The influence of subclinical ketosis and possible risk factors on the first service conception success in US dairy cows

and the association of SCK and season, parity, body condition score, retained placenta, metritis, mastitis, respiratory diseases, displacement abomasum, lameness, clinical endometritis, cyclicity and AI estrus.

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Research project Florida (N-America), 5th of December 2013 – 5th of March 2014
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Prefatory note

In the master Farm Animal Health of Veterinary Medicine at the University of Utrecht all students have to do a research project of 3 months. During this project the student will learn how to write a research proposal, how to collect and analyze the data and how to write a report. This paper is the final report of the BHBA research project carried out by Mariska Bosman at the Department of Farm Animal Health of the University of Utrecht. The BHBA project was supervised by Klibs Galvão at the Department of Large Animal Clinical Sciences of the University of Florida and Peter Vos at the Department of Farm Animal Health of the University of Utrecht.

Contents

Abstract

The objectives of this study were to 1.) determine if a higher betahydroxybutyrate (BHBA) level (≥1.2 mmol/L) measured at 5 days in milk (DIM) results in a lower first service conception success; 2.) study the relationship of BHBA ≥ 1.2 mmol/L measured at 5 DIM with the following variables: season, parity, body condition score at calving (BCSCavC) and 40 DIM (BCS40C), retained placenta (RP), metritis, mastitis, respiratory diseases (Resp), displacement abomasum (DA), lameness (Lame), clinical endometritis (CE), cyclic and AI at estrus. The null hypothesis is that there is no association between BHBA ≥ 1.2 mmol/L and first service conception success.

From November 2012 to august 2013, a sample set of 1197 (474 primiparous and 723 multiparous) Holstein-Friesian cows at the Alliance farm were studied. Cows were enrolled at calving till the second pregnancy check on 60 ± 3 days after first insemination. Cows were excluded from study if sold or dead within 60 DIM (days in milk) or when data was incomplete. The definition of P32AI1 is the presence of an amniotic vesicle holding an embryo with heartbeat at 32±3 days after first artificial insemination (AI). In this study subclinical ketosis (SCK) is defined as serum concentrations of BHBA ≥ 1.2 mmol/L. Cows were inseminated by an expert inseminator when standing heat was observed or after timed artificial insemination (TAI). All cows had serum blood collected in evacuated tubes without anticoagulant on day 5 post-partum from the coccygeal artery or vein. All serum samples were analyzed for concentrations of 3-OH-butyrate (BHBA; enzymatic assay using a commercial kit, Autokit 3-HB, Wako) in the laboratory of the department of Large Animal Clinical Sciences in Florida. For the statistical analyses 1041 (ROC curve used 1050 cows) Holstein-Friesian cows were included. All analyses were performed with the SAS system version 9.3 (SAS/STAT, SAS Institute inc., Cary, NC) and MedCalc (version 12.7).

Elevated BHBA (≥1.2 mmol/L) concentrations measured with the Wako-kit on 5 DIM after calving are not associated with pregnancy after first service. The logistic regression model with P32AI1 as response variable selected by backward elimination includes 2 variables that were significantly associated; RP and mastitis; 3 variables that have a tendency toward statistical differences; DA, Lameness, and CE. So, the probability to become pregnant after first service with RP, mastitis, DA, lameness or CE is smaller than to become pregnant after first service without these diseases.

The logistic regression model with BHBA (≥1.2mmol/L) as response variable shows a statistically significantly relationship between season, parity, BCS at calving, mastitis, DA, and a tendency forstatistical difference for BSC at 40 DIM and CE. The odds of having SCK in summer was 1.7 times greater than the odds of having SCK in winter. Primiparous had lesser (0.2 times) odds of SCK than multiparous. Cows with a high (≥3.5) BCS at calving have 2.4 times greater odds of SCK than cows with a BCS lower than 3.0. Cows without a DA were 0.5 times as likely to develop SCK as cows with a DA. The odds of SCK in cows with BCS ≥ 3.5 at 40 DIM was 0.5 times less than the odds of SCK in cows with BCS < 3.0 at 40 DIM. The odds of SCK in cows without CE was 0.7 times smaller than the odds of SCK with CE. The odds of SCK without mastitis is 1.8 times more than the odds of SCK with mastitis. This is the first study that reported such a relationship between SCK and mastitis. More research should be done to better understand this association.

Keywords: subclinical ketosis, 3-hydroxybutyricacid, first service AI, dairy cow, post partum disease

Introduction

After parturition a dairy cow will experience a negative energy balance in which the energy demand for maintenance, milk production, and growth cannot be met by energy from the dry matter intake. Over the past 40 years the milk yield of dairy cattle has doubled. An increase in milk yield will also increase the susceptibility to metabolic diseases mainly related to hypocalcaemia, ketosis, and fatty liver. The early lactation period is characterized by an increase in the mobilization of body fat in the form of nonesterified fatty acids (NEFA). Incomplete oxidation of NEFA will result in acetoacetate, the first product of ketogenesis in the liver (Thomas Laeger et al., 2012), which is reduced to betahydroxybutyrate (BHBA), a ketone. These NEFA's and their products can be used as indicators to measure the energy balance (Galvão et al., 2010 en 2012). Another metabolic pathway to generate BHBA is by the oxidation of mainly butyrate in ruminal epithelial cells and the liver (Thomas Laeger et al., 2012).

In this study subclinical ketosis (SCK) is defined as serum concentrations of BHBA ≥ 1.2 mmol/L. This is a common threshold for SCK for predicting health risks in early lactation cows (Nielen et al, 1994; Duffield et al. 2009; Le Blanc, 2005). McArt et al. 2012 and Oetzel, 2004 defined SCK as a blood BHBA concentration of 1.2 to 2.9 mmol/L. A high BHBA level is an associated risk factor of several subsequent diseases; for example, displacement abomasum (Le Blanc et al, 2005; Dufield et al., 2009), (endo)metritis (Duffield et al., 2009; Galvão et al., 2012) lameness (Suthar et al., 2013). SCK negatively affects milk yield (Ospina et al., 2010b; Duffield et al. 2009), reproductive performance (Koller et al., 2003; Walsh et al., 2007a,b; Ospina et al., 2010b).

A major concern in the dairy industry is the economic losses caused by decreased fertility (J.Frossling et al., 2012). Methods to identify cows at risk of impaired fertility are needed to optimize fertility status in a herd. Blood BHBA concentrations may be a valuable tool and powerful predictor of conception to first service success. Laboratory measurement of BHBA is considered the gold standard for evaluating ketone body concentrations in blood (Duffield et al., 1997)

The objectives of this study were to 1.) determine if a higher betahydroxybutyrate (BHBA) level (≥1.2 mmol/L) measured at 5 days in milk (DIM) results in a lower first service conception success, 2.) study the relationship of BHBA ≥ 1.2 mmol/L measured at 5 DIM with season, parity, body condition score at calving (BCSCavC) and 40 DIM (BCS40C), retained placenta (RP), metritis, mastitis, respiratory diseases (Resp), displacement abomasum (DA), lameness (Lame), clinical endometritis (CE), cyclic and AI estrus.

The null hypothesis is that there is no association between BHBA ≥ 1.2 mmol/L and first service conception success. The alternative hypothesis is that there is an association.

Materials and methods

This study was approved by the University of Florida department of Large Animal Clinical Sciences, Florida.

Animals, housing and diets

The present study was conducted on a single commercial dairy farm located in Florida named Alliance Farm. The herd consists of ± 5000 milking cows and is milked three times a day. The rolling herd average was approximately 10205 kg of milk. From November 2012 to august 2013, a sample set of 1197 (474 primiparous and 723 multiparous) Holstein-Friesian cows at the Alliance farm were studied. For this study cows were enrolled at calving till the second pregnancy check on 60 ± 3 days after first artificial insemination (AI).

Cows were housed in free stall barns with sand-bedded stalls and equipment with fans and sprinklers for cooling. Primiparous and multiparous cows were housed separately. Cows were fed diets of complete mixed rations twice daily in the pre- and postpartum periods. The herd nutritionist formulated the diet using NRC guidelines (2001) for a lactating Holstein-Friesian cow producing 45 kg/day of milk with 3.5% fat and 3.2% true protein when dry matter intake is 25 kg/day. Sixty days before expected calving date the cows were moved to the far-off pens, and at twenty-one days they were moved to the close-up group. The cows were moved to the calving pens three days before expected calving or signs of calving. The fresh cows were relocated to the fresh cow pen for approximately thirty days and were next moved to the final mid-lactation pen during the experiment.

Blood collection

All cows had serum blood collected in evacuated tubes without anticoagulant on day 5 post-partum from the coccygeal artery or vein with a 20 gauge yellow blood collecting needle. Cows had their head locked into headlock stanchions during blood collection. The skin was disinfected using alcohol solution. Immediately after collecting the samples were placed in ice and blood tubes were centrifuged at 2,200 x *g* for 15 minutes at 4 ºC for plasma separation within 8 hours of collection. Plasma samples were collected in a freezer at -20 ºC until assayed. Finally all serum samples were analyzed for concentrations of 3-OH-butyrate (BHBA; enzymatic assay using a commercial kit, Autokit 3-HB, Wako) in the laboratory of the department of Large Animal Clinical Sciences in Florida. For the BHBA assay the protocol for high values was used (see appendix). For all the assays the quadratic response fitted better than the linear response. Therefore the quadratic response was used to convert it from result (mg/dL) to BHBA (mmol/l) by dividing the result by 10.41 (molecular mass of BHBA). Minitab 15.0 was used to make a fitted line plot for the values that were out of range. With this fitted line plot the missing values could be calculated and converted to a BHBA mmol/l.

Data collection

Data collection was standardized and included a list with definitions. For consistency, the definitions of **Subclinical ketosis (SCK)**: BHBA ≥ 1.2 mmol/L; **First insemination success (P32AI1):** the presence of an amniotic vesicle holding an embryo with heartbeat at 32±3 days after first AI; **Season**: Winter = 30th of November 2012 till 2th of January 2013 or Summer = 13th of June 2013 till 23th of July 2013; **Parity**: Pimiparous versus multiparous; **Body condition**: < 3.0 is low, 3.0-3.25 is medium, and ≥ 3.5 is high; On the day of calving (**BCSCavC)**(d 0) and again at 40±3 postpartum (**BSC40C)** , cows were scored using a 5-point system for dairy cows (1= emaciated, 5=obese with 0.25 increments (BCS; Ferguson et al., 1994)); **Calving Problems (CalvProb)**: If the cow had dystocia (with or without birth assistance), twins, stillbirth (calves that were born dead or died in the first 6 hours postpartum) or abortion alone or in a combination of these factors; **Retained placenta (RP)**: failure to pass the fetal membranes within 24 h after parturition diagnosed by farm personnel; **Metritis**: On the Alliance farm a daily examination (by the hospital staff) of cows from calving to 14 DIM for signs of metritis was done. Measurements of rectal temperatures were recorded using an electronic thermometer (GLA agricultural electronics, St. Louis Obispo, CA) (fever= ≥39,5). Cows with fever were evaluated for uterine discharge. Metricheck (evaluating uterine contents and odor) was used on days 4, 7 and 10 postpartum based on the scoring system of Sheldon et al. (2006) 1= clean mucus, 2= flecks, 3= <50% purulent, 4= > 50% purulent, 5= watery reddish discharge with a foul smell. Cows with score 5 were classified as metritis; **Mastitis**: Incidence of mastitis was monitored by personnel daily during milking based on presence of abnormal milk (pus/flakes) and/or swelling/ redness of the mammary gland; **Respiratory diseases (Resp):** based on herd personnel; **Displacement abomasum (DA)**: a veterinary diagnosis of a left or right side displacement based on auscultation of the characteristic 'ping' sound within 30 DIM; **Clinical endometritis (CE)**: the vaginal mucus were evaluated at 33±3 days DIM, vaginal discharge score equal or above 3 was considered as CE (Sheldon et al 2006**)**; **Lameness (Lame)**: Lame = Locomotion score ≥ 3 (1-5) at 40 ±3 DIM (locomotion score 1= normal, 2= mildly lame, 3= moderately lame, 4= lame and 5= severely lame); **Cyclic**: Cows with the presence of a corpus luteum (CL=1). If no CL was detected on day 33 or 49, the cows were considered not cycling (0); **AI estrus**: Timed AI (TAI) or Estrus (heat detection); were supplied on the excel sheet.

Farm personnel was instructed to document any stillbirth, twins, dystocia, RP, lameness, metritis, respiratory problems and displacement of abomasum according to farm standard operating procedures. In the first 10 days postpartum the research team recorded daily and afterwards recording was done by the herd personnel.

Reproductive management for first AI Postpartum:

The dairy farm used a reproductive program for synchronization of estrus and ovulation (TAI; double ovsynch). $PGF_{2\alpha}$ (5ml of lutalyse; Zoetis) was injected on day 50 ± 3 DIM and 64 ± 3 DIM. Cows tailheads were painted with chalk at the second $PGF_{2\alpha}$ injection to detect estrus. Cows received AI on the same morning if she was identified in estrus by removal of tail chalk. Cows were enrolled in the 5-days timed AI program at 76 ± 3 DIM if she was not observed in estrus within 12 days of the second $PGF_{2\alpha}$ treatment. This protocol included an intramuscular injection of GnRH (2ml of Cystorelin sterile solutions; Merial), subsequently an injection of $PGF_{2\alpha}$ on day 5 and 6. At 72 hours after the first $PGF_{2\alpha}$ a second injection of GnRH was directed at the same time as AI. Cows were inseminated by an expert inseminator when standing heat was observed or after TAI. Transrectal ultrasound equipped with a 7.5 MHz linear transducer (Easi-Scan, BCF Technology, Rochester, MN) was used to diagnose pregnancy on days 32±3 and 60±3 after first AI. The criteria used to conclude pregnancy was the presence of an amniotic vesicle holding an embryo with heartbeat.

Figure 1. *Illustration of TAI; presynch- ovsynch protocol*

TAI	Presynchronization										
Injection	PGF2α	14 days	PGF2α	12 days	GnRH	5 days	PGF2α	1 day	PGF2α	2 days	GnRH + AI
DIM (± 3)	50		64		76		81		82		84

Inclusion criteria

Data records of cows were excluded from study if cows were sold, dead, culled or were classified as "do not breed" within 60 DIM. Cows with missing records were excluded. For the statistical analyses 1041(ROC curve used 1050 cows) Holstein-Friesian cows were included.

Statistical analyses

All analyses were performed with the SAS system version 9.3 (SAS/STAT, SAS Institute inc., Cary, NC) and MedCalc (version 12.7). Season, parity, BCSCavC, BCS40C, CalvProb, RP, metritis, mastitis, Resp, DA, Lame, CE, cyclic and AI estrus were entered into Excel (Microsoft office Excel 2007) spreadsheets dichotomized (i.e., 1 = with disease, 0 = without disease). Categorical data were analyzed by logistic regression using the GLIMMIX procedure of SAS version 9.3 (SAS/STAT, SAS Institute inc., Cary, NC). A correlation spearman (in appendix C) was performed using the CORR procedure of SAS.

ROC curve

The main risk factor of interest in the assessment of development of postpartum diseases and poor reproduction outcomes (reduced first insemination success) was considered to be the concentration of blood BHBA on day 5 post partum. The receiver operator characteristics (ROC) analysis was used to identify optimum thresholds of blood BHBA concentrations for the occurrence of reduced first service success. The ROC curve is plotted as a means of determining the best cut-off value for BHBA (mmol/L). It plots the sensitivity (the true positive rate) against one minus specificity (the false positive rate) for different cut-off values (see appendix B) (Petrie & Watson, 2006). The highest combined sensitivity and 1-specificity is the point in an ROC curve that is closest to the upper left corner of the graph and is considered as the optimum threshold for BHBA. Sensitivity measured the proportion of actual positives (true positive rate) which are correctly identified as such. Specificity (true negative rate)is the percentage of healthy cows who are correctly identified as not having the condition. The area under curve (AUC) is used for the interpretation of this optimum threshold. An AUC of 0.5 was considered no discrimination exists; if $0.5 < AUC \leq 0.7$, it was accurate; if $0.7 < AUC \leq 0.9$, it was very accurate; if $0.9 < AUC$ it is highly accurate and; if AUC = 1, then it was considered perfect (Swets, 1988). This analysis was performed to investigate if other cut-off values would have a better accuracy than the chosen cut-off of 1.2 mmol/L.

Univariable Chi-square analyses

Cross tabs were used to provide a basic picture of the interrelation between two variables and helped to find interactions between them. The chi-square statistic was used for testing the statistical significance of the tables using the FREQ procedure of SAS (Version 9.3, SAS institute Inc., Cary, NC).

Multivariate logistic regression models

The procedure of manual backward stepwise elimination was used to eliminate variables from the model. Variables with a significance level of $P \leq 0.1$ were retained in the model (see table 5 and 6). Two models were constructed in our study. Model 1 (see table 5) was produced to evaluate association P32AI1 and the variables entered. Model 2 (see table 6) was constructed to evaluate the association of SCK (BHBA ≥1.2 mmol/L) and their odds of later developing clinical diseases. Differences with a P-value ≤ 0.05 were considered statistically significant, and a P-value in between 0.05 and 0.10 was considered as a tendency towards statistical difference, in univariable as well in multivariable analyses.

Results

Overall, 1197 Holstein-Friesian cows from the Alliance farm were initially enrolled. After excluding 147 cows, a total of 1050 records were used for the ROC curve and for some cross tabs. Reasons for excluding 7.4% (88), 3.0% (36) and 1,9% (23) (total 12.3%) cows from analyses were sold or dead cows within 60 days of milk, never bred and missing information, respectively. Of the 1050 cows used in the ROC curve, 1041 cows were used for the final analyses with the logistic regression model. The reason for excluding 9 more cows was of missing data for the independent variables.

ROC curve

The ROC curve is plotted as a means of determining the best cut-off value for BHBA. Variable is BHBA (mmol/L) and the classification variable was open at 32 days after first service (P32AI1). The sample size used is 1050 cows. 324 (30,9%) cows were pregnant after first insemination and 726 (69,1%) were not pregnant after first service.

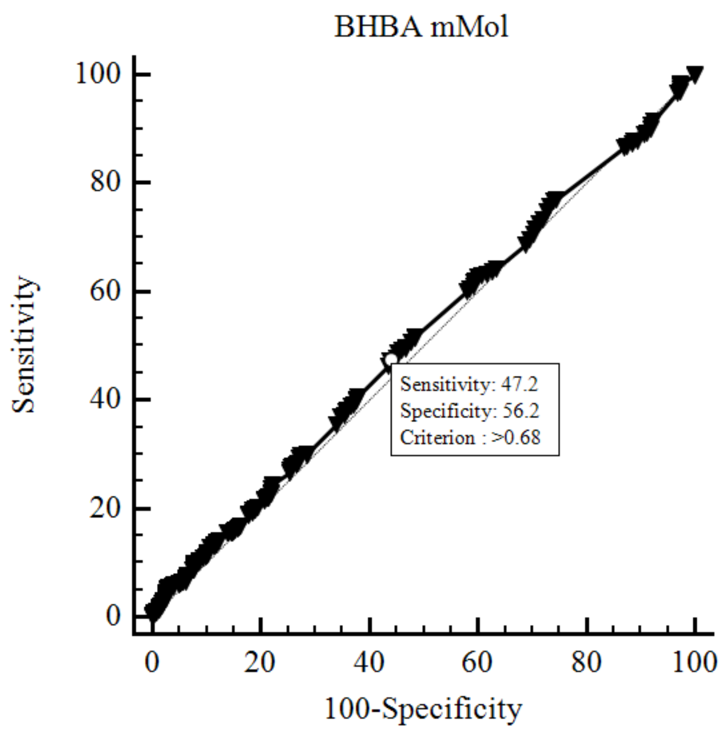

Figure 2. *ROC curve; Variable is BHBA (mMol/L), classification variable is P32AI1*

The area under the ROC curve (AUC) is 0.513. The AUC was close to 0.5; therefore showing no discrimination. The optimized cut-off was > 0.68 with 47.2 Se and 56.2 Sp; P = 0.50. The cut-off value of ≥ 1.2 mmol/L had Se of 16% and Sp of 86%. Because 1.2 mmol/L is a commonly used cut-off in the literature we proceeded with the statistical analysis using this cut-off. Of the 1050 cows used in ROC curve, 158 (15.05%) were diagnosed with a BHBA level ≥ 1.2 mmol/L and classified as SCK for the statistical analyses.

Univariable Chi-square analysis

SCK by P32AI1

Univariable Chi-square analyses were performed to find an association between subclinical ketosis and first service conception success, and are shown in table 1, 2, and 3. A P-value ≤ 0.05 was considered statistically significant. A P-value in between 0.05 and 0.1 was considered a tendency towards statistical difference. None of the cross tabs were significantly different between SCK 1.0, 1.2, 1.4 and P32AI1.

	P32AI1	No P32AI1	
SCK1.0	66 (6.29%)	156 (14.86%)	**222 (21.14%)**
No SCK1.0	258 (24.57%)	570 (54.29%)	**828 (78.86%)**
	324 (30.86%)	**726 (69.14%)**	

Table 1. SCK1.0 by P32AI1
1050 cows, Chi-square = 0.1677, df =1, OR= 0.93, 95%CI 0.677:1.291, P = 0.6821.
There is no association between BHBA 1.0 mmol/L and pregnancy at first service.

	P32AI1	No P32AI1	
SCK1.2	45 (4.32%)	112 (10.76%)	**157 (15.08%)**
No SCK1.2	273 (26.22%)	611 (58.69%)	**884 (84.92%)**
	318 (30.55%)	**723 (69.45%)**	

Table 2. SCK1.2 by P32AI1
1041 cows, Chi-square = 0.3097, df =1, OR= 0.90, 95%CI 0.619:1.307, P = 0.5779.
There is no association between BHBA 1.2 mmol/L and pregnancy at first service.

	P32AI1	No P32AI1	
SCK1.4	31 (2.95%)	80 (7.62%)	**111 (10.57%)**
No SCK1.4	293 (27.90%)	646 (61.52%)	**939 (89.43%)**
	324 (30.86%)	**726 (69.14%)**	

Table 3. SCK1.4 by P32AI1
1050 cows, Chi-square = 0.4992, df =1, OR= 0.85, 95%CI 0.552:1.323, P = 0.4799
There is no association between BHBA 1.4 mmol/L and pregnancy at first service.

Multivariable regression models

The logistic procedure 1: P32AI1 as response variable

In this logistic model the first insemination success was the response variable. Total number of observations used is 1041. A total of 318 (69.5%) cows were pregnant after first service. Model included the effects of season (summer = 1, winter = 0), parity (primiparous = 1, multiparous = 0), BCSCavC (high (≥3.5), med (3.0 or 3.25), low (≤2.75)), BCS40C (high (≥3.5), med (3.0 or 3.25), low (≤2.75)), CalvProb (Yes=1, No=0), RP (Yes=1, No=0), metritis (Yes=1, No=0), mastitis (Yes=1, No=0), resp (Yes=1, No=0), DA (Yes=1, No=0), SCK1.2 (Yes=1, No=0), Lame (Yes=1, No=0), CE (Yes=1, No=0), cyclic (Yes=1, No=0), and AIestrus (Yes=1, No=0). In table 4 the prevalence of the variables in this herd is shown.

Table 4. Prevalence table

Prevalence of postpartum diseases and season, parity, BCS and Calving problems in 1041 dairy cows of the Alliance Farm in Florida.

Total 1041 cows	BHBA ≥ 1.2	P32AI1	RP	Metritis	Mastitis	Resp	DA	Lame	CE
%	15.08	30.55	10.85	34.74	17,0	8.17	3.75	10.85	31.89
No	157	318	113	361	177	85	39	113	332

	Cyclic	AIestrus	Season	BCSCavC high	BCS40C high	CalvProb
%	77.71	40.73	50.34	23,5	10,8	17,2
No	809	424	524	245	112	179

The manual backward elimination procedure was performed, and unimportant variables were deleted, one at the time when the p-value was > 0.10. The least important predictor left the model first. The next effects were removed from the model during backward elimination in the following order; BCS40C (df =2, Pr>ChiSq = 0.9654), SCK1.2 (df =1, Pr>ChiSq = 0.9540), Cyclic (df =1, Pr>ChiSq = 0.7503), Resp (df =1, Pr>ChiSq = 0.5400), AIEstrus (df =1, Pr>ChiSq = 0.4861), Metritis (df =1, Pr>ChiSq = 0.4128), BCSCavC (df =2, Pr>ChiSq = 0.2102), Season (df =1, Pr>ChiSq = 0.1864), Parity (df =1, Pr>ChiSq = 0.1382) and CalvProb (df =1, Pr>ChiSq = 0.1571). A P-value ≤ 0.05 was considered statistically significant. A P-value in between 0.05 and 0.1 was considered a tendency towards statistical difference.

Table 5. Summary of logistic procedure with P32AI1 as response variable

Results of multivariate analysis for the relationship of pregnancy at first service with different covariates. The model summary selected by backward elimination includes 5 predictors; RP, mastitis, DA, Lame and CE. P <0.1. Wald Confidence intervals of 95%. 95% of the CI's will include the true population OR. Intercept (df =1, estimate = -3.2034, SE = 0.5805, Pr>ChiSq = <.0001)

Variable	Level	number	% pregnant at first service	Odds ratio	95% confidence interval	P- value
Retained placenta	No	928	31.90	1.650	1.001 - 2.722	0.0496
	Yes	113	19.47	Referent	-	-
Mastitis	No	864	26.99	1.704	1.150 - 2.526	0.0079
	Yes	177	20.90	Referent	-	-
Displacement abomasum	No	1002	31.24	2.471	0.946 - 6.451	0.0648
	Yes	39	12.82	Referent	-	-
Lameness	No	928	31.68	1.571	0.974 - 2.534	0.0638
	Yes	113	21.24	Referent	-	-
Clinical endometritis	No	709	33.15	1.312	0.969 - 1.776	0.0795
	Yes	332	25.00	Referent	-	-

Cows without a retained placenta (n=928, 89.15%) were 1.7 times more likely (*P*=0.0496, 95%CI: 1.001 – 2.722) to become pregnant (31.90%) at first service than cows that had retained placenta. Mastitis occurred in 17.0% (see prevalence table). Only 20.90% of them became pregnant at first service. Cows that did not have a mastitis were 1.7 times more likely (*P*=0.0079, 95% CI: 1.150 – 2.526) to have a pregnancy at 32 days ± 3 days after first insemination (n= 864, 26.99%). Cows without displacement abomasum (n=1002, 96.25%), lameness(n=928, 89.15%) , and clinical endometritis (n=709, 68.11%) were 2.5, 1.6, and 1.3 times more likely to become pregnant at first service than cows that had DA, lameness or CE, respectively.

The logistic procedure 2: SCK 1.2 mmol/L as response variable

In this multivariable model SCK 1.2 mmol/L was the response variable. Total number of observations used is 1041. A total of 157 (15.1%) cows had a BHBA level ≥ 1.2 mmol/L, classified as having sub clinical ketosis. The following effects were entered; season (summer = 1, winter = 0), parity (primiparous (=1) vs. multiparous (=0)), BCSCavC (high (≥3.5), med (3.0 or 3.25), low (≤2.75)), BCS40C (high (≥3.5), med (3.0 or 3.25), low (≤2.75)), CalvProb, RP, metritis, mastitis, resp, DA, SCK1.2, Lame, CE, cyclic and AIestrus. See for prevalence table 4.

The backward elimination procedure deletes unimportant variables. The effects which were removed during backward elimination; Cyclic (df =1, Pr>ChiSq = 0.9473), CalvProb (df =1, Pr>ChiSq = 0.6759), Lame (df =1, Pr>ChiSq= 0.5492), RP (df =1, Pr>ChiSq = 0.4976), AIEstrus (df =1, Pr>ChiSq = 0.3230), Metritis (df =1, Pr>ChiSq = 0.3160) and Resp (df =1, Pr>ChiSq = 0.1812).

Table 6. Summary of logistic procedure with SCK 1.2 as response variable

Results of multivariate analysis for the relationship of BHBA ≥ 1.2 mMol/L with different covariates. Seven predictor variables retained in the final model summarized in the table below. P <0.1. Wald Confidence intervals of 95%. 95% of the CI's will include the true population OR.

Variable	Level	number	% ≥ 1.2 mmol/L BHBA	Odds ratio	95% confidence interval	P- value
Season	Summer	524	16.60	1.659	1.132 - 2.431	0.0094
	Winter	517	13.54	Referent	-	-
Parity	Primiparous	428	3.97	0.159	0.093 - 0.272	<.0001
	Multiparous	613	22.84	Referent	-	-
BCS at calving	≥ 3.50	245	24.90	2.402	1.339 - 4.311	0.0044
	3.0-3.25	517	13.35	-	-	-
	< 3.00	279	9.86	Referent	-	-
BCS at 40 DIM	≥ 3.50	112	12.50	0.458	0.228 - 0.920	0.0793
	3.0-3.25	436	14.60	-	-	-
	< 3.00	493	14.60	Referent	-	-
Mastitis	No	864	15.97	1.830	1.069 - 3.134	0.0276
	Yes	177	10.73	Referent	-	-
Displacement abomasum	No	1002	14.17	0.466	0.225 - 0.964	0.0396
	Yes	39	38.46	Referent	-	-
Clinical endometritis	No	709	14.10	0.695	0.469 - 1.031	0.0705
	Yes	332	17.17	Referent	-	-

SCK (16.60%) in summer (n=524, 50.34%), was 1.66 times the odds in the winter (P=0.0094, 95%CI: 1.132 - 2.431). Primiparous (n=428, 41.11%) has lesser odds of SCK than multiparous (P=<.0001, 95%CI: 0.093 - 0.272). Cows with a high BCS at calving (n=245, 23.54%) have greater odds of SCK than cows <3.00 BCS (P=0.0044, 95%CI: 1.339 - 4.311). The odds of SCK without mastitis (n=864, 83%) is 1.83 times greater than the odds of SCK with mastitis (P=0.0276, 95%CI: 1.069 - 3.134). Cows without a DA (n= 1002, 96.25%) were 0.47 times less likely to develop SCK (2.71%) than cows with a DA (P=0.0396, 95%CI: 0.225 - 0.964). The odds of SCK with a low BCS at 40 DIM (n=112, 10.76%) is 0.46 times smaller than the odds of SCK with a high BCS at 40 DIM (P=0.0793, 95%CI: 0.228 - 0.920). The odds of SCK without CE were 0.696 times smaller than the odds of SCK with CE (P=0.0705, 95%CI: 0.469 - 1.031).

Overall, calving in summer, primiparous cows, a high BCS at calving. mastitis, and displacement abomasum are significantly associated with SCK. Cows with a low BCS at 40 DIM and cows with CE tended to have a negative effect on developing SCK.

Discussion

The objectives of this study were to 1.) determine if a higher betahydroxybutyrate (BHBA) level (≥1.2 mmol/L) measured at 5 days in milk (DIM) results in lower first service conception success; 2.) study the relationship of BHBA ≥ 1.2 mmol/L measured at 5 DIM with the following variables: season, parity, body condition score at calving (BCSCavC) and 40 DIM (BCS40C), retained placenta (RP), metritis, mastitis, respiratory diseases (Resp), displacement abomasum (DA), lameness (Lame), clinical endometritis (CE), cyclic and AI estrus. The null hypothesis is that there is no association between BHBA ≥ 1.2 mmol/L and first service conception success.

There are 3 main reasons to enter a state of negative energy balance (NEB) in the transition period; the increased demands of energy at parturition, the decrease of dry matter intake shortly before calving, and the lack of dry matter intake compared to the energy demands due to milk production. The liver oxidized or re-esterified mobilized NEFA from stored fat into triglycerides that are exported as very low density lipoproteins or stored. In the periparturient period high rates of NEFA enter the liver and sometimes overdo the capacity of the liver to secrete triglycerides as very low density lipoproteins. This results in the accumulation of triglycerides. The liver regulates the ketogenesis, through removing of NEFA to BHBA. It is important to search for relations between NEB and diseases to help farmers prevent diseases proactively by focusing on management and nutritional strategies (Ospina et al, 2010). The measurement of BHBA concentrations in blood in our study could be an ability to predict at the individual cow level which animals are more likely to develop a specific type of disease.

Cut-off

In this study, BHBA level was unable to discriminate pregnancy status at 32 d after AI. Therefore, the threshold of 1.2 mmol/L which is commonly used in the literature was used (Duffield et al. 2009; Nielen et al. 1994; Garro et al. 2013; McArt et al. 2013). Nielen et al. (1994) had a threshold for SCK of 1.2 mmol/L based on health impairment and production results. Duffield et al. (2009) and Le Blanc et al.(2005) begins at a concentration of BHBA ≥ 1.2 mmol/L as an important threshold for defining hyperketonemia for predicting health risks in early lactation dairy cows.

Definition of SCK

McArt et al. (2012) defined SCK as a blood BHBA concentration of 1.2 to 2.9 mmol/L. Cows with blood BHBA concentrations of ≥ 3.0 mmol/L should show clinical signs of ketosis (Oetzel, 2004). In our study 11 cows of the 1050 used for the statistic analyses had a BHBA value of ≥ 3.0 mmol/L. However no diagnosis of clinical ketosis were recorded in this study and therefore no upper limit of BHBA concentration was applied to define SCK. Cows are more likely to suffer from SCK, an excess of circulating ketone bodies without symptoms (decreased appetite, weight loss, and milk production) of clinical ketosis (Andersson, 1988). Garro et al. (2013) and McArt et al. (2013) considered that a cow presented hyperketonemia when BHBA concentration in blood was ≥ 1.2 mmol/L, like we did.

Blood collection

SCK was classified based on the plasma concentration of BHBA ≥ 1.2 mmol/L at day 5 post partum. The collection of blood was only done once for each cow after milking when the cows had their head locked into headlock stanchions. It is important to note that blood samples were obtained at fixed times from cows in our study, because of the diurnal variations in concentrations of BHBA. In the large field study of McArt et al. (2012) the peak prevalence of SCK occurred remarkably soon after calving at 5 DIM (see figure 3).

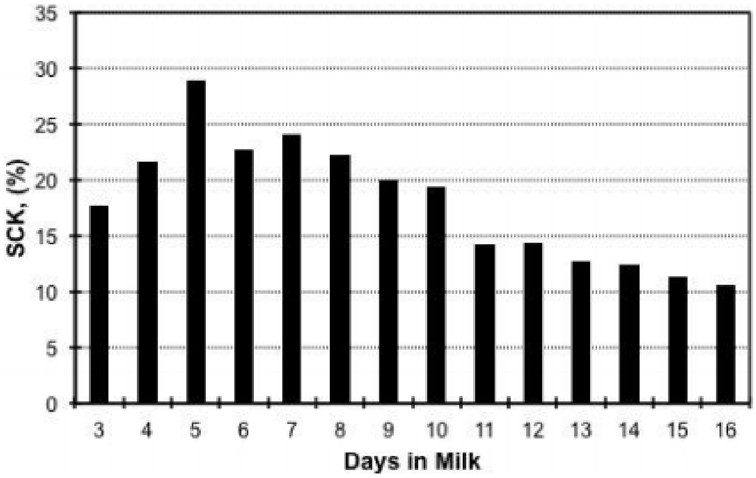

Figure 3. *Histogram of prevalence of SCK in 1.717 Holstein dairy cows undergoing repeated testing for ketosis from 3 to 6 DIM. A positive test for SCK was defined as a blood BHBA concentration of 1.2 to 2.9 mmol/L (results of McArt et al, 2012)*

Her research was used to determine the collection date for BHBA in our project. Moreover Duffield et al. (2009) showed that elevated BHBA levels in the first week after calving has more impact on both milk yield reduction and cow health than elevation later in lactation. The significance of the results of our study, however, is limited to a single collection of blood for all the cows on day 5 post partum. Further research is required to investigate if SCK diagnosed on another day still gives significant differences.

Prevalence of SCK

The prevalence of SCK (≥ 1.2 mmol/L) in 1041 Holstein Frisian cows at the Alliance Farm was 15%. Prevalence describes the proportion of animals that is tested positive for SCK at the time of testing. In the study of V.S. Suthar et al. (2012) the overall prevalence of SCK (blood BHBA ≥1.2mmol/L) within 10 European countries was 21.8%, ranging from 11.2-36.6%. In the study of McArt et al. 2012 the prevalence of SCK was 28.9% at 5 DIM. Compton et al. (2014) reported a prevalence of SCK using a cut-point of 1.2 mmol/L BHBA at day 7-12 post-calving of 23.8%. The prevalence of SCK in 107 cows was 10.3% between 4 and 19 DIM in the study of Garro et al. (2013). The prevalence of SCK can be used for monitoring of herds over time and can be used as an outcome indicator for changes in the management of dry or fresh cows (McArt et al., 2012). Our prevalence of SCK is comparable with the other studies done. However, comparisons should be made with caution, because methodological differences may exist and can affect prevalence reported. Diets can have different glucogenic potential, even with the same energy content. The different diets used in experiments can affect the relationship between concentrations of ketone bodies and energy balance, because the level of ketone bodies is influenced by as well plasma glucose as the energy balance (Andersson, 1988).

SCK and first service conception success

In this study elevated BHBA (≥1.2 mmol/L) concentrations measured with the Wako-kit on 5 DIM after calving were not associated with pregnancy after first service. A reason why investigations of blood BHBA levels at 5 DIM may not have detected significant relationships with fertility is that cows may not have been sampled at the most relevant time point (Karimi Dehkordi et al., 2012). However in our study this is unlikely because we collected blood samples at fixed times. More studies are in agreement with our findings and did not find an association of SCK and first conception success. The study of Snijders et al. (2001) reported no effect of SCK on cow fertility. Chapinal et al. (2012) found no relationship between blood BHBA after calving and first service conception rates, and McArt et al. (2012) observed no difference in BHBA concentration at first positive test (BHBA ≥ 1.2 mmol/L = positive) and conception to first service (0.1 mmol/L increase relative risk= 1.1, 95%CI 0.5:2.3, P=0.84). Cows had the same risk of conception with a BHBA concentration of 1.2 mmol/L as with a concentration of 1.3 or 2.4 mmol/L (McArt et al., 2012).

A few studies, like Walsh et al.(2007) found that a depression of first service conception rate occurred when blood concentrations BHBA measured ≥ 1.0 mmol/L at 1 week post-calving. They also reported that the first service conception risk had declined by 50% in cows with serum concentrations of BHBA ≥1.4mmol/L in the second week post partum. Ospina et al. (2010) showed for BHBA a significant herd level effect of 0.8% decrease in pregnancy rates. In our research project a different cut-off was used. Even though we did not find an association with BHBA 1.0 and 1.4mmol/L with P32AI1 (see table 1 and 3) on day 5 in milk. These relationships need to be evaluated at several stages in lactation to overcome such limitations.

Not all cows were bred on the same moment during this research project and different seasonal dependent variables can affect the reproductive outcome. Moreover it is less likely to breed a cow in estrus if the cow has a disease. Indeed, cows with BHBA ≥ 1.2 mmol/L were less likely to be AI in estrus (33.8 vs. 42.0%; *P* = 0.05) hence more received TAI (66.2 vs. 58.0%; *P* = 0.05). Cows going to TAI had longer voluntary waiting period which is known to improve first service conception rate. This could be a reason for reporting no significantly relationship between SCK and P32AI1.

Risk factors on first service conception success

The logistic regression model with P32AI1 as response variable selected by backward elimination includes 2 variables that are significantly associated; RP (Pr>ChiSq = 0.0496), and mastitis(Pr>ChiSq = 0.0079); 3 variables that have a tendency toward statistical differences; DA (Pr>ChiSq = 0.0648), Lameness (Pr>ChiSq = 0.0638), and CE (Pr>ChiSq = 0.0795). When using a logistic regression model with multiple variables, the explanatory variables should not be highly correlated with one another because this could cause problems with estimation (Bewick et al., 2005). In this research project only weak correlations were found (see appendix C) and probably did not affect the final outcomes of the model. The odds to become pregnant with RP, mastitis, DA, lameness and CE is smaller than the odds to become pregnant without these diseases.

Retained placenta and P32AI1

Cows without RP were 1.7 times more likely to become pregnant at first service than cows with RP. Our study is in agreement with the study of Tillard et al. (2008) who reported that RP was significantly (P=<0.05) related with a lower risk of conception. Fourichon et al. (2000) did a meta-analysis on the effect of disease on reproduction in dairy cows. They mentioned that retained placenta was associated with a 10% lower conception rate at first service, resulting in 6 to 12 more days to conception. In our study the exact route by which retained placenta affects reproductive performance has not been clarified.

Mastitis and P32AI1

Cows without mastitis have 1.7 times higher odds of pregnancy at first AI than cows with mastitis. In several studies the relation between mastitis and reproduction outcome is evaluated. Schrick et al. (2001) reported that cows with clinical mastitis in the period before first service had increased days to first service (from 67.8 ± 2.2. to 77.3 ± 2.7), days open (from 85.4 ± 5.8 to 110.0 ± 6.9), and services per conception (from 1.6 ± 0.2 to 2.1 ± 0.2) compared to control cows (P=<0.05). In the study of Pinedo et al. (2009) it was concluded that subclinical mastitis had a significant effect on reproduction performance in Chilean dairy cattle. Mastitis could affect follicular development and oocyte maturation via alterations in LH and FSH activity or function and therefore influence reproduction outcome (Schrick et al., 2001). Elevated body temperature is often a symptom associated with clinical mastitis. Cows exposed to heat stress experienced increased mortality of the embryo. It is a possibility that cows became pregnant after first AI and lost the pregnancy before the first confirmation was done at 32 days.

Displacement abomasum and P32AI1

A cow without a DA has 2.5 times higher odds of first conception AI than a cow with a DA. In our study this risk factor had a tendency towards statistical difference. No effect on reproductive performance of DA was reported in the study of Fourichon et al. (2000).

Lameness and P32AI1

Cows without a lameness (locomotion score ≥ 3 (1-5) at 40 ±3 DIM) have 1.6 times higher odds of pregnancy at first AI than cows with a lameness. Locomotion disorders have a wide variation based on lesions and stage of occurrence. It has been associated with an average of 12 more days to conception, possibly due a decrease in conception at first service (Fourichon et al. 2000).

Clinical endometritis and P32AI1

Cows without CE have 1.3 times higher odds of pregnancy at first AI than cows with CE. Endometritis is a uterine disease that is highly prevalent in high producing dairy cows and has also been shown to be associated with reduced pregnancy per AI in the study of Galvão (2012). Function of follicular cells, oocyte maturation and ovulation are negatively influenced by endogenous mediators of inflammation and bacterial products. Affected oocytes may have impaired development potential. The primary effects of mild endometritis might be at the ovarian level (Gilbert 2012).

Negative impacts of SCK

The logistic regression model with BHBA (≥1.2mmol/L) as response variable shows a statistically significantly relationship (α= <0.1) between season (Pr>ChiSq = 0.0094), parity (Pr>ChiSq = <.0001), high body condition score at calving (Pr>ChiSq = 0.0044), mastitis (Pr>ChiSq = 0.0276), and DA (Pr>ChiSq = 0.0396). A tendency towards statistical differences was found in the following two variables; BCS at 40 DIM (Pr>ChiSq = 0.0793), and CE (Pr>ChiSq = 0.0705). Internationally, several risk factors have been reported for SCK. Important risk factors are season and parity (Andersson, 1988). The development of SCK in cows very early in lactation within the first week postpartum were more likely to have adverse health events and produce less milk than cows that develop SCK after the first week of lactation (McArt et al, 2012).

Season

During summer ($13^{th}$of June 2013 till 23^{th} of July 2013) the odds of SCK was 1.7 times the odds of winter (30^{th} of November 2012 till 2^{th} of January 2013). In summer the rates of SCK may be highest due to suppressed feed intake (heat stress), changes in forage, and reduced intensity of management. In our study only calving season was used and we did not take into account the humidity or temperature. One half of the cows enrolled in our study calved in summer (n=524) and the other half in winter (n=517).

Parity

Primiparous has lesser odds (0.16) of SCK than multiparous cows. Several studies have found that SCK is more frequent in multiparous cows compared with primiparous cows (Dahoo et al. 1984). These results are in agreement with the study of McArt et al. (2013). They reported that cows in parity 1 and parity 2 were less likely to develop hyperketonemia than cows in parity ≥ 3. On different farms the odds of hyperketonemia in parity ≥ 3 were in between 1.4 - 2.0 times greater than cows in parity 1 (McArt et al. 2013).Berge and Vertenten (2014) reported that the lowest prevalence of ketosis (≥1.2 mmol/L) was found in cows of parity 1. This was significantly higher in cows of parity 2, and highest in cows of parity 3 to 7. In our study we did not categorized parity in primiparous, biparous and multiparous cows, like McArt et al. (2013) did. In future research it is recommended to make this categorization.

Compton et al. (2014) described the opposite findings. In a previous study of them they found 75% of heifers were in severe negative energy balance within 0-5 days of calving. An explanation given by them for the increased risk of SCK in heifers comes from evidence that heifers regularly erupt their primary permanent incisor teeth directly prior to calving perhaps with impairment of feeding. Moreover it is possible that less dominant heifers competed less effectively for limited feed when mixed with all age groups. This results in greater prevalence of SCK in primiparous cows. In our study primiparous cows and multiparous cows were kept in different pens and this could be a reason that we reported different results.

BCS at calving

In our study, BCS as high or higher than 3.5 at calving was associated with increased OR for SCK. Cows with a BCS ≥ 3.5 were approximately 2.4 times more likely to become ketotic than cows with scores at calving lower than 3.0. Gillund et al. (2001) reported similar results, a cow with a score ≥ 3.5 was 2.5 times more likely to develop ketosis than cows with scores as low or lower than 3.25 at calving. Compton et al.(2014) reported that cows with a higher BCS have been found to have increased probability of developing SCK during early lactation. His paper refers to Duffield et al. (1998) were is recorded that fat cows had 1.6 times greater chance of developing SCK and thin cows 0.33 lower risk compared to medium condition cows. A possible explanation for this association is the reduction of feed intake during the transition period and therefore the aggravation of NEB. Under conditions of stress or NEB cows with a high BCS have a tendency to mobilize body fat very rapidly. A lot of NEFA can enter the liver and accumulate which can cause hepatic lipidosis. Hepatic lipidosis is also associated with an increased BHBA concentration in blood (Garro et al. 2013). To minimize the risk of occurrence of post partum SCK monitoring of BCS and proper nutrition management can help.

BCS is a subjective method. In our study we have take into account that BCS were done by three to four persons. This could have led to biased data.

Increased risk for DA

Cows without a DA were 0.5 times less likely to develop SCK than cows with a DA. SCK has a highly significant weak correlation with displacement abomasum (r=0.12889; p=<.0001; n=1041). Reduced feed intake is usually the first sign of ketosis. According to Doll et al. (2009), the development of DA can be explained by an inadequately filled rumen, which creates more space in the abdomen. Usually an adequately filled rumen serves as a natural barrier in preventing of DA. All animals with a decrease in feed intake due to other illnesses are more affected (Doll et al., 2009). Another risk factor that may increase DA is the influence of reduced insulin and glucose concentrations on abomasum gases and motility during negative energy balance described in the study of Van Winden et al. (2003). A reduction of insulin and glucose concentrations occur along with increased levels of BHBA.

The relationship of SCK and DA is investigated in various studies. Ospina et al. (2010) reported a higher incidence of DA (1.8% higher) in cows with increased BHBA concentrations and reported a 6.9 times higher risk for DA(95% CI 3.7:12.9) for cows with BHBA concentrations of ≥ 1.0 mmol/L post calving. In the first week post partum with BHBA ≥ 1.2 mmol/L increased the odds for DA by 2.6 (95% CI 1.3:5.2). McArt et al. (2012) investigated if the effect of days in milk at the first onset of SCK also affects the risk of DA and reported that cows who developed SCK between 3 and 5 DIM were 6.1 times more likely to develop DA compared to cows developed SCK between 6 and 16 DIM (Oetzel, 2004). In the field study of Oetzel et al. (2004) cows with SCK had 19.3 times more chance to develop a DA than cows without SCK (95% CI 13.8:27). Our study findings support numerous other studies indicating that SCK is associated with increased risks of DA in the first weeks after calving.

Relationship between SCK and mastitis

The odds of SCK without mastitis is 1.8 times greater than the odds of SCK with mastitis. To our knowledge, this is the first study that reported such a relationship between SCK and mastitis. Usually studies report the opposite finding and describe the possible impairment of udder defense mechanisms because of experiencing negative energy balance (Hammon et al. 2006; Suriyasathaporn et al. 2000; Berge and Vertenten 2014). Environmental risk factors, udder defense mechanisms, and exposure to microbes are three components for developing a mastitis infection. To protect and minimize the clinical symptoms in infected mammary glands udder defense mechanisms plays a crucial role. Important components of udder defense are both quality (capacity of the phagocytosis) and quantity of polymorphonuclear leukocytes (PMN) and macrophages. A low number of PMN in blood is related to increased severity of experimental E.coli mastitis (Kremer et al., 1993).Incubated milk PMN and macrophages in acetone or BHBA have lower phagocytosis of bacteria than in cultures of cells without ketone bodies (Klucinski et al., 1988). Both, in vitro and in vivo, it is clear that the killing capacity of leukocytes is impaired by ketone bodies (Suriyasathaporn et al., 2000). Suriyasathaporn et al. hypothesized that in hyperketonemic cows the generation of chemoattractant is reduced. Normal cows have higher amounts of cytokines produced by lymphocytes than ketotic cows. Moreover, the number of leukocytes in healthy cows were higher than that of ketotic cows. In ketotic cows the migration rate of leukocytes was reduced. Mechanisms of impairment due to high levels of ketone bodies have not been fully investigated. More exploration in mechanisms is necessary to clarify the impaired function of leukocytes in negative energy balanced cows.

In our study the relation of SCK and mastitis is not clear. Data of mastitis was collected by farm personnel, assuming data were filled in correctly. The prevalence of mastitis in this herd was 17%. More research should be done to better understand this association between SCK and mastitis.

Increased risk for clinical endometritis

The odds of SCK without CE were 0.7 times smaller than the odds of SCK with CE. A tendency towards statistical difference was found (P=0.07) in our study. According to Giuliodori et al. (2013) clinical endometritis had no effect on metabolic status. Affected cows had similar levels of metabolites (BCS, NEFA, and BHBA) than healthy cows. CE is restricted to the uterus without systemic signs of illness, which could be a reason to the lack of effect of CE on metabolic status.

In our study, CE had a weak correlation with calving problems (r=0.0998, P=0.0013, n=1041), retained placenta (r=0.2250, P=<.0001, n=1041), and metritis (r=0.1879, P=<0.0001, n=1041) (see table 11 in appendix C). Our data support the study of Giuliodori et al. (2013) that have found RP, metritis, and dystocia as predisposing factors for clinical endometritis.

A negative energy balance may have negative effects on neutrophil functions, and therefore on uterine health. Hammon et al (2006) reported that cows with cytological endometritis had worse neutrophil function than unaffected cows. They also reported that increasing NEFA concentration is associated with reduced neutrophil function. Galvão et al. (2010) found associations of increased NEFA and BHBA concentrations near calving with the risk of cytological endometritis.

Conclusion

Elevated BHBA (≥1.2 mmol/L) concentrations measured with the Wako-kit on 5 DIM after calving are not associated with pregnancy after first service. The null hypothesis could not be rejected. Although there is no proved relationship between BHBA and P32AI1, several other variables are significantly associated with P32AI1. The logistic regression model with P32AI1 as response variable selected by backward elimination includes 2 variables that are significantly associated; RP (Pr>ChiSq = 0.0496), and mastitis(Pr>ChiSq = 0.0079); 3 variables that have a tendency toward statistical differences; DA (Pr>ChiSq = 0.0648), Lameness (Pr>ChiSq = 0.0638), and CE (Pr>ChiSq = 0.0795).

In conclusion, cows without a retained placenta were 1.7 times more likely to become pregnant at first service than cows that had retained placenta. Cows that did not have a mastitis were 1.7 times more likely to have a pregnancy at 32 days ± 3 days after first insemination. Cows without displacement abomasum, lameness, and clinical endometritis were 2.5, 1.6, and 1.3 times more likely to become pregnant at first service than cows that had DA, lameness or CE, respectively.

The logistic regression model with BHBA (≥1.2mmol/L) as response variable shows a statistically significantly relationship between season (Pr>ChiSq = 0.0094), parity (Pr>ChiSq = <.0001), high body condition score at calving (Pr>ChiSq = 0.0044), mastitis (Pr>ChiSq = 0.0276), and DA (Pr>ChiSq = 0.0396). A tendency towards statistical differences was found in the following two variables; BCS after 40 DIM (Pr>ChiSq = 0.0793), and CE (Pr>ChiSq = 0.0705).

SCK in summer was 1.7 times the odds in the winter. Primiparous has lesser odds of SCK than multiparous. Cows with a high BCS at calving have greater odds of SCK than cows with a BCS lower than 3.0. Cows without a DA were 0.5 times less likely to develop SCK than cows with a DA. The odds of SCK without a high BCS at 40 DIM is 0.5 times smaller than the odds of SCK with a high BCS at 40 DIM. The odds of SCK without CE were 0.7 times smaller than the odds of SCK with CE. The odds of SCK without mastitis is 1.8 times greater than the odds of SCK with mastitis. This is the first study that reported such a relationship between SCK and mastitis. More research should be done to better understand this association.

Cows that develop SCK at 5 DIM may require special attention to decrease their probability of adverse events during the lactation period.

Acknowledgement

This research project is based upon collected data supported by students of the University of Florida. I thank the owner and staff of Alliance Dairies (Trenton, FL) for the use of their facilities and cows. I would like to express my deep gratitude to Professor Klibs Galvão and Professor Peter Vos, my research supervisors, for their guidance, enthusiastic encouragement and useful critiques of this research work along with the opportunity to fulfill my research project at a place outside the University of Utrecht.

I would also like to thank all those who have helped in carrying out the research. Special thanks to Sergei Sennikov for his assistance in the laboratory, Eduardo Ribeiro for his BHBA protocol for high values and Rodolfo Daetz for sharing his collected data. Advice given by Klibs Galvão and Hans Vernooij has been a great help in the statistical analysis. I am particularly grateful for the help and valuable support given by Nienke van de Burgwal as a colleague during this project.

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Appendix A. BHBA analyse method

Eduardo Ribeiro- BHBA Protocol for Postpartum Cows (High values)

Standards:
Weighing 24 mg of D,L beta-OH butyric acid sodium into 50ml of water results in 19.8 mg/100ml concentration.

(24 x 0.825 x 0.5 = 9.9 mg in 50ml or 19.8 mg/ 100ml)

19.8mg of D-BHBA/100ml = 24mg of D,L BHBA sodium into 50ml of water = **STOCK SOLUTION**

- **16.97mg**/100ml = 6 ml of stock solution + 1 ml water;
- **14.85mg**/100ml = 3 ml of stock solution + 1 ml water;
- **13.2mg**/100ml = 2 ml of stock solution + 1 ml water;
- **9.9mg**/100mg = 1 ml of stock solution + 1 ml water;
- **7.92mg**/100mg = 1 ml of stock solution + 1.5 ml water;
- **4.95mg**/100mg = 1 ml of stock solution + 3 ml water;
- **1.98mg**/100mg = 1 ml of stock solution + 9 ml water;
- **0mg**/100ml = blank

The quadratic response normally fits better to this assay.

Beta Hydroxy Butyric Acid (BHBA) Analysis
May 22, 2007

Wako Diagnostics
1600 Bellwood Road
Richmond, VA 23237
977-714-1924

Molecular weight of D,L Beta-OH-butyric acid sodium = 126 g per mole

Linear reaction up to 1000 umoles/L according to WAKO
1000 um/L = 1 mmole/L = (0.1 mmole/100 ml) x (104 mg of BHBA/mmole of BHBA) = 10.4 mg of BHBA/100 ml of plasma; so high standard below is prepared to 9.9 mg/100 ml

Standards:
Weighing 24 mg of D,L beta-OH butyric acid sodium into 100 ml of water results in a 9.9 mg of D-beta-OH butyric acid concentration if the D,L standard is a 50:50 mix of D and L form.
24 mg of BHBA sodium * 82.5% BHBA * 50% D-form = 9.9 mg of D-BHBA.

9.9 mg of D-BHBA / 100 ml = 24 mg of D,L BHBA sodium into 100 ml of water.
7.92 mg/100 ml = 4 ml of stock std + 1 ml of water
6.60 mg/100 ml = 2 ml of stock std + 1 ml of water
4.95 ~~4.50~~ mg/100 ml = 1 ml of stock std + 1 ml of water
~~1.98 mg/100 ml = 1 ml of stock standard + 4 ml of water~~
0 mg/100 ml = blank

This procedure is published by WAKO "Microtiter procedure for Wako Autokit 3-HB."

1. Pipette 4 ul of plasma or standard into well.
2. Add 150 ul of R1 to each well.
3. Mix, incubate at 37°C for 5 minutes in plate reader.
4. Add 50 ul of R2 to each well.
5. Mix and incubate at 37°C for 2 minutes in plate reader.
6. Take initial readings at 405 nm after this 2 minute period (T=0). Continue to take readings every 30 seconds for 2 additional minutes (T = 2).
7. Determine the change in OD/min by subtraction.

This is a modification of the WAKO procedure in order to do more samples per kit.
The plate reader in Dr. ealy's lab was used and a program was created by Idania and Sergei for this assay. The plate reader is warmed to 37°C to run this procedure.

We validated this procedure by spiking plasma with std and by running dilutions of plasma. The dilutions and the std can be prepared using water or 0.9% saline. Both dilutions gave the same results. One kit will run about 380 samples; that is 57 ml of R1 in a kit divided by 0.15 ml per sample of R1.

Wako Diagnostics
1600 Bellwood Road
Richmond, VA 23237
Phone: 877-714-1924
Fax: 804-271-7791
www.wakousa.com

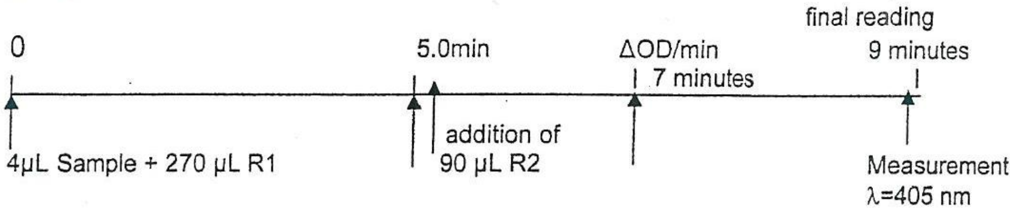
Microtiter procedure for Wako Autokit 3-HB

1. Accurately pipette 4 uL of sample or standard into the test tubes.*
2. Add 270 μL of prepared R1 to each well.
3. Mix, incubate for 5 minutes at 37°C.
4. Add 90 μL of prepared R2 to each well.
5. Mix, incubate at 37°C for 2 minutes. Take initial readings at 405nm after this 2 minute period (T=0). Continue to take readings every 30 seconds for 2 additional minutes (T=2min).
6. Determine the Δ OD/min by subtraction.
7. Calculate the 3-HB concentration by comparing to the calibrator's value. See equation below:

$$\text{Sample conc. } (\mu\text{mol/L}) = \text{calibrator concentration} \times \frac{\text{Sample } \Delta \text{ OD/min}}{\text{Calibrator } \Delta \text{ OD/min}}$$

The basic procedure outline is the following:

37°C



* To increase the sensitivity of the method, increase sample volume to 17 uL.

Wako

Code No. 417-73501
413-73601

Autokit 3-HB

(Cyclic Enzymatic Method)
For Research Use Only. Not for use in diagnostic procedures.

Intended use

The Autokit 3-HB is an *in vitro* assay for the quantitative determination of 3-hydroxybutyrate (3-HB) in serum or plasma.

Summary and explanation of the test

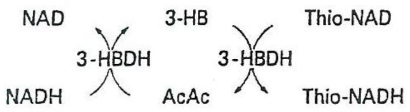
The Autokit 3-HB is reagent to measure 3-HB with high sensitivity and high specificity by utilizing cyclic enzymatic reactions.

Principle of the method

When a sample is mixed with R1, acetoacetone (AcAc) in the sample is broken down to acetone by acetoacetone decarboxylase (AADC).

$$AcAc \xrightarrow{AADC} Acetone$$

Upon addition of R2, 3-HB in the sample is oxidized in the presence of 3-HBDH and Thio-NAD. This oxidation triggers the cyclic reactions. Since the original AcAc in the sample has been removed, only 3-HB is assayed by measuring the rate of Thio-NADH production spectrophotometrically.



Reagents

(1) Thio-NAD — 2 x for 27 mL
When reconstituted
4.27 mmol/L β-Thionicotinamide adenine dinucleotide, oxidized form (Thio-NAD)
Store at 2-10℃.

(2) Buffer — 2 x 27 mL
20 mmol/L Phosphate buffer, pH 7.0, containing 5 IU/mL acetoacetate decarboxylase (AADC) from *Bacillus*
0.018% sodium azide
Store at 2-10℃.

(3) Enzyme — 2 x for 9 mL
When reconstituted
3200 IU/mL 3-Hydroxybutyrate dehydrogenase (3-HBDH), from *Alcaligenes*
2.65 mmol/L β-nicotinamide adenine dinucleotide disodium, reduced form (NADH)
Store at 2-10℃.

(4) Diluent — 2 x 9 mL
0.2 mol/L Good's buffer, pH 9.0, containing 0.053% sodium azide
Store at 2-10℃.

Warnings and precautions

(1) For Research Use Only. Not for use in diagnostic procedures.
(2) Do not use the reagents described above in any procedures other than those described herein. Performance cannot be guaranteed if the reagents are used in other procedures or for other purposes.
(3) Operate the instruments according to operator's manuals under appropriate conditions. Consult the instrument manufacturer for details.
(4) Store the reagents under the specified conditions. Do not use reagents past the expiration date stated on each reagent container label.
(5) Do not use reagents which were frozen in error. Such reagents may give false results.
(6) After opening the reagents, it is recommended to use them immediately. When the opened reagents are stored, cap the bottles and keep them under the specified conditions.
(7) Do not use the containers and other materials in the package for any purpose other than those described herein.
(8) Use Wako's Ketone Body Calibrator for preparation of a calibration curve. Read the instruction sheet in the package of the calibrator thoroughly before use.
(9) When discarding the reagents, dispose of them according to local or national regulations.
(10) The Buffer and Diluent contain 0.018%, 0.053% sodium azide respectively, as a preservative. Sodium azide may react with copper or lead plumbing to form explosive compounds. Even though the reagents contain minute quantity of sodium azide, drains should be flushed well with a large amount of water, when discarding the reagents.
(11) If the reagents come in contact with the mouth, eyes or skin, wash off immediately with a large amount of water. Consult a physician if necessary.
(12) Be careful not to cut yourself with the aluminum cap when removing it from the vial.

Physical or chemical indications of instability

The presence of precipitates in the reagents or values of control sera outside the manufacturer's acceptable range may be an indication of reagent instability.

Instruments

The reagent is designed to be used on commercially available automated analyzers such as Hitachi 917s analyzer.
Refer to the operating manual for a description of instrument operation, specifications and calibration.

Specimen collection and preparation

(1) Samples
  (a) Perform the 3-HB assay immediately after blood collection. Store samples in a refrigerator or a freezer if immediate assay cannot be done.
  (b) Hemolysis gives slightly falsely negative results.
  (c) Ascorbic acid and bilirubin do not have a significant effect on the assay.
(2) Interfering substances
  (a) Heparin, citrate, oxalate, EDTA, and sodium fluoride do not affect measurements when they are used in their respective usual quantities.

Procedure for Hitachi 917s analyzer

Used Plate Reader instead

Materials supplied

Refer to the section entitled "Reagents."

Materials required but not supplied

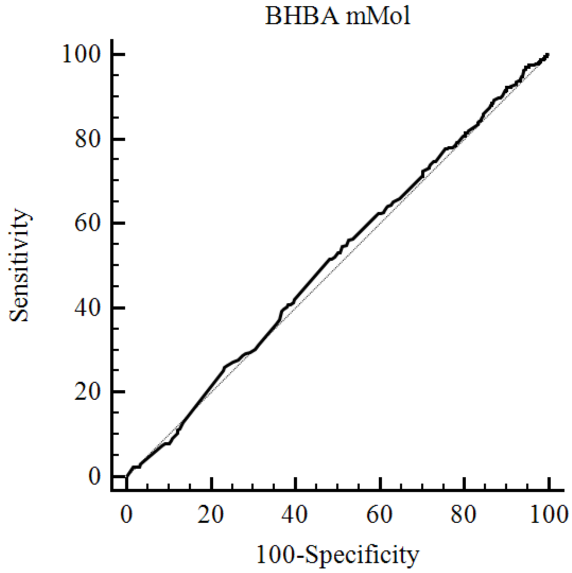
Hitachi 917s analyzer
Quality control material
Ketone Body Calibrator
Catalog No. 412-73791 300μmol/L
Catalog No. 418-73891 40μmol/L

All analyzer applications should be validated in accordance with CLIA recommendations. For further assistance call Wako Diagnostics Technical Service Department at 1-877-714-1924.

Reagent preparation

Reagent 1 Dissolve one bottle of Thio-NAD with one bottle of Buffer. The reconstituted solution is stable for 3 weeks at 2-10℃.
Reagent 2 Dissolve one bottle of Enzyme with one bottle of Diluent. The reconstituted solution is stable for 3 weeks at 2-10℃.

Appendix B. ROC curve BHBA and P32AI1



Variable	BHBA_mMol BHBA mMol	
Classification variable	P32AI1	
Sample size		1050
Positive group :	P32AI1 = 1	324
Negative group :	P32AI1 = 0	726
Disease prevalence (%)		unknown

Area under the ROC curve (AUC)

Area under the ROC curve (AUC)	0.513
Standard Error[a]	0.0192
95% Confidence interval[b]	0.482 to 0.544
z statistic	0.679
Significance level P (Area=0.5)	0.4973

[a] DeLong et al., 1988
[b] Binomial exact

Youden index

Youden index J	0.03418
Associated criterion	≤0.68

Criterion values and coordinates of the ROC curve

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
<0.07	0.00	0.0 - 1.1	100.00	99.5 - 100.0		1.00
≤0.07	0.00	0.0 - 1.1	99.86	99.2 - 100.0	0.00	1.00
≤0.2	2.47	1.1 - 4.8	98.35	97.1 - 99.1	1.49	0.99
≤0.27	2.47	1.1 - 4.8	96.97	95.4 - 98.1	0.81	1.01
≤0.29	3.09	1.5 - 5.6	96.69	95.1 - 97.9	0.93	1.00
≤0.3	7.41	4.8 - 10.8	91.60	89.3 - 93.5	0.88	1.01
≤0.31	8.02	5.3 - 11.5	90.77	88.4 - 92.8	0.87	1.01

≤0.32	8.02	5.3 - 11.5	89.94	87.5 - 92.0	0.80	1.02
≤0.33	8.64	5.8 - 12.2	89.26	86.8 - 91.4	0.80	1.02
≤0.34	9.26	6.3 - 13.0	88.98	86.5 - 91.2	0.84	1.02
≤0.35	10.49	7.4 - 14.4	87.88	85.3 - 90.2	0.87	1.02
≤0.36	11.11	7.9 - 15.0	87.74	85.1 - 90.0	0.91	1.01
≤0.37	11.42	8.2 - 15.4	87.33	84.7 - 89.7	0.90	1.01
≤0.38	12.35	9.0 - 16.4	86.78	84.1 - 89.2	0.93	1.01
≤0.39	12.96	9.5 - 17.1	86.50	83.8 - 88.9	0.96	1.01
≤0.4	25.31	20.7 - 30.4	77.00	73.8 - 80.0	1.10	0.97
≤0.41	25.93	21.2 - 31.1	76.86	73.6 - 79.9	1.12	0.96
≤0.42	26.54	21.8 - 31.7	75.90	72.6 - 79.0	1.10	0.97
≤0.43	27.16	22.4 - 32.4	74.79	71.5 - 77.9	1.08	0.97
≤0.44	27.78	23.0 - 33.0	73.42	70.0 - 76.6	1.04	0.98
≤0.45	28.70	23.8 - 34.0	72.59	69.2 - 75.8	1.05	0.98
≤0.46	29.32	24.4 - 34.6	71.63	68.2 - 74.9	1.03	0.99
≤0.47	29.63	24.7 - 34.9	70.66	67.2 - 74.0	1.01	1.00
≤0.48	30.25	25.3 - 35.6	69.56	66.1 - 72.9	0.99	1.00
≤0.49	31.17	26.2 - 36.5	68.60	65.1 - 72.0	0.99	1.00
≤0.5	36.42	31.2 - 41.9	64.33	60.7 - 67.8	1.02	0.99
≤0.51	37.35	32.1 - 42.9	63.77	60.2 - 67.3	1.03	0.98
≤0.52	38.27	33.0 - 43.8	63.36	59.7 - 66.9	1.04	0.97
≤0.53	39.20	33.8 - 44.7	63.09	59.5 - 66.6	1.06	0.96
≤0.54	39.81	34.4 - 45.4	62.81	59.2 - 66.3	1.07	0.96
≤0.55	40.43	35.0 - 46.0	61.98	58.3 - 65.5	1.06	0.96
≤0.56	40.74	35.3 - 46.3	61.71	58.1 - 65.3	1.06	0.96
≤0.57	40.74	35.3 - 46.3	61.16	57.5 - 64.7	1.05	0.97
≤0.58	41.36	35.9 - 46.9	60.47	56.8 - 64.0	1.05	0.97
≤0.59	41.98	36.5 - 47.6	60.19	56.5 - 63.8	1.05	0.96
≤0.6	51.54	46.0 - 57.1	51.79	48.1 - 55.5	1.07	0.94
≤0.61	51.54	46.0 - 57.1	51.38	47.7 - 55.1	1.06	0.94
≤0.62	52.16	46.6 - 57.7	50.69	47.0 - 54.4	1.06	0.94
≤0.63	53.09	47.5 - 58.6	49.86	46.2 - 53.6	1.06	0.94
≤0.64	53.09	47.5 - 58.6	49.45	45.8 - 53.2	1.05	0.95
≤0.65	54.01	48.4 - 59.5	49.17	45.5 - 52.9	1.06	0.94
≤0.66	54.63	49.0 - 60.1	48.76	45.1 - 52.5	1.07	0.93
≤0.67	54.94	49.3 - 60.4	47.80	44.1 - 51.5	1.05	0.94
≤0.68	56.17	50.6 - 61.7	47.25	43.6 - 51.0	1.06	0.93
≤0.69	56.48	50.9 - 62.0	46.28	42.6 - 50.0	1.05	0.94
≤0.7	62.04	56.5 - 67.3	40.77	37.2 - 44.4	1.05	0.93
≤0.71	62.35	56.8 - 67.6	40.22	36.6 - 43.9	1.04	0.94
≤0.72	62.35	56.8 - 67.6	39.94	36.4 - 43.6	1.04	0.94
≤0.73	62.65	57.1 - 67.9	39.12	35.6 - 42.8	1.03	0.95
≤0.74	63.27	57.8 - 68.5	38.84	35.3 - 42.5	1.03	0.95
≤0.75	63.89	58.4 - 69.1	38.29	34.7 - 41.9	1.04	0.94
≤0.76	64.20	58.7 - 69.4	38.02	34.5 - 41.7	1.04	0.94
≤0.77	64.51	59.0 - 69.7	37.33	33.8 - 41.0	1.03	0.95
≤0.78	65.12	59.7 - 70.3	36.91	33.4 - 40.5	1.03	0.94
≤0.79	66.05	60.6 - 71.2	35.40	31.9 - 39.0	1.02	0.96
≤0.8	71.30	66.0 - 76.2	30.03	26.7 - 33.5	1.02	0.96
≤0.81	71.30	66.0 - 76.2	29.89	26.6 - 33.4	1.02	0.96
≤0.82	72.53	67.3 - 77.3	29.75	26.4 - 33.2	1.03	0.92
≤0.83	72.84	67.6 - 77.6	29.34	26.0 - 32.8	1.03	0.93
≤0.84	73.15	68.0 - 77.9	28.37	25.1 - 31.8	1.02	0.95

≤0.85	73.46	68.3 - 78.2	28.24	25.0 - 31.7	1.02	0.94
≤0.86	74.07	68.9 - 78.8	27.96	24.7 - 31.4	1.03	0.93
≤0.87	74.38	69.3 - 79.0	27.55	24.3 - 31.0	1.03	0.93
≤0.88	74.69	69.6 - 79.3	27.27	24.1 - 30.7	1.03	0.93
≤0.89	74.69	69.6 - 79.3	26.72	23.5 - 30.1	1.02	0.95
≤0.9	77.78	72.9 - 82.2	24.38	21.3 - 27.7	1.03	0.91
≤0.91	77.78	72.9 - 82.2	24.10	21.0 - 27.4	1.02	0.92
≤0.92	78.09	73.2 - 82.5	23.83	20.8 - 27.1	1.03	0.92
≤0.94	78.09	73.2 - 82.5	22.73	19.7 - 26.0	1.01	0.96
≤0.95	78.40	73.5 - 82.8	22.31	19.3 - 25.5	1.01	0.97
≤0.96	78.40	73.5 - 82.8	22.04	19.1 - 25.2	1.01	0.98
≤0.97	78.70	73.8 - 83.0	21.90	18.9 - 25.1	1.01	0.97
≤0.98	79.32	74.5 - 83.6	21.63	18.7 - 24.8	1.01	0.96
≤0.99	79.32	74.5 - 83.6	21.49	18.6 - 24.7	1.01	0.96
≤1	80.56	75.8 - 84.7	20.39	17.5 - 23.5	1.01	0.95
≤1.01	80.86	76.2 - 85.0	20.11	17.3 - 23.2	1.01	0.95
≤1.03	80.86	76.2 - 85.0	19.83	17.0 - 22.9	1.01	0.96
≤1.04	81.17	76.5 - 85.3	19.70	16.9 - 22.8	1.01	0.96
≤1.06	81.48	76.8 - 85.6	19.70	16.9 - 22.8	1.01	0.94
≤1.07	81.48	76.8 - 85.6	19.28	16.5 - 22.3	1.01	0.96
≤1.08	82.10	77.5 - 86.1	19.01	16.2 - 22.1	1.01	0.94
≤1.09	82.10	77.5 - 86.1	18.87	16.1 - 21.9	1.01	0.95
≤1.1	83.64	79.2 - 87.5	16.80	14.2 - 19.7	1.01	0.97
≤1.12	83.95	79.5 - 87.8	16.67	14.0 - 19.6	1.01	0.96
≤1.13	84.26	79.8 - 88.0	16.39	13.8 - 19.3	1.01	0.96
≤1.14	84.57	80.2 - 88.3	15.98	13.4 - 18.8	1.01	0.97
≤1.15	84.88	80.5 - 88.6	15.84	13.3 - 18.7	1.01	0.95
≤1.16	85.19	80.8 - 88.9	15.70	13.1 - 18.6	1.01	0.94
≤1.17	85.49	81.2 - 89.1	15.56	13.0 - 18.4	1.01	0.93
≤1.19	86.11	81.9 - 89.7	15.56	13.0 - 18.4	1.02	0.89
≤1.2	87.65	83.6 - 91.0	14.05	11.6 - 16.8	1.02	0.88
≤1.21	87.96	83.9 - 91.3	13.77	11.3 - 16.5	1.02	0.87
≤1.24	87.96	83.9 - 91.3	13.50	11.1 - 16.2	1.02	0.89
≤1.25	88.27	84.3 - 91.6	13.36	11.0 - 16.1	1.02	0.88
≤1.26	88.58	84.6 - 91.8	13.36	11.0 - 16.1	1.02	0.85
≤1.28	88.58	84.6 - 91.8	13.22	10.8 - 15.9	1.02	0.86
≤1.29	89.20	85.3 - 92.4	12.95	10.6 - 15.6	1.02	0.83
≤1.3	89.81	86.0 - 92.9	11.98	9.7 - 14.6	1.02	0.85
≤1.35	89.81	86.0 - 92.9	11.43	9.2 - 14.0	1.01	0.89
≤1.36	90.12	86.3 - 93.1	11.16	9.0 - 13.7	1.01	0.89
≤1.38	90.43	86.7 - 93.4	11.02	8.8 - 13.5	1.02	0.87
≤1.4	91.36	87.8 - 94.2	10.33	8.2 - 12.8	1.02	0.84
≤1.41	91.36	87.8 - 94.2	10.19	8.1 - 12.6	1.02	0.85
≤1.44	91.67	88.1 - 94.4	10.06	8.0 - 12.5	1.02	0.83
≤1.45	91.67	88.1 - 94.4	9.92	7.8 - 12.3	1.02	0.84
≤1.47	92.28	88.8 - 94.9	9.92	7.8 - 12.3	1.02	0.78
≤1.57	92.28	88.8 - 94.9	8.82	6.9 - 11.1	1.01	0.88
≤1.58	92.59	89.2 - 95.2	8.82	6.9 - 11.1	1.02	0.84
≤1.6	93.21	89.9 - 95.7	7.71	5.9 - 9.9	1.01	0.88
≤1.63	93.52	90.3 - 95.9	7.71	5.9 - 9.9	1.01	0.84
≤1.64	93.52	90.3 - 95.9	7.58	5.8 - 9.7	1.01	0.86
≤1.7	93.83	90.6 - 96.2	7.16	5.4 - 9.3	1.01	0.86
≤1.76	93.83	90.6 - 96.2	6.61	4.9 - 8.7	1.00	0.93

≤1.78	94.44	91.4 - 96.7	6.61	4.9 - 8.7	1.01	0.84
≤1.8	94.75	91.7 - 96.9	6.34	4.7 - 8.4	1.01	0.83
≤1.82	94.75	91.7 - 96.9	6.20	4.6 - 8.2	1.01	0.85
≤1.84	95.06	92.1 - 97.2	6.20	4.6 - 8.2	1.01	0.80
≤1.89	95.06	92.1 - 97.2	6.06	4.4 - 8.1	1.01	0.81
≤1.9	95.99	93.2 - 97.8	5.92	4.3 - 7.9	1.02	0.68
≤1.96	95.99	93.2 - 97.8	5.79	4.2 - 7.7	1.02	0.69
≤1.97	96.30	93.6 - 98.1	5.79	4.2 - 7.7	1.02	0.64
≤1.98	96.60	94.0 - 98.3	5.51	4.0 - 7.4	1.02	0.62
≤2	96.91	94.4 - 98.5	5.51	4.0 - 7.4	1.03	0.56
≤2.02	97.22	94.8 - 98.7	5.37	3.8 - 7.3	1.03	0.52
≤2.1	97.22	94.8 - 98.7	4.55	3.1 - 6.3	1.02	0.61
≤2.11	97.53	95.2 - 98.9	4.55	3.1 - 6.3	1.02	0.54
≤2.29	97.53	95.2 - 98.9	3.31	2.1 - 4.9	1.01	0.75
≤2.3	97.84	95.6 - 99.1	3.03	1.9 - 4.6	1.01	0.71
≤2.35	97.84	95.6 - 99.1	2.48	1.5 - 3.9	1.00	0.87
≤2.36	98.15	96.0 - 99.3	2.48	1.5 - 3.9	1.01	0.75
≤2.4	98.15	96.0 - 99.3	2.20	1.3 - 3.6	1.00	0.84
≤2.44	98.46	96.4 - 99.5	2.20	1.3 - 3.6	1.01	0.70
≤2.46	98.46	96.4 - 99.5	1.93	1.1 - 3.2	1.00	0.80
≤2.58	98.77	96.9 - 99.7	1.93	1.1 - 3.2	1.01	0.64
≤2.7	98.77	96.9 - 99.7	1.24	0.6 - 2.3	1.00	1.00
≤2.9	99.07	97.3 - 99.8	1.10	0.5 - 2.2	1.00	0.84
≤3	99.07	97.3 - 99.8	0.96	0.4 - 2.0	1.00	0.96
≤3.01	99.38	97.8 - 99.9	0.96	0.4 - 2.0	1.00	0.64
≤3.1	99.69	98.3 - 100.0	0.83	0.3 - 1.8	1.01	0.37
≤4	99.69	98.3 - 100.0	0.41	0.09 - 1.2	1.00	0.75
≤4.1	100.00	98.9 - 100.0	0.28	0.03 - 1.0	1.00	0.00
≤4.8	100.00	98.9 - 100.0	0.00	0.0 - 0.5	1.00	

Appendix C. Cross tabs of P32AI1 and correlations spearmann

Table 6. RP by P32AI1

	P32AI1	No P32AI1	Total
RP	22 (2.11%)	91 (8.74%)	**113 (10.85%)**
No RP	296 (28.43%)	632 (60.71%)	**928 (89.15%)**
Total	**318 (30.55%)**	**723 (69.45%)**	**1041**

Table 7. Mastitis by P32AI1

	P32AI1	No P32AI1	Total
Mastitis	37 (3.55%)	140 (13.45%)	**177 (17.0%)**
No Mastitis	281 (26.99%)	583 (56.0%)	**864 (83.0%)**
Total	**318 (30.55%)**	**723 (69.45%)**	**1041**

Table 8. DA by P32AI1

	P32AI1	No P32AI1	Total
DA	5 (0.48%)	34 (3.27%)	**39 (3.75%)**
No DA	313 (30.07%)	689 (66.19%)	**1002 (96.25%)**
Total	**318 (30.55%)**	**723 (69.45%)**	**1041**

Table 9. Lame by P32AI1

	P32AI1	No P32AI1	Total
Lame	24 (2.31%)	89 (8.55%)	**113 (10.85%)**
No Lame	294 (28.24%)	634 (60.90%)	**928 (89.15%)**
Total	**318 30.55%)**	**723 (69.45%)**	**1041**

Table 10. CE by P32AI1

	P32AI1	No P32AI1	Total
CE	83 (7.97%)	249 (23.92%)	**332 (31.89%)**
No CE	235 (22.57%)	474 (45.53%)	**709 (68.11%)**
Total	**318 (30.55%)**	**723 (69.45%)**	**1041**

Table 11. Correlations Spearman

1041 observations used, 10 indicator independent variables entered.

	SCK1.2	CalvProb	RP	Metritis	Mastitis	DA	Lame	CE	Cyclic	AIEstrus
SCK1.2	1.000	-0.0345	0.0773	-0.0191	-0.0550	0.1289	0.0514	0.0399	0.0257	-0.0598
		0.2655	0.0126	0.5380	0.0762	<.0.0001	0.0947	0.1983	0.4069	0.0537
CalvProb	-.0345	1.000	0.1450	0.1664	0.0457	-0.0224	0.0712	0.0998	-0.0449	-0.0026
	0.2655		<.0001	<.0001	0.1402	0.4699	0.0216	0.0013	0.1473	0.9334
RP	0.0773	0.1450	1.000	0.3480	0.0394	0.0938	0.0470	0.2250	0.0162	-0.0379
	0.0126	<.0001		<.0001	0.2046	0.0025	0.1297	<.0001	0.6016	0.2222
Metritis	-0.0191	0.1664	0.3480	1.000	0.0491	0.0127	0.0208	0.1879	-0.0337	-0.0258
	0.5380	<.0001	<.0001		0.1136	0.6834	0.5033	<.0001	0.2767	0.4059
Mastitis	-0.0550	0.0457	0.0394	0.0491	1.000	0.0588	0.0805	0.0524	0.0335	0.0776
	0.0762	0.1402	0.2046	0.1136		0.0578	0.0094	0.0911	0.2807	0.0123
DA	0.1289	-0.0224	0.0938	0.0127	0.0588	1.000	0.0450	0.1146	-0.0038	0.0218
	<.0001	0.4699	0.0025	0.6834	0.0578		0.1469	0.0002	0.9039	0.4827
Lame	0.0514	0.0712	0.0470	0.0208	0.0805	0.0450	1.000	0.0461	-0.0506	-0.0064
	0.0974	0.0216	0.1297	0.5033	0.0094	0.1469		0.1369	0.1029	0.8355
CE	0.0399	0.0998	0.2250	0.1879	0.0524	0.1146	0.0461	1.000	-0.0496	0.0997
	0.1983	0.0013	<.0001	<.0001	0.0911	0.0002	0.1396		0.1099	0.0013
Cyclic	0.0257	-0.0449	0.0162	-0.0337	0.0335	-0.0038	-0.0506	-0.0496	1.000	0.1949
	0.4069	0.1473	0.6016	0.2767	0.2807	0.9039	0.1029	0.1099		<.0001
AIEstrus	-0.0598	-0.0026	-0.0379	-0.0258	0.0776	0.0218	-0.0064	0.0997	0.1949	1.000
	0.0537	0.9334	0.2222	0.4059	0.0123	0.4827	0.8355	0.0013	<.0001	

SCK has a positive significant weak correlation with retained placenta (r=0.07730; p=0.0126; n=1041) and a highly significant weak correlation with displacement abomasum (r=0.12889; p=<.0001; n=1041), where α=<0.05. Calving problems have a weak positive significant correlation with retained placenta, metritis, lameness and CE.

Appendix D. Cross tabs of SCK1.2 mmol/L

Table 13. Season by SCK1.2

	SCK1.2	No SCK1.2	Total
Summer	87 (8.36%)	437 (41.98%)	**524 (50.34%)**
Winter	70 (6.72%)	447 (42.94%)	**517 (49.66%)**
Total	**157 (15.08%)**	**884 (84.92%)**	**1041**

Table 14. Parity by SCK1.2

	SCK1.2	No SCK1.2	Total
Primiparous	17 (1.63%)	411 (39.48%)	**428 (41.11%)**
Multiparous	140 (13.45%)	473 (45.44%)	**613 (58.89%)**
Total	**157 (15.08%)**	**884 (84.92%)**	**1041**

Table 15. SCK1.2 by BCS at calving

	SCK1.2	No SCK1.2	Total
BSCCavC high	61 (5.86%)	184 (17.68%)	**245 (23.54%)**
BCSCavC med	69 (6.63%)	448 (43.04%)	**517 (49.66%)**
BCSCavC low	27 (2.59%)	252 (24.21%)	**279 (26.80%)**
Total	**157 (15.08%)**	**884 (84.92%)**	**1041**

Table 16. SCK1.2 by Mastitis

	Mastitis	No Mastitis	Total
SCK1.2	19 (1.83%)	138 (13.26%)	**157 (15.08%)**
No SCK1.2	158 (15.18%)	726 (69.74%)	**884 (84.92%)**
Total	**177 (17%)**	**864 (83%)**	**1041**

Table 17. SCK1.2 by DA

	DA	No DA	Total
SCK1.2	15 (1.44%)	142 (13.64%)	**157 (15.08%)**
No SCK1.2	24 (2.31%)	860 (82.61%)	**884 (84.92%)**
Total	**39 (3.75%)**	**1002 (96.25%)**	**1041**

Table 18. SCK1.2 by CE

	CE	No CE	Total
SCK1.2	57 (5.48%)	100 (9.61%)	**157 (15.08%)**
No SCK1.2	275 (26.42%)	609 (58.50%)	**884 (84.92%)**
Total	**332 (31.89%)**	**709 (68.11%)**	**1041**

Table 19. SCK1.2 by BCS 40 DIM

	BCS40 high	BCS40 med	BCS40 low	Total
SCK1.2	14 (1.34%)	71 (6.82%)	72 (6.92%)	**157 (15.08%)**
No SCK1.2	98 (9.41%)	365 (35.06%)	421 (40.44%)	**884 (84.92%)**
Total	**112 (10.76%)**	**436 (41.88%)**	**493 (47.36%)**	**1041**

Appendix E. Logistic procedure with SCK1.2 as response variable

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                     The LOGISTIC Procedure

                       Model Information

        Data Set                      WORK.MARISKASTUDY
        Response Variable             SCK1.2
        Number of Response Levels     2
        Model                         binary logit
        Optimization Technique        Fisher's scoring


            Number of Observations Read        1041
            Number of Observations Used        1041


                        Response Profile

               Ordered                     Total
                 Value     SCK1.2      Frequency

                     1     1                 157
                     2     0                 884

              Probability modeled is SCK1.2='1'.


                 Backward Elimination Procedure


                    Class Level Information

                                         Design
               Class        Value      Variables

               Season       Summer       1
                            Winter       0

               Parity       Mult         0
                            Prim         1

               BCSCavC      High         1      0
                            Low          0      0
                            Med          0      1

               BCS40C       High         1      0
                            Low          0      0
                            Med          0      1

               CalvProb     0            1
                            1            0

               RP           0            1
                            1            0
```

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The LOGISTIC Procedure

Class Level Information

Class	Value	Design Variables
Metritis	0	1
	1	0
Mastitis	0	1
	1	0
Resp	0	1
	1	0
DA	0	1
	1	0
Lame	0	1
	1	0
CE	0	1
	1	0
Cyclic	0	0
	1	1
AIEstrus	0	0
	1	1

Step 0. The following effects were entered:

Intercept Season Parity BCSCavC BCS40C CalvProb RP Metritis Mastitis Resp DA Lame CE Cyclic AIEstrus

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	885.024	799.723
SC	889.972	883.838
-2 Log L	883.024	765.723

The LOGISTIC Procedure

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	117.3010	16	<.0001
Score	108.9592	16	<.0001
Wald	86.5278	16	<.0001

Step 1. Effect Cyclic is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	885.024	797.727
SC	889.972	876.894
-2 Log L	883.024	765.727

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	117.2966	15	<.0001
Score	108.9568	15	<.0001
Wald	86.5320	15	<.0001

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
0.0044	1	0.9473

Step 2. Effect CalvProb is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

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                      The LOGISTIC Procedure

                       Model Fit Statistics

                                        Intercept
                          Intercept           and
             Criterion         Only    Covariates

             AIC            885.024       795.904
             SC             889.972       870.123
             -2 Log L       883.024       765.904


            Testing Global Null Hypothesis: BETA=0

     Test                 Chi-Square       DF     Pr > ChiSq

     Likelihood Ratio       117.1194       14         <.0001
     Score                  108.7963       14         <.0001
     Wald                    86.3587       14         <.0001


                    Residual Chi-Square Test

               Chi-Square       DF     Pr > ChiSq

                   0.1792        2         0.9143

Step  3. Effect Lame is removed:


                    Model Convergence Status

          Convergence criterion (GCONV=1E-8) satisfied.


                       Model Fit Statistics

                                        Intercept
                          Intercept           and
             Criterion         Only    Covariates

             AIC            885.024       794.257
             SC             889.972       863.528
             -2 Log L       883.024       766.257
```

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                     The LOGISTIC Procedure

            Testing Global Null Hypothesis: BETA=0

    Test                 Chi-Square       DF     Pr > ChiSq

    Likelihood Ratio       116.7671       13         <.0001
    Score                  108.4642       13         <.0001
    Wald                    86.1514       13         <.0001


                   Residual Chi-Square Test

              Chi-Square       DF     Pr > ChiSq

                  0.5388        3         0.9103


Step  4. Effect RP is removed:


                   Model Convergence Status

         Convergence criterion (GCONV=1E-8) satisfied.


                     Model Fit Statistics

                                            Intercept
                             Intercept            and
              Criterion           Only     Covariates

              AIC              885.024        792.712
              SC               889.972        857.035
              -2 Log L         883.024        766.712


            Testing Global Null Hypothesis: BETA=0

    Test                 Chi-Square       DF     Pr > ChiSq

    Likelihood Ratio       116.3118       12         <.0001
    Score                  107.4980       12         <.0001
    Wald                    85.6271       12         <.0001


                   Residual Chi-Square Test

              Chi-Square       DF     Pr > ChiSq

                  0.9988        4         0.9100
```

The LOGISTIC Procedure

Step 5. Effect AIEstrus is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	885.024	791.689
SC	889.972	851.064
-2 Log L	883.024	767.689

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	115.3347	11	<.0001
Score	106.5929	11	<.0001
Wald	84.8421	11	<.0001

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
1.9824	5	0.8516

Step 6. Effect Metritis is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

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                      The LOGISTIC Procedure

                       Model Fit Statistics

                                             Intercept
                              Intercept            and
             Criterion             Only     Covariates

             AIC                885.024        790.688
             SC                 889.972        845.115
             -2 Log L           883.024        768.688


             Testing Global Null Hypothesis: BETA=0

    Test                 Chi-Square       DF     Pr > ChiSq

    Likelihood Ratio       114.3356       10         <.0001
    Score                  105.8064       10         <.0001
    Wald                    84.1380       10         <.0001


                    Residual Chi-Square Test

                 Chi-Square       DF     Pr > ChiSq

                     3.0055        6         0.8082

Step  7. Effect Resp is removed:


                    Model Convergence Status

         Convergence criterion (GCONV=1E-8) satisfied.


                     Model Fit Statistics

                                             Intercept
                              Intercept            and
             Criterion             Only     Covariates

             AIC                885.024        790.395
             SC                 889.972        839.874
             -2 Log L           883.024        770.395
```

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The LOGISTIC Procedure

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	112.6287	9	<.0001
Score	103.7800	9	<.0001
Wald	82.5180	9	<.0001

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
4.8157	7	0.6824

NOTE: No (additional) effects met the 0.1 significance level for removal from the model.

Summary of Backward Elimination

Step	Effect Removed	DF	Number In	Wald Chi-Square	Pr > ChiSq
1	Cyclic	1	13	0.0044	0.9473
2	CalvProb	1	12	0.1748	0.6759
3	Lame	1	11	0.3588	0.5492
4	RP	1	10	0.4600	0.4976
5	AIEstrus	1	9	0.9769	0.3230
6	Metritis	1	8	1.0053	0.3160
7	Resp	1	7	1.7874	0.1812

Type 3 Analysis of Effects

Effect	DF	Wald Chi-Square	Pr > ChiSq
Season	1	6.7371	0.0094
Parity	1	45.1975	<.0001
BCSCavC	2	10.8405	0.0044
BCS40C	2	5.0690	0.0793
Mastitis	1	4.8499	0.0276
DA	1	4.2353	0.0396
CE	1	3.2724	0.0705

The LOGISTIC Procedure

Analysis of Maximum Likelihood Estimates

Parameter		DF	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
Intercept		1	-1.3306	0.4712	7.9761	0.0047
Season	Summer	1	0.5060	0.1949	6.7371	0.0094
Parity	Prim	1	-1.8372	0.2733	45.1975	<.0001
BCSCavC	High	1	0.8765	0.2984	8.6284	0.0033
BCSCavC	Med	1	0.2502	0.2602	0.9245	0.3363
BCS40C	High	1	-0.7802	0.3553	4.8218	0.0281
BCS40C	Med	1	-0.0859	0.2109	0.1658	0.6839
Mastitis	0	1	0.6044	0.2744	4.8499	0.0276
DA	0	1	-0.7639	0.3712	4.2353	0.0396
CE	0	1	-0.3631	0.2007	3.2724	0.0705

Association of Predicted Probabilities and Observed Responses

Percent Concordant	73.8	Somers' D	0.497
Percent Discordant	24.2	Gamma	0.507
Percent Tied	2.0	Tau-a	0.127
Pairs	138788	c	0.748

Odds Ratio Estimates and Profile-Likelihood Confidence Intervals

Effect		Unit	Estimate	95% Confidence Limits	
Season	Summer vs Winter	1.0000	1.659	1.134	2.439
Parity	Prim vs Mult	1.0000	0.159	0.090	0.265
BCSCavC	High vs Low	1.0000	2.402	1.349	4.355
BCSCavC	Med vs Low	1.0000	1.284	0.778	2.165
BCS40C	High vs Low	1.0000	0.458	0.222	0.898
BCS40C	Med vs Low	1.0000	0.918	0.606	1.387
Mastitis	0 vs 1	1.0000	1.830	1.092	3.219
DA	0 vs 1	1.0000	0.466	0.227	0.981
CE	0 vs 1	1.0000	0.695	0.470	1.034

Odds Ratio Estimates and Wald Confidence Intervals

Effect		Unit	Estimate	95% Confidence Limits	
Season	Summer vs Winter	1.0000	1.659	1.132	2.431
Parity	Prim vs Mult	1.0000	0.159	0.093	0.272
BCSCavC	High vs Low	1.0000	2.402	1.339	4.311
BCSCavC	Med vs Low	1.0000	1.284	0.771	2.139
BCS40C	High vs Low	1.0000	0.458	0.228	0.920
BCS40C	Med vs Low	1.0000	0.918	0.607	1.388
Mastitis	0 vs 1	1.0000	1.830	1.069	3.134
DA	0 vs 1	1.0000	0.466	0.225	0.964
CE	0 vs 1	1.0000	0.695	0.469	1.031

Appendix F. Logistic procedure with P32AI1 as response variable

```
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               The LOGISTIC Procedure

                  Model Information

Data Set                      WORK.MARIELSTUDY
Response Variable             P32AI1
Number of Response Levels     2
Model                         binary logit
Optimization Technique        Fisher's scoring


   Number of Observations Read        1041
   Number of Observations Used        1041


                Response Profile

      Ordered                    Total
        Value     P32AI1       Frequency

            1     1                  318
            2     0                  723

      Probability modeled is P32AI1='1'.


        Backward Elimination Procedure


           Class Level Information

                                Design
      Class         Value      Variables

      Season        Summer      1
                    Winter      0

      Parity        Mult        0
                    Prim        1

      BCSCavC       High        1      0
                    Low         0      0
                    Med         0      1

      BCS40C        High        1      0
                    Low         0      0
                    Med         0      1

      CalvProb      0           1
                    1           0

      RP            0           1
                    1           0
```

```
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                                     The LOGISTIC Procedure

                                    Class Level Information

                                                      Design
                               Class        Value     Variables

                               Metritis     0           1
                                            1           0

                               Mastitis     0           1
                                            1           0

                               Resp         0           1
                                            1           0

                               DA           0           1
                                            1           0

                               SCK1.2      0           1
                                            1           0

                               Lame         0           1
                                            1           0

                               CE           0           1
                                            1           0

                               Cyclic       0           0
                                            1           1

                               AIEstrus     0           0
                                            1           1

Step  0. The following effects were entered:

Intercept  Season  Parity  BCSCavC  BCS40C  CalvProb  RP  Metritis  Mastitis  Resp  DA  Lame  CE
Cyclic  AIEstrus  SCK1.2


                                   Model Convergence Status

                         Convergence criterion (GCONV=1E-8) satisfied.
```

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The LOGISTIC Procedure

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	1283.331	1277.088
SC	1288.278	1366.151
-2 Log L	1281.331	1241.088

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	40.2423	17	0.0012
Score	36.8773	17	0.0035
Wald	34.9943	17	0.0062

Step 1. Effect BCS40C is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	1283.331	1273.159
SC	1288.278	1352.326
-2 Log L	1281.331	1241.159

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	40.1718	15	0.0004
Score	36.8162	15	0.0013
Wald	34.9413	15	0.0025

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The LOGISTIC Procedure

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
0.0704	2	0.9654

Step 2. Effect SCK1.2 is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	1283.331	1271.162
SC	1288.278	1345.381
-2 Log L	1281.331	1241.162

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	40.1685	14	0.0002
Score	36.8125	14	0.0008
Wald	34.9388	14	0.0015

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
0.0737	3	0.9948

Step 3. Effect Cyclic is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

```
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                     The LOGISTIC Procedure

                      Model Fit Statistics

                                          Intercept
                            Intercept           and
               Criterion         Only    Covariates

               AIC           1283.331      1269.264
               SC            1288.278      1338.535
               -2 Log L      1281.331      1241.264


              Testing Global Null Hypothesis: BETA=0

      Test                 Chi-Square       DF     Pr > ChiSq

      Likelihood Ratio        40.0669       13         0.0001
      Score                   36.7305       13         0.0005
      Wald                    34.8652       13         0.0009


                    Residual Chi-Square Test

                 Chi-Square       DF     Pr > ChiSq

                     0.1750        4         0.9964


Step  4. Effect Resp is removed:


                    Model Convergence Status

           Convergence criterion (GCONV=1E-8) satisfied.


                      Model Fit Statistics

                                          Intercept
                            Intercept           and
               Criterion         Only    Covariates

               AIC           1283.331      1267.635
               SC            1288.278      1331.958
               -2 Log L      1281.331      1241.635
```

The LOGISTIC Procedure

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	39.6959	12	<.0001
Score	36.4188	12	0.0003
Wald	34.5756	12	0.0005

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
0.5509	5	0.9901

Step 5. Effect AIEstrus is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	1283.331	1266.120
SC	1288.278	1325.495
-2 Log L	1281.331	1242.120

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	39.2105	11	<.0001
Score	35.9815	11	0.0002
Wald	34.1751	11	0.0003

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
1.0361	6	0.9842

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The LOGISTIC Procedure

Step 6. Effect Metritis is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	1283.331	1264.789
SC	1288.278	1319.217
-2 Log L	1281.331	1242.789

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	38.5413	10	<.0001
Score	35.3526	10	0.0001
Wald	33.5739	10	0.0002

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
1.7066	7	0.9743

Step 7. Effect BCSCavC is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

The LOGISTIC Procedure

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	1283.331	1263.966
SC	1288.278	1308.498
-2 Log L	1281.331	1245.966

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	35.3643	8	<.0001
Score	32.3986	8	<.0001
Wald	30.8079	8	0.0002

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
4.8291	9	0.8489

Step 8. Effect Season is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	1283.331	1263.715
SC	1288.278	1303.299
-2 Log L	1281.331	1247.715

The LOGISTIC Procedure

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	33.6155	7	<.0001
Score	30.7466	7	<.0001
Wald	29.2283	7	0.0001

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
6.5780	10	0.7646

Step 9. Effect Parity is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	1283.331	1263.910
SC	1288.278	1298.545
-2 Log L	1281.331	1249.910

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	31.4210	6	<.0001
Score	28.5918	6	<.0001
Wald	27.1674	6	0.0001

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
8.7497	11	0.6450

The LOGISTIC Procedure

Step 10. Effect CalvProb is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	1283.331	1263.967
SC	1288.278	1293.654
-2 Log L	1281.331	1251.967

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	29.3640	5	<.0001
Score	26.6683	5	<.0001
Wald	25.3386	5	0.0001

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
10.7404	12	0.5513

NOTE: No (additional) effects met the 0.1 significance level for removal from the model.

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The LOGISTIC Procedure

Summary of Backward Elimination

Step	Effect Removed	DF	Number In	Wald Chi-Square	Pr > ChiSq
1	BCS40C	2	14	0.0704	0.9654
2	SCK1.2	1	13	0.0033	0.9540
3	Cyclic	1	12	0.1013	0.7503
4	Resp	1	11	0.3755	0.5400
5	AIEstrus	1	10	0.4851	0.4861
6	Metritis	1	9	0.6707	0.4128
7	BCSCavC	2	8	3.1197	0.2102
8	Season	1	7	1.7457	0.1864
9	Parity	1	6	2.1981	0.1382
10	CalvProb	1	5	2.0014	0.1571

Type 3 Analysis of Effects

Effect	DF	Wald Chi-Square	Pr > ChiSq
RP	1	3.8552	0.0496
Mastitis	1	7.0457	0.0079
DA	1	3.4113	0.0648
Lame	1	3.4346	0.0638
CE	1	3.0741	0.0795

Analysis of Maximum Likelihood Estimates

Parameter		DF	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
Intercept		1	-3.2034	0.5805	30.4483	<.0001
RP	0	1	0.5011	0.2552	3.8552	0.0496
Mastitis	0	1	0.5330	0.2008	7.0457	0.0079
DA	0	1	0.9044	0.4897	3.4113	0.0648
Lame	0	1	0.4520	0.2439	3.4346	0.0638
CE	0	1	0.2713	0.1547	3.0741	0.0795

Association of Predicted Probabilities and Observed Responses

Percent Concordant	42.7	Somers' D	0.149
Percent Discordant	27.8	Gamma	0.211
Percent Tied	29.5	Tau-a	0.063
Pairs	229914	c	0.575

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The LOGISTIC Procedure

Odds Ratio Estimates and Profile-Likelihood Confidence Intervals

Effect		Unit	Estimate	95% Confidence Limits	
RP	0 vs 1	1.0000	1.650	1.017	2.779
Mastitis	0 vs 1	1.0000	1.704	1.161	2.555
DA	0 vs 1	1.0000	2.471	1.031	7.326
Lame	0 vs 1	1.0000	1.571	0.989	2.583
CE	0 vs 1	1.0000	1.312	0.971	1.782

Odds Ratio Estimates and Wald Confidence Intervals

Effect		Unit	Estimate	95% Confidence Limits	
RP	0 vs 1	1.0000	1.650	1.001	2.722
Mastitis	0 vs 1	1.0000	1.704	1.150	2.526
DA	0 vs 1	1.0000	2.471	0.946	6.451
Lame	0 vs 1	1.0000	1.571	0.974	2.534
CE	0 vs 1	1.0000	1.312	0.969	1.776

Appendix G. Definition table of variables

Variable	Description
SCK	BHBA ≥ 1.2 mmol/L
P32AI1	The presence of amniotic vesicle holding an embryo with heartbeat at d32±3 after AI1
Season	Winter = 11/30/12 till 01/02/13 or Summer = 06/13/13 till 07/23/13
Parity	Primiparous versus multiparous
BCSCavC	d 0, 1= emaciated, 5=obese with 0.25 increments (BCS; Ferguson et al., 1994)
BCS40C	d40±3 p.p., 1= emaciated, 5=obese with 0.25 increments (BCS; Ferguson et al., 1994)
CalvProb	Dystocia (with or without birth assistance), twins, stillbirth (dead or died within 6h p.p.) or abortion alone or in combination
RP	Failure to pass the fetal membranes within 24 h p.p. diagnosed by farm personnel
Metritis	A daily examination (by the hospital staff) from calving to 14 DIM. Cows with fever= ≥39,5 were evaluated for uterine discharge. Metricheck on d4, 7 and 10 p.p. (Sheldon et al.,2006) 1= clean mucus, 2= flecks, 3= <50% purulent, 4= > 50% purulent, 5= watery reddish discharge with a foul smell. Score 5 = metritis
Mastitis	The presence of abnormal milk (pus/flakes), swelling/ redness of the mammary gland. Daily examination by herd personnel
Resp	Based on herd personnel
DA	A veterinary diagnosis based on auscultation of the 'ping' sound within 30 DIM
CE	d33±3 DIM, vaginal discharge score ≥3 was considered as CE (Sheldon et al., 2006)
Lame	Lame = Locomotion score ≥ 3 (1-5) at 40 ±3 DIM (1= normal, 2= mildly lame, 3= moderately lame, 4= lame and 5= severely lame)
Cyclic	The presence of a corpus luteum (CL=1). No CL on d33 or 49 = not cycling
AI estrus	Timed AI (TAI) or Estrus (heat detection)