It was shown, however, that the period in which early lactation dairy cows are at risk for hyperketonemia lasts at least until week 6 postpartum (Mahrt et al., 2015). There is limited evidence for the effect of

hyperketonemia from week 3 to 6 postpartum on milk production, reproduction, and health. Therefore, the aim of the second study of this thesis was to evaluate the effects of hyperketonemia on milk production, reproduction and early lactation culling risk in respect of the occurrence (early vs. late onset hyperketonemia).

Besides BHBA, blood glucose concentrations seem to play a role for ketosis classification (Gordon, 2013). According to the theory of type I and II ketosis, early onset ketosis (week 1 to 2 postpartum) is the hyperglycaemic-hyperinsulinaemic form whereas late onset ketosis (week 3 to 6 postpartum) is the hypoglycaemic-hypoinsulinaemic form. However, there is a lack of science-based information that describes the association between hypoglycemia and hyperketonemia considering the ketosis classification. Therefore, the aim of the third study was 1) to describe the associations between hypoglycemia and hyperketonemia within the first six weeks of lactation, 2) to evaluate the effects of body condition score (**BCS**) at calving on glucose concentration, and 3) to evaluate the effects of hypoglycemia on milk production.

The overall aim of this thesis was to improve the on-farm diagnosis of subclinical ketosis pre- and postpartum and to gain further knowledge about the effects of subclinical ketosis in respect of its onset and classification within the first six weeks of lactation.

2 RESEARCH PAPERS

2.1 Associations of β-hydroxybutyrate, cholesterol, triglycerides and high-density lipoproteins to non-esterified fatty acids pre- and postpartum

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2.1.1 **Abstract**

While laboratory tests for measuring the concentration of NEFA in serum are well established, a point of care test to determine NEFA on farm is not available. Several hand-held meters, however, have been validated for measuring ß-hydroxybutyrate (BHBA) in cattle or cholesterol, triglycerides (TAG), and high-density lipoproteins (HDL) in human medicine, respectively. The objective of this study was to evaluate the association between NEFA and different parameters related to lipid metabolism. Specifically, we set out to determine if it is feasible to predict the concentration of NEFA by means of surrogate measures. The concentration of BHBA was determined by a hand-held meter evaluated for the use in cows, whereas the concentrations of the other parameters were determined by laboratory analysis because hand-held meters for cholesterol, TAG and HDL are only evaluated for human medicine so far. A total of 254 cows were included in the trial. One blood sample was taken from each cow between d 10 and d 1 prepartum. Second and third samples were collected on d 2 and d 10 postpartum, respectively. The coefficients of correlation between parameters were calculated and a receiver-operator characteristic curve analysis has been used. The prediction of NEFA concentrations using only one of the parameters was insufficient. However, a NEFA concentration ≥ 0.5 mEq/L could be predicted with a high sensitivity (i.e., Se = 0.88) and specificity (i.e., Sp = 0.93) from d 3 to d 1 prepartum and a NEFA concentration ≥ 0.7 mEq/L could be reliably predicted on d 2 postpartum (i.e., AUC = 0.89, Se = 0.89, Sp = 0.76) when using a combination of BHBA, cholesterol and TAG as surrogates. Overall, our results suggest that a combination of different parameters of lipid metabolism could be used as surrogates for NEFA.

Keywords: nonesterified fatty acids; ß-hydroxybutyrate; lipid metabolism; hand-held device; dairy cow

2.1.2 **Introduction**

Most dairy cows experience a phase of negative energy balance during the transition period (Herdt, 2000). Lipolysis, a consequence of a negative energy balance, can be measured by elevated serum concentrations of NEFA and BHBA (Herdt et al. 2000; McArt et al. 2013). Both are risk factors for several postpartum diseases (McArt et al. 2013). Critical thresholds for NEFA and BHBA have been determined for the prepartum (i.e., NEFA = 0.3 mEq/L to 0.5 mEq/L, BHBA = 0.8 mmol/L) and postpartum (i.e., NEFA = 0.7 mEq/L to 1.0 mEq/L, BHBA = 1.2 mmol/L to 1.4 mmol/L) periods (McArt et al. 2013), respectively. Although BHBA is a commonly used parameter, NEFA concentrations, both pre- and postpartum, offer more information on odds of negative health outcomes than BHBA (Chapinal et al. 2011). Several electronic meters have been validated for on farm testing of BHBA in dairy cows (Iwersen et al. 2009; Iwersen et al. 2013; Mahrt et al. 2014). Unfortunately, there is no electronic meter for on farm testing for NEFA available so far.

Besides BHBA, other metabolites in the lipid metabolism have been shown to be associated with NEFA and periparturient disease. Prepartum levels of cholesterol are a predictor of retained placenta in dairy cows in addition to NEFA (Quiroz-Rocha et al. 2009). Furthermore, triglycerides (TAG) and NEFA are both associated with fatty liver (Bobe et al. 2004) and an increase in postpartum liver TAG concentrations is accompanied by an increase in plasma NEFA concentrations (van den Top et al. 1995). Besides cholesterol and TAG, high-density lipoproteins (HDL) are related to the lipid metabolism during the transition period (Kurpinska et al. 2015).

Hand-held meters for measuring cholesterol, triglycerides (TAG), and high-density lipoproteins (HDL) are commercially available in human medicine (Accutrend® Plus system, Roche Diagnostics, Indianapolis, USA; CardioChek® PA, PTS Diagnostics, Indianapolis, USA). These meters have been shown to be a valid alternative to laboratory analysis (Ferreira et al. 2015; Coqueiro et al. 2013).

Therefore, the objective of this study was to evaluate the association between different parameters related to lipid metabolism and to find out if those parameters could be used to predict the concentration of NEFA. Specifically, we set out to determine the relationship of BHBA, cholesterol, TAG and HDL to NEFA and to study, if BHBA, cholesterol, TAG and HDL could serve as surrogates for NEFA both before and after calving by means of point of care systems.

2.1.3 **Materials and Methods**

A total of 254 primi- (n = 88) and multiparous (n = 166) Holstein dairy cows from a commercial dairy farm in Saxony-Anhalt, Germany were included in the trial. Sixty-six prepartum samples had to be excluded from cows that did not calve within 10 d after first sample collection, resulting in 188 prepartum samples. The farm housed approximately 1,200 cows in a freestall barn with an average 305d-lactation of 10,124 kg (4.1% fat, 3.4% protein). Close-up cows were housed in groups of approximately 12 cows with deep-straw bedding and fed a total mixed ration once a day at 0700 h. Feed was pushed up several times a day. Fresh cows were housed in groups of approximately 20 cows with slatted floors and cubicles equipped with rubber mats. A total mixed ration was delivered over a conveyer belt system 10 times per day. Cows were milked three times daily in a 52 stall rotary milking parlor.

Data was collected between May and August 2010. One blood sample was taken from each cow between d 10 and d 1 prepartum. Second and third samples were collected on d 2 and d 10 postpartum, respectively. All experimental procedures reported herein were conducted with the approval of the Institutional Animal Care and Use Committee of Freie Universität Berlin.

Blood samples were collected from the coccygeal vein using sterile 9 ml tubes without anticoagulant (Venoject II, Termumo Europe N.V., Leuven, Belgium). The measurement of BHBA was conducted within 30 seconds after blood sampling on farm with an electronic BHBA meter (Precision Xceed, Abbott Diabetes Care Ltd., Witney, UK) that had been validated and used in several studies (Iwersen et al. 2009; Endecott et al. 2012; McArt et al. 2012; Mahrt et al. 2014). After BHBA measurement blood was allowed to clot. For NEFA, cholesterol, TAG and HDL the concentration was measured by laboratory analysis because hand-held meters for these parameters are not validated yet for the use in dairy cattle. Blood samples were centrifuged within 2h after sampling at 1000 × g for 10 min at ambient temperature and serum was separated and stored at -22 °C until analysis. The serum samples were sent to an accredited, commercial lab (Synlab Laboratories, EU accreditation number: D-PL-14016-01-00, accreditation body: DAkkS, Berlin, Germany). The concentration of NEFA was measured with a clinical chemistry analyser (AU 680, Beckman Coulter, Krefeld, Germany) and a testkit from Randox by photometric measurement. The lower limit of quantitation was 0.07 mmol/L. The inter- and intraassay coefficients of variation were 2.3 and 2.9%, respectively, for NEFA. For the determination of cholesterol an enzymatic assay with cholesterol oxidase, phenol and 4-aminophenazone was used based on the formation of a

quinoneimine dye. The rate of color development was measured spectrometrically at 540/600 nm. In order to determine TAG an enzymatic assay with glycerol-3-phosphate oxidase, phenol and 4-aminophenazone was used producing a chromophore which was read at 660/800 nm. The inter- and intraassay coefficients of variation were 2.7 and 0.7%, respectively, for cholesterol and 3.9 and 1.8%, respectively, for TAG. For the determination of HDL an enzymatic assay with cholesterol esterase, cholesterol oxidase and a chromogen system was used to yield a blue color complex which was measured bichromatically at 600/700nm. For HDL the inter- and intraassay coefficients of variation were validated for two different concentrations but for humans only. The inter- and intraassay coefficients of variation were 3.08 and 1.32%, respectively, for a low concentration and 4.79 and 4.79%, respectively, for a high concentration.

Statistical Analysis

A total of 188 cows were sampled between d 10 and d 1 prepartum. Of those, 181 samples had sufficient laboratory results and were used for statistical analysis. In total, 254 cows were sampled postpartum. Of those, 246 cows had laboratory results from d 2 postpartum, 245 cows had results from d 10 postpartum and were used for statistical analysis. Data were entered into Excel spreadsheets (version 2010; Microsoft Corporation, Redmond, WA) and statistical analyses were performed with IBM SPSS Statistics for Windows software (version 20.0; IBM Deutschland GmbH, Ehningen, Germany) and MedCalc for Windows (version 12.0.3.0., MedCalc Software bvba, Mariakerke, Belgium). The level of significance for all statistical tests was set at $P < 0.05$. The normal distribution of NEFA, BHBA, Cholesterol, TAG and HDL was assessed by plotting and visually examining the data and performing a Kolmogorov-Smirnoff-test.

All statistical analyses were carried out separately for 4 different sample sets. In order to include a longer time frame to take samples prepartum, which would be beneficial in practice, an early prepartum sample set (i.e. d 10 to 1; n = 181) was defined. To find out if the predictive value improved before calving a late prepartum sample set (i.e. d 3 to 1; n = 91) was defined which included eligible samples of the early prepartum sample set. After calving, an early postpartum (i.e. d 2 postpartum; n = 246) and a late postpartum (i.e. d 10 postpartum; n = 245) sample set was defined and analyzed. The degree of association between BHBA, cholesterol, TAG, and HDL with NEFA was determined by computing Spearman's rank correlation coefficients. For the prediction, if the NEFA concentration exceeded a certain value and for the determination of thresholds of the predicting variables, receiver operator

characteristic analysis (ROC) was used. The predicting variables were either single analytes (i.e., BHBA, cholesterol, TAG, HDL) or a combination of these analytes utilizing a modified procedure. The aim of the second approach was to determine thresholds for a combination of predicting variables and to calculate Se and Sp for the determination of NEFA if 1, 2, 3 or 4 of the predicting variables were higher than the thresholds. Because the ROC analysis only allows 1 predicting variable, BHBA, Cholesterol, HDL and TAG concentrations were transformed into binary variables (i.e., 0 and 1) and a dummy variable was calculated to be used for the ROC analysis. The dummy variable equaled the sum of the recoded BHBA, Cholesterol, HDL and TAG values and could range from 0 (i.e., all variable below the threshold) to 4 (i.e., all variables above the threshold). Thresholds for the recoding of BHBA, cholesterol, HDL and TAG were chosen at random and the calculations were repeated until the highest area under the curve (AUC) for the prediction of NEFA was achieved. The Se was defined as the proportion of positives that were correctly identified as cows over the critical NEFA threshold. The Sp was defined as the proportion of negatives that were correctly identified as cows below the critical NEFA threshold. In accordance with McArt et al. (2013) the following critical thresholds for NEFA were chosen: 0.3 mEq/L and 0.5 mEq/L prepartum and 0.7 mEq/L and 1.0 mEq/L postpartum. Additionally, the AUC, positive predictive value (PPV) and the negative predictive value (NPV) were calculated utilizing the prevalence in the study population. The calculations were conducted for three different scenarios. Firstly, a combination in which BHBA, cholesterol and TAG were treated equally was analyzed. Secondly, BHBA was set as a fixed parameter (i.e., BHBA level had to exceed a certain threshold for the sum of predicting variables to be positive) and combined with cholesterol and TAG. We used BHBA as a fixed parameter because it is the most common metabolite to assess negative energy balance in transition cows and hand-held meters are already validated in cows. Thirdly, a combination of only BHBA and cholesterol was tested.

2.1.4 **Results and Discussion**

Prepartum mean NEFA concentration was 0.22 ± 0.24 mEq/L (min = 0.07 mEq/L; max = 2.51 mEq/L). Postpartum NEFA averaged 0.65 ± 0.49 mEq/L (min = 0.07 mEq/L; max = 2.65 mEq/L). Before and after calving NEFA concentrations were weakly or moderately correlated with BHBA, TAG and cholesterol ($P < 0.05$; Table 1). No significant correlation was found between NEFA and HDL ($P > 0.05$).

With BHBA as a surrogate measure the prediction of a NEFA concentration ≥ 0.3 mEq/L prepartum was not significant (P > 0.05). A NEFA concentration ≥ 0.5 mEq/L prepartum could be predicted with an AUC = 0.77 – 0.81; however, the positive predictive value (PPV) was low (i.e., PPV = 0.17 – 0.27). Considering these results, a high percentage of cows predicted with a NEFA concentration ≥ 0.5 mEq/L would be false positives and would undergo unnecessary treatment. Postpartum a NEFA concentration ≥ 0.7 and 1.0 mEq/L could be predicted with a PPV = 0.31 – 0.57 (AUC = 0.64 – 0.78).

When using cholesterol prepartum a NEFA concentration ≥ 0.3 and 0.5 mEq/L could be predicted with a high sensitivity (i.e., Se = 0.9 – 1.0), however, the specificity was low (i.e., Sp = 0.37 – 0.48; AUC = 0.59 – 0.65). Postpartum, the best results were found on d 2 after calving for predicting a NEFA concentration ≥ 0.7 mEq/L (i.e., AUC = 0.77; Se = 0.86; Sp = 0.66; PPV = 0.6; NPV = 0.89).

The usability for TAG as a prepartum predictor was low (i.e., AUC = 0.54 – 0.57). Better results were found for TAG on d 2 postpartum (i.e., AUC = 0.84; Se = 0.77; Sp = 0.86; PPV = 0.77; NPV = 0.87).

Neither pre- nor postpartum NEFA concentrations could be predicted utilizing HDL (P > 0.05).

The prediction of NEFA by only one parameter was not sufficient, because either Se, Sp, or PPV was low. With a low Se, a high percentage of ill cows would not be found and remain without treatment. With a low specificity, a high percentage of healthy cows would be identified as ill and falsely treated.

The weak correlation between transition period NEFA and BHBA is in accordance with the results found by McCarthy et al. (2015) who identified a low correlation between postpartum NEFA AUC and postpartum BHBA AUC (r = 0.26). No significant correlation was found between prepartum BHBA AUC and prepartum NEFA AUC (P > 0.05). Although early lactation studies suggested that the peak of mean NEFA concentration occurs before the peak of the mean BHBA (Hammon et al. 2009; Janovick et al. 2011), McCarthy et al. (2015) observed that the largest proportion of cows had maximum NEFA and BHBA concentrations on the same day. It was suggested that the reason for the low correlation might be that ketone synthesis not only depends on energy balance and availability of intermediates in the Krebs cycle, but could also be related to individual differences in hepatic gluconeogenic capacity (Reynolds et al. 2003; McCarthy et al. 2015). Furthermore, sources of carbon other than NEFA (e.g., lactate and ketogenic amino acids) contributing to BHBA synthesis might be an

explanation for the weak relationships between blood concentrations of NEFA and BHBA (McCarthy et al. 2015). The low associations between NEFA, cholesterol and HDL, respectively, might be due to physiological changes in lipid metabolism during the transition period. Kurpińska et al. (2015) observed a decrease of total cholesterol and HDL in the last month of pregnancy and an increase in the first two months of lactation. An increased requirement of foetal tissues as well as maternal glands for steroid hormones synthesis is likely to be the reason for the decrease of cholesterol during the last stage of pregnancy (Turk et al. 2005). In contrast to cholesterol and HDL, the concentration of TAG was rather stable during lactation (Kurpińska et al. 2015).

For the second part of our analysis, we combined the parameters mentioned above in order to achieve higher AUC, Se and Sp. Because a correlation between NEFA and HDL was not found and the prediction of a certain NEFA concentration by ROC analysis was not significant, HDL was not used in these analyses and the dummy variable was only calculated as the sum of BHBA, cholesterol and TAG (Table 2). A NEFA concentration ≥ 0.5 mEq/L between d 3 and d 1 prepartum could be predicted with a high reliability (i.e. Se = 0.88; Sp = 0.93; AUC = 0.93), if two or more parameters exceeded the calculated cut points (i.e., BHBA ≥0.7 mmol/l; cholesterol ≥1.76 mmol/l; TAG ≥0.39 mmol/l). After calving predictive values improved as well compared to the calculations using only one parameter (Table 2). After calving, the highest Se and Sp was found on d 2 postpartum for the prediction of a NEFA concentration ≥ 1.0 mEq/L by a combination of BHBA ≥0.7 mmol/l, cholesterol ≥0.9 mmol/l and TAG ≥0.15 mmol/l in which the parameters were treated equally (i.e. Se = 0.91; Sp = 0.76; AUC = 0.87).

If BHBA was chosen as a fixed parameter, results were inferior to the approach using all parameters in an equal combination as mentioned above. The highest Se and Sp (i.e. Se = 0.75; Sp = 0.94; AUC = 0.83) was found between d 3 and d 1 prepartum for the prediction of a NEFA concentration ≥ 0.5 mEq/L by a combination of BHBA ≥0.7 mmol/l, cholesterol ≥1.76 mmol/l and TAG ≥0.39 mmol/l.

In our last approach, BHBA was combined only with cholesterol, because it would be more convenient in practice to just measure two parameters instead of 3 and for both of which hand-held meters are commercially available. However, those results were overall lower than the equal combination approach. The highest Se and Sp (i.e. Se = 0.75; Sp = 0.94; AUC = 0.83) was found between d 3 and d 1 prepartum for the prediction of a NEFA concentration ≥ 0.5 mEq/L by a combination of BHBA ≥0.7 mmol/l and cholesterol ≥1.80 mmol/l.

Based on our data the equal combination of all three parameters (BHBA, cholesterol and TAG) showed the best results to determine if NEFA concentrations exceeded a certain threshold.

The approach to combine different variables to improve ROC analysis was used in other studies before. In human medicine Pepe et al. (2006) combined multiple biomarkers for cancer in order to maximize the AUC. In another study, clinical signs were combined and a standard operating procedure established in order to predict the time of parturition in cattle (Streyl et al. 2011). For the prediction of NEFA the combination of multiple surrogate measures (i.e. BHBA, cholesterol and TAG) has not been used before, but could be an alternative to the prediction by only one parameter. For some of our calculations we used BHBA as a fixed parameter because hand-held meters for BHBA have been already validated for the use in cattle. However, we found that an equal combination of BHBA, cholesterol and TAG generated better results and thus the use of hand-held meters for cholesterol and TAG in cows should be considered. Hand-held meters for cholesterol and TAG are already validated for the use in human medicine (Ferreira et al. 2015; Coqueiro et al. 2013) and have been used in cats to measure blood TAG concentrations (Kluger et al. 2010). For our purpose, however, the measuring ranges in human medicine are too high (Kluger et al. 2010) and have to be adapted to cows. Therefore, in our study the concentration of cholesterol and TAG was measured by laboratory analysis. We are aware of the fact that this method is not suitable for daily use in practice. However, we suggest that further research for the usage of human medicine hand-held meters for cholesterol and TAG in cows should be conducted based on our findings.

The objective of this study was to determine if 4 parameters related to lipid metabolism (i.e., BHBA, cholesterol, TAG and HDL), for which hand-held meters are commercially available for cows (i.e. BHBA) and in human medicine (i.e. cholesterol, TAG and HDL), correlate with the concentration of NEFA and to evaluate if those meters could potentially serve as surrogate measures for NEFA. In conclusion, prepartum a NEFA concentration ≥0.5 mEq/L could be predicted with a high accuracy when using a combination of BHBA, cholesterol and TAG as surrogates. Although this method would not predict the exact NEFA value, it can predict if NEFA is over a certain threshold and therefore provides insight in the lipid metabolism and helps to identify high risk cows. Further investigations about the usage of hand-held meters for cholesterol and TAG which are only used and validated in human medicine so far are necessary before this method can be applied in practice considering the test characteristics of the hand-held meters. The study was conducted

on only one farm and breed during a relatively short season. These circumstances are a limitation and further research is warranted considering multiple farms and longer time periods. In the future, the combination of BHBA, cholesterol and TAG might serve as an alternative to laboratory analysis in order to predict if NEFA concentrations are above a certain threshold.

Table 1. Coefficients of correlation between the concentration of nonesterified fatty acids (NEFA in mEq/L) and the concentrations of β-hydroxybutyrate (BHBA in mmol/L), cholesterol (Chol in mmol/L), triglycerides (TAG in mmol/L) and high-density lipoproteins (HDL in mmol/L).

Time relative to calving	Number of matched pairs	Parameter	Coefficient of correlation	*P*-value
Days before calving				
-10 to -1	181	BHBA	0.15	0.049
	181	Chol	0.49	<0.001
	181	TAG	0.35	<0.001
	181	HDL	0.01	0.890
-3 to -1	91	BHBA	0.27	0.010
	91	Chol	0.48	<0.001
	91	TAG	0.23	0.027
	91	HDL	0.01	0.893
Days after calving				
2	246	BHBA	0.29	<0.001
	246	Chol	0.61	<0.001
	246	TAG	0.67	<0.001
	246	HDL	-0.03	0.680
10	245	BHBA	0.44	<0.001
	245	Chol	0.39	<0.001
	245	TAG	0.50	<0.001
	245	HDL	0.04	0.520

Table 2. Receiver-operator characteristic curve determination of critical β-hydroxybutyrate (BHBA in mmol/L), cholesterol (Chol in mmol/L) and triglycerides (TAG in mmol/L) thresholds as predictors of nonesterified fatty acid (NEFA in mEq/L) concentrations considering a combination in which β-hydroxybutyrate, cholesterol and triglycerides were treated equally.

Time relative to calving	NEFA	BHBA	Chol	TAG	AUC[1]	Se[2]	Sp[3]	PPV[4]	NPV[5]	Crit.[6]	*P*-value
Days before calving											
-10 to -1	≥0.3	≥0.4	≥1.37	≥0.11	0.66	0.74	0.54	0.30	0.89	3	<0.001
	≥0.5	≥0.7	≥1.76	≥0.43	0.82	0.70	0.92	0.33	0.98	2	<0.001
-3 to -1	≥0.3	≥0.4	≥1.35	≥0.11	0.69	0.79	0.56	0.44	0.85	3	<0.001
	≥0.5	≥0.7	≥1.76	≥0.39	0.93	0.88	0.93	0.54	0.99	2	<0.001
Days after calving											
2	≥0.7	≥0.5	≥1.40	≥0.11	0.89	0.89	0.76	0.69	0.92	2	<0.001
	≥1.0	≥0.7	≥0.90	≥0.15	0.87	0.91	0.76	0.47	0.97	2	<0.001
10	≥0.7	≥0.7	≥1.20	≥0.15	0.82	0.79	0.79	0.71	0.85	2	<0.001
	≥1.0	≥0.7	≥0.98	≥0.20	0.76	0.84	0.64	0.38	0.94	2	<0.001

[1]AUC = area under the curve
[2]Se = sensitivity
[3]Sp = specificity
[4]PPV = positive predictive value
[5]NPV = negative predictive value
[6]Crit. = number of parameters above threshold

2.1.5 **Acknowledgements**

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2.2 Effects of Hyperketonemia within the First Six Weeks of Lactation on Milk Production and Reproductive Performance

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2.2.1 **Abstract**

Hyperketonemia is a common disease in early lactating dairy cows and diagnosed by measurement of blood β-hydroxybutyric acid. The objectives of our study were to describe the occurrence of hyperketonemia within the first six weeks of lactation and to evaluate the effects of hyperketonemia on milk production (1st test day milk yield and 100 DIM milk yield), reproductive performance (time to first service, first service conception risk, and time to pregnancy within 200 DIM) and early lactation culling risk.

A total of 655 Holstein dairy cows from 6 commercial dairy farms in Germany were enrolled between 1 and 4 DIM. Cows were tested twice weekly using an electronic handheld meter for β-hydroxybutyric acid for an examination period of 42 days resulting in 12 test results per cow. Hyperketonemia was defined as a β-hydroxybutyric acid concentration ≥ 1.2 mmol/l. The onset of hyperketonemia was described as early onset (first hyperketonemia event within the first 2 weeks postpartum) and late onset (first hyperketonemia event in week 3 to 6 postpartum). Prevalence and incidence of hyperketonemia were assessed based on the 12 examinations.

Cumulative incidence of hyperketonemia was 48% and 72% for primiparous and multiparous cows, respectively. Mean prevalence was 17.5%. Early onset ketotic cows had a higher 1st test day milk yield (+ 3.0 kg/d, $P < 0.001$) and 100 DIM milk production (+ 301.6 kg; $P < 0.001$) compared to non-ketotic cows. There was no effect of late onset of ketosis on milk production. There were no effects of hyperketonemia on reproductive performance and culling risk, irrespective of onset of ketosis.

Keywords: Hyperketonemia; Transition; Dairy cow; Adaptation; Milk production; Reproduction

2.2.2 **Introduction**

The transition period of dairy cows is often accompanied by hyperketonemia (HYK) caused by a poor adaptation to negative energy balance [1]. Negative energy balance (NEB) is a result of an imbalance between energy input and output because the energy requirements for milk production and maintenance exceed the available energy from feed intake [2]. Some authors speculated that there are two different types of HYK differing in their onset and pathophysiology [3, 4]. Type I ketosis was described as spontaneous or underfeeding ketosis. It occurs 3 to 6 weeks postpartum when milk secretion is so extensive that the demand for glucose exceeds the capacity for gluconeogenesis in the liver. The plasma levels of glucose and insulin are low, the levels of ketone bodies are high. Type II generally occurs earlier in lactation as a result of insulin resistance and excessive body fat mobilization prior to or at calving and is also known as "fat cow syndrome". Blood insulin and glucose concentrations are high, whereas blood ketone concentrations are lower in type II ketosis than in type I [3, 5].

Several studies evaluated the effects of HYK on milk yield [6-10] and reproductive performance [7, 9, 11] within 2 wk after parturition. Most studies showed that milk production in cows with HYK in early lactation is decreased compared to cows with lower β-hydroxybutyric acid (BHBA) values [1]. It was also shown, however, that in the long-term HYK was not associated with a milk loss but that cows with HYK within the first 2 wk postpartum produced more milk across four Dairy Herd Improvement Association (DHIA) tests [8] and that cows with HYK in the second week postpartum produced more milk at the third DHIA test [6].

No consistent results have been found for the evaluation of associations between elevated postpartum BHBA and reproductive measures [1]. An odds ratio (OR) of 0.7 was found for the outcome of pregnancy to 1st service for a BHBA cut-point ≥ 1.0 mmol/L ($P = 0.04$) [11]. The results of other studies investigating the association between BHBA (for BHBA ≥ 1.0 or 1.2 mmol/L) and pregnancy to 1st service [8-10] and the time to pregnancy [7, 9] were not significant ($P > 0.05$).

The first 14 to 16 days after calving have been described as the main risk period for hyperketonemia [9, 12]. It was shown, however, that the period in which early lactation dairy cows are at risk for hyperketonemia lasts at least until 42 days in milk (DIM) [13]. To the author's knowledge, there is limited evidence for the effect of HYK from week 3 to 6 on milk production, reproduction, and health.

Therefore, the objectives of our study were (1) to describe the occurrence of hyperketonemia within the first six weeks of lactation, (2) to evaluate the effect of onset of hyperketonemia within the first six weeks of lactation on milk production and reproduction and (3) to evaluate the effect of onset of hyperketonemia within the first six weeks of lactation on early lactation culling risk.

2.2.3 **Materials and Methods**

A total The experimental procedures reported herein were conducted with the approval of the Institutional Animal Care and Use Committee of Freie Universität Berlin. Cows were managed according to the guidelines set by the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medical Products [14].

Study Population

This study was conducted between June 2013 and July 2015. A total of 655 Holstein dairy cows from 6 different commercial dairy farms in Brandenburg, Sachsen and Sachsen-Anhalt, Germany, were included in the trial. All farms kept at least 600 dairy cows in freestall barns, fed a total mixed ration (TMR) and recorded medical treatments using computer based farm management programs (HerdeW, version 5.8, dsp-Agrosoft Ltd., Ketzin, Germany). The energy-corrected milk yield (ECM) was calculated using the following formula: ECM (kg) = (0.38 × fat %+0.21 × protein %+1.05)/3.28 × milk yield (kg) [15].

Data of farms 1 to 3 were reported previously [13]. Farm 4 kept approximately 1,600 cows in a freestall barn with slatted floors and cubicles equipped with rubber mats. Feed was delivered once daily and pushed up 3 times a day. Cows were milked 2 times a day in a rotary parlor. The average annual milk yield was 8,465 kg (ECM). Farm 5 kept approximately 1,300 cows in a freestall barn with concrete solid floors and cubicles equipped with rubber mats. Feed was delivered once daily and pushed up 5 times a day. Cows were milked 3 times a day in a side-by-side parlor, the average annual milk yield was 9,509 kg (ECM). Farm 6 kept approximately 1,400 cows in a freestall barn with slatted floors and cubicles equipped with rubber mats. Feed was delivered over a conveyer belt system 6 to 8 times per day. Cows were milked 3 times a day in a rotary milking parlor, the average annual milk yield was 9,785 kg (ECM). Some cows received a prophylactic treatment against ketosis (Table 1).

Milk Recording

All participating herds were enrolled in a DHIA milk recording program in which milk production and other data were recorded on-farm at intervals of approximately 35 to 40 d. We used test day milk yield 1 to 4 and a projection of milk yield in the first 100 DIM based on test day milk yield 1 to 3.

Reproductive Management

The management of reproduction of farms 1 to 6 is shown in Table 2. Pregnancy diagnosis was performed by the herd veterinarian using transrectal ultrasonography between day 28 and 42 after breeding. A positive pregnancy diagnosis was based on visualization of an embryo with a heartbeat.

Breeding dates and results of pregnancy diagnoses were obtained from the on-farm computer system.

Experimental Design

Cows were enrolled between 1 and 4 DIM and were tested for BHBA twice weekly at intervals of 3 to 4 days for an examination period of 42 days, resulting in 12 test results per cow. The term lactation week was used to describe the time of sample collection relative to calving (e.g., lactation week 0.5 for the first measurement on DIM 1 to 4, lactation week 1 for the second measurement on DIM 4 to 7, lactation week 1.5 for the third measurement on DIM 8 to 11). Hyperketonemia was defined as a BHBA concentration ≥ 1.2 mmol/l. The onset of HYK was categorized as early (first HYK event within lactation week 0.5 to 2.0) and late onset (first HYK event within lactation week 2.5 to 6.0).

The collection of blood samples and the measurement of BHBA by an electronic BHBA meter (NovaVet, Nova Biomedical, Waltham, USA) were described in the study by Mahrt et al. [13] for farm 1 to 3. The same methods were used for farm 4 to 6.

A total of 32 cows were excluded from analysis because they received a treatment of intravenous applications of a butafosfan-cyanocobalamin combination (Catosal, Bayer Animal Health, Leverkusen, Germany), dextrose or dexamethasone (Dexatat ad us.vet., aniMedica, Senden-Bösensell, Germany), oral administration of propylene glycol, monensin (Kexxtone, Elanco Animal Health, Hampshire, UK) or combinations of these therapies as

effects on blood BHBA concentrations are possible [16]. Animals that received routinely prophylaxes with dextrose or dexamethasone against disorders in energy metabolism on DIM 0 or 1 (n = 115) were not excluded. The exclusion of cows due to treatment was discussed in more detail in a previous study by Mahrt et al. [13]. Farms monitored during this study implemented different feeding and disease prevention strategies. This is inevitable when conducting a study on multiple commercial dairy farms, as has been discussed previously [17] and is comparable to the situation in the field. Furthermore, two cows were excluded due to an abortion. After exclusion, 621 cows (195 primiparous; 426 multiparous) were used for the final analyses.

For the analysis of milk production and reproductive performance some cows had to be excluded due to incomplete data recording on farm, culling, or the decision to not breed anymore. In total, 593 and 533 cows were analyzed for 1st test day milk yield and 100 DIM milk production, respectively. For the evaluation of reproduction 614, 558 and 544 cows were analyzed for insemination within 100 DIM, first service conception risk and pregnancy within 200 DIM, respectively.

For the evaluation of incidence, number of HYK events per cow and time from first positive HYK test to one blood BHBA concentration of < 1.2 mmol/L only cows with a complete set of test results (i.e. 12 tests) were considered. In total, 458 cows had 12 test results and were considered for the analysis of incidence and number of HYK events. Of those cows, 294 cows were ketotic and were considered in the analysis for time from first positive HYK test to one blood BHBA concentration of < 1.2 mmol/L.

Statistical Analysis

Data from the cowside evaluation of BHBA and data from the on farm computer system were exported to Excel spreadsheets and analyzed using SPSS for Windows (version 22.0, SPSS Inc., IBM, Ehningen, Germany).

To evaluate the effect of ketosis status on milk yield in early lactation linear regression analysis was performed using the GENLINMIXED procedure of SPSS. The outcome variable was either 1st test day milk yield (kg/ d) or a projection of cumulative 100 d milk yield (kg). Cow was the experimental unit and herd was considered as a random effect. According to the model-building strategies described by Dohoo et al. [18] each parameter considered for the mixed model should be separately analyzed in a univariate model, including the parameter as a fixed factor (i.e., categorical parameter) or covariate (i.e., continuous parameter). Only

parameters resulting in univariate models with $P \leq 0.2$ should be included in the final mixed model. The initial model contained the following explanatory variables as fixed effects: ketosis status (non-ketotic, early onset, late onset), parity (primiparous vs. multiparous), and DIM at test day. Selection of the model that best fit the data was performed by testing each effect separately in a univariate model and finding the model with the lowest value for the Akaike information criterion using a backward elimination procedure that removed all variables with $P > 0.10$ from the model. Regardless of the significance level ketosis status was forced to remain in the model.

The analysis of pregnancy per artificial insemination at first artificial insemination (AI) was performed by logistic regression using the GENLINMIXED procedure of SPSS. Cow was the experimental unit and herd was considered as a random effect. The initial model contained the following explanatory variables as fixed effects: ketosis status (non-ketotic, early onset, late onset) and parity (primiparous vs. multiparous). Model building and selection of the model that best fit the data was performed as described earlier. Regardless of the significance level ketosis status was forced to remain in the model.

Survival analysis was conducted to evaluate the effect of ketosis status on the hazard of insemination within 100 DIM and pregnancy within 200 DIM by creating Cox semiparametric proportional hazard model using SPSS. The outcome variable for the Cox model for insemination within 100 DIM was DIM at first insemination and for pregnancy within 200 DIM was the DIM at which cows conceived. The models included ketosis status, herd, and parity as categorical explanatory variables. Selection of the model that best fit the data was performed by using a backward elimination procedure that removed all variables with $P > 0.10$ from the model. Regardless of the significance level, ketosis status was forced to remain in the model.

Survival analysis was conducted to evaluate the effect of ketosis status on the hazard of culling (live or dead) within the study period (42 DIM) by creating Cox semiparametric proportional hazard model using SPSS. The outcome variable was DIM at culling within 42 DIM. The models included ketosis status, herd, and parity as categorical explanatory variables. Selection of the model that best fit the data was performed as described earlier. Regardless of the significance level, ketosis status was forced to remain in the model.

Kaplan-Meier survival analysis curves were constructed to illustrate the rate at which cows resolved HYK, received first AI, conceived until 200 DIM, and were culled within 42

DIM by using the Kaplan-Meier survival analysis option of MedCalc (version 12.5.0.0, MedCalc Software, Mariakerke, Belgium).

A significant difference between the levels of a classification variable was declared when $P < 0.05$, whereas differences between $P \geq 0.05$ and $P \leq 0.10$ were considered a statistical tendency.

2.2.4 **Results**

Primiparous Of 655 cows enrolled in the trial, 34 (5.2%) were excluded from analysis as described above; 621 cows remained for final analysis. Of these cows, 195 (31.4%) were in first lactation, 173 (27.9%) were in second lactation, and 253 (40.7%) were in the third or greater lactation. Overall, 235 cows were non-ketotic, 224 cows were early onset-ketotic, and 162 cows were late onset-ketotic.

Occurrence of HYK within the First Six Weeks of Lactation

Only cows with 12 test results were considered in the analysis for incidence (n = 458). Cumulative incidence of HYK was 48% and 72% for primiparous and multiparous cows, respectively. The peak incidence of HYK for primiparous cows occurred in wk 1.0 of lactation whereas for multiparous cows it was found in wk 2.5 (Figure 1).

Mean prevalence was 17.5%, ranging from 9.9% in lactation wk 0.5 and to 20.8% in lactation wk 4.5. Overall prevalence stayed on the same level within the first six weeks of lactation (Figure 2). Peak prevalence occurred in week 4.5 and 2.5 for primiparous (15.1%) and multiparous cows (25.5%), respectively. Multiparous cows had a higher mean prevalence of HYK (21%) within the first six weeks of lactation than primiparous cows (10%).

The number of HYK events within the first six weeks of lactation is shown in Figure 3. Only cows with 12 test results (n = 458) were considered. Of those, 164 cows (35.8%) had no positive BHBA test result within the first six weeks of lactation, 104 cows (22.7%) had one event of HYK only. A total of 50 cows (10.9%) had 6 or more HYK events. Figure 4 shows the time from the first positive test for HYK (i.e., BHBA concentration ≥ 1.2 mmol/L) to one blood BHBA concentration of <1.2 mmol/L in 294 cows with HYK. Only ketotic cows with 12 test events were included. Because of the testing scheme (i.e., Tuesday and Friday or Monday and Thursday, respectively) time between two test events was either 3 or 4 days. In

total, 72% of HYK positive cows were test negative 3 to 4 days after the initial diagnosis of HYK.

Effect of Onset of HYK within the First Six Weeks of Lactation on Milk Production

In total, 593 and 533 cows were used for the analysis for the 1st test day milk production and the 100 DIM milk production, respectively.

The effects of onset of HYK on 1st test day and 100 DIM milk production are shown in Table 3 and 4, respectively. Early onset ketotic cows produced 3.0 kg more milk on the 1st test day ($P < 0.001$) and 301.6 kg more milk in the first 100 d of lactation ($P < 0.001$) than non-ketotic cows. There was no significant effect of late onset of ketosis on 1st test day milk yield (+ 1.2 kg/d; $P = 0.1$). Cows with late onset of ketosis showed a trend to a higher 100 DIM milk yield (+ 118.6 kg; $P = 0.075$) than non-ketotic cows.

Graphs of milk yield by test day for non-ketotic, early onset and late onset ketotic cows were plotted for DHIA test 1 to 4 for primiparous and multiparous cows, respectively (Figures 5 and 6). Both, early and late onset of HYK were associated with an increased milk yield on each DHIA test day. This applied for primiparous as well as for multiparous cows. The increase in milk production was higher for early onset HYK than for late onset HYK.

Effect of Onset of HYK within the First Six Weeks of Lactation on Reproduction

The association between ketosis status and reproductive performance was evaluated using the time to first service within 100 DIM, first service conception risk (FSCR), and time to pregnancy within 200 DIM.

In total, 614 cows had data concerning insemination within 100 DIM. The risk for insemination within 100 DIM did not differ between early onset ketotic cows (HR = 0.9; 95% CI = 0.7 to 1.1; $P = 0.110$) and late onset ketotic cows (HR = 1.0; 95% CI = 0.8 to 1.3; $P = 0.976$) compared to non-ketotic cows.

A total of 558 cows were used in the analysis for the effect of ketosis status on FSCR. An odds ratio < 1 indicates a reduced risk for pregnancy at first AI. Multiparous cows had a lower risk for conceiving at first artificial insemination compared to primiparous cows (OR = 0.64, 95% CI = 0.4 to 1.0; $P = 0.030$). There was no effect of HYK on the risk for conceiving

at first artificial insemination, neither for early onset-ketotic cows (OR = 0.9; 95% CI = 0.6 to 1.4; P = 0.604) nor for late onset-ketotic cows (OR = 1.0; 95% CI = 0.6 to 1.6; P = 0.945).

A total of 544 cows were used for analysis of pregnancy risk within 200 DIM. The risk to become pregnant within 200 DIM did not differ between ketotic and non-ketotic cows (Table 5). Risk for pregnancy within 200 DIM was not decreased for early onset ketotic cows (HR = 0.8; 95% CI = 0.7 to 1.1; P = 0.197) nor for late onset ketotic cows (HR = 1.0; 95% CI = 0.8 to 1.3; P = 0.821) compared to non-ketotic cows.

Effect of Onset of HYK within the First Six Weeks of Lactation on Early Lactation Culling Risk

The association between ketosis status and culling risk within 42 DIM was evaluated for 621 cows using survival analysis. Culling risk was comparable for early onset ketotic cows (HR = 1.5; 95% CI = 0.8 to 2.7; P = 0.223) compared to non-ketotic cows. Cox proportional hazard analysis for culling risk determined that culling risk for cows with late onset HYK was numerically lower (HR = 0.4; 95% CI = 0.1 to 1.0; P = 0.062).

Table 1. Description of the 6 study herds.

	Herd					
Parameter	1	2	3	4	5	6
Enrolled cows, n	124	121	60	136	104	110
Herd size, n	1,200	1,800	600	1,600	1,300	1,400
Annual milk production[1], kg	10,733	10,900	11,127	8,465	9,509	9,785
Ketosis prophylaxis	All cows: TMR including 300 g glycerol per cow/d and oral 300 g dextrose at 0 DIM 3rd lactation and higher: 0.04 mg/kg dexamethasone at 0 DIM	All cows: TMR including 300 g glycerol per cow/d 3rd lactation and higher:160 g dextrose per cow intravenous at 0 DIM	All cows: TMR including 300 g propylene glycol per cow/d during the first 21 DIM	2nd lactation and higher: oral 35 g calcium propionate and 21 g sodium propionate at 0 DIM	2nd lactation and higher: 500 ml dextrose (40%) intravenous on 1st DIM	All cows: TMR including 100 g propylene glycol and 150 g glycerol per cow/d for cows between 7-42 DIM

[1]Energy corrected milk (4.0% fat; 3.4% protein)

Table 2. Description of reproductive management.

	Herd					
Parameter	1	2	3	4	5	6
VWP[1]	50	50	42	40	42	42
TAI (DIM)[2]	Presynch (70)	No	No	No	Ovsynch (80)	No
21d pregnancy rate	13	15	21	17	13	24
Time of PD[3]	35	30	28	38	39	42

[1]VWP = Voluntary Waiting Period

[2]TAI (DIM) = Synchronization protocol for the first AI and responding DIM of fixed AI.

[3]Time of pregnancy diagnosis in days after insemination

Table 3. Effect of ketosis status within the first six weeks of lactation on 1st test day milk production in Holstein dairy cows (n = 593).

Variable	Estimate[1]	SE[2]	95% Confidence Interval		*P*
	Kg/d		Lower CI	Upper CI	
Parity					
Primiparous	Referent				
Multiparous	+ 9.4	0.6	+ 8.1	+ 10.6	< 0.001
Ketosis status[3]					
Non-ketotic	Referent				
Early onset	+ 3.0	0.7	+1.6	+ 4.4	< 0.001
Late onset	+ 1.2	0.8	- 0.2	+ 2.7	0.1

[1] Model adjusted for the random effect of herd and the fixed effect of parity.

[2] SE = Standard error of the estimate.

[3] Ketosis status: Non-ketotic = no HYK event in the first six weeks of lactation; Early onset = first HYK event within the first two weeks; Late onset = first HYK event in week 3 to 6 postpartum

Table 4. Effect of ketosis status within the first six weeks of lactation on 100 DIM milk production in Holstein dairy cows (n = 533).

Variable	Estimate[1]	SE[2]	95% Confidence Interval		*P*
	kg		Lower CI	Upper CI	
Parity					
Primiparous	Referent				
Multiparous	+853.9	57.3	+741.3	+966.6	<0.001
Ketosis status[3]					
Non-ketotic	Referent				
Early onset	+301.6	64.1	+175.7	+427.6	<0.001
Late onset	+118.6	66.5	-12.0	+249.2	0.075

[1] Model adjusted for the random effect of herd and the fixed effect of parity.

[2] SE = Standard error of the estimate.

[3] Ketosis status: Non-ketotic = no HYK event in the first six weeks of lactation; early onset = first HYK event within the first two weeks; late onset = first HYK event in week 3 to 6 postpartum.

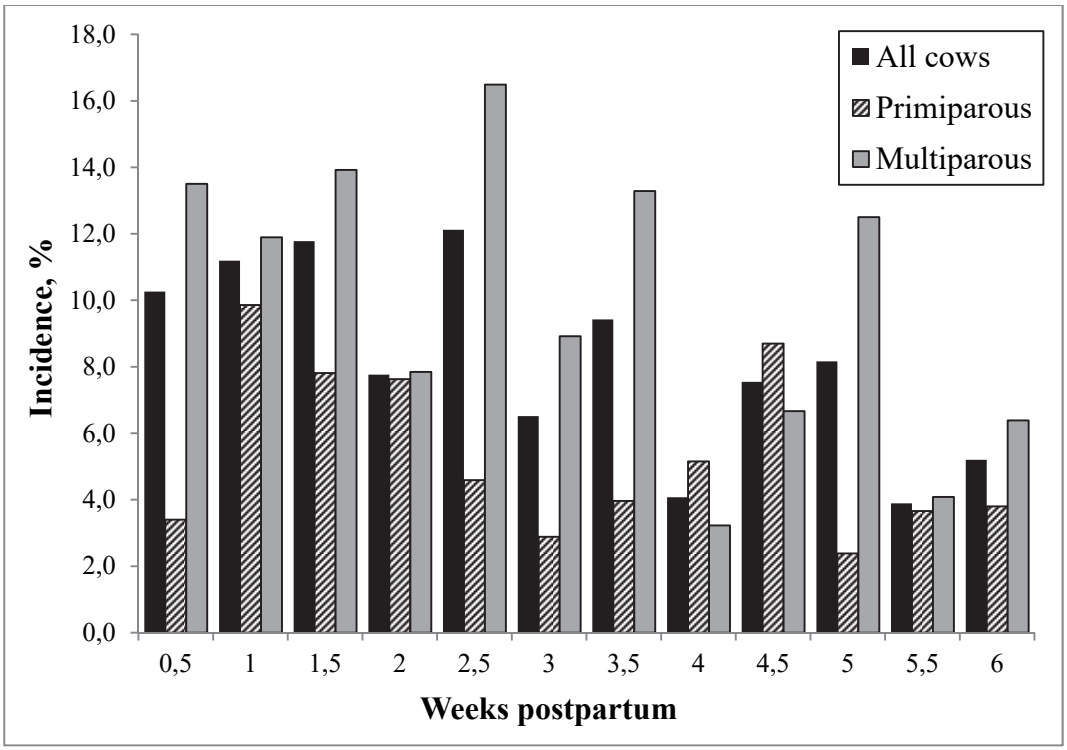
Table 5. Cox proportional hazard analysis for pregnancy within 200 DIM for 544 Holstein dairy cows.

Variable	Estimate	SE[1]	*P* - value	HR[2]	95% CI
Parity					
Primiparous	Referent				
Multiparous	- 0.279	0.109	0.011	0.757	0.611 – 0.937
Ketosis status[3]					
Non-Ketotic	Referent				
Early onset	- 0.164	0.127	0.197	0.848	0.662 – 1.088
Late onset	- 0.030	0.131	0.821	0.971	0.753 – 1.252

[1] SE = Standard error of the estimate.

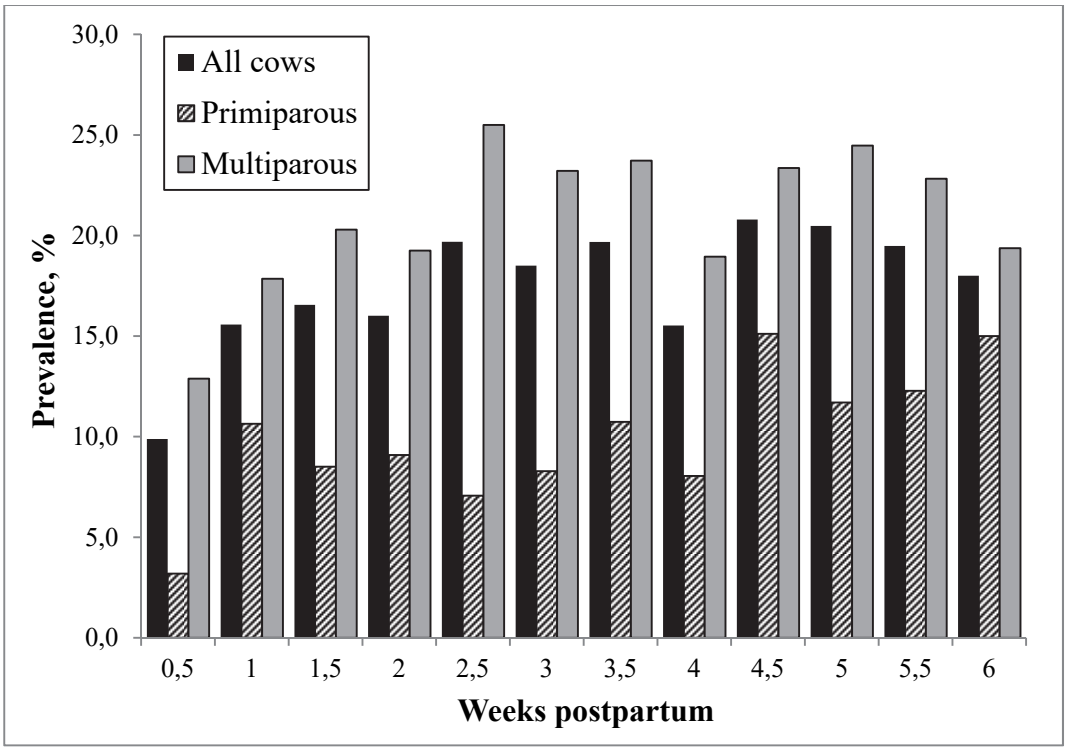
[2] HR = Hazard ratio for becoming pregnant within 200 DIM. HR < 1 indicating decreased risk for pregnancy within 200 DIM.

[3] Ketosis status: Non-ketotic = no HYK event in the first six weeks of lactation; early onset = first HYK event within the first two weeks; late onset = first HYK event in week 3 to 6 postpartum.



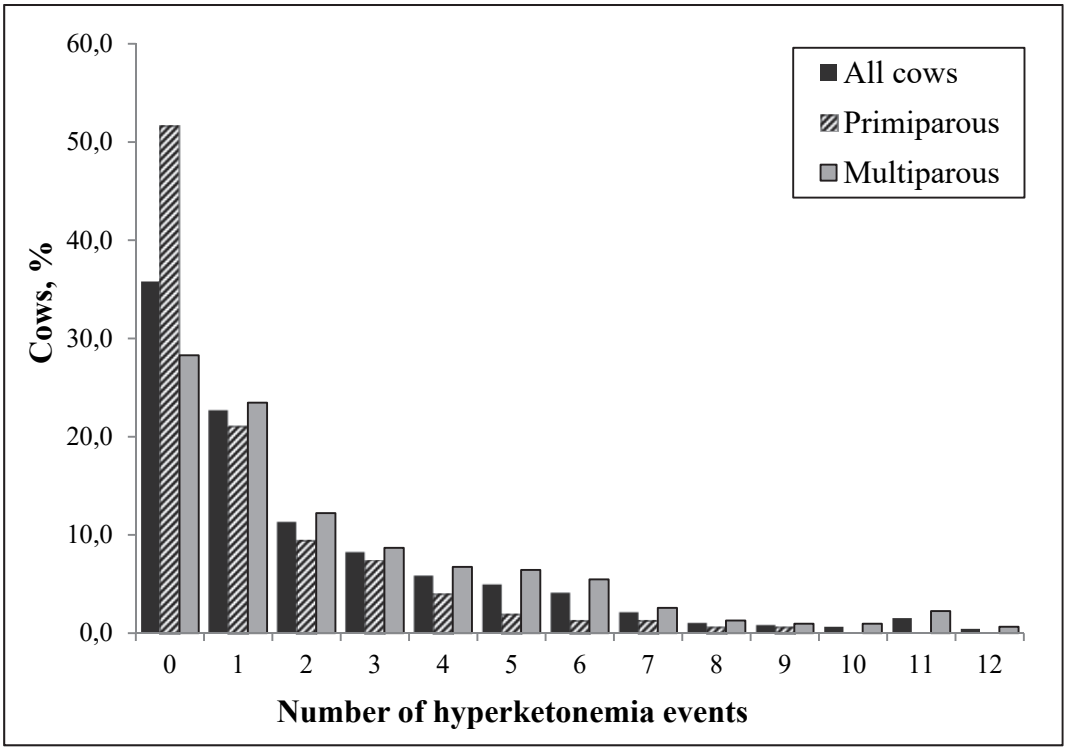
Ruoff. Figure 1

Figure 1. Incidence of hyperketonemia (BHBA concentration ≥ 1.2 mmol/L) in primiparous, multiparous, and overall Holstein dairy cows during the first 6 wk of lactation (n = 458).



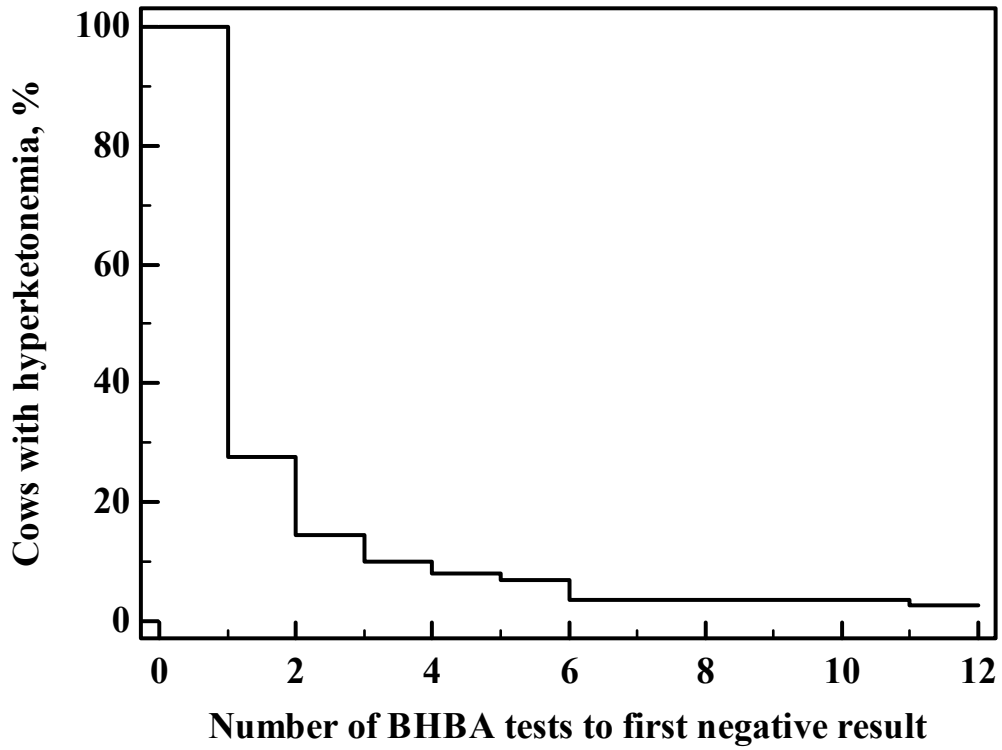
Ruoff. Figure 2

Figure 2. Prevalence of hyperketonemia (BHBA concentration ≥ 1.2 mmol/L) in primiparous, multiparous, and overall Holstein dairy cows during the first 6 wk of lactation (n = 621).



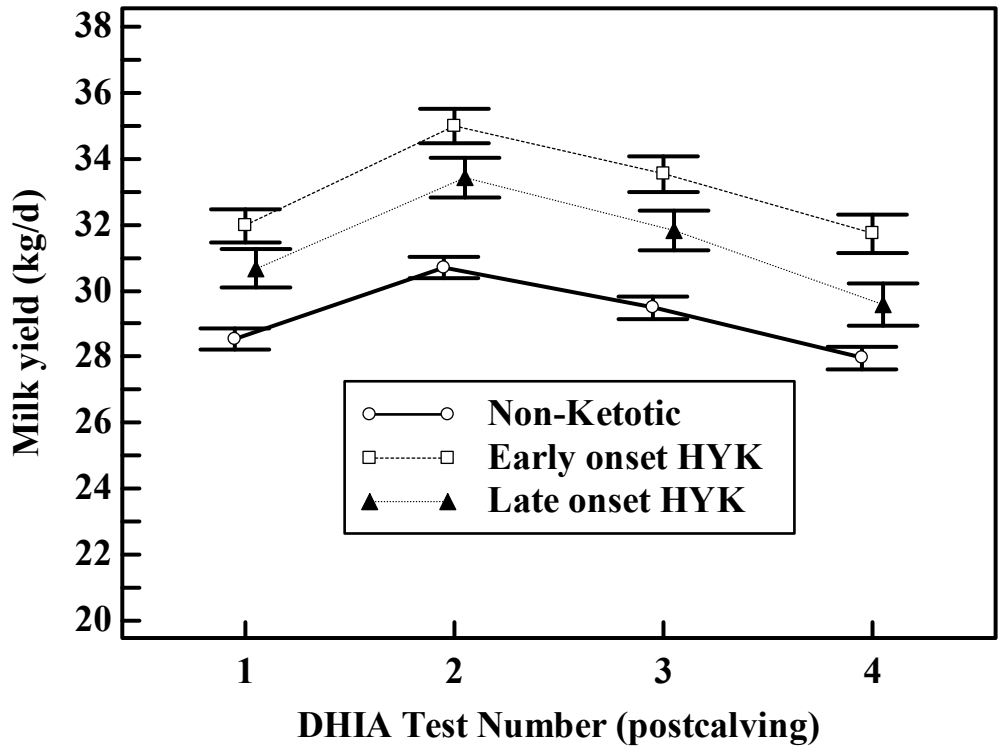
Ruoff. Figure 3

Figure 3. Number of hyperketonemia (BHBA concentration ≥ 1.2 mmol/L) events per cow within the first 6 weeks of lactation (n = 458).



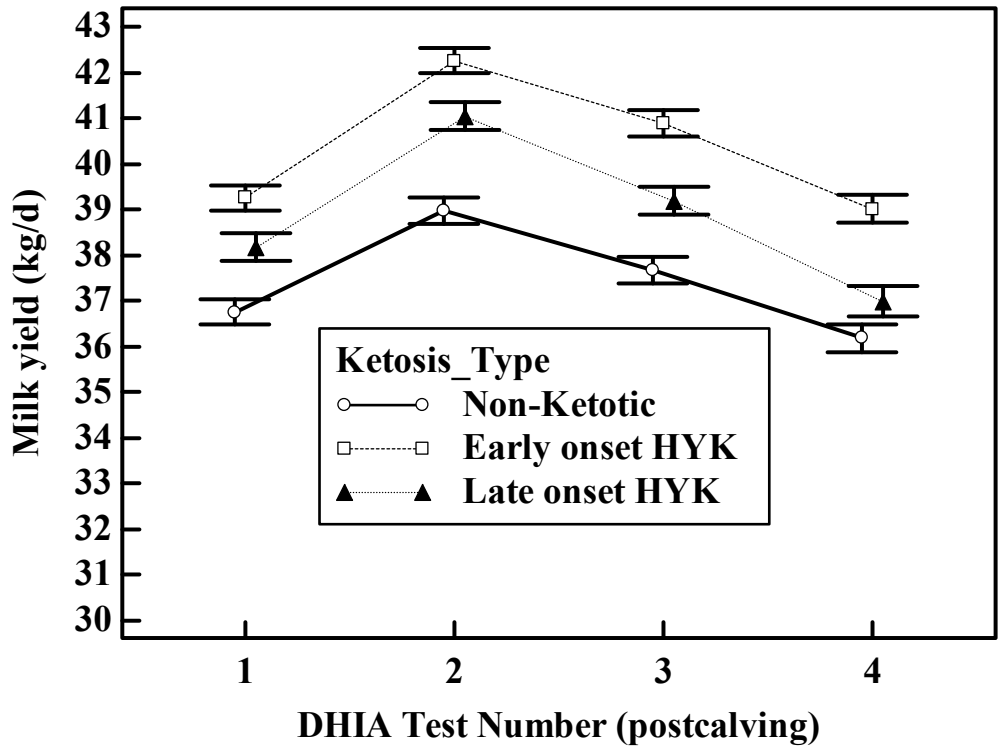
Ruoff. Figure 4

Figure 4. Kaplan-Meier curve of time from first positive test for hyperketonemia (BHBA concentration ≥ 1.2 mmol/L) to one blood BHBA concentration of < 1.2 mmol/L in 294 Holstein dairy cows undergoing repeated testing for ketosis in the first 6 weeks postpartum.



Ruoff. Figure 5

Figure 5. Least squares means ± SE of milk yield (kg/d) from the mixed procedure in primiparous cows, stratified by test day for non-ketotic cows (n = 102), cows with early onset (n = 50; wk 0 to wk2), and cows with late onset of hyperketonemia (n = 44; wk 3 to wk 6).



Ruoff. Figure 6

Figure 6. Least squares means ± SE of milk yield (kg/d) from the mixed procedure in multiparous cows, stratified by test day for non-ketotic cows (n = 140), cows with early onset (n = 199; wk 0 to wk2), and cows with late onset of hyperketonemia (n = 120; wk 3 to wk 6).

2.2.5 **Discussion**

This study was conducted to describe the occurrence of early and late onset of HYK and to evaluate the effects of early and late onset of HYK on milk production, reproduction and culling risk. To our knowledge this is the first study that evaluates the effects of early and late onset of HYK within the first 6 wk of lactation.

Our results indicated that early onset HYK was associated with higher milk production. Cows were at risk for HYK for at least 6 wk postpartum. There were, however, no effects on reproductive performance and culling, irrespective of the onset of HYK.

Occurrence of Hyperketonemia

In our study, incidence, prevalence and the time from the first positive HYK event to a negative BHBA test (i.e., < 1.2mmol/L) event were evaluated for cows within the first 42 DIM. Incidence describes the percentage of cows with new cases of HYK in a group of cows frequently tested during the risk period, whereas prevalence presents the percentage of all affected cows at a certain moment (i.e., a snapshot; [19]). Time to first negative test event provides information about the self-cure rate of HYK.

The cumulative incidence of HYK within 42 DIM was 48% and 72% for primiparous and multiparous cows, respectively. McArt et al. [20] reported an incidence of approximately 44% when cows were tested 3 times per week for 3-16 DIM. Duffield et al. [12] found a cumulative incidence of 59% when cows were tested once weekly for 1-63 DIM. Incidence depends on the frequency and time of testing. McArt et al. [9] speculated that Duffield et al. [12] most likely underestimated the true incidence as cows were only tested once weekly. The peak incidence in our study was found in wk 1.0 (4-7 DIM) for primiparous cows and in wk 2.5 (15-18 DIM) for multiparous cows. In a study by McArt et al. [1] in which cows were tested 3 times per week between 3 to 16 DIM the peak incidence was found on 5 DIM. Our results confirm that multiparous cows have a higher risk of HYK in early lactation. Also the results indicate that the risk for multiparous cows to develop new cases of HYK extends further into lactation. It has to be noted that the prophylactic treatment on some farms for multiparous cows could have influenced our results and that the true incidence for multiparous cows on the first testing could be higher than our results indicate. External factors that could influence metabolic conditions such as different feeding and disease prevention strategies on multiple commercial dairy farms have been discussed in detail in a previous study [13].

Different sampling times relative to feeding may also explain some variation among studies. However, it was shown that sampling time of continuously TMR fed dairy cows does not affect the concentration of BHBA [21].

The mean prevalence in our study was 17.5% which is close to the mean prevalence for Germany described by Suthar et al. [22] and in the middle of the prevalence range of 12% to 31% described in a recent review [1]. In our study the peak prevalence was found in wk 4.5 whereas McArt et al. [1] found the peak prevalence to be on 5 DIM. In that study, however, cows were tested until 16 DIM and the period further in lactation was not evaluated. In fact, prevalence increased in our study during the first 2 weeks of lactation and then stayed on a level of approximately 20% until 42 DIM.

Many cows (22.7%) tested positive for HYK had only one single HYK event and 72% of all HYK positive cows were tested negative within 3 to 4 d after the initial diagnose of HYK. These results indicate a high self-cure rate of HYK positive cows which should be considered in future studies when evaluating the effects of a treatment on the cure-risk of HYK. Instead of using only cure (BHBA < 1.2mmol/L) from HYK, studies should also use health and production outcomes with an economic value to the dairy farmer (e.g., reduction of disease or culling risk) to evaluate a treatment effect.

Furthermore, we wanted to investigate the theory of type I and II ketosis by Holtenius et Holtenius [3] as the theory has never been confirmed in a large scale study using commercial dairy herds. It was proposed that cows with elevated BHBA concentrations within the first 2 wk postpartum would most likely have type II ketosis which generally occurs earlier in lactation as a result of body fat mobilization prior to or at calving especially in obese cows [5]. Cows with elevated BHBA concentrations between 3 to 6 wk postpartum would probably have type I ketosis because milk secretion is so extensive that the demand for glucose exceeds the capacity of gluconeogenesis in the liver [3-5]. Our results indicate that early onset of HYK seems to be primarily an adaptational response to a high metabolic load caused by higher milk production in the beginning of lactation without the negative side effects on health.

Milk Production

Several studies evaluated the effect of HYK on milk production but findings were inconsistent. Some studies reported a decrease in milk yield for cows with BHBA ≥ 1.2 or 1.4 mmol/L [6, 8, 9]. Chapinal et al. [8] found, however, that the milk loss concerned only the

first DHIA test. Across four DHIA tests cows with HYK were found to produce more milk. A similar result was demonstrated by Duffield et al. [6] who showed that cows with HYK in wk 2 had a higher milk yield on the third DHIA test. McArt et al. [1] suggested that higher producing cows might have impaired production in the short term that might be overcome by mid-lactation. Our findings confirm that early onset HYK was associated with a higher 100 d milk production. In contrast to Chapinal et al. [8] and Duffield et al. [6], however, we found a higher milk production already on the first DHIA test for early onset ketotic cows. This observation is in accordance with a most recent study [23]. We suppose that the severity of SCK and the individual ability to adapt could be the reason for the inconsistent results. In our study some cows (n = 32) were excluded due to individual treatment decisions by the farm personnel. The exclusion of treated animals with clinical symptoms might have masked the negative effects of HYK. It was shown by Duffield et al. [6] that an increasing threshold of BHBA has a more negative impact on 305-d milk yield (i.e. 15.7 kg yield loss for BHBA ≥ 1.2 mmol/L vs. 333.7 kg yield loss for BHBA ≥ 1.8 mmol/L.

Hyperketonemia is part of a physiological strategy of ruminants of adaptation to NEB in early lactation [4]. In early lactation, an excessive BHBA concentration is considered as a continuous risk measure for maladaptation of energy and fat metabolism to negative energy balance [2]. Our intense testing schedule (i.e., twice a week for 6 wk) and the exclusion of some cows (e.g., treated cows) with more severe HYK might have led to the inclusion of cows that had a disproportionally high metabolic load (i.e., high milk production) but were able to cope with it. We can only speculate that the adaptational response to NEB is animal specific and that some cows with HYK might still be able to undergo a successful adaptation and consequently do not suffer from negative effects whereas other animals with a high metabolic load might not be successful. In the first case the cow would show HYK as a physiological adaptational response to NEB whereas in the second case the HYK is accompanied by negative outcomes such as diseases and reduced milk yield representing maladaptation to NEB.

Reproduction

Previous studies evaluating the association between HYK and reproductive performance are also inconsistent. A meta-analysis concluded that the precision of results regarding reproductive performance was low and that most relationships between different reproductive indicators were quantified in only 1 or 2 studies [24].

Walsh et al. [11] reported a decreased probability of pregnancy to 1st service for a BHBA concentration ≥ 1.0 mmol/L in week 1 postpartum (OR = 0.7; P = 0.04). In other studies, however, pregnancy to 1st service did not differ between ketotic and non-ketotic cows [8-10]. In our study we neither detected an effect of early onset HYK nor late onset of HYK on pregnancy to 1st service. The statistical power in our study for the effect of HYK on 1st service pregnancy risk, however, was limited due to the overall sample size and the different categories of HYK (i.e., early vs. late onset of HYK) and therefore has to be interpreted with caution.

To our knowledge, only one study evaluated time to first service [11] and reported that cows with HYK in either of the first 2 wk after calving were inseminated 8 d later than animals that were never HYK positive (HR = 0.85; P = 0.04). Our data do not confirm this relationship.

In the study by Walsh et al. [11] the median time to pregnancy for animals never classified as HYK positive was 108 d relative to 124 d for animals above the threshold in either wk 1 (BHBA ≥ 1.0 mmol/L) or wk 2 (BHBA ≥ 1.4 mmol/L). In other studies time to pregnancy was not different [7, 9].

Culling Risk

In contrast to other studies we did not find an effect of HYK on culling risk, neither for early nor for late onset of HYK. Several studies described that HYK cows were more likely to be removed from the herd [9, 25].

A possible explanation for the inconsistent results could be handling of severely sick cows in our study and previous studies. In our study sick cows were treated at the discretion of the farm personnel and consequently excluded form analysis. Those cows were likely to suffer more intensely from HYK as a direct result of a disease. Unfortunately, the reporting of those cows is unspecific in previous research.

It was shown in the past that a high milk yield is protective against culling [26]. In our study HYK was associated with high milk yield. As discussed above we suggest that cows with a high milk production can have HYK as a result of a successful adaptation without necessarily developing short term diseases that might lead to culling.

2.2.6 **Conclusions**

Hyperketonemia in the first 2 weeks of lactation was associated with increased milk production. We could not detect a negative impact of HYK on reproductive performance or culling risk in early lactation. As HYK was more pronounced in high producing cows it seems to be part of a physiological adaptational response to negative energy balance in transition dairy cows. Further research is required to determine the effects of occurrence of hyperketonemia considering duration and severity of HYK on subsequent disease risk.

2.2.7 **Acknowledgements**

We gratefully thank the farm personnel of the dairy farm for the kind cooperation.
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3 ADDITIONAL UNPUBLISHED DATA

3.1. *Short communication:* Associations between blood glucose concentration, onset of hyperketonemia and milk production in early lactation dairy cows. (Formatted for Journal of Dairy Science.)

3.1 *Short communication:* Associations between blood glucose concentration, onset of hyperketonemia and milk production in early lactation dairy cows.

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3.1.1 **Abstract**

The objectives of this study were to describe the associations between hypoglycemia and the onset of hyperketonemia (HYK) within the first six weeks of lactation, to evaluate the effects of body condition score at calving on glucose concentration, and to study the effects of hypoglycemia on milk production. A total of 655 dairy cows from 6 commercial dairy farms in Germany were enrolled between 1 and 4 days in milk (DIM). Cows were tested twice weekly using an electronic handheld meter for glucose and β-hydroxybutyrate (BHBA), respectively, for a period of 42 days. Hypoglycemia was defined as glucose concentration ≤ 2.2 mmol/L. Hyperketonemia was defined as a BHBA concentration ≥ 1.2 mmol/l. The onset of HYK was described as early onset (first HYK event within the first 2 weeks postpartum) and late onset (first HYK event in week 3 to 6 postpartum). The effect of ketosis status on blood glucose within 42 DIM was evaluated using a generalized linear mixed model. There was no effect of HYK on glucose concentration in primiparous cows ($P > 0.1$). Multiparous cows with early onset HYK had a lower glucose concentration (-0.21 mmol/L) compared to non-ketotic cows ($P = 0.041$). Overall, primiparous cows had a lower prevalence and incidence of hypoglycemia than multiparous cows. Hypoglycemia in multiparous cows was associated with higher 1st test day milk production and 100 DIM milk production. In conclusion, hypoglycemia mainly occurred in multiparous cows with early onset HYK whereas primiparous were at a lower risk for hypoglycemia presumably due to their lower metabolic load.

Key words: ß-hydroxybutyrate, glucose, body condition score, milk production, transition

3.1.2 **Short communication**

The transition period is characterized by a sudden increase in energy requirements in order to support milk production (Drackley et al., 2001). Because the demand for glucose cannot be met by the diet alone, gluconeogenesis is required to supply the organism with adequate amounts of glucose (Herdt, 2000, Jorritsma et al., 2003). Therefore, the mobilization of body stores and various metabolic and endocrine adaptations are necessary (Herdt, 2000). Those coordinated changes in metabolism of body tissues were described as homeorrhetic regulation and are necessary to support a physiologic state (Bauman and Currie, 1980; Bauman, 2000). During this process a moderate increase in circulating ketone bodies is considered to be part of a normal metabolic response, whereas a poor adaptive response can lead to an inadequate increase in β-hydroxybutyrate (**BHBA**) which can be measured in blood (Duffield et al., 2009).

Two different types of hyperketonemia **(HYK)** differing in their onset and pathophysiology were hypothesized. Type I ketosis was described as hyperketonemia occurring 3 to 6 weeks postpartum when milk secretion is so extensive that the demand for glucose exceeds the capacity for glucose production. The plasma levels of glucose and insulin are low, the levels of ketone bodies are high (Holtenius and Holtenius, 1996; Herdt, 2000). Type II was described as ketosis occurring earlier in lactation as a result of body fat mobilization prior to or at calving. Blood insulin and glucose concentrations are high, whereas blood ketone concentrations are lower in type II ketosis than in type I ketosis (Holtenius and Holtenius, 1996; Herdt, 2000).

Hand-held devices have been evaluated for the measurement of BHBA (Bach et al., 2016) and glucose (Wittrock et al., 2013) on farm. Cut-points for BHBA were defined based on production or health impairment (McArt et al., 2013). A cut-point for blood glucose was determined by Gordon (2013) as blood glucose ≤ 2.2 mmol/L. In that study animals that had blood glucose ≤ 2.2 mmol/L at the time of ketosis diagnosis were more likely to cure and produced more milk when treated with insulin, butaphosphan-cyanocobalamin, or extended duration of propylene glycol than untreated controls with blood glucose ≤ 2.2 mmol/L. Treatment benefits did not extend to animals with blood glucose > 2.2 mmol/L at the time of enrollment. The authors suggested that animals with low blood glucose would be more likely to respond to the stimulation of gluconeogenic pathways, whereas animals with normal blood glucose would not have a strong homeorhetic drive to increase glucose production (Holtenius and Holtenius, 1996; Gordon, 2013).

There is a lack of science-based information that describes the association between hypoglycemia and hyperketonemia considering the ketosis classification. Therefore, the objectives of our study were 1) to describe the associations between hypoglycemia and hyperketonemia within the first six weeks of lactation, 2) to evaluate the effects of body condition score (**BCS**) at calving on glucose concentration, and 3) to evaluate the effects of hypoglycemia on milk production.

The experimental procedures reported herein were conducted with the approval of the Institutional Animal Care and Use Committee of Freie Universität Berlin. Cows were managed according to the guidelines set by the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medical Products (Hellmann and Radeloff, 2000).

A total of 655 dairy cows from 6 different commercial dairy farms in Germany were included in the trial. All farms kept at least 600 Holstein dairy cows in freestall barns, fed a TMR and recorded medical treatments using computer based farm management programs (HerdeW, version 5.8, dsp-Agrosoft Ltd., Ketzin, Germany). An overview of herd size and milk production can be found in Table 1.

Cows were enrolled between 1 and 4 DIM and were tested for BHBA and glucose twice weekly at intervals of 3 to 4 days for 42 days, resulting in 12 test results per cow. At each test BCS was determined on a 5-point scale with 0.25-point increments (Edmonson et al., 1989) by a trained investigator. The term lactation week was used to describe the time of sample collection relative to calving (e.g., lactation week 0.5 for the first measurement on DIM 1 to 4, lactation week 1 for the second measurement on DIM 4 to 7, lactation week 1.5 for the third measurement on DIM 8 to 11).

The collection of blood samples and the measurement of BHBA by an electronic BHBA meter (NovaVet, Nova Biomedical, Waltham, USA) were described in the study by Mahrt et al. (2015). Hyperketonemia was defined as BHBA $\geq$ 1.2 mmol/L (Suthar et al., 2013). The measurement of glucose was conducted with a hand-held electronic glucometer (Precision Xtra; Abbott Diabetes Care Inc., Mississauga, ON, Canada) which was evaluated for cow-side use in dairy cattle (Wittrock et al., 2013) using blood from the coccygeal vessels. Hypoglycemia was defined as blood glucose $\leq$ 2.2 mmol/L.

A total of 32 cows were excluded from analysis because they received a treatment of intravenous applications of a butaphosphan-cyanocobalamin combination (Catosal, Bayer Animal Health, Leverkusen, Germany), dextrose or dexamethasone (Dexatat ad us.vet.,

aniMedica, Senden-Bösensell, Germany), oral administration of propylene glycol, monensin (Kexxtone, Elanco Animal Health, Hampshire, UK) or combinations of these therapies as effects on blood BHBA concentrations were possible (Gordon, 2013; Mann et al., 2015). Animals that received a blanket prophylaxis with dextrose or dexamethasone against disorders in energy metabolism on DIM 0 or 1 (n = 115) were not excluded because a one-time intravenous bolus administration of 50% dextrose in postpartum dairy cows is unlikely to prevent or resolve hyperketonemia (Gordon, 2013) and a single treatment with glucocorticoids is considered equivocal (Gordon, 2013). However, an effect of a second treatment or a treatment in combination with other therapies is possible and therefore cows that received a second treatment or a combination were excluded from the trial as described above. Two cows were excluded due to an abortion.

After exclusion, 621 cows (195 primiparous; 426 multiparous) were used for final analysis.

Data from the cow-side evaluation of BHBA and glucose and data from the on farm computer system were exported to Excel spreadsheets and analyzed using SPSS for Windows (version 22.0, SPSS Inc., IBM, Ehningen, Germany).

To evaluate the effect of ketosis status (i.e., non-ketotic = no HYK event within the first six weeks of lactation; early onset = first HYK event within the first two weeks; late onset = first HYK event within week 3 to 6 postpartum) and body condition score on blood glucose within 42 DIM repeated measures ANOVA with first-order autoregressive covariance was performed using the GENLINMIXED procedure of SPSS. The outcome variable was blood glucose (mmol/l). Cow was the experimental unit and herd was considered as a random effect. According to the model-building strategies described by Dohoo et al. (2009) each parameter considered for the mixed model should be separately analyzed in a univariate model, including the parameter as a fixed factor (i.e., categorical parameter) or covariate (i.e., continuous parameter). Only parameters resulting in univariate models with $P \leq 0.2$ should be included in the final mixed model. The initial model contained the following explanatory variables as fixed effects: ketosis status (non-ketotic, early onset, late onset), body condition score (1.00 to 5.00) at the first and second BHBA test, parity (primiparous vs. multiparous) and farm. Selection of the model that best fit the data was performed by testing each effect separately in an univariate model and finding the model with the lowest value for the Akaike information criterion (AIC) using a backward elimination procedure that removed all

variables with $P > 0.10$ from the model. Regardless of the significance level ketosis status was forced to remain in the model.

To evaluate the effect of ketosis status and glucose on milk yield in multiparous cows linear regression analysis was performed using the GENLINMIXED procedure of SPSS. The outcome variable was either first test day milk yield (kg/ d) or a projection of cumulative 100 d milk yield (kg). Cow was the experimental unit and herd was considered as a random effect. The initial model contained the following explanatory variables as fixed effects: ketosis and glucose status (non-ketotic, hypoglycemia only, hyperketonemia only and both hyperketonemia and hypoglycemia) and DIM at test day. Model building and selection of the model that best fit the data was performed as described earlier. Regardless of the significance level ketosis status was forced to remain in the model. Primiparous cows had a low prevalence and incidence of hypoglycemia and therefore effects of hypoglycemia on milk production were only evaluated for multiparous cows.

A significant difference between the levels of a classification variable was declared when $P < 0.05$, whereas differences between $P \geq 0.05$ and $P \leq 0.10$ were considered a statistical tendency. As there was a marked difference in the occurrence of hypoglycemia in primiparous vs. multiparous cows, the two groups were evaluated separately.

The concentration of serum glucose in primi- and multiparous cows is shown in Figure 1 and 2, respectively. The effect of ketosis status on blood glucose concentration is shown in Table 2. Multiparous cows with early onset HYK had a lower glucose concentration (-0.21 mmol/L) compared to multiparous non-ketotic cows ($P = 0.041$). Multiparous cows with late onset HYK showed no difference in glucose concentration compared to multiparous non-ketotic cows ($P > 0.1$). There was no effect of HYK on glucose concentration in primiparous cows irrespective of their HYK onset ($P > 0.1$). There was also no effect of BCS at calving on glucose concentration in primi- and multiparous cows, respectively ($P > 0.05$). Prevalence of hypoglycemia in primi- and multiparous cows and of hypoglycemia incidence by week postpartum are shown in Figures 3, 4 and 5, respectively. Overall, primiparous cows had a lower prevalence and incidence of hypoglycemia than multiparous cows. Peak incidence of hypoglycemia in multiparous cows occurred in lactation wk 1.5, when 13.1% of cows had their first hypoglycemic test result. In multiparous cows, peak prevalence of hypoglycemia only and of hypoglycemia combined with HYK occurred in wk 2.0 (7.3%) and in wk 3.0 (9.2%), respectively.

Estimated means of 1st test day milk yield and cumulative 100 DIM milk yield in multiparous cows, stratified by metabolic status (i.e., non-ketotic cows, cows with hypoglycemia only, cows with hyperketonemia only, and cows with hyperketonemia and hypoglycemia) are shown in Figure 6 and 7, respectively. Hypoglycemia was associated with a higher 1st test day milk yield as well as with a higher cumulative 100 DIM milk yield.

This study was conducted to describe the association between hypoglycemia and the onset of hyperketonemia (ketosis type) within the first six weeks of lactation. To our knowledge, this is the first multi-site study conducted on freestall dairy farms with TMR fed cows that evaluates the theory of type I and II ketosis by Holtenius and Holtenius (1996).

Our results indicated that the concept of type I and II ketosis cannot be transferred to modern, commercial TMR fed dairy farms. According to Holtenius and Holtenius (1996) type I ketosis (lactation week 3 to 6) is associated with hypoglycemia. Our results showed that early onset HYK (lactation week 1 to 2) was associated with hypoglycemia in multiparous cows, whereas late onset HYK (lactation week 3 to 6) was not associated with hypoglycemia. Primiparous cows were at lower risk for hypoglycemia than multiparous cows within 6 weeks of lactation. Hypoglycemia was also associated with higher milk production.

Glucose demand is high during early lactation due to the high requirements for milk synthesis (Herdt and Emery, 1992). Glucose discrepancy of supply from digestible energy and output must be made up by increased gluconeogenesis from intestinally absorbed amino acids, lactate and glycerol (Drackley, 2001). To ensure the proper nutrient supply to the mammary gland, coordinated regulation of the metabolism of different organs and tissues take place (Bauman, 2000). Those metabolic adaptations occurring with the onset of lactation are related to hormonal changes occurring throughout this period (Bauman, 2000). Hormones that signal for the increases in gluconeogenesis in the liver of periparturient dairy cows may include insulin, glucagon, somatotropin and cortisol (Drackley et al., 2001). Somatotropin is the homeorhetic control that coordinates adaptations in body tissues by alterations in tissue responses to homeostatic signals and allows a chronic alteration of nutrient utilization (Bauman, 2000). The net effect is that the increase in mammary gland metabolic rate and nutrient use coincides with alterations in the metabolism of other body tissues so that an adequate quantity and pattern of nutrients to support milk synthesis is ensured (Bauman, 2000).

Part of these changes is an increased liver glucose release after calving due to changes in the metabolism of splanchnic tissues (Reynolds et al., 2003) and a substantial increase in splanchnic tissue mass (Gibert et al., 1992).

According to Holtenius and Holtenius (1996) early onset HYK (type II) is the hyperglycaemic-hyperinsulinaemic form whereas late onset HYK (type I) is the hypoglycaemic-hypoinsulinaemic form. Hypoglycemia did not occur, however, between wk 3 to 6 postpartum near peak lactation (late onset HYK), but could mainly be found in multiparous cows in wk 1 to 2 after calving (early onset HYK) when major physiological, nutritional, metabolic and immunological changes occur (Bell, 1995; Goff, 1997; Sordillo et Raphael, 2013). A sudden increase in the demand for hepatic gluconeogenesis is required for milk synthesis and secretion after parturition (Drackley et al., 2001). But although gluconeogenic pathways in the liver are maximally stimulated the demand for glucose exceeds the gluconeogenic ability of the liver in that early stage. Changes in the response to insulin have the effect that non-mammary tissues spare glucose to ensure the proper nutrient supply to the mammary gland (Bauman, 2000).

Interestingly, glucose concentration increased after the first 2 weeks although milk production was increasing. We suggest that this is due to the homeorhetic controls as described above which lead to an increased liver glucose release. As previously described the majority of this increase occurred in the first days after calving and release of glucose was even greater than requirements with increasing surplus evident as lactation progressed (Reynolds et al., 2003).

In our study multiparous cows had a low glucose concentration presumably due to a higher metabolic load that overcomes homeostatic regulation whereas primiparous cows had a higher glucose concentration overall.

It remains unclear to which extent early onset ketotic cows with hypoglycemia are affected by negative health and production outcomes or if this can be seen as a physiological adaptational response. The complexity of the metabolic changes makes it difficult to evaluate the success of adaptation by one specific parameter. As shown in another study (Ruoff et al., 2016) the majority of cows had one HYK event only and a high self-cure rate. It could be possible that those cows are more able to cope with the process of adaptation and an increased BHBA concentration could therefore be seen as a sign of a physiological process.

The objective of this study was to evaluate the associations between hypoglycemia and onset of hyperketonemia and to evaluate the concept of type I and II ketosis. In conclusion, hypoglycemia mainly occurred in multiparous cows with early onset of HYK. Primiparous cows seemed to show a different adaptational response, probably due to their lower metabolic load. This should be considered for future treatment and studies. Although HYK still occurs until week 6 postpartum, the first 2 weeks after calving seem to be the most challenging weeks for multiparous cows as the sudden increase in milk production leads to a sudden drop in glucose concentrations. It has to be evaluated if hypoglycemia in high producing cows leads to negative effects and if glucose monitoring in addition to BHBA should be considered for evaluation of fresh cows.

TABLES AND FIGURES

Table 1. Description of the 6 study herds.

	Herd					
Parameter	1	2	3	4	5	6
Enrolled cows, n	124	121	60	136	104	110
Herd size, n	1,200	1,800	600	1,600	1,300	1,400
Annual milk production[1], kg	10,733	10,900	11,127	8,465	9,509	9,785
Ketosis prophylaxis	All cows: TMR including 300 g glycerol per cow/d and oral 300 g dextrose at 0 DIM 3rd lactation and higher: 0.04 mg/kg dexamethasone at 0 DIM	All cows: TMR including 300 g glycerol per cow/d 3rd lactation and higher:160 g dextrose per cow intravenous at 0 DIM	All cows: TMR including 300 g propylene glycol per cow/d during the first 21 DIM	2nd lactation and higher: oral 35 g calcium propionate and 21 g sodium propionate at 0 DIM	2nd lactation and higher: 500 ml dextrose (40%) intravenous on 1st DIM	All cows: TMR including 100 g propylene glycol and 150 g glycerol per cow/d for cows between 7-42 DIM

[1]Energy corrected milk (4.0% fat; 3.4% protein)

Table 2. Effect of ketosis type[1] within the first six weeks of lactation on glucose concentration in Holstein dairy cows stratified by parity.

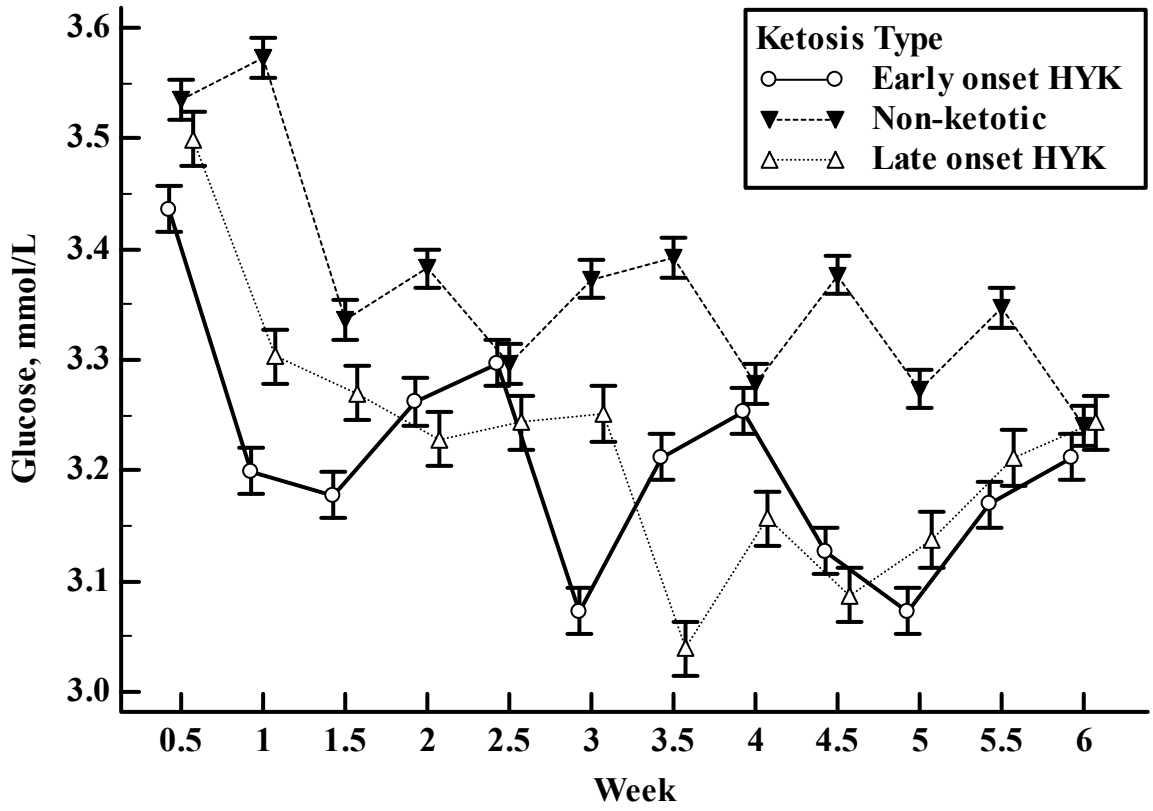
Variable	Estimate[2]	SE[3]	95% Confidence Interval		*P*
	mmol/L		Lower CI	Upper CI	
Primiparous (n = 195)					
Non-ketotic	Referent				
Early onset HYK[4]	-0.16	0.15	-0.46	0.13	0.281
Late onset HYK	-0.04	0.16	-0.35	0.28	0.813
Multiparous (n = 426)					
Non-ketotic	Referent				
Early onset HYK	-0.21	0.10	-0.41	-0.01	0.041
Late onset HYK	+0.06	0.11	-0.16	0.28	0.571

[1] Ketosis type: Non-ketotic = no hyperketonemia event within the first six weeks of lactation; early onset = first hyperketonemia event within the first two weeks; late onset = first hyperketonemia event within week 3 to 6 postpartum.

[2] Model adjusted for the random effect of herd.

[3] SE = Standard error of the estimate.

[4] HYK = hyperketonemia



Ruoff. Figure 1

Figure 1. Serum glucose concentrations in primiparous Holstein dairy cows (n = 195) within the first 6 weeks of lactation for different ketosis types: non-ketotic cows = no hyperketonemia (HYK) event within the first six weeks of lactation; early onset HYK = first HYK event within the first two weeks of lactation; late onset HYK = first HYK event in week 3 to 6 postpartum.

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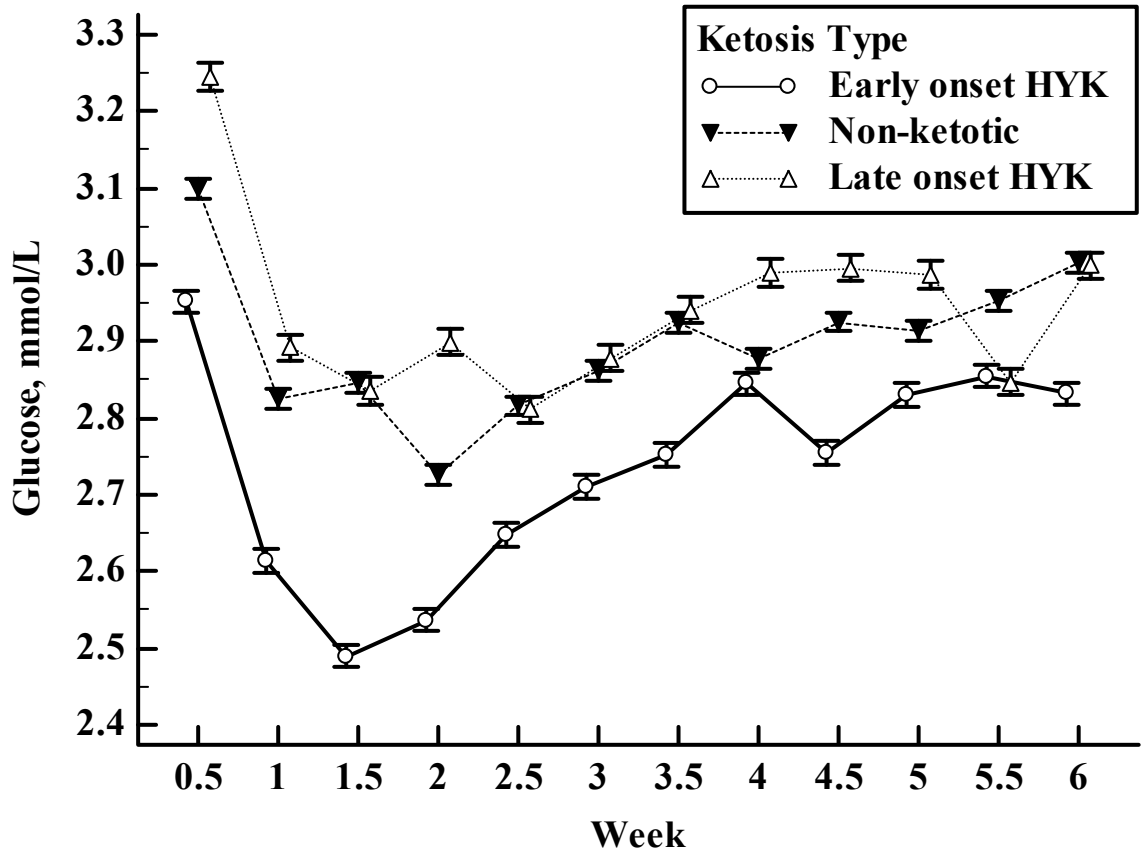
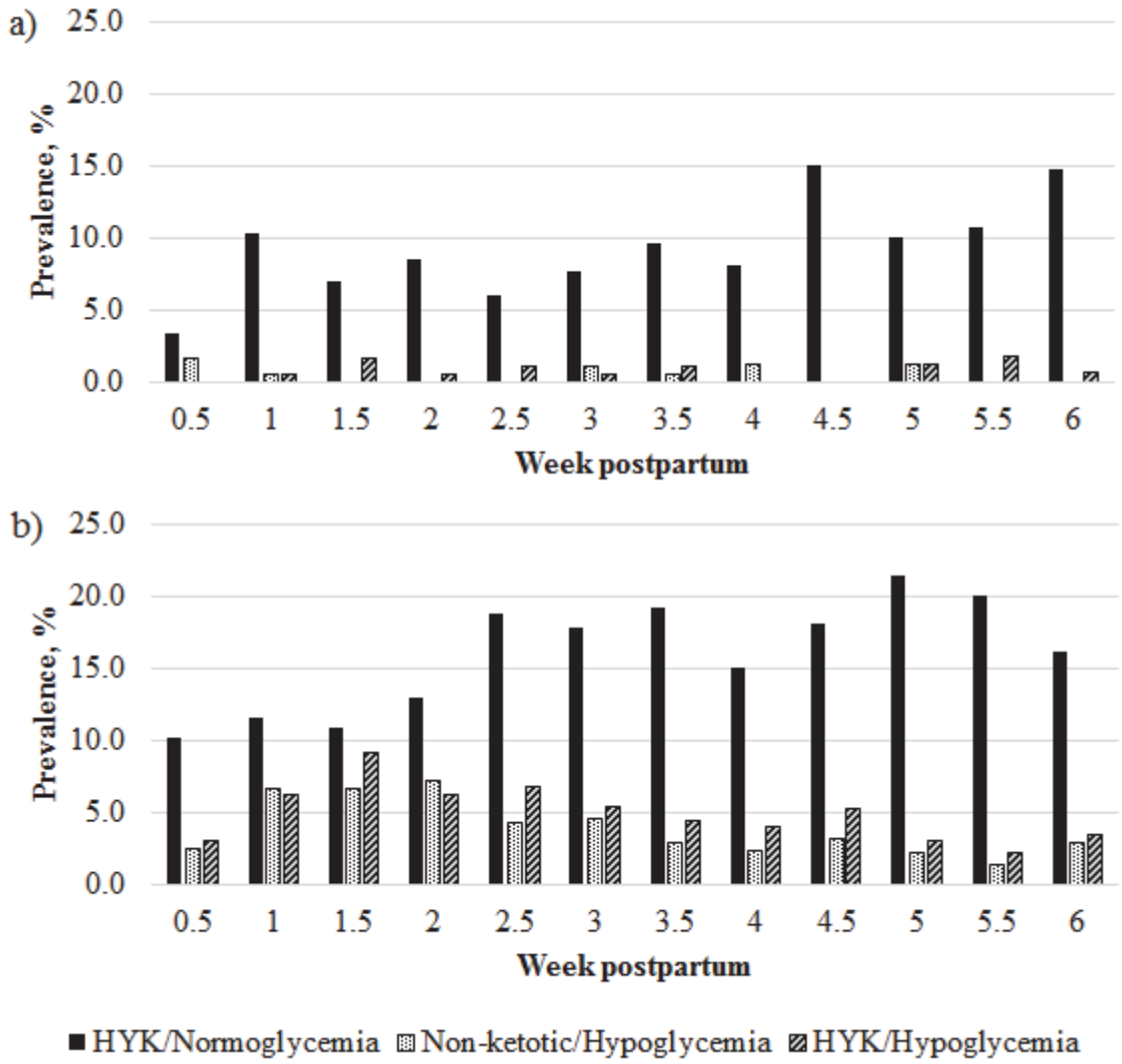


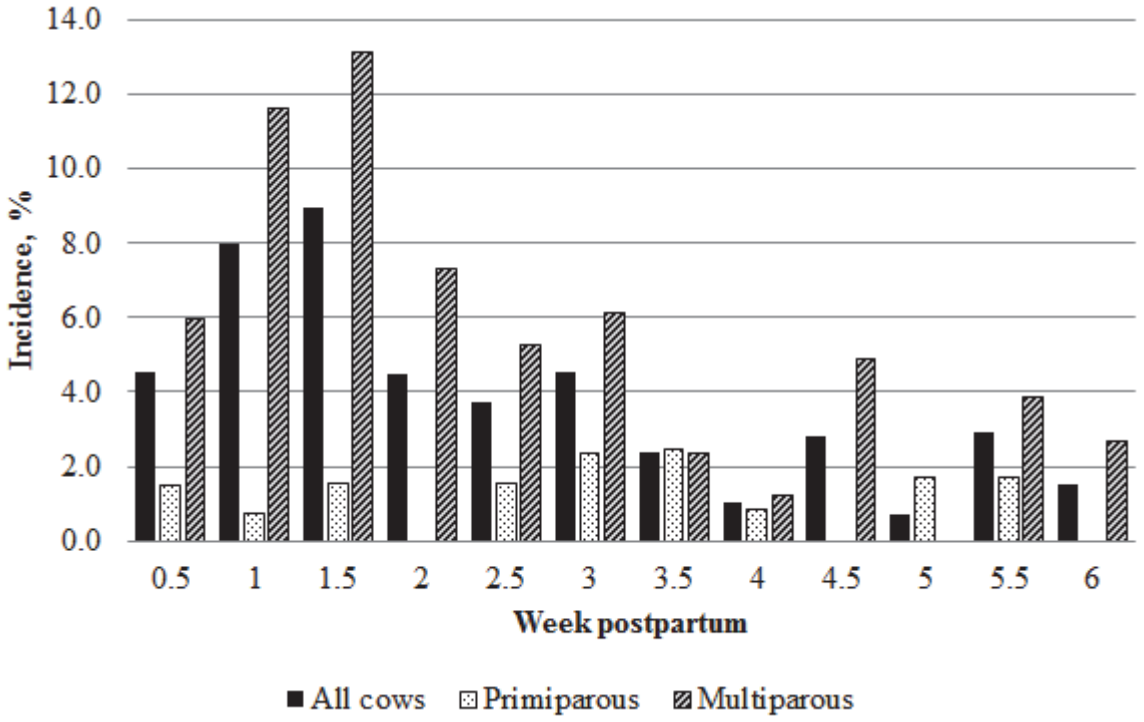
Ruoff. Figure 2

Figure 2. Serum glucose concentrations in multiparous Holstein dairy cows (n = 426) within the first 6 weeks of lactation for different ketosis types: non-ketotic cows = no hyperketonemia (HYK) event within the first six weeks of lactation; early onset HYK = first HYK event within the first two weeks of lactation; late onset HYK = first HYK event in week 3 to 6 postpartum.



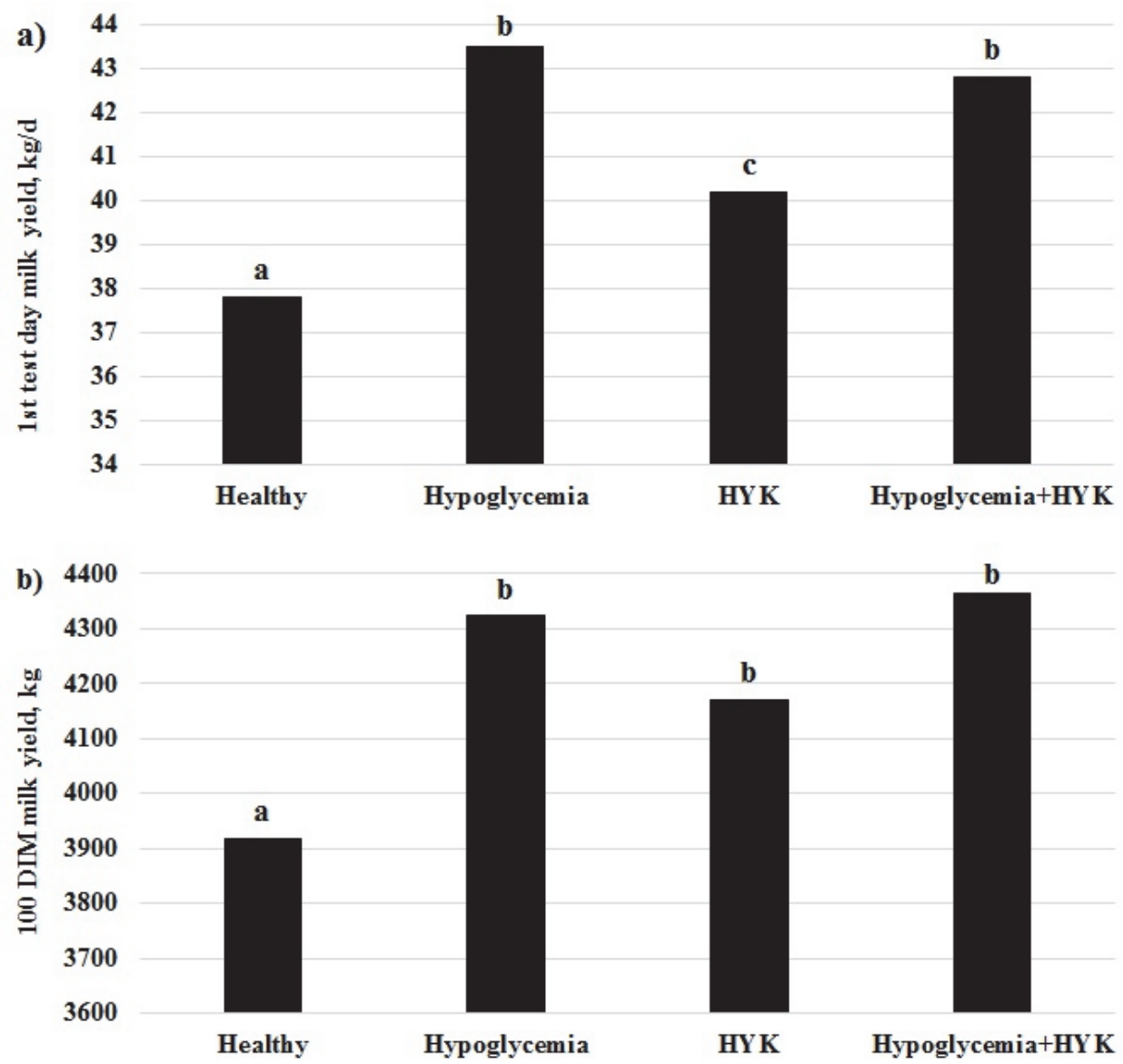
Ruoff. Figure 3

Figure 3. Prevalence of hyperketonemia (BHBA concentration ≥ 1.2 mmol/L) and hypoglycemia (Glucose concentration ≤ 2.2 mmol/L) in (a) primiparous (n = 177) and (b) multiparous (n = 386) Holstein dairy cows during the first 6 weeks of lactation.



Ruoff. Figure 4

Figure 4. Incidence of hypoglycemia (glucose concentration ≤ 2.2 mmol/L) in primiparous and multiparous Holstein dairy cows during the first 6 weeks of lactation. Only cows with 12 test results were considered (n = 417).



Ruoff. Figure 6

Figure 6. Estimated marginal means of (a) 1st test day milk yield (kg/d) and (b) cumulative 100 DIM milk yield (kg) from the mixed procedure in multiparous cows, stratified by non-ketotic and normoglycemic cows (n = 190; n = 174), cows with hypoglycemia only (n = 46; n = 40), cows with hyperketonemia only (n = 93; n = 81), and cows with hyperketonemia and hypoglycemia (n = 62; n = 59) within the first two weeks of lactation. [abc]Columns with different superscripts differ significantly ($P < 0.05$).

3.1.3 **References**

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4 DISCUSSION

The overall objective of this thesis was to improve on-farm testing strategies of subclinical ketosis and to gain futher knowledge about the effects of ketosis in respect of its onset and classification.

The aim of the first study was to find out if other parameters of fat metabolism could serve as surrogates for NEFA. Those parameters could be measured by hand-held devices and therefore improve on-farm diagnosis.

The results of the first study indicated that a combination of BHBA, cholesterol and TAG as surrogates showed the best results. Prepartum, a NEFA concentration ≥ 0.5 mEq/L could be predicted with a high sensitivity (i.e., Se = 0.88) and specificity (i.e., Sp = 0.93) from d 3 to d 1 prepartum. Postpartum, a NEFA concentration ≥ 0.7 mEq/L could be predicted most precisely on d 2 postpartum (i.e., Se = 0.89, Sp = 0.76). The prediction of NEFA by only one parameter would be preferable because generally on-farm testing with hand-held devices is associated with costs for test strips and labor (McArt et al., 2014). However, the prediction by one parameter was not sufficient, because either sensitivity or specificity was low. With a low sensitivity a high percentage of ill cows would not be found and would remain without an advantageous treatment. With a low specificity a high percentage of healthy cows would be identified as ill and falsely treated with no benefit. In another approach, BHBA was set as a fixed parameter and combined with cholesterol only, because several hand-held meters for BHBA measurement are already evaluated for the use in cows (Bach et al., 2016). However, those results were overall lower than the equal combination of BHBA, cholesterol and TAG.

Based on our data the equal combination of all three parameters (i.e., BHBA, cholesterol and TAG) showed the best results to determine if NEFA concentrations exceeded a certain threshold. The use of hand-held meters for cholesterol and TAG in cows should be considered as it could improve on-farm monitoring and treatment of disease. Hand-held meters for cholesterol and TAG are already validated for the use in human medicine (Coqueiro et al. 2013; Ferreira et al. 2015) and have been used in other animals to measure blood TAG concentrations (Kluger et al. 2010). However, the measuring ranges of those meters would have to be adapted for the use in cows first and the meters would have to be validated against the gold standard for bovine cholesterol and TAG concentration in blood in the same way as it was done for hand-held meters for BHBA (Carrier et al., 2004; Iwersen et al., 2009; Mahrt et al., 2014). The method is therefore not suitable for daily use in practice

yet. Further research for the usage of human medicine hand-held meters for cholesterol and TAG in cows should be conducted based on the findings of this study. In the future, the combination of BHBA, cholesterol and TAG might serve as an alternative to laboratory analysis in order to predict NEFA concentrations on-farm.

The aim of the second study was to describe the occurrence of hyperketonemia within the first six weeks of lactation and to evaluate the effect of hyperketonemia in respect of its onset on milk production, reproduction and culling risk.

It was shown in another study that hyperketonemia occurred not only within the first 2 weeks of lactation but until lactation week 6 (Mahrt et al., 2015). Our results indicated that early onset hyperketonemia was associated with a higher milk production. There were, however, no effects on reproductive performance and culling risk, irrespective of the onset of hyperketonemia. Several studies evaluated the effects of hyperketonemia within the first 2 weeks of lactation on milk production, however, the results were inconsistent. In contrast to our results, some studies found that milk production was reduced for ketotic cows (Ospina et al., 2010; Chapinal et al., 2012). However, it was also shown that although cows with hyperketonemia had a lower milk production in the short term, they produced more milk in the long term (Chapinal, Duffield). McArt et al. (2013) suggested that higher producing cows might have impaired production in the short term that might be overcome by mid-lactation. In accordance with our findings a recent study showed that hyperketonemia in early lactation was associated with a higher milk production (Vanholder et al., 2015). The results of different studies regarding reproductive performance were also inconsistent. Whereas some studies found that hyperketonemia was associated with a decreased reproductive performance (Walsh et al., 2007) other studies did not find an association (Ospina et al., 2010; Chapinal et al., 2012; McArt et al., 2012). In contrast to other studies, no effect was found in my study on culling risk, neither for early nor for late onset hyperketonemia. Several studies described that ketotic cows were more likely to be removed from the herd (McArt et al., 2012; Roberts et al., 2012). The results of my study indicated that a high milk production is associated with hyperketonemia but the risk for an impaired reproductive performance and culling did not increase for ketotic cows. An explanation for the inconsistent results could be that the adaptational response to negative energy balance is an individual process. Whereas in some animals hyperketonemia could be a sign of a physiological and successful adaptational response without any negative effects, in other animals excessive BHBA production could be due to a poor adaptative ability. Further research should be done on the animal-specific

strategies to respond to negative energy balance and on diagnostic tools to identify cows that are on risk for a less successful adaptation. It should be investigated if other additional parameters to BHBA could help to improve the differentiation and overall on-farm monitoring.

In total, 72% of all cows with hyperketonemia had a negative test result within 3 to 4 days after the initial diagnosis in my study. Treatment studies often use a negative BHBA test result as a sign for a successful treatment (Rollin et al., 2010; Compton et al., 2015). However, the results of my study indicated a high self-cure rate which should be considered in future treatment studies. Instead of only using a negative test result (BHBA < 1.2mmol/L) as a sign for a successful treatment, studies should also use health and production outcomes with an economic value to the dairy farmer (e.g., reduction of disease or culling risk) to evaluate a treatment effect (McArt et al., 2012).

Furthermore, the aim of this thesis was to investigate the theory of type I and II ketosis by Holtenius et Holtenius (1996) which has never been confirmed in a large scale study using multiple commercial dairy herds. The theory suggested that cows with elevated BHBA concentrations within the first 2 weeks postpartum have type II ketosis which generally occurs earlier in lactation as a result of body fat mobilization prior to or at calving. Cows with elevated BHBA concentrations between 3 to 6 weeks postpartum near peak lactation have type I ketosis because milk secretion is so extensive that the demand for glucose exceeds the capacity of gluconeogenesis in the liver (Holtenius and Holtenius, 1996; Herdt, 2000). In contrast to this theory, the results of the second study of this thesis indicated that early onset hyperketonemia was associated with a higher milk production whereas late onset hyperketonemia was not.

Therefore, a third study was conducted in order to further investigate the theory of type I and II ketosis. The aim of the third study was to evaluate the associations between hypoglycemia and the onset of hyperketonemia. According to Holtenius and Holtenius (1996) early onset HYK (type II) is the hyperglycaemic-hyperinsulinaemic form whereas late onset HYK (type I) is the hypoglycaemic-hypoinsulinaemic form. Our results indicated that the concept of type I and II ketosis cannot be transferred to modern, commercial TMR fed dairy farms. According to Holtenius and Holtenius (1996) type I ketosis (lactation week 3 to 6) is associated with hypoglycemia. Our results, however, showed that late onset HYK (lactation week 3 to 6) was not associated with hypoglycemia. The study showed that hypoglycemia mainly occurred in

multiparous cows with early onset HYK. Primiparous cows seemed to show a different adaptational response, probably due to their lower metabolic load. Although HYK still occurred until week 6 postpartum, the first 2 weeks after calving seem to be the most challenging weeks for multiparous cows as the increase in milk production leads to a sudden drop in glucose concentrations. Overall, glucose concentration increased after the first 2 weeks although milk production was increasing. We suggest that this is due to homeorhetic controls which lead to an increased liver glucose release (Bauman, 2000; Reynolds et al., 2003).

Further research is required to find out if the measurement of glucose in addition to BHBA would help to identify cows at risk for a failed adaptation and should be considered in future monitoring strategies. The complexity of the metabolic changes makes it difficult to evaluate the success of adaptation by only one specific parameter. It remains unclear if early onset ketotic cows with hypoglycemia are affected by negative health and production outcomes or if it can be seen as a physiological adaptational response.

5 SUMMARY

5.1 Investigation on diagnosis, effects and classification of subclinical ketosis in dairy cows

The overall aim of this thesis was to improve the on-farm diagnosis of subclinical ketosis pre- and postpartum and to gain further knowledge about the effects and classification of subclinical ketosis within the first six weeks of lactation. To evaluate the metabolic situation of dairy herds and to detect affected cows, the measurement of non-esterified fatty acids (NEFA) and ß-hydroxybutyrate (BHBA) was shown to be a useful diagnostic tool. Circulating concentrations of NEFA and BHBA measure aspects of the success in adaptation to a state of negative energy balance.

Prepartum measurement of NEFA on-farm would be a useful alternative to laboratory analysis, however, hand-held devices for the measurement of NEFA are not available yet. Therefore, the aim of the first study was to evaluate if different parameters of fat metabolism could serve as surrogates for NEFA. Specifically, it was determined if it is feasible to predict the concentration of NEFA by the measurement of BHBA, cholesterol, triglycerides (TAG) and HDL as surrogates. A total of 254 cows were included in the trial. One blood sample was taken from each cow between d 10 and d 1 prepartum. Second and third samples were collected on d 2 and d 10 postpartum, respectively. The coefficients of correlation between parameters were calculated and a receiver-operator characteristic curve analysis was conducted. The prediction of NEFA concentrations using only one of the parameters was insufficient. However, a NEFA concentration ≥ 0.5 mEq/L could be predicted with a high sensitivity (i.e., Se = 0.88) and specificity (i.e., Sp = 0.93) from d 3 to d 1 prepartum and a NEFA concentration ≥ 0.7 mEq/L could be reliably predicted on d 2 postpartum (i.e., AUC = 0.89, Se = 0.89, Sp = 0.76) when using a combination of BHBA, cholesterol and TAG as surrogates. Overall, the results suggest that a combination of different parameters of lipid metabolism could be used as surrogates for NEFA. However, hand-held devices for cholesterol and TAG would have to be evaluated for the usage in cows before this method could be used in practice.

Postpartum the measurement of blood BHBA is a common tool for on-farm diagnosis of subclinical ketosis. Some authors speculated that there are two different types of ketosis differing in their onset and pathophysiology. According to that theory type I ketosis, the spontaneous type, occurs 3 to 6 weeks postpartum, whereas type II ketosis, known as “fat cow

syndrome" occurs in week 1 to 2 postpartum. Several studies evaluated the effects of hyperketonemia within the first 2 weeks after parturition. However, there is no evidence if hyperketonemia occurring in week 3 to 6 of lactation has the same impact on health and production as the early type. Therefore, the aim of the second study was to evaluate the effects of hyperketonemia on milk production (1st test day milk yield and 100 DIM milk yield), reproductive performance (time to first service, first service conception risk, and time to pregnancy within 200 DIM) and early lactation culling risk within the first six weeks of lactation. A total of 655 Holstein dairy cows from 6 commercial dairy farms in Germany were enrolled between 1 and 4 DIM. Cows were tested twice weekly using an electronic handheld meter for BHBA for an examination period of 42 days resulting in 12 test results per cow. Hyperketonemia was defined as a BHBA concentration ≥ 1.2 mmol/l. The onset of hyperketonemia was described as early onset (first hyperketonemia event within the first 2 weeks postpartum) and late onset (first hyperketonemia event in week 3 to 6 postpartum). Prevalence and incidence of hyperketonemia were assessed based on the 12 examinations. Cumulative incidence of hyperketonemia was 48% and 72% for primiparous and multiparous cows, respectively. Mean prevalence was 17.5%. Early onset ketotic cows had a higher 1st test day milk yield (+ 3.0 kg/d, $P < 0.001$) and 100 DIM milk production (+ 301.6 kg; $P < 0.001$) compared to non-ketotic cows. There was no effect of late onset of ketosis on milk production. There were no effects of hyperketonemia on reproductive performance and culling risk, irrespective of onset of ketosis.

In order to further investigate the hypothesis of type I and II ketosis a third study was conducted to evaluate the associations between hyperketonemia and hypoglycemia in early lactation. According to the theory of type I and II ketosis, early onset ketosis (week 1 to 2 postpartum) is the hyperglycaemic-hyperinsulinaemic form whereas late onset ketosis (week 3 to 6 postpartum) is the hypoglycaemic-hypoinsulinaemic form. A total of 655 dairy cows from 6 commercial dairy farms in Germany were enrolled between 1 and 4 days in milk (DIM). Cows were tested twice weekly for 6 weeks using an electronic handheld meter for glucose and BHBA, respectively. Hypoglycemia was defined as glucose concentration ≤ 2.2 mmol/L. Hyperketonemia was defined as a BHBA concentration ≥ 1.2 mmol/l. As described above, the onset of hyperketonemia was described as early onset (first HYK event within the first 2 weeks postpartum) and late onset (first HYK event in week 3 to 6 postpartum). The effect of ketosis status on blood glucose within 42 DIM was evaluated using a generalized linear mixed model. There was no effect of HYK on glucose concentration in primiparous cows ($P > 0.1$). Multiparous cows with early onset HYK had a lower glucose concentration (-

0.21 mmol/L) compared to non-ketotic cows (P = 0.041). Unlike the theory of type I and II ketosis, hypoglycemia was not associated with late onset HYK but with early onset HYK. Overall, primiparous cows had a lower prevalence and incidence of hypoglycemia than multiparous cows. Hypoglycemia in multiparous cows was associated with higher 1st test day milk production and 100 DIM milk production. In conclusion, hypoglycemia mainly occurred in multiparous cows with early onset HYK whereas primiparous were at a lower risk for hypoglycemia, presumably due to their lower metabolic load.

6 ZUSAMMENFASSUNG

6.1 Untersuchung zur Diagnose, den Auswirkungen und der Einteilung der subklinischen Ketose bei Milchkühen

Das Ziel dieser Arbeit war es, die Diagnostik der subklinischen Ketose vor und nach der Abkalbung zu verbessern und weitere Erkenntnisse über die Auswirkungen und die Einteilung der subklinischen Ketose innerhalb der ersten sechs Laktationswochen zu gewinnen. Zur Bewertung der Stoffwechsellage von Milchviehbeständen und zum Auffinden betroffener Kühe hat sich die Messung von freien Fettsäuren (NEFA) und ß-Hydroxybutyrat (BHBA) als nützliches Diagnostikum herausgestellt. Die Messung der Konzentrationen von NEFA und BHBA im Blut können zur Bewertung einer erfolgreichen Adaptation an einen Zustand der negativen Energiebilanz herangezogen werden.

Die prepartale Messung von NEFA direkt auf dem Betrieb mithilfe von Handmessgeräten wäre eine nützliche Alternative zur Laboranalyse. Allerdings sind solche Geräte für die Messung von NEFA derzeit noch nicht verfügbar. Daher war das Ziel der ersten Studie herauszufinden, ob andere Parameter des Fettstoffwechsels als Alternative zur NEFA-Messung dienen können. Insbesondere wurde bestimmt, ob es möglich ist, die Konzentration von NEFA durch die kombinierte Messung von BHBA, Cholesterol, Triglyceriden (TAG) und High Density Lipoproteinen (HDL) vorherzusagen. Insgesamt wurden 254 Kühe in die Studie aufgenommen. Von jeder Kuh wurde zwischen dem 10. und 1. Tag vor der Abkalbung eine Blutprobe entnommen. Eine weitere Probe wurde jeweils am 2. und 10. Tag nach der Abkalbung entnommen. Die Korrelationskoeffizienten zwischen den Parametern wurden berechnet und eine ROC-Kurven-Analyse wurde durchgeführt. Die Vorhersage der NEFA-Konzentration mithilfe eines Parameters war unzureichend. Jedoch konnte bei Verwendung einer Kombination von BHBA, Cholesterol und TAG zwischen dem 3. und dem 1. Tag prepartum eine NEFA-Konzentration ≥ 0,5 mEq / L mit einer hohen Sensitivität (Se = 0,88) und Spezifität (Sp = 0,93) vorhergesagt werden und am 2. Tag postpartum eine NEFA-Konzentration ≥ 0,7 mEq / L vorhergesagt werden (AUC = 0,89; Se = 0,89; Sp = 0,76). Insgesamt deuten die Ergebnisse darauf hin, dass eine Kombination verschiedener Parameter des Lipidstoffwechsels als Ersatz für die NEFA-Messung verwendet werden könnte. Allerdings müssen Handmessgeräte für Cholesterin und TAG für die Verwendung bei Kühen angepasst werden, bevor dieses Verfahren in der Praxis angewandt werden kann.

Postpartum ist die Messung von BHBA im Blut eine weit verbreitete Methode, um subklinische Ketose vor Ort zu diagnostizieren. Es wird vermutet, dass es zwei verschiedene Arten von Ketose gibt, welche sich in ihrem zeitlichen Auftreten und der Pathophysiologie unterscheiden. Laut dieser Theorie gibt es zwei Formen der Ketose: Typ I, der spontane Typ, welcher 3 bis 6 Wochen nach der Abkalbung auftritt und Typ II, auch bekannt unter dem Begriff "Fat Cow Syndrome", welcher zwischen der ersten und zweiten Woche nach der Abkalbung vorkommt. In zahlreichen Studien wurden die Auswirkungen subklinischer Ketose innerhalb der ersten zwei Wochen nach der Geburt untersucht. Jedoch ist bisher nicht bekannt, ob die späte Form der subklinischen Ketose (3. bis 6. Woche postpartum) vergleichbare Auswirkungen auf Gesundheit und Leistung hat wie die frühe Form. Ziel der zweiten Studie war es daher, die Auswirkungen der subklinischen Ketose innerhalb der ersten 6 Wochen postpartum auf die Milchleistung (Einsatzleistung und 100-Tage-Leistung), die Fruchtbarkeit (Rastzeit, Erstbesamungserfolg und Güstzeit) und die Abgangsrate zu untersuchen. Insgesamt wurden 655 Holsteinkühe aus 6 kommerziellen Milchviehbetrieben in Deutschland zwischen dem 1. und 4. Tag postpartum in die Studie aufgenommen. Kühe wurden zweimal wöchentlich mit einem elektronischen Handmessgerät für BHBA für einen Untersuchungszeitraum von 42 Tagen getestet, sodass pro Kuh 12 Testergebnissen vorlagen. Hyperketonämie wurde als BHBA-Konzentration ≥ 1,2 mmol / L definiert. Je nachdem wann Hyperketonämie zum ersten mal vorkam, wurde diese als frühes Auftreten (Woche 1 bis 2 postpartum) bzw. spätes Auftreten (Woche 2 bis 6 postpartum) definiert. Prävalenz und Inzidenz von Hyperketonämie wurde basierend auf den 12 Untersuchungsergebnissen berechnet. Die kumulative Inzidenz von Hyperketonämie betrug 48% bzw. 72% für primipare bzw. multipare Kühe. Die mittlere Prävalenz betrug 17,5%. Kühe mit früh auftretender Ketose hatten im Vergleich zu nicht ketotischen Kühen eine höhere Einsatzleistung (+ 3,0 kg / d, P <0,001) und 100-Tage-Leistung (+ 301,6 kg; P <0,001). Spät auftretender Ketose hatte keine Auswirkungen auf die Milchproduktion. Unabhängig vom zeitlichen Auftreten, hatte Hyperketonämie keine Auswirkungen auf die Fruchtbarkeit und Abgangsrate.

Zur weiteren Untersuchung der Theorie zu Typ I und II Ketose wurde eine dritte Studie durchgeführt, um den Zusammenhang zwischen Hyperketonämie und Hypoglykämie in der Frühlaktation zu untersuchen. Laut dieser Theorie handelt es sich bei früh auftretender Ketose (1. bis 2. Woche postpartum) um die hyperglykämisch-hyperinsulinämische Form und bei der spät auftretenden Ketose (3. bis 6. Woche postpartum) um die hypoglykämisch-hypoinsulinämische Form. Insgesamt wurden 655 Milchkühe aus 6 kommerziellen Milchviehbetrieben in Deutschland zwischen dem 1. und 4. Tag nach der Abkalbung in die

Studie aufgenommen. Die Kühe wurden zweimal wöchentlich für 6 Wochen mit jeweils einem elektronischen Handmessgerät für Glukose und BHBA getestet. Hypoglykämie wurde als Glucosekonzentration ≤ 2,2 mmol / L definiert. Hyperketonämie wurde als BHBA-Konzentration ≥ 1,2 mmol / l definiert. Wie oben beschrieben wurde Hyperketonämie in frühes Auftreten (Woche 1 bis 2 postpartum) bzw. spätes Auftreten (Woche 2 bis 6 postpartum) eingeteilt. Die Auswirkung des Erkrankungszeitpunktes auf die Glukosekonzentration im Blut innerhalb von 42 Tagen in Milch wurde unter Verwendung eines generalisierten linearen gemischten Modells ausgewertet. Bei erstlaktierenden Kühen war kein Effekt von Hyperketonämie auf die Glukosekonzentration zu beobachten (P> 0,1). Kühe ab der 2. Laktation mit früh auftretender Ketose hatten eine geringere Glukosekonzentration (-0,21 mmol / L) im Vergleich zu nicht ketotischen Kühen (P = 0,041). Im Unterschied zur Theorie der Typ I und II Ketose, gab es keinen Zusammenhang zwischen Hypoglykämie und spät auftretender Hyperketonämie, stattdessen war Hypoglykämie mit früh auftretender Hyperketonämie assoziiert. Die Prävalenz und Inzidenz der Hypoglykämie war bei erstlaktierenden Kühen insgesamt niedriger als bei Kühen ab der 2. Laktation. Hypoglykämie bei Kühen ab der 2. Laktation war mit einer höheren Einsatzleistung und 100-Tage-Leistung verbunden. Zusammenfassend zeigen die Ergebnisse, dass Hypoglykämie vor allem bei Kühen ab der 2. Laktation mit früh auftretender Hyperketonämie vorkam, während erstlaktierende Kühe ein geringeres Risiko für Hypoglykämie hatten, vermutlich aufgrund der geringeren metabolischen Belastung.

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8 PUBLICATIONS

8.1 Research articles

J. Ruoff, S. Bertulat, O. Burfeind and W. Heuwieser (2016):

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8.2 Oral presentations at conferences

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10 DECLARATION OF INDEPENDENCE

Hiermit erkläre ich, dass ich, Julia Ruoff, alle Studien selbständig durchgeführt und die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Tabelle 1. Eigener Anteil[1] an den Forschungsprojekten der vorliegenden Dissertation

	Studie 1[a]	Studie 2[b]	Studie 3[c]
Studienplanung	+++	+++	+++
Datenerhebung	+++	+++	+++
Datenanalyse	+++	+++	+++
Verfassen des Manuskripts	+++	+++	+++
Editieren des Manuskripts	++	++	++

[1]Legende: +++: > 70%

++: 50−70%

+: < 50%

[a] Associations of β-hydroxybutyrate, cholesterol, triglycerides and high-density lipoproteins to non-esterified fatty acids pre- and postpartum

[b] Effects of hyperketonemia within the first six weeks of lactation on milk production and reproductive performance

[c] *Short communication:* Associations between blood glucose concentration, onset of hyperketonemia and milk production in early lactation dairy cows

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