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Identifying poor metabolic adaptation during early lactation in dairy cows using cluster analysis

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ABSTRACT

Currently, cows with poor metabolic adaptation during early lactation, or poor metabolic adaptation syndrome (PMAS), are often identified based on detection of hyperketonemia. Unfortunately, elevated blood ketones do not manifest consistently with indications of PMAS. Expected indicators of PMAS include elevated liver enzymes and bilirubin, decreased rumen fill, reduced rumen contractions, and a decrease in milk production. Cows with PMAS typically are higher producing, older cows that are earlier in lactation and have greater body condition score at the start of lactation. It was our aim to evaluate commonly used measures of metabolic health (input variables) that were available [i.e., blood β-hydroxybutyrate acid, milk fat: protein ratio, blood nonesterified fatty acids (NEFA)] to characterize PMAS. Bavarian farms (n = 26) with robotic milking systems were enrolled for weekly visits for an average of 6.7 wk. Physical examinations of the cows (5–50 d in milk) were performed by veterinarians during each visit, and blood and milk samples were collected. Resulting data included 790 observations from 312 cows (309 Simmental, 1 Red Holstein, 2 Holstein). Principal component analysis was conducted on the 3 input variables, followed by K-means cluster analysis of the first 2 orthogonal components. The 5 resulting clusters were then ascribed to low, intermediate, or high PMAS classes based on their degree of agreement with expected PMAS indicators and characteristics in comparison with other clusters. Results revealed that PMAS classes were most significantly associated with blood NEFA levels. Next, we evaluated NEFA values that classify observations into appropriate PMAS classes in this data set, which we called separation values. Our resulting NEFA separation values [<0.39 mmol/L (95% confidence limits = 0.360–0.410) to identify low PMAS observations and ≥0.7 mmol/L (95% confidence limits = 0.650–0.775) to identify high PMAS observations] were similar to values determined for Holsteins in conventional milking settings diagnosed with hyperketonemia and clinical symptoms such as anorexia and a reduction in milk yield, as reported in the literature. Future studies evaluating additional clinical and laboratory data, breeds, and milking systems are needed to validate these finding. The aim of future studies would be to build a PMAS prediction model to alert producers of cows needing attention and help evaluate on-farm metabolic health management at the herd level.

Key words: metabolic adaptation, cluster analysis, negative energy balance, nonesterified fatty acid

INTRODUCTION

Cows with poor metabolic adaptation during early lactation, or poor metabolic adaptation syndrome (**PMAS**), are often identified based on detection of hyperketonemia (blood BHB ≥1.2 mmol/L). Despite initial observations (Sjollema and Van der Zande, 1923; Shaw, 1956), elevated blood ketone levels do not manifest consistently with indications of poor metabolic adaptation during early lactation (Andersson, 1984; Simensen et al., 1990; Duffield et al., 2009). The indications for poor metabolic adaptation to negative energy balance (**NEB**) during early lactation are secondary to the high energy demands of milk production and a concurrent decrease in DMI that is independent of milk production demands (Baird, 1982). Expected indications of PMAS include elevated liver enzymes and bilirubin, decreased rumen fill, reduced rumen contractions, and a decrease in milk production (Ghanem et al., 2016; Issi et al., 2016; Cao et al., 2017). Cows with PMAS typically are higher producing, older cows that are earlier in lactation and have greater BCS at the start of lactation

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(Baird, 1982; Rukkwamsuk et al., 1999; Andrews et al., 2004; Ghanem et al., 2016).

The need for an accurate measurement associated with PMAS has not been addressed. It was our aim to re-evaluate the commonly used measures of metabolic health (input variables) that were available [i.e., blood BHB acid, milk fat:protein ratio, blood nonesterified fatty acids (**NEFA**)] to characterize patterns of PMAS. Unlike some infectious diseases with clear case definitions (present or absent), cases of metabolic disease are more defined as syndromes observed on a spectrum of signs. A strictly binomial outcome variable such as "diseased or healthy" can be difficult to define for the purpose of prediction models. Principal component analysis (**PCA**) and cluster analysis do not require an outcome variable. A PCA detects important patterns among cases by generating linear combinations of meaningful potential predictors that represent the data's variance associated with disease. The PCA is followed by a cluster analysis that systematically groups the most similar observations into clusters that best explain the data's variance and therefore disease states (Borcard et al., 2011).

We hypothesized that performing a PCA and a cluster analysis using the input variables would differentiate groups of cattle with regards to patterns of PMAS. A clear understanding of PMAS is needed to further study the underlying mechanisms, possible prevention, and treatment options and to provide better indicators of genetic selection for metabolic health.

MATERIALS AND METHODS

Data Collection

Sixty farms equipped with Lely (Lely Industries N.V., Maassluis, the Netherlands) or Lemmer-Fullwood (Lemmer-Fullwood GmbH, Lohmar, Germany) automatic milking systems up to 70 km from Munich were asked to participate in the study. Twenty-six Bavarian farms (10 Lely, 16 Lemmer-Fullwood) were enrolled between May 2015 and December 2015. Data were collected between May 2015 and February 2016 as farms were enrolled. On average, farms were visited for 6.65 (SD 1.16) consecutive weeks (range: 3–10).

Up to 8 early-lactation cows between 5 and 50 DIM were evaluated during each visit. If more than 8 cows were between 5 and 50 DIM, the 8 cows earliest in their lactation were sampled. There was no minimum number of cows sampled to be included in the analysis. Milk samples were collected from all milkings on the day before the visit using an automatic sample collecting system attached to the automatic milking system for a minimum duration of 12 h (0700–1900 h or 0800–2000 h). Milk collection had to be from voluntary milkings (samples were not collected by hand, and cows were not fetched into the milking robot for collection).

Physical exams of the cows and blood sample collection were performed by the same 2 veterinarians (SP and HL). To screen animals for negative health conditions other than PMAS, physical exams included evaluating behavior, hygiene, and conformation; measuring internal body temperature, heart rate, and respiration rate; and performing heart auscultation, lung auscultation, complete udder examination, abdominal auscultation, percussion, and rectal palpation. Farm and cow identification numbers, date, DIM, breed, and lactation number were recorded. Clinical information documented for use in the analysis included the frequency of rumen contractions as described by Dirksen (1979), milk reduction compared with the day before, back fat measured by ultrasound as described by Staufenbiel (1992), change in back fat in 1 wk, and rumen fill (scored between 1 and 5, with 5 representing the most fill; Zaaijer and Noordhuizen, 2003; Appendix, Table A1).

Blood samples were analyzed using the Cobas c311-Analyzer (Roche Diagnostics, Rotkreuz, Switzerland) for total blood protein, albumin, cortisol, bilirubin, aspartate aminotransferase (**AST**), gamma-glutamyl transferase (**GGT**), glutamate dehydrogenase (**GLDH**), creatine kinase (**CK**), BHB, and NEFA. Milk production (kg) was calculated using the automatic milking system mid-24-h milk production measurement. Corresponding milk samples were analyzed for milk fat and protein percentage, urea, and lactose using the MilkoScan FT-6000 (Foss GmbH, Hamburg, Germany), and SCC was measured using the Fossomatic 5000 (Foss GmbH).

Data Editing

Several criteria were used to select data for the analysis. Observations were removed if any non-PMAS-related health event was suspected or diagnosed at the time of the physical exam and if milk data were not collected from the robot. The earlier observations were removed if multiple milk samples were collected from a cow within the previous 12-h period. Outliers, most likely due to data entry errors, were identified by visual inspection of each variable's histogram. Finally, observations were removed if they had a missing value for an input variable. The descriptive statistics (mean, standard deviation, and number of missing values) and variable descriptions of the final data set were examined.

PCA and Cluster Analysis

All analyses were performed using the program R version 3.0.1 (R Development Core Team, 2013). The princomp and kmeans functions were used to perform the PCA and cluster analysis, respectively. The assumption of PCA is that input variables are normally distributed and that they have linear relationships (Borcard et al., 2011). The statistical assumption about the independence of observations can be relaxed with heuristic procedures (noninference methods) such as PCA and cluster analysis (Jolliffe, 2002). The 3 input variables [NEFA, BHB, and milk fat:protein ratio (**FPR**)] were scaled and centered to standardize the data using the scale function in R. The scale function subtracts the mean of each variable from all the variable's values and then divides each value by the variable's standard deviation. Furthermore, scatter plots of the input variables were inspected for nonlinear relationships.

A PCA was performed to transform the data into several orthogonal principal components (**PC**; Borcard et al., 2011). The PC are ordered in descending order based on the amount of the variance they explain. The PCA results were examined by means of a scree plot that shows the decreasing amount of variance explained by PC sorted by the amount of variance explained. The "elbow rule" was applied to determine how many PC would be used in the cluster analysis. Briefly, the elbow rule selects PC up until the elbow of the plot, where the slope between PC begins to increase most prominently (Jackson, 1993; Johnson and Wichern, 2002).

A cluster analysis was performed using K-means, a least squares method. K-means is a linear method and as such requires normally distributed input variables that are not highly correlated (Borcard et al., 2011). The resulting PC were visually inspected for normality by creating histograms, and pairwise Pearson correlations were calculated. The wrapper cascadeKM() calculated the simple structure index (**SSI**) criterion 1,000 times per cluster number between 2 and 10 clusters (Borcard et al., 2011). The final number of clusters was selected by applying the elbow rule to the SSI plot. This was done to balance the minimum number of clusters with the maximum SSI criteria (Hothorn and Everitt, 2014). The silhouette plot was used to identify misclassifications (any observations with negative silhouette widths) and to evaluate the distribution of observations among clusters.

Comparison of External Variables per Cluster or PMAS Class

External variables are all the variables available that were not used as input variables for the PCA and cluster analysis: DIM, lactation, clinical information, and blood and milk data excluding BHB, FPR, and NEFA. Linear mixed-effect regression models were used to test for statistically significant associations between each of the external variables and the clusters with an α of 0.05. Cow ID and Farm ID were included as random effects on the intercept. Because there was no within-cow variation in lactation number, duplicate cow–cluster observations were removed and only farm ID was included as a random effect when modeling lactation number. A fixed effect of DIM and an interaction between DIM and cluster number were added if they significantly improved the models' goodness of fit using a log-likelihood ratio test. Goodness of fit was evaluated using diagnostic plots of the residuals among which the predicted versus fitted values plot. External variables were log-transformed to normalize residuals, but the model estimates were transformed into the original scale for reporting the results. Results were presented as least squares means and standard errors per cluster, and post hoc comparisons among clusters' estimates were adjusted for multiple comparisons using Tukey's honest significant difference method (Gelman and Hill, 2006). The significance of cluster number as a fixed effect was based on type III sum of squares and used an α level of 0.05 to determine significance. Post hoc estimates for back fat were also reported at the beginning of lactation (DIM = 5) when the interaction between cluster number and DIM was significant. Linear mixed-effect regression models were used again to quantify associations among each of the external variables and the 3 PMAS classes described in the next paragraph.

Classification of Clusters to PMAS Classes

The clusters' external variable characteristics were compared with expected indicators and characteristics of PMAS, including elevated liver enzymes and bilirubin, decreased rumen fill, reduced rumen contractions, and a decrease in milk production (Ghanem et al., 2016; Issi et al., 2016; Cao et al., 2017). Cows with PMAS typically are higher producing, older cows that are earlier in lactation and have greater BCS at the start of lactation (Baird, 1982; Rukkwamsuk et al., 1999; Andrews et al., 2004; Ghanem et al., 2016). The clusters were then ascribed to low, intermediate, or high PMAS classes based on their degree of agreement with expected PMAS indicators in comparison with other clusters.

Separation of PMAS Classes

The PCA biplot was examined to identify how the input variables influenced the cluster separation and how

clusters separated into the new PMAS classifications. The most influential input variable(s) was selected as the PMAS measure to be used to identify values that classify observations into appropriate PMAS classes in this data set, which we called separation values. Separation values that maximized the accuracy of classification were selected. Accuracy is the proportion of correctly classified observations out of all observations (Dohoo et al., 2012). First, separation values of the selected PMAS measure were evaluated for correctly predicting the PMAS classifications of intermediate PMAS observations compared with low PMAS observations in this data set. Second, separation values of the PMAS measure were evaluated for correctly predicting the PMAS classifications of the high PMAS observations compared with intermediate PMAS observations in this data set.

RESULTS

Data Collection

On average, 14.65 (SD 3.68) cows were sampled per farm (range: 9–21). A total of 381 cows were evaluated. An average of 57.88 (SD 20.50) observations were collected per farm (range: 22–116). Each cow was evaluated on average 3.95 times (SD 2.50).

Table 1. Descriptive statistics of all variables in a data set of n = 790 observations originating from 312 cows and 26 Bavarian herds sampled between 5 and 50 DIM

Variable	Mean	SD	Missing values (no.)
Lactation no.	3.00	1.60	0
DIM	27.5	12.0	0
Milk production,[1] kg	32.0	7.1	0
Milk fat, %	4.16	0.83	0
Milk protein, %	3.27	0.32	0
Milk fat:protein ratio	1.28	0.25	0
SCC, 1,000 cells/mL	158.8	488.4	0
Urea, mg/dL	23.8	8.7	0
Lactose, %	4.83	0.17	0
Blood protein, g/L	71.2	5.1	0
Albumin, g/L	36.5	2.8	0
Bilirubin, μmol/L	1.21	1.08	0
Aspartate aminotransferase, U/L	84.2	25.1	0
Gamma-glutamyl transferase, U/L	19.8	6.1	0
Glutamate dehydrogenase, U/L	12.4	11.2	0
Creatine kinase, U/L	281	452	0
BHB, mmol/L	0.80	0.38	0
Nonesterified fatty acids, mmol/L	0.45	0.35	0
Cortisol, ng/mL	26.0	20.2	1
Rumen contractions, no./2 min	2.02	0.33	0
Diagnostic rumen fill score[2]	3.08	0.68	1
Back fat,[3] mm	12.1	3.9	15
Milk production reduction in 1 d, kg	0.012	0.055	15
Difference in back fat in 1 wk, mm	−0.63	2.37	260

[1]Mid-24-h milk calculated from robot data.
[2]Scoring system described in the Appendix. Theoretical range = 1–5.
[3]Measured by ultrasound.

Data Editing

The starting data set contained 1,505 observations; 427 observations were removed due to a negative health condition other than PMAS being suspected. Examples of such conditions include mastitis, retained placenta, milk fever, and displaced abomasum. In addition, 254 observations were removed because of multiple milk samples corresponding to a blood sample, and 30 observations were removed due to missing milk data from the robot. Outlier observations were removed, including 2 observations with CK values above 12,000 U/L and 1 outlier sample with a blood protein value less than 5 g/L. One sample was removed due to a missing NEFA value.

The resulting data set contained 790 observations from 26 farms and represented 312 cows, of which 309 were German Simmental cows, 1 was a Red Holstein cow, and 2 were Holstein cows. On average, 12 (SD 2.99) cows were sampled per farm (range: 8–19). An average of 30.38 (SD 7.81) observations were collected per farm (range: 13–42). Each cow was evaluated an average of 2.53 times (SD 1.32). Of those, 67 cows were in their first lactations, 81 cows were in their second lactations, and 164 cows were in their third or later lactations. There were 260 missing change in back fat values because the calculation of this value depended on having 2 consecutive measurements.

The descriptive statistics (mean, standard deviation, and number of missing values) of the final data set are shown in Table 1. On average, cows in this study were 27.51 DIM (SD 12.01) and produced 32.02 kg of milk/d (SD 7.10). Mean FPR was 1.28 (SD 0.25), BHB mean was 0.80 mmol/L (SD 0.38), and NEFA mean was 0.45 mmol /L (SD 0.35).

PCA and Cluster Analysis

The standardized input variables (i.e., BHB, FPR, NEFA) met the linearity assumption and were then transformed into PC by means of a PCA to be used in the cluster analysis. The first and second components (PC1, PC2) explained 76.5% of the variance in the data, and the second component was identified as the elbow in the scree plot. The loadings of NEFA, BHB, and FPR were −0.55, −0.59, and −0.59, respectively, in PC1 and 0.84, −0.38, and −0.40, respectively, in PC2.

A feature of PCA is that the resulting orthogonal PC are normally distributed and not correlated (Borcard et al., 2011); therefore, PC1 and PC2 met the assump-

tions for cluster analysis. The cluster analysis results were visualized by means of an SSI plot. Based on the elbow rule, the elbow in the SSI plot was identified at 5 clusters (SSI = 1.21). Therefore, 5 clusters were selected for our final clustering results. No misclassifications were recognized in the silhouette plot, and the number of observations and silhouette widths were similar among clusters. Cluster 1 included 234 observations, cluster 2 included 157 observations, cluster 3 included 137 observations, cluster 4 included 142 observations, and cluster 5 included 120 observations. Boxplots of the input variables per cluster number are described in Table 2. On average, a cow had observations in 1.776 different clusters (SD 0.838).

Comparison of External Variables per Cluster

Somatic cell count, GLDH, CK, and cortisol were log-transformed to normalize residuals. All regression models of the external variables included DIM as a fixed effect except DIM, FPR, BHB, rumen fill, and change in back fat. The only regression models that included an interaction between cluster number and DIM were milk protein, bilirubin, AST, GLDH, NEFA, and back fat. All external variables, with the exception of urea, SCC, albumin, GGT, CK, rumen contractions, and milk production reduction, were significantly associated with cluster assignment ($P < 0.05$; Table 2). The input variables' linear mixed-effects regression model results were also reported for comparison (Table 2), although it is to be expected that they would be significantly associated with the cluster classifications (Legendre and Legendre, 2012).

Classification of Clusters to PMAS Classes

Clusters 1 and 2 had the greatest rumen fill and consisted of younger cows and cows later in lactation compared with the other clusters (Table 2). Clusters 1 and 2 had low bilirubin, AST, GLDH, CK, and

Table 2. Results of the linear mixed-effects regression models including least squares means and standard errors (in parentheses) by cluster number and type III sum of squares *P*-values[1]

	Cluster					
Variable	1	2	3	4	5	*P*-value
Lactation[2]	2.70[a] (0.17)	2.84[a] (0.19)	3.11[a] (0.20)	3.09[a] (0.19)	2.87[a] (0.19)	0.16
DIM	30.6[a] (1.03)	30.5[a] (1.17)	23.2[b] (1.24)	23.5[b] (1.20)	26.3[b] (1.26)	<0.001
Milk production, kg	31.2[bc] (0.73)	31.4[bc] (0.74)	32.7[a] (0.76)	32.1[ab] (0.75)	30.9[c] (0.75)	<0.001
Milk fat, %	3.76[d] (0.053)	4.44[b] (0.060)	4.23[c] (0.064)	4.89[a] (0.062)	3.56[e] (0.064)	<0.001
Milk protein,[3] %	3.34[a] (0.031)	3.24[b] (0.033)	3.18[bc] (0.035)	3.11[c] (0.034)	3.36[a] (0.034)	<0.001
Milk fat:protein ratio[4]	1.12[c] (0.012)	1.36[b] (0.014)	1.33[b] (0.015)	1.57[a] (0.015)	1.06[d] (0.016)	<0.001
SCC, 1,000 cells/mL	66.7[a] (6.70)	81.5[a] (8.87)	66.0[a] (7.59)	72.3[a] (8.04)	74.6[a] (8.45)	0.17
Urea, mg/dL	23.9[a] (1.11)	23.5[a] (1.16)	23.7[a] (1.20)	23.7[a] (1.18)	22.8[a] (1.20)	0.68
Lactose, %	4.85[a] (0.013)	4.82[ab] (0.015)	4.82[ab] (0.016)	4.80[b] (0.016)	4.83[ab] (0.016)	0.058
Blood protein, g/L	71.2[a] (0.47)	70.7[a] (0.50)	71.5[a] (0.52)	70.7[a] (0.51)	71.8[a] (0.52)	0.061
Albumin, g/L	36.1[a] (0.25)	36.1[a] (0.27)	36.6[a] (0.28)	36.3[a] (0.27)	36.2[a] (0.27)	0.15
Bilirubin,[3] μmol/L	0.86[c] (0.071)	0.77[c] (0.086)	1.90[a] (0.092)	1.33[b] (0.091)	1.38[b] (0.092)	<0.001
Aspartate aminotransferase,[3] U/L	81.7[b] (1.97)	79.9[b] (2.24)	89.8[a] (2.41)	89.2[a] (2.31)	83.8[ab] (2.38)	<0.001
Gamma-glutamyl transferase, U/L	20.0[a] (0.38)	19.3[a] (0.42)	20.3[a] (0.44)	19.9[a] (0.43)	20.0[a] (0.43)	0.15
Glutamate dehydrogenase,[3] U/L	9.28[a] (0.47)	9.48[a] (0.53)	10.24[a] (0.60)	10.50[a] (0.60)	9.71[a] (0.55)	<0.001
Creatine kinase, U/L	179[a] (9.7)	175[a] (11.3)	205[a] (14.4)	205[a] (13.8)	186[a] (13.2)	0.24
Blood BHB,[4] mmol/L	0.68[c] (0.029)	0.86[b] (0.032)	0.79[b] (0.034)	1.11[a] (0.033)	0.60[c] (0.034)	<0.001
Blood nonesterified fatty acids,[3,4] mmol/L	0.264[c] (0.016)	0.242[c] (0.020)	0.889[a] (0.021)	0.516[b] (0.021)	0.490[b] (0.021)	<0.001
Cortisol, ng/mL	18.6[a] (1.51)	17.8[ab] (1.62)	23.1[a] (2.24)	14.2[b] (1.33)	19.5[a] (1.89)	<0.001
Rumen contractions (no./2 min)	2.00[a] (0.024)	2.02[a] (0.029)	1.99[a] (0.031)	2.01[a] (0.030)	2.06[a] (0.032)	0.52
Rumen fill[5]	3.17[a] (0.058)	3.21[a] (0.065)	2.92[b] (0.070)	3.09[ab] (0.067)	2.95[b] (0.069)	<0.001
Back fat,[3] mm	12.2[a] (0.41)	12.3[a] (0.43)	12.5[a] (0.44)	12.1[a] (0.43)	12.1[a] (0.44)	<0.001
DIM = 5	13.2[bc] (0.54)	12.8[c] (0.64)	15.1[a] (0.57)	14.7[ab] (0.57)	13.8[abc] (0.56)	
Milk production reduction, kg	0.019[a] (0.004)	0.005[a] (0.004)	0.010[a] (0.005)	0.012[a] (0.005)	0.010[a] (0.005)	0.18
Change in back fat, mm	−0.18[b] (0.189)	−0.51[ab] (0.217)	−0.87[ab] (0.244)	−1.25[a] (0.248)	−0.74[ab] (0.273)	0.010

[a–e]Means within a row with different superscripts differ ($P < 0.05$).

[1]Multiple comparisons among cluster numbers are adjusted using Tukey's honestly significant difference method. The data set originated from 312 cows and 26 Bavarian herds sampled 5 to 50 DIM (n = 790).

[2]Duplicate cluster–cow combinations removed due to a lack in variance per cow (n = 554).

[3]Significant interaction between cluster and DIM ($P < 0.05$).

[4]These variables were used as input variables for the cluster analysis and are therefore expected to be significantly associated among clusters.

[5]The description of the scoring system is available in the Appendix. Theoretical range = 1–5.

NEFA. These characteristics align with characteristics of healthy cows. Cluster 3 had greater milk production, greater back fat at the beginning of lactation (DIM = 5), and earlier DIM compared with clusters 1 and 2 (Table 2). These risk factors, in addition to decreased rumen fill and elevated bilirubin, AST, GLDH, CK, and NEFA, align with expected characteristics of cows with PMAS. Clusters 4 and 5 had intermediate back fat at the beginning of lactation (DIM = 5), rumen fill, bilirubin, and NEFA (Table 2). These intermediate levels of liver values and clinical results during early lactation placed clusters 4 and 5 between the levels of agreement of the other clusters. Therefore, clusters 1 and 2 were classified together as low, clusters 4 and 5 were classified as intermediate, and cluster 3 was redefined as the only cluster with high agreement with expected PMAS indicators.

On average, cows had observations in 1.532 PMAS classes (SD 0.641). Eighty-seven cows had at least 1 observation classified in the high PMAS class. Thirty-one cows had more than 1 observation classified in the high PMAS class.

Comparison of External Variables per PMAS Class

Somatic cell count, GLDH, CK, and cortisol were log-transformed to normalize residuals. All regression models of the external variables included DIM as a fixed effect except DIM, rumen fill, and change in back fat. The only regression models that included an interaction between cluster number and DIM were milk protein, bilirubin, AST, GLDH, BHB, NEFA, and back fat. All external variables, with the exception of lactation, urea, SCC, lactose, blood protein, GGT, CK, rumen contractions, and milk production reduction, were significantly associated with the PMAS classifications ($P < 0.05$; Table 3). The input variables' linear mixed-effects regression model results were also reported for comparison, although it is to be expected that they would be significantly associated with the PMAS classifications (Legendre and Legendre, 2012). The low PMAS class had significantly lower average FPR, bilirubin, AST, and NEFA compared with the intermediate and high PMAS classes (Table 3). The low PMAS class also had significantly greater DIM, rumen fill, and milk protein compared with the intermediate and high PMAS classes (Table 3). Although not significantly different, the low PMAS class had lower average lactation number, milk production, and albumin compared with the intermediate and high PMAS classes. The high PMAS class had significantly lower BHB and greater milk production, bilirubin, NEFA, and cortisol compared with the intermediate PMAS class (Table 3). Although not significantly different, the high PMAS class had the lowest rumen fill and greatest back fat at beginning of lactation compared with low and intermediate PMAS classes. Bilirubin and NEFA were the only variables that were significantly different among all 3 PMAS classifications.

Separation of PMAS Classes

Examining the biplot of the PCA, it is apparent that NEFA's direction of influence is what separated out the 3 PMAS classifications in our data set (Figure 1). The influence of BHB was in the same direction as the one of FPR (arrows overlap in Figure 1). The direction of influence of BHB and FPR separated out cluster 1 from cluster 2 and cluster 4 from cluster 5 within their own classification of low and intermediate PMAS, respectively. Nonesterified fatty acids was selected as the PMAS measure for this data set because NEFA's direction of influence in the biplot was responsible for separating out low, intermediate, and high PMAS classifications, and NEFA was the only input variable significantly different among all 3 PMAS classifications. The greatest accuracy of separation between low and intermediate PMAS observations was at a value of 0.390 (95% CI: 0.360–0.410) mmol/L of NEFA (Figure 2). The greatest accuracy of separation between intermediate and high PMAS observations in this data set was at a value of 0.700 (95% CI: 0.650–0.775) mmol/L of NEFA (Figure 3).

DISCUSSION

Metabolic Adaptation to NEB

The 3 levels of agreement with expected PMAS indicators did not follow differences in BHB levels. This was highlighted by the differences between clusters 3 and 4 wherein cluster 3 had the highest agreement with expected PMAS indicators, whereas cluster 4 had the highest BHB values. The contrast between PMAS classes and BHB measurements may be due to the fact that ketogenesis, and resulting ketonemia, are normal physiological responses to compensate for NEB and do not necessarily reflect pathological changes. Indeed, keto-adaptation is a well-known phenomenon; in humans, ketones become the major fuel source following a period of adaptation to low carbohydrate intake. Furthermore, endurance athletes have been shown to be in an almost constant state of ketonemia during NEB (Volek et al., 2016). As ketonemia does not necessarily reflect pathology, it becomes important for veterinary

clinicians to be able to distinguish between appropriate and inappropriate responses to NEB.

Klein et al. (2012) proposed that cows may compensate for NEB in 1 of 2 ways: by reducing fat in milk or by increasing fat mobilization from adipose tissue. Only the latter group consistently developed hyperketonemia (Klein et al., 2012). Although more research is needed in this area, our data are in agreement with this hypothesis. Cluster 5, a group with intermediate NEFA levels, had low milk fat but no elevation in BHB compared with cluster 4, which had similar NEFA levels. This suggests that cluster 5 adapts to NEB either by limiting milk fat or by being limited in ketogenesis, which in turn limits milk fat (Baumgard et al., 2000). Cluster 4 had the highest BHB level as well as the highest milk fat of any cluster. This suggests that cluster 4 adapts to NEB by increasing ketogenesis and not by limiting milk fat. Cluster 3 had the highest agreement with expected PMAS indicators. These observations did not have decreased milk fat like cluster 5 or mobilized ketones like cluster 4, which suggests that they did not adapt appropriately to NEB. At the same time, cluster 3 exhibited higher NEFA values than clusters 4 or 5.

NEFA Separation Values

Currently, NEFA values are used during the prepartum period to indicate the success of transition cow management programs (Oetzel, 2007). The majority of studies have focused on the use of NEFA values to predict negative sequellae during lactation (e.g., displaced abomasum, retained placenta, metritis, culling, reduced reproduction performance, and so on). These outcomes can result from elevated NEFA, which can impair immune, liver, and ovarian function (Adewuyi et al., 2005). Furthermore, NEFA values above 0.4 mmol/L during the prepartum period are associated with negative outcomes during the subsequent lactation

Table 3. Results of the linear mixed-effects regression models including least squares means and standard errors (in parentheses) by poor metabolic adaptation syndrome (PMAS) classification and type III sum of squares *P*-values[1]

	PMAS classification[2]			
Variable	Low	Intermediate	High	*P*-value
Lactation[3]	2.76[a] (0.158)	2.97[a] (0.164)	3.12[a] (0.202)	0.15
DIM	30.5[a] (0.93)	24.8[b] (1.00)	23.2[b] (1.24)	<0.001
Milk production, kg	31.3[b] (0.72)	31.5[b] (0.72)	32.7[a] (0.76)	<0.001
Milk fat, %	4.03[b] (0.064)	4.27[a] (0.068)	4.20[ab] (0.084)	<0.001
Milk protein,[4] %	3.30[a] (0.030)	3.24[b] (0.031)	3.18[b] (0.035)	0.028
Milk fat:protein ratio[5]	1.22[b] (0.018)	1.33[a] (0.019)	1.32[a] (0.025)	<0.001
SCC, 1,000 cells/mL	72.0[a] (6.71)	73.4[a] (7.13)	65.8[a] (7.56)	0.51
Urea, mg/dL	23.8[a] (1.08)	23.3[a] (1.10)	23.7[a] (1.20)	0.69
Lactose, %	4.84[a] (0.012)	4.81[a] (0.013)	4.82[a] (0.016)	0.12
Blood protein, g/L	71.0[a] (0.45)	71.3[a] (0.46)	71.5[a] (0.53)	0.41
Albumin, g/L	36.1[b] (0.24)	36.3[ab] (0.25)	36.6[a] (0.28)	0.037
Bilirubin,[4] μmol/L	0.83[c] (0.060)	1.38[b] (0.068)	1.90[a] (0.093)	<0.001
Aspartate aminotransferase,[4] U/L	80.4[b] (1.76)	86.3[a] (1.90)	87.9[a] (2.44)	<0.001
Gamma-glutamyl transferase, U/L	19.7[a] (0.35)	20.0[a] (0.37)	20.3[a] (0.44)	0.31
Glutamate dehydrogenase,[4] U/L	9.38[a] (0.456)	10.10[a] (0.508)	10.21[a] (0.602)	<0.001
Creatine kinase, U/L	177[a] (8.1)	196[a] (10.1)	204[a] (14.3)	0.12
Blood BHB,[5] mmol/L	0.761[b] (0.032)	0.847[a] (0.033)	0.771[ab] (0.041)	<0.001
Blood nonesterified fatty acids,[4,5] mmol/L	0.256[c] (0.014)	0.507[b] (0.015)	0.889[a] (0.021)	<0.001
Cortisol, ng/mL	18.1[b] (1.35)	16.5[b] (1.31)	23.3[a] (2.28)	<0.001
Rumen contractions, no./2 min	2.01[a] (0.020)	2.03[a] (0.023)	1.99[a] (0.031)	0.46
Rumen fill[6]	3.19[a] (0.054)	3.02[b] (0.057)	2.92[b] (0.070)	<0.001
Back fat,[4] mm	12.2[a] (0.40)	12.0[a] (0.40)	12.4[a] (0.44)	<0.001
DIM = 5	13.1[b] (0.49)	14.1[a] (0.48)	15.1[a] (0.56)	
Milk production reduction, kg	0.013[a] (0.003)	0.011[a] (0.003)	0.010[a] (0.005)	0.77
Change in back fat, mm	−0.32[b] (0.143)	−1.02[a] (0.184)	−0.87[ab] (0.244)	0.007

[a–c]Means within a row with different superscripts differ ($P < 0.05$).

[1]Multiple comparisons among PMAS classification are adjusted using Tukey's honestly significant difference method. The data set originated from 312 cows and 26 Bavarian herds sampled 5 to 50 DIM (n = 790).

[2]PMAS classification = degree of agreement with expected PMAS indicators in comparison with other clusters.

[3]Duplicate cluster–cow combinations removed due to a lack in variance per cow (n = 478).

[4]Significant interaction ($P < 0.05$).

[5]These variables were used as input variables for the cluster analysis and are therefore expected to be significantly associated among clusters.

[6]The description of the scoring system is available in the Appendix. Theoretical range = 1–5.

(Whitaker, 2004; McArt et al., 2013). When measured during the postpartum period, the NEFA cut-off value used to predict negative outcomes is >0.7 mmol/L (Whitaker, 2004; McArt et al., 2013). The separation values we determined for these data [NEFA <0.39 (95% CI: 0.360–0.410) mmol/L to identify low PMAS observations and ≥0.7 (95% CI: 0.650–0.775) mmol/L to identify high PMAS observations) were similar to those values used to predict negative health outcomes later during lactation.

Cao et al. (2017) suggested NEFA values greater than 0.82 mmol/L as the cut-off for diagnosing cows with BHB greater than 1.2 mmol/L and clinical symptoms such as anorexia and a reduction in milk yield. Considering that Cao et al. (2017) examined Holsteins exclusively and used a case definition of cows with BHB greater than 1.2 mmol/L and clinical symptoms, their reported cut-off values for NEFA were surprisingly similar to the high PMAS separation value determined in our study that examined predominantly Simmentals. However, 0.82 mmol/L is not included in our separation value's confidence intervals of 0.650 to 0.775 mmol/L. In addition to differences in breed and case definitions, the difference in NEFA separation values between Cao et al. (2017) and our study could be due to the difference in ability to identify subtle indications of PMAS of the individual performing the exam.

Outlook

The number of rumen contractions was not significantly associated with the clusters in our study. This

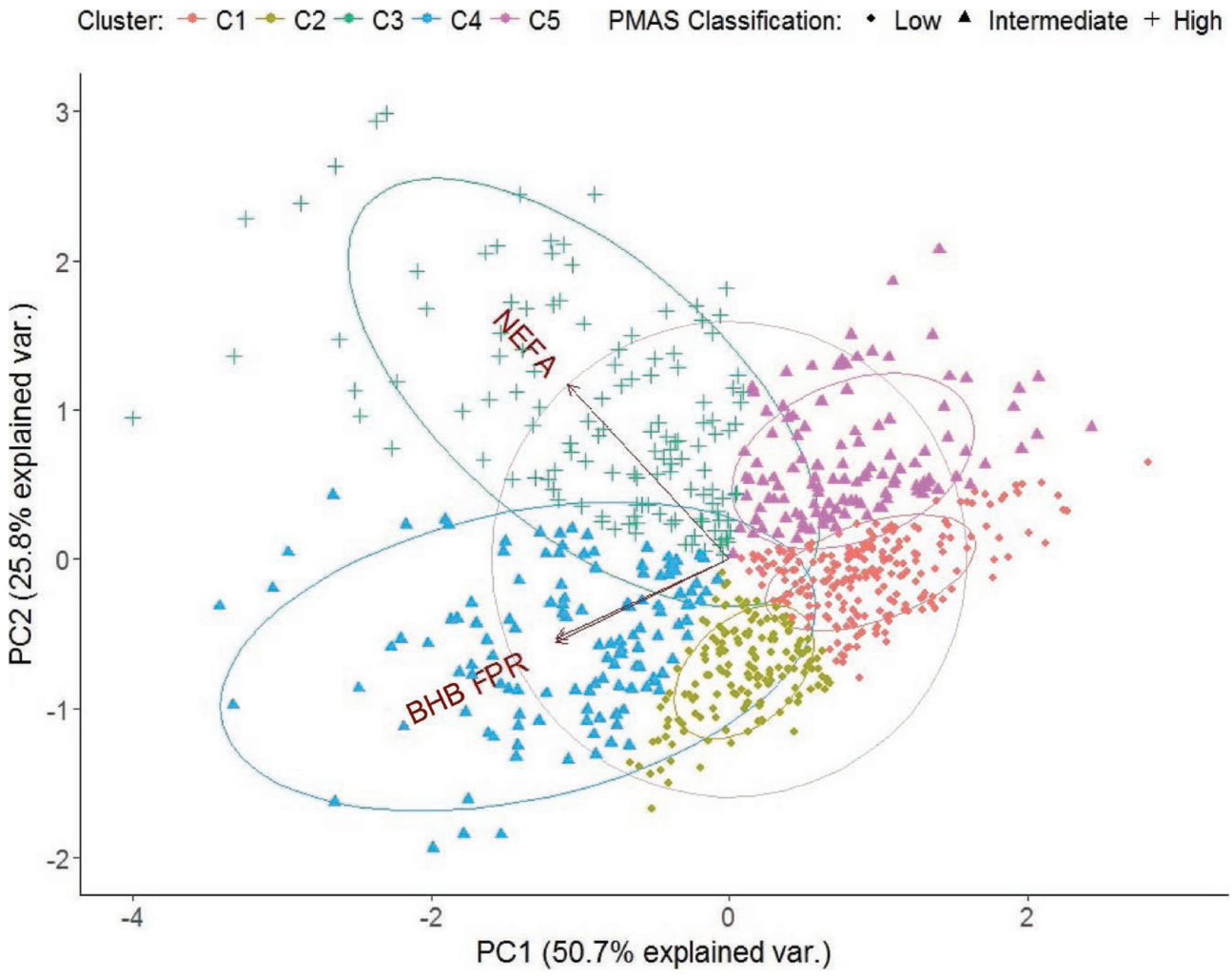

Figure 1. Biplot of the principal component analysis results by cluster (C) number and assigned poor metabolic adaptation syndrome (PMAS) classification. PC = principal component; var. = variance; NEFA = nonesterified fatty acids; FPR = milk fat:protein ratio. PMAS classification refers to the degree of agreement with expected PMAS indicators in comparison with other clusters. The data set originated from 312 cows and 26 Bavarian herds sampled 5 to 50 DIM (n = 790). Color version available online.

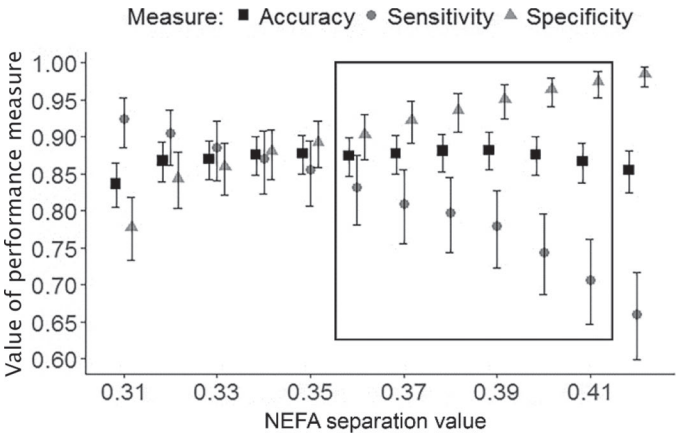

Figure 2. Classification performance measures (accuracy, sensitivity, and specificity) for classifying low poor metabolic adaptation syndrome (PMAS) and intermediate PMAS observations by nonesterified fatty acid (NEFA) value. The box surrounds values that have overlapping confidence intervals with the separation value that has greatest accuracy. The data set originated from 312 cows and 26 Bavarian herds sampled 5 to 50 DIM (n = 790). Error bars indicate 95% CI.

finding was surprising and could be caused by several factors, including large individual variation among cows, differences in time between feeding and sampling, and differences in nutrition. The most likely reason for the lack of detectable difference in rumen contractions among clusters is short intervals of measurements of 2 min as described by Dirksen (1979) versus 5 min used by Issi et al. (2016) used when describing a significant difference in rumen contractions. Reduced milk production was not significantly associated with PMAS

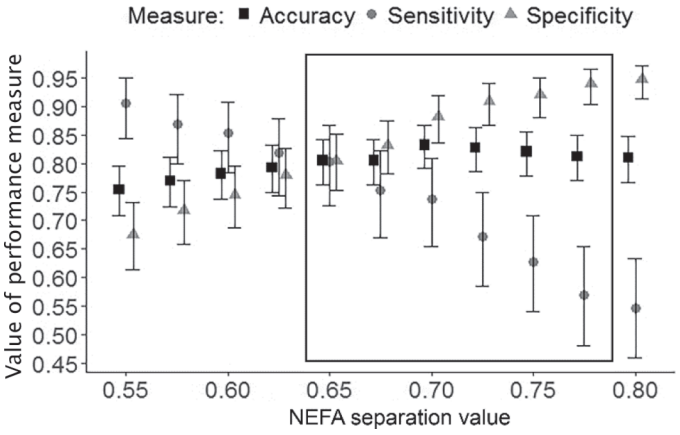

Figure 3. Classification performance measures (accuracy, sensitivity, and specificity) for classifying intermediate poor metabolic adaptation syndrome (PMAS) and high PMAS observations by nonesterified fatty acid (NEFA) values. The box surrounds separation values that have overlapping confidence intervals with the point that has greatest accuracy. Error bars indicate 95% CI. The data set originated from 312 cows and 26 Bavarian herds sampled 5 to 50 DIM (n = 790).

classifications in our study, although it was an expected indication of PMAS (Ghanem et al., 2016). The lack of an association between PMAS and reductions in milk production in our study may be due to fluctuations in milk production that were not detected during weekly visits or because the differences in milk production were not adjusted for the expected milk production of each cow. To better characterize the clusters, future studies should count the number of rumen contractions for at least 5 min and record milk production every day to improve the ability to detect reduced milk production.

Our study was limited by the fact that we did not include observations from cows experiencing negative health conditions other than PMAS. It is possible that other health events could also cause elevated NEFA, in which case the NEFA values from these cows could affect the accuracy of the chosen separation values to identify PMAS cows. In addition, this study was unable to determine the possible effect of previous health events or concurrent health events on PMAS classification. In our final data set, all cows were Simmental cows except 3, and these data were only from automatic milking system herds. Thus, it is possible that our findings are particular to this breed and milking system. In this analysis we did not consider feed intake; time between feeding and sampling of cows; or previous treatments, interventions, and health events because these data were not available in the provided data set. These missing variables would have been useful to characterize the clusters in more detail and could have a significant influence on cluster classification.

It is necessary to further investigate the effects of genetics on the development of PMAS as well as the various physiological mechanisms by which cows compensate for NEB to develop selection criteria against cows that are predisposed to developing PMAS. The most appropriate management strategy may vary depending on the physiological compensation mechanism. Our resulting NEFA separation values are similar to those determined for Holsteins with BHB greater than 1.2 mmol/L and clinical symptoms in conventional milking settings, but follow-up analyses are required to determine whether these separation values should be adjusted further to account for additional variables such as location, DIM, breed, milking system, and season. Further adjustments may also be necessary to differentiate PMAS from other health conditions. The selection of separation values should result in a balance between the need for high sensitivity or high specificity or both. Finally, future studies are needed to validate these findings in different populations, breeds, seasons, and locations. Because NEFA is expensive to measure, future studies could also evaluate milk Fouri-

er-transform infrared spectroscopy data for its ability to distinguish PMAS classes. This would allow routine in-line measurements to be used for PMAS prediction. Beyond individual cow detection, these separation values should be tested at the herd detection level as well to determine a herd prevalence alarm level.

CONCLUSIONS

A cluster analysis was able to differentiate groups of cattle in terms of NEB compensation mechanisms and PMAS classifications: low, intermediate, and high. Nonesterified fatty acids were the best indicator of PMAS classifications for these data, and separation values were selected at <0.39 (95% CI: 0.360–0.410) mmol/L to identify low PMAS observations and ≥ 0.7 (95% CI: 0.650–0.775) mmol/L to identify high PMAS observations. Future prospective studies are needed to validate these findings and to evaluate other possible predictors for metabolic health, such as milk Fourier-transform infrared data from milk. The aim of future studies would be to build a prediction model for PMAS to alert producers of cows needing attention in addition to helping evaluate on-farm metabolic health management (e.g., transition cow management, nutrition).

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APPENDIX

Table A1. Description of the rumen fill scoring system from Zaaijer and Noordhuizen (2003)[1]

Rumen fill score	Description
1	The para lumbar fossa[2] cavitates more than a hand's width behind the last rib and a hand's width inside under the transversal processes.
2	The para lumbar fossa cavitates a hand's width behind the last rib and to a lesser extent inside under the transversal processes.
3	The para lumbar fossa cavitates less than a hand's width behind the last rib and falls about a hand's width vertically downward from the transversal processes and then bulges out.
4	The para lumbar fossa skin covers the area behind the last rib and arches immediately outside below the transversal processes due to an extended rumen.
5	The rumen is quite distended and almost obliterates the fossa; the last rib and the transversal processes are not visible.

[1]The rumen fill scoring system was developed and described by Zaaijer and Noordhuizen (2003). Scoring was performed when standing at the left hind side of the cow. Refer to Zaaijer and Noordhuizen (2003) for more information and example photographs.

[2]The para lumbar fossa is between the last rib, the transversal processes, and the hip bone.