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The relationship between postpartum vaginal discharge symptoms and progesterone profile characteristics in lactating dairy cows in Denmark

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Abstract

In this paper, the effect of clinical symptoms of uterine inflammation on progesterone profile characteristics was quantified in dairy cows. A continuous scale based on visual observation of vaginal discharge (the previously developed D-index) was used to describe the clinical symptoms. Progesterone profiles in milk were used to describe the ovarian cycles, and to determine the distinguishing features of these profiles, a multivariate statistical procedure (principal component analysis) was performed.

Significant negative effects of the D-index were seen during the first and second postpartum ovarian cycles. The D-index had a significant effect on the shape of progesterone profiles and the length of the ovarian cycles but it only accounted for a small proportion of the variation in these ovarian cycle features. The D-index was not a significant risk factor for the length of postpartum anovulatory period in the present study.

1. Introduction

The effects of bacterial infection in the uterus are much wider reaching than simply provoking a uterine inflammation. Bacterial endotoxin and immune mediators can disturb hormonal interactions, both systemically at the level of hypothalamus and pituitary gland, and also locally with effects on follicular selection, growth and function [1]. Uterine inflammation can also be associated with prolonged postpartum anestrus or ovarian cysts, as well as abnormalities in progesterone profiles [2–5]. However, studies of the relationship between progesterone profiles and the clinical findings of uterine inflammation have revealed differing results ranging from strong to no relationship [2,3,6–9].

A possible reason for these differing reports relates to the different methods used to characterize uterine inflammation, especially under field conditions. Under such conditions, the most useful, simple and noninvasive method for estimating uterine inflammation is to evaluate the vaginal discharge [10–12]. Vaginal discharge has been reported to accurately reflect uterine bacterial infection and immune response [13], and presence of purulent vaginal discharge was associated with the presence of pathogenic bacteria in the uterus [6,13,14]. However, most vaginal discharge scoring systems can only distinguish severe from less severe cases, and are usually limited to one category of infec-

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Table 1
Definitions of scores of vaginal discharge features used in this study. The system used is that of Gorzecka et al [15].

Discharge features	Score			
	0*	1	2	3
Proportion of purulent material	no	flecks	≤50%	>50%
Amount	no	<1 handful	1 handful	>1 handful
Consistency	no	watery	semi-viscous	mucus-like
Smell	no	yes	—	—
Color	no blood	pink	brownish (red/brown/black)	—

* Score amount = 0 was given when there was no discharge present in the vagina, in these cases the other features could not be evaluated and were all given score 0.

tion. As such, they are not well suited to finding quantitative relationships to progesterone profile characteristics. Recently, by using a multivariate analysis of vaginal discharge features, we have created a more finely graded index for vaginal discharge symptoms (the D-index) that is universal in the sense that it can be applied to any type of uterine infection at any time from one to six weeks postpartum in dairy cows [15].

Over the last decade, milk progesterone profiles have become increasingly common not only in research herds but also, with the advent of automated in-line monitoring systems, in commercial dairy herds [16,17]. Such monitoring systems are expected to provide reliable information on dairy cow fertility, so effects of uterine inflammation on ovarian cycle features should be included. Thus, the aim of the present study was to quantify the relationship between vaginal discharge symptoms, evaluated using the new D-index, and progesterone profile characteristics in milk.

2. Materials and methods

The data for this study were collected from 2002 to 2009 in one dairy herd at the Danish Cattle Research Center in Foulum, Denmark. This herd included three breeds (Danish Holstein, Danish Jersey, Danish Red) until 2008, when the Danish Red cows were excluded. The cows were kept in a loose housing system and fed *ad libitum* with a total mixed ration containing 50 percent roughage (approximately fifty/fifty corn silage and grass silage) and 50 percent standard dairy concentrate. The cows were milked by VMS (Voluntary Milking System, DeLaval, Tumba, Sweden) and the average milk yield per year was 9640 kg ECM (energy-corrected milk yield).

In this study two separate data sets were used: one data set with uterine inflammation measurements and milk progesterone measurements collected during the present trial, and a second data set with only milk progesterone measurements collected from previous studies and used here to characterize the profiles.

2.1. Vaginal discharge symptoms

2.1.1. Animals

The data were collected from January to December 2008 from a total of 75 cows in their first to fifth lactation: 48 Danish Holstein kept in two groups and 27 Danish Jersey kept in one group.

2.1.2. Clinical examination

The cows were examined once weekly by the same person, from week one until week six after calving. At each examination rectal temperature was measured and vaginal discharge was collected by hand with a rectal glove and lubricant, after cleaning the vulva with paper towels. Each sample of collected discharge was scored separately for five features, according to the system of Gorzecka et al [15] (Table 1): proportion of purulent material, amount, consistency, smell and color (content of blood).

2.1.3. Index of vaginal discharge symptoms (D-index)

After scoring (Table 1), the D-index value was calculated for each sample of discharge as described in detail by Gorzecka et al [15]. Briefly, the D-index was derived from a multivariate analysis (principal component analysis) of vaginal discharge features and rectal temperature. The D-index reflects the severity of vaginal discharge symptoms and ranges from zero to ten. It can be used under field conditions without any adjustment from one to six weeks post calving, for any type of vaginal discharge and without defining the type of infection or differentiating between infection and contamination.

The D-index was calculated by summing the D-coefficients for each discharge feature score, rectal temperature and an offset (Table 2). Rectal temperature, as a non-class variable, needed to be first multiplied by the D-coefficient. For example, a cow

Table 2
Coefficients used for calculating the value of D-index according to scores for discharge features and rectal temperature. The derivation of these coefficients is described in Gorzecka et al [15].

Parameter		Coeff.
Rectal temperature	Value	0.28
Smell	Yes	1.14
Color	no blood	−1.02
	pink	1.06
	brownish	0.74
Consistency	watery	0.35
	semi-visc	1.15
	mucus-like	−1.22
Amount	<1 handful	−0.96
	1 handful	0.32
	>1 handful	0.82
Purulent material	No	−1.06
	Flecks	−0.30
	⩽50%	0.23
	>50%	1.07
offset		−6.41

recorded as: > handful vaginal discharge, > 50% purulent material, semi-viscous consistency, smell—yes, pink color and temperature = 39.5 °C will have a D-index value = 9.89 (see Table 3).

2.2. Progesterone profiles

2.2.1. Animals

The data collection was done from 2002 to 2009, and the resulting data represents 785 lactations from 554 cows in their first to fifth lactation: 240 Danish Holstein, 178 Danish Red and 136 Danish Jersey.

2.2.2. Milk collection and progesterone analysis

Milk progesterone measurements were made on proportional whole milk samples collected automatically from the cows during their milking in a robotic milking system with free traffic (mean no. of visits/day = 2.4). One progesterone measure was made daily during the first 120 d from calving, while it was made every second day for the remainder of lactation. The actual average intervals between progesterone samples before and after 120 d from calving were 1.4 and 2.4 d, respectively.

Progesterone was analyzed using the Ridgeway ELISA-kit (Ridgeway Science Ltd, Gloucestershire, UK). Milk samples were pipetted, diluted and distributed using a Biomek 2000 © (Laboratory Automation workstation, Beckman Coulter, Fullerton, CA, USA). Milk samples (25 μL, diluted 1:2 with water) were handled according to the manufacturer's instructions, however, incubation with substrate-buffer was increased to 1 h 30 min. Plates were read using a spectrophotometer/fluorometer at 575 nm (Fluostar© BMG Labtechnologies, Offenburg, Germany). Analyses were performed in 96-well plates; two sets of seven standards (0–30 ng/mL), locally made using milk from an ovariectomized cow and ethanolic progesterone solutions, and two sets of two control samples were used for every analysis and plate. For the low and high controls, the intra-assay precision (CV %) was 14.9 and 1.4, respectively; the inter-assay precision (CV %) was 32.7 and 20.1, respectively; and the average inaccuracy (bias) was ± 0.82 and 0.60 ng/mL, respectively.

The time series of milk progesterone measurements were examined for gaps longer than 14 d with no progesterone measurements. All such gaps were caused by technical reasons and all data following such gaps were excluded. Further, the entire lactation was excluded if there were insufficient data to identify the end of the postpartum anovulatory period (i.e., the time series did not start at calving and there were less than five measurements before the cow was detected to have elevated progesterone and resumed ovarian cycles).

2.2.3. Extraction of progesterone profiles and their features

In order to obtain features of the progesterone profiles (e.g., duration of the luteal phase and rate of decline in progesterone at the end of the luteal phase) in a consistent manner, a computerized extraction procedure was used. The progesterone profile features were extracted by fitting a spline curve to the progesterone data and then using the first and second derivatives of the spline function (hereafter referred to as velocity and acceleration, respectively) to identify the peaks and troughs of the progesterone profile, and the points with greatest rates of decrease or increase in the profile. Smoothing was carried out using the functional data analysis approach of Ramsay and Silverman [18] im-

Table 3
An example of calculation of D-index value for a given set of vaginal discharge scores.

Parameter		D-coefficient
Purulent material	>50%	1.07
Amount	>1 handful	0.82
Consistency	Semi-viscous	1.15
Smell	Yes	1.14
Color	Pink	1.06
Rectal temperature	39.5	39.5*0.28 = 11.06
Offset		−6.41
		D-index = 9.89

plemented in R [19]. The smoothing spline was a fifth order polynomial B-spline with knots placed at each data point and a roughness penalty imposed on the second derivative (see [20]). At the peaks and the troughs of the smoothed profiles, velocity, i.e., the rate of change in progesterone, is zero and when the velocity is at a local maximum or minimum, the acceleration is zero.

In a first step, all points were identified where velocity = 0 and velocity had exceeded a lower or upper velocity threshold of ± 1 ng/mL/d in the interval since the preceding zero velocity was identified. The velocity threshold was imposed to filter out zero velocity values associated with trivial undulations in the profile. Likewise, points of zero acceleration were identified using an acceleration threshold of ± 0.35 ng/mL/d/d.

In a second step, to extract progesterone profile features, the following cyclicity marks were set on each smoothed profile, based on the following six definitions:

1) Luteal phase peaks (LutProgPeak) were defined as a point of zero velocity crossing from positive to negative velocity values and a smoothed progesterone value of > 8 ng/mL. When more than 1 such point occurred in a luteal phase (the interval between two estrus marks), the last occurrence was chosen as the luteal phase peak as this marks the end of the luteal phase. The threshold of >8 ng/mL was determined from the data of confirmed pregnant cows, as progesterone never fell below 8 ng/mL during the luteal phase of pregnancy in these animals.
2) Estrus marks were defined as a point of zero velocity with velocity crossing from negative to positive values and a smoothed progesterone value of < 8 ng/mL.
3) The end of the postpartum anovulatory period was defined as the first point on the smoothed profile > 3 ng/mL immediately prior to the first two consecutive progesterone measurements >3 ng/mL [21].
4) The pre-estrus maximum rate of decline in progesterone (Down Rate) was defined as the velocity of the smoothed profile at the point of zero acceleration crossing from negative to positive acceleration in the period from luteal peak to estrus mark. If there was more than one such point, then the point with the minimum (i.e., most negative) velocity was chosen.
5) The post-estrus maximum rate of increase in progesterone (Up Rate) was defined as the velocity of the smoothed profile at the point of zero acceleration crossing from positive to negative acceleration in the period from an estrus mark to a luteal peak. If there was more than one such point, then the point with the maximum (i.e., most positive) velocity was chosen. In the last period (usually pregnancy) after the luteal peak mark, acceleration marks were excluded.
6) The length of the ovarian cycle was calculated as the number of days between two subsequent estrus marks.

Two further features, "Longer" and "Shorter", were created for the each ovarian cycle as the absolute value of the negative and positive deviation from the median cycle length, respectively. The median cycle length (based on progesterone profiles) was calculated to be 23 d (25% = 20 600, 75% = 26 400) from the 2046 ovarian cycles (cycle number first to ninth) available in the data set. For the 737 first postpartum ovarian cycles, the median was 22.5 d, but 23 d was used as the median length of the cycle in all analyses. For each ovarian cycle shorter than 23 d, a value of zero was attributed to the feature "Longer". Similarly, for the cycles longer than 23 d, a value of zero was attributed to the feature "Shorter".

2.3. Statistical analysis

2.3.1. Principal component analysis (PCA) of progesterone profile features

In order to describe the relationships between the different progesterone profile features, and through this to describe the different progesterone profiles, PCA was used as this deals appropriately with correlations between variables. The following progesterone profile features were analyzed using LatentiX 2.00 (Latent5, Copenhagen, Denmark, www.latentix.com): LutProgPeak, DownRate, UpRate, Longer and Shorter. The PCA model was based on 1827 ovarian cycles, which had available data concerning all mentioned features. Before creating the PCA model, the data were auto-scaled to mean 0 and SD 1. Using these scaled data the loading coefficients for each variable and for each principal component were calculated. Loading coefficients, i.e., describing the relationship between all variables, were used to calculate the scores, i.e., the position of all observations in the PCA model [22].

2.3.2. The relationship between D-index and progesterone profile characteristics

The relationship between the D-index and progesterone profile characteristics for the postpartum ovar-

Table 4
The correlations between breed, parity, last D-index and milk yield (at the first day of ovarian cycling).

	Breed	Parity	Last D-index	Milk yield
Last D-index	−0.191	−0.021	—	—
	P = 0.033	P = 0.812		
Milk yield	−0.503	−0.026	−0.108	—
	P = 0.000	P = 0.784	P = 0.258	
BCS	−0.074	−0.239	0.083	−0.060
	P = 0.424	P = 0.008	P = 0.369	P = 0.544

ian cycles were estimated by including the "D-index" variable in the PCA model described above and by regressing individual progesterone-based ovarian cycle features on the D-index. For this analysis, 75 first and 50 second ovarian cycles were available. Because of the short-term action of uterine inflammation on ovarian cycle features [1], the last D-index value before the onset of the particular ovarian cycle was used for this analysis (last D-index). Addition to the regressions of earlier values of the D-index did not alter the relationships found, and no significant effect of earlier values of the D-index was observed. This "D-index history" had no predictive value in this study.

2.3.2.1. Principal component analysis of progesterone profile features and D-index. In order to investigate the relationship between progesterone-based ovarian cycle features and last D-index, the variable "D-index" was added to the existing PCA model of progesterone-based ovarian cycle features. The resulting PCA model was based on 94 ovarian cycles that had available values of last D-index. These 94 ovarian cycles came from 75 cows that were clinically examined for uterine inflammation and thus had calculated values of D-index.

2.3.2.2. Regression. The effect of D-index on individual progesterone-based ovarian cycle features was examined by multiple linear regression with stepwise reduction of non-significant variables, using Minitab 9 software (www.minitab.com). Three descriptors of the progesterone-based ovarian cycle features were examined: the first principal component (PC#1) that described the shape of the progesterone profile, and the variables "Longer" and "Shorter" that described the length of the ovarian cycle. In these analyses, the variables "Longer" than and "Shorter" than typical in this study ovarian cycle length (median = 23 d) were used rather than PC#2 because there are good *a priori* reasons for not assuming that the factors affecting longer and shorter cycles are the same. Given that the length parameters comprising PC#2 were found to be independent of the shape parameters comprising PC#1 this presented no methodological difficulties.

To account for possible breed effects, D-index value was adjusted for breed (i.e., residuals values of last D-index from a one-way analysis of variance with breed as the only explanatory factor were used). Other possible factors affecting progesterone-based ovarian cycle features were also included in the regressions: breed, parity, milk yield (at the first day of ovarian cycling) and body condition score (BCS). Milk yield and BCS were adjusted for breed as described above (correlations between breed, milk yield, parity, D-index and BCS are shown in Table 4). Available data for BCS did not allow the calculation of the rate of change of body condition and therefore the last measurement before the onset of the ovarian cycles was used.

BCS was scored manually every two weeks based on the system described by Kristensen [23] using a one to five point scale with 0.25 point intervals, where one is very thin and five is obese.

Regressions were done for three groups of ovarian cycles: all ovarian cycles, first postpartum ovarian cycles and second postpartum ovarian cycles.

2.4. The length of postpartum anovulatory period

The relationship between the length of postpartum anovulatory period and possible risk factors was estimated using the regression model in Minitab 9 software. Predictors for the length of postpartum anovulatory period were: breed, parity, average milk yield from calving to the first ovarian cycle, BCS at calving and maximum D-index during the time from calving to the first ovarian cycle. Variables were adjusted for the effect of breed by using the residuals for maximum D-index, average milk yield, BCS at calving from one way analysis of variance with breed as the only explanatory factor.

Table 5
The relationships between progesterone profile characteristics expressed in terms of loading coefficients in a principal component analysis of 2046 estrous cycles. The cumulative percent of explained variance is presented for each successive principal component (PC#1 to PC#5) with those having the highest percent explained to the left.

Loadings[a]	PC#1	PC#2	PC#3	PC#4	PC#5
UpRate	0.474582	0.345376	−0.35179	0.679354	0.26497
LutProgPeak	0.615835	−0.06731	0.037355	−0.07212	−0.78078
DownRate	−0.56307	−0.11284	0.018163	0.652722	−0.49381
Longer	0.019794	−0.69537	−0.71527	−0.04857	0.045822
Shorter	−0.27943	0.616373	−0.60242	−0.32384	−0.27244
Cumulative % explained variance	47.4117	73.4462	87.8417	96.9926	100

[a] "Up Rate" is the maximum rate of increase in progesterone post-estrus, "LutProgPeak" is luteal phase peak of progesterone, "Down Rate" is the maximum rate of decline in progesterone pre-estrus, "Longer" is the length the cycle longer than median cycle length, "Shorter" is the length the cycle shorter than median cycle length.

3. Results

3.1. PCA model of progesterone profile features during ovarian cycles

3.1.1. General description of relationship between progesterone features

PCA was used to characterize the relationships between different features of the progesterone profile during ovarian cycles, and to identify the most influential feature combinations. The results of this analysis are shown in Table 5. In Table 5 it can be seen that the principal components first and second (PC#1, PC#2) account for 73.4 percent of the total variance in progesterone profile features (respectively 47.4 and 26.0 percent). The numerically highest values of the loading coefficients indicate the features that have the greatest influence in the PCs. For PC#1 these features are the ones that describe the shape of the progesterone profiles during the ovarian cycle, since LutProgPeak, DownRate and UpRate have the highest values. This can also be seen in Figure 1, where the progesterone features most strongly influencing PC#1 are those furthest from zero on the horizontal axis of the loadings plot, i.e., LutProgPeak, UpRate, and DownRate. These loadings indicate that less negative values of DownRate (i.e., a shallow downward slope), low progesterone peaks and less positive values of UpRate (i.e., a shallow upward slope) are characteristic of observations on the left side of the scores plot in Figure 1. Steeper downward slopes (more negative DownRate), high progesterone peaks and steeper upward slopes (more positive UpRate) are characteristic of observations on the right side of the scores plot in Figure 1.

In contrast, PC#2 is mostly influenced by the length of the ovarian cycle, with Longer and Shorter having the highest values and being furthest from zero on the vertical axis on Figure 1. Negative scores for PC#2 indicate cycles longer than 23 d; the more negative the values, the longer cycles. Similarly, positive values of PC#2 indicate cycles shorter than 23 d; the more positive the values, the shorter the cycle (Figure 1). In this study, within the 1827 ovarian cycles, there was a high positive correlation between the length of the cycle and the length of the luteal phase (0.927, $P < 0.0001$). It is striking that the ovarian cycle length features (Shorter, Longer) are close to zero on PC#1 and are thus largely uncorrelated with the shape features, which are close to zero on the vertical axis describing PC#2. Ovarian cycles, with the length 23 d (median cycle length of all

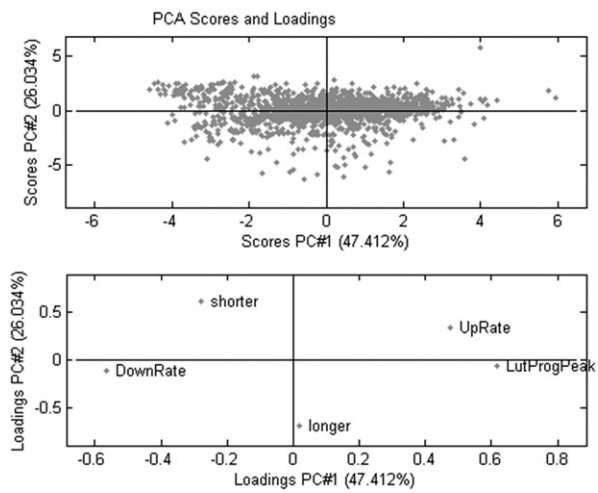

Fig. 1. PCA model of progesterone profile features. The scores plot (upper panel) presents the position of the observed scores for all ovarian cycles (i.e; the data from which the loadings were derived). The loadings plot (lower panel) presents how the variables are correlated with each other. PC#1 describes a shape of the profile, contrasting low-progesterone and flat profiles (on the left side) with high-progesterone and steep profiles (on the right side). PC#2 describes the length of the ovarian cycle, contrasting longer cycles with shorter cycles.

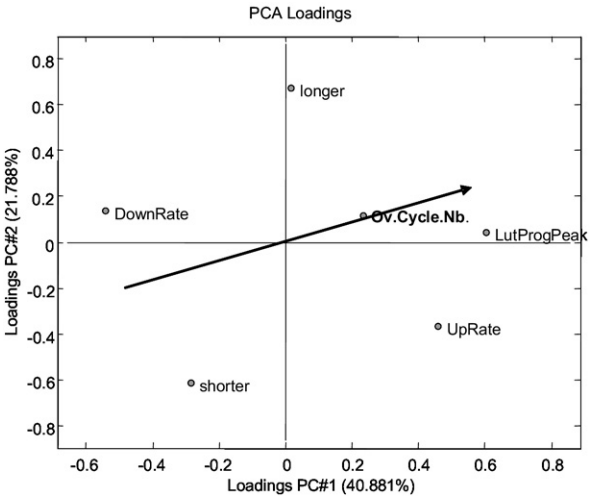

Fig. 2. The loadings plot for the PCA model of progesterone profile features after including the variable "Ov.Cycle.Nb." Adding "Ov.Cycle.Nb." did not impair the relationships between the other variables. The proportion of high, steep and longer ovarian cycles increases with ovarian cycle number greater than one and the proportion of low, flat and shorter ovarian cycles increases with first ovarian cycle. Note that due to the rotational ambiguity of PCA, the sign of the loadings is reversed.

ovarian cycles) have in PC#2 a value = 0. As the first two principal components accounted for the majority of the total variance, these were thus used in the subsequent analysis. PC#3–PC#5 accounted for too small a percent of total variance, and they largely represent the noise part of the model [22].

3.2. Effect of ovarian cycle number

To study the relationship between progesterone features and ovarian cycle number (Ov.Cycle.Nb), this variable was added to the PCA model (Fig. 2). Adding this variable did not adversely affect the relationship between the other variables, which strongly suggests that the associations between progesterone profile features during ovarian cycles are unaffected by cycle number. Indeed, coding the scores in Figure 1 to distinguish first ovarian cycles (coded as zero in the PCA) from second to ninth ovarian cycle number (coded as one) showed that both first and greater ovarian cycle numbers occurred throughout the whole space of the model (data not shown). Because the loading for Ov.Cycle.Nb. is positive for PC#1 and PC#2 this indicates that the proportion of high, steep and longer ovarian cycles increases with ovarian cycle number higher than one and that the proportion of low, flat and shorter ovarian cycles increases with first ovarian cycle. However, because it is placed close to zero both in PC#1 and in PC#2, it does not have any meaningful influence on the shape of progesterone profiles and length of the cycle.

3.3. The relationship between D-index and progesterone profile features

3.3.1. PCA model of progesterone features and last D-index

For exploratory purposes, the D-index was added to the PCA model, as shown in Figure 3. In this model, the two most influential components (PC#1 and PC#2) together accounted for 70.1 percent of a total variance. Figure 4 presents the scores plot (i.e., the PC values calculated for each observation) shaded according to the value of last D-index. Low values of last D-index (healthy) were correlated with high and steep profiles and with ovarian cycles of typical length in this study (median cycle length = 23 d). High values of last D-index were correlated with low and flat ovarian cycles, and prolonged or shortened cycle duration (Fig. 3). The effect of last D-index on the progesterone profile features is relatively small, because the "D-index" variable is placed close to zero in PC#1 and PC#2.

3.3.2. Regression of progesterone profile features and last D-index

For the purpose of quantifying the effect of D-index and other risk factors on the distinguishing features of progesterone profiles during ovarian cycles (PC#1, lon-

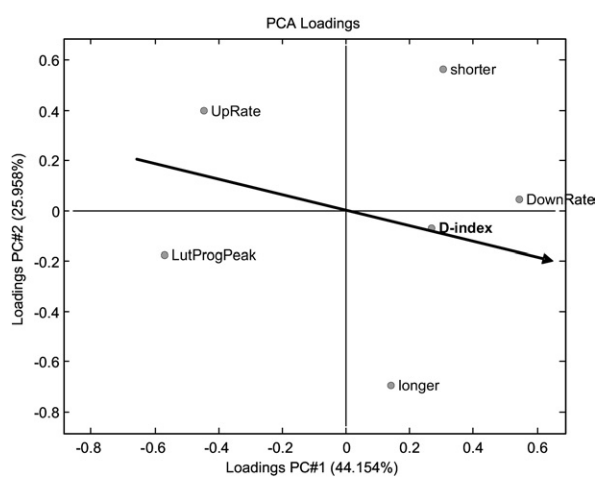

Fig. 3. The loadings plot for the PCA model of progesterone profile features after adding the variable "D-index". Low values of last D-index were correlated with high and steep profiles with typical in this study cycle length (median = 23 days). High values of last D-index were correlated with low and flat profiles, and prolonged or shortened cycle length. Note that due to the rotational ambiguity of PCA, the sign of the loadings is reversed.

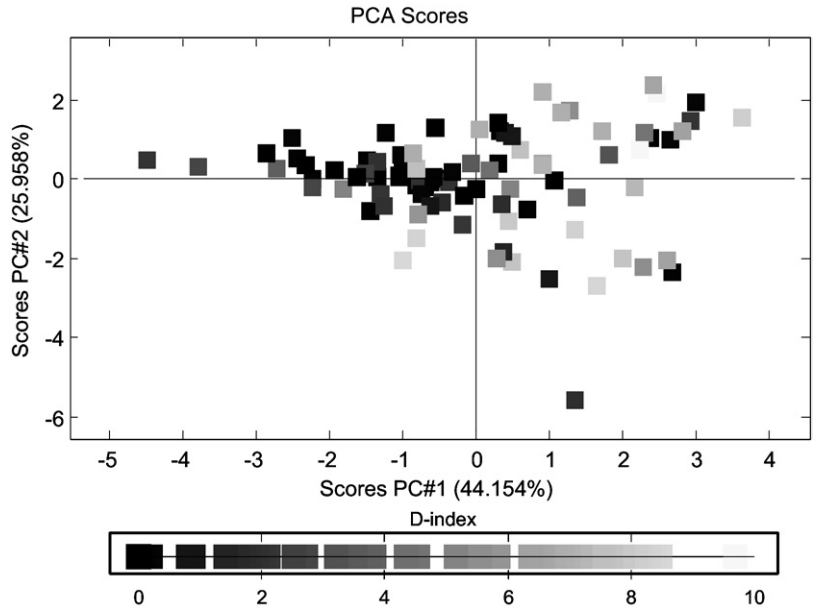

Fig. 4. PCA-scores of progesterone profile features shaded according to the value of D-index. The shading scale is given below the main plot. Low values of last D-index were clearly correlated with high and steep profiles with typical length of the cycle. High values of last D-index were correlated with low and flat profiles, and prolonged or shortened length of the cycle.

ger, shorter) stepwise linear regression was carried out. The results of these regressions analyses are presented in Tables 6–8.

The regression on PC#1 values (describing the shape of the profile) of breed, parity, adjusted last D-index, adjusted last milk yield and adjusted BCS, resulted - after stepwise removal of non-significant terms - in an equation with two significant predictors: adjusted last D-index and adjusted last milk yield. PC#1 values diminish with increasing last D-index and last milk yield, which means that the progesterone profile shape becomes flatter during the following ovarian cycle when the D-index increases. The coefficients for these factors were significant for both first postpartum ovarian cycles and all ovarian cycles (first + second), but not for second ovarian cycles (Table 6).

The regression on "Longer" (indicating the prolongation of cycle length) resulted in equations with three predictors: last D-index, breed and parity. The length of the cycle extends with an increase in last D-index, parity, and in Holstein cows (breed = 0). The parity effect was not significant for all groups and the effect of last D-index was not significant in the group of second ovarian cycles (Table 7).

The stepwise regression on "Shorter" (indicating the shortening of cycle length) resulted in the equations with three predictors: last D-index, breed and parity.

Table 6
The regression coefficients of the relationship PC#1 = a + b*lastD-index + c*lastMilkYield for three groups of estrous cycles (all, only 1st and only 2nd). Last D-index is the latest value of the vaginal discharge score (the D-index) before the ovarian cycle in question. Last milk yield is the week average milk yield (kg/d) at the corresponding time. Last D-index and last milk yield are both adjusted for breed effects. The significance values (P) for each coefficient, the R^2, and the residual standard deviation of the regressions are also presented.

PC1	a	b	c	R^2	Residual SD
All estrous cycles	−0.484	−0.163	−0.0455	10.8%	1.557
	P = 0.002	P = 0.002	P = 0.047		
1st estrous cycles	−0.775	−0.139	−0.0615	10.5%	1.640
	P = 0.001	P = 0.044	P = 0.036		
2nd estrous cycles	−0.062	−0.118	−0.0154	5.5%	1.350
	P = 0.794	P = 0.159	P = 0.666		

Table 7
The coefficients of relationship between last D-index, breed, parity and the variable "Longer" in three groups of ovarian cycles (Longer = a + b*lastD-ind + c*breed + d*parity). Last D-index was adjusted for breed effects. The breed effect represents the difference for Jersey relative to Holstein. The parity effect represents the difference of multiparous relative to primaparous.

Longer	a	B	c	d	R^2	Residual SD
All ovarian cycles	2.52	0.333	−2.30	1.19	14.6%	3.907
	P = 0.000	P = 0.004	P = 0.003	P = 0.111		
1st ovarian cycles	2.62	0.458	−2.56	1.24	16.4%	4.595
	P = 0.003	P = 0.009	P = 0.027	P = 0.258		
2nd ovarian cycles	1.38	−0.133	−1.25	0.632	12.5%	2.089
	P = 0.008	P = 0.296	P = 0.072	P = 0.366		

The length of the cycle shortens with an increase in last D-index, with parity one and in Jersey cows (Table 8).

3.4. The relationship between the length of postpartum anovulatory period, D-index and other risk factors

The regression model indicated that postpartum anovulatory period was longer in Holstein than in Jersey cows and the length of anovulatory period extended with: parity number greater than one, increasing average milk yield, maximum D-index, and lower BCS at calving. However, only average milk yield was a significant factor for the length of the postpartum anovulatory period in this study.

4. Discussion

The aim of this study was to quantify the relationship between clinical symptoms of uterine inflammation and progesterone profile characteristics of the ovarian cycle. To quantify severity of inflammation symptoms we used a continuous scale based on visual observation of vaginal discharge, the D-index, developed and described by Gorzecka et al [15]. Williams et al [13] have shown that vaginal discharge accurately reflects uterine bacterial infection and immune response. With regard to ovarian cycle features we chose to examine the aspects that are reflected in progesterone profiles because they provide objective measurements throughout the ovarian cycle. However, from the literature it is not clear which aspects of the progesterone profile during ovarian cycles are likely to be of importance. Therefore rather than make a priori assumptions we used a multivariate statistical procedure, PCA, to determine the distinguishing features of these progesterone profiles, i.e., which measurements are correlated and which measurements account for the variation in the shape of progesterone profiles and length of the cycles. Such an approach has not been previously reported.

4.1. PCA model of progesterone profile features during ovarian cycles

The major cause of variation, PC#1, were features describing the shape of the progesterone profile (a combination of peak progesterone concentration and the upward and downward slopes at the beginning and end of the luteal phase) that contrast low-progesterone and flat profiles (indicating low *corpus luteum* function) with high-progesterone and steep profiles (indicating elevated *corpus luteum* function). Importantly, the shape parameters were largely independent of cycle length parameters.

Abnormal luteal function, associated with reduced progesterone concentration after insemination [24–26],

Table 8
The coefficients of relationship between last D-index, breed, parity and the variable "shorter" in three groups of estruses (shorter = a + b*lastD-ind + c*breed + d*parity). Last D-index was adjusted for breed effects. The breed effect represents the difference for Jersey relative to Holstein. The parity effect represents the difference of multiparous relative to primaparous.

SHORTER	a	b	c	d	R^2	Residual SD
All ovarian cycles	1.80	0.193	1.93	−1.12	14.8%	2.884
	P = 0.000	P = 0.024	P = 0.001	P = 0.043		
1st ovarian cycles	1.5853	0.1501	2.3971	−0.8152	16.0%	2.965
	P = 0.005	P = 0.174	P = 0.002	P = 0.248		
2nd ovarian cycles	2.36	0.304	1.19	−1.56	17.3%	2.795
	P = 0.001	P = 0.078	P = 0.198	P = 0.100		

and also before insemination [27–29], has previously been correlated with reduced fertility. It has also been shown that pregnancy is more likely to be maintained in cows with high luteal phase progesterone [24]. These findings suggests that this combination of progesterone profile features, discriminating low and flat progesterone profiles (reduced quality) from high and steep profiles (improved quality), describes one valuable aspect of ovarian cycle characteristics.

The second major axis of variation in progesterone profile features, PC#2, related to the cycle length parameters, Longer and Shorter, independently of the shape parameters. It has been shown that both shortened [30,31] and prolonged luteal phases [7,21,32,33] are associated with poorer reproductive performance, suggesting that progesterone derived cycle durations represent another valuable aspect of ovarian cycle characteristics. Different mechanisms are responsible for these two abnormalities in cycle length, such as premature release or lack of release of PGF2α [34,35] and late embryonic mortality [36].

The PCA model revealed that this clustering of ovarian cycle features was the same regardless of ovarian cycle number (from first to fifth ovarian cycle postpartum). However, there was an indication that the proportion of low, flat and shorter ovarian cycles was greater with first ovarian cycle and the proportion of high, steep and longer ovarian cycles increases with ovarian cycle number greater than one. Accordingly, we looked at first and second ovarian cycles separately.

4.2. Relationship between D-index and progesterone profile features during ovarian cycles

There was a significant relationship between D-index and progesterone profile features during ovarian cycles that was consistent with the hypothesis that uterine inflammation adversely affects ovarian cycle features. The PCA model of progesterone profile features together with the D-index revealed the general, biological pattern of the relationship (Fig. 3), which was confirmed and quantified by the regression analyses (Tables 6, 7, and 8). Low values of last D-index, i.e., from healthier cows, were correlated with a high and steep shape of the profile and with cycles of typical duration in this study (median = 23 d). High values of last D-index, i.e., from less healthy cows, were correlated with low and flat ovarian cycles and prolonged or shortened duration of the ovarian cycles.

However, although the D-index had a significant effect on the shape of progesterone profiles and the length of the cycles, it only accounted for a small proportion of the variation in these progesterone-based ovarian cycle features (the R^2 of the regressions are low and when the D-index is included in the PCA, this variable is placed close to zero in both PC#1 and PC#2). This suggests that one should not overestimate the practical effect of clinical findings of uterine inflammation on ovarian activity postpartum, which is also suggested by McCoy [7] who found no significant relationship between vaginal discharge and abnormal progesterone profiles. Further, Shrestha at al [8] observed abnormal cervico-vaginal discharge in 20 percent of cows with normal resumption of ovarian activity, and abnormal discharge was detected in 47 percent (8/17) of cows with a normal progesterone profile [9]. In a trial investigating relationship between uterine infection involution and postpartum ovarian activity, Mateus et al [2] found that ovulation took place in the presence of a heavily contaminated uterus (determined by bacteriology and clinical signs of infection) in 45 percent of the medium endometritis and in 38 percent of the severe endometritis cows (see also [6]). These studies indicate that uterine inflammation symptoms do not preclude ovarian activity although as we have shown, clinical signs of uterine inflammation negatively affect progesterone-based ovarian cycle features.

Although it only accounted for a small proportion of the variation (R^2 in Table 7 and Table 8), we were able to quantify the effect of vaginal discharge symptoms on progesterone profile characteristics by means of the D-index. Thus, an increase by one unit of D-index was associated with an increase in average cycle length of the prolonged cycles of 0.3 d, and a decrease in the average length of shortened cycles of 0.2 d. The negative effects of D-index were consistently significant. Because the D-index applies throughout week one to six post calving, for any type of vaginal discharge, without the need to define the type of infection or differentiate between infection and contamination [15], this result can be attributed a certain generality. The result of the present study is in agreement with the literature indicating that uterine bacterial inflammation affects ovarian cycle features via bacterial endotoxin and immune mediators, which disturb follicular selection, growth and function and hormonal interactions on the level of hypothalamus and pituitary. Observations in several species indicate that endotoxin or intermediary cytokines, such as IL-1 or TNFα, can inhibit the pulsatile and surge modes of GnRH secretion, for instance in ewes [37], goats [38] or rats [39]. It has also been shown that endotoxin inhibits pituitary responsiveness to GnRH in ewes [40]. In cattle, endotoxin may suppress luteinizing hormone (LH) surges [41], interrupt the preovulatory rise

Table 9
The coefficients of relationship between the length of post-partum anovulatory period and breed, parity, average milk yield, maximum D-index and BCS at calving (Y = a + b*breed + c*parity + d*average milk yield + e*maximum D-index + f*BCS). Maximum D-index was adjusted for breed effects. The breed effect represents the difference for Jersey relative to Holstein. The parity effect represents the difference of multiparous relative to primaparous.

Y	a	b	c	d	e	f	R^2	Residual SD
The length of post-partum anovulatory period	20.6	−1.33	2.80	0.98	0.91	−0.96	25.7%	11.19
	P = 0.00	P = 0.65	P = 0.39	P = 0.00	P = 0.10	P = 0.80		

in estradiol and delay LH surge and subsequent ovulation [42,43], and infections with gram negative bacteria producing endotoxin, such as *Escherichia Coli*, suppress estradiol production by granulosa cells in the follicle [44]. In the study of Williams et al [5] cows with high pathogen growth density at Day 7 postpartum had a smaller diameter of the first postpartum dominant follicle and lower plasma estradiol concentrations.

Moreover, bacterial endotoxin modulates prostaglandin secretion by the endometrial cells [45]. Changes in the relative balance of PGF2α and PGE2α following bacterial infection may decide the fate of the *corpus luteum*, with luteolysis if PGF2α dominates and persistence if PGE2α dominates [46]. These mechanisms lead to a shortened [4] or prolonged luteal phases, which are the most frequent cyclic abnormalities in the postpartum period [3,8,9].

It is known that uterine inflammation is not the only cause of poorer features of the ovarian cycles, as, e.g., milk yield and negative energy balance can be also responsible [24,47]. In the present study, milk yield was a significant factor affecting the shape of the progesterone profile during ovarian cycles. Higher milk yield was also associated with a longer postpartum anovulatory period. In addition, we included BCS in our regression models as an indicator of the cow's energy status. However, this factor did not have any significant effect as an explanatory variable for variation in ovarian cycle features. We also found a significant relationship between the breed and the duration of the cycle. Holstein cows were more likely to have either prolonged or shortened cycles, compared with Jersey cows. This may be explained by lower average milk yield [21] and less negative energy balance [48] of the Jersey cows in this study. They also had on average lower D-index value, indicating enhanced uterine health than Holstein cows.

4.3. The duration of postpartum anovulatory period

The regression model indicated that postpartum anovulatory period was significantly longer in Holstein than in Jersey cows and was shorter for cows in first parity. In addition, even after adjustment for breed differences, the duration of the postpartum anovulatory period was increased by an increase in average milk yield. However, there was no significant effect of maximum D-index or of BCS at calving on the duration of the postpartum anovulatory period. The effect of milk yield is in agreement with the literature [24,49], and it has been suggested that the unfavorable association between milk yield and the duration of postpartum anovulatory period may be an effect of increased negative energy balance in cows with higher milk production [50].

To examine possible effects of uterine inflammation on postpartum anovulatory period we used the maximum value of D-index before the first ovulation. However, the regression coefficient for maximum D-index was not significant. We also found that constant high D-index values preceding the first ovarian cycle did not prevent ovulation (data not shown). This indicates that uterine inflammation was not a significant risk factor for the duration of postpartum anovulatory period in the examined herd and uterine inflammation was more likely to affect the quality of progesterone-based ovarian cycle features rather than to prevent cyclicity. This finding is supported by some previous studies, where authors suggest that uterine inflammation may be more responsible for prolonged luteal phase whilst negative energy balance might be more responsible for delayed resumption of ovarian activity [7–9].

The finding that BCS at calving had no effect on postpartum anovulatory period may seem surprising since BCS at calving is often correlated with negative energy balance (NEB) [51] and NEB affects postpartum anovulatory period [52]. However, it is increasingly accepted that there is a normal, natural NEB in early lactation [53,54]. In most mammals, independent of feeding level, there is an increase in body fatness during pregnancy and a decrease in body fatness during early lactation. Thus, a certain degree of NEB in early lactation cannot be prevented and it is supposed that this natural level of energy mobilization does not affect health and reproduction. It

may well have been the case that this natural level was not exceeded in this study [48].

5. Conclusions

Increasing severity of symptoms of uterine inflammation, as reflected by the D-index, was associated with abnormalities in all ovarian cycles examined. D-index had a significant negative effect on the shape of the progesterone profiles during ovarian cycles. It also significantly increased the frequency of prolonged and shortened ovarian cycles. Although the D-index had a significant effect on the shape and the length of progesterone profiles, it accounted for only a small proportion of the variance, suggesting that one should not overestimate the practical effect of clinical findings of uterine inflammation on ovarian activity in postpartum cows.

The D-index was not a significant risk factor for the length of postpartum anovulatory period in this herd, and was more likely to decrease the quality of progesterone-based ovarian cycle features than to prevent cyclicity. Table 9.

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