Genetic analysis of new progesterone-based fertility traits in dairy cows measured on-farm

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ABSTRACT

The objectives of this study were (1) to analyze the agreement of a standard laboratory ELISA for progesterone (P4) with an automated on-farm ELISA kit operated under commercial conditions in 1,297 milk samples from 50 dairy cows; (2) to study the influence of the method of detection of luteal activity on genetic parameters of fertility traits based on P4 measured with an automated on-farm ELISA once weekly from wk 3 to 9 postpartum in the milk of 1,304 cows; and (3) to study the influence of sampling frequency (once or twice weekly from wk 3 to 9) on the same traits from 296 cows. Luteal activity can be detected when there is an active corpus luteum in the ovary producing P4 and indicating the onset of reproductive cyclicity after calving. The on-farm ELISA overestimated P4 contents by a mean square error of prediction of 2.76 ng/mL and had an intermediate Spearman correlation with the laboratory kit (0.54). For the second objective, the postpartum interval to the commencement of luteal activity (C-LA), proportion of luteal activity between d 15 and 63 postpartum (P-LA), calculated as the number of samples above the threshold for high P4 values divided by the number of all samples, and delay of first ovulation (DOV1), defined as C-LA occurring later than d 45 postpartum, were derived from the P4 profiles. Both C-LA and DOV1 were determined by (a) thorough qualitative visual inspection of the profile, (b) the profile's mean as threshold for the first increase in P4 postpartum, indicating commencement of luteal activity, and (c) 3 ng/mL as threshold for the first increase in P4, a value that has been used by

many other studies. Similarly, P-LA was determined by using methods (b) and (c). Estimates of heritability were 0.04 to 0.13 for C-LA, 0.12 to 0.23 for P-LA, and 0.03 to 0.07 for DOV1. Genetic correlation of P-LA with C-LA and with the profile's mean P4 was -1.00 . The profile's mean had a higher estimate of heritability (0.11–0.12) than C-LA or DOV1. It can be calculated as the arithmetic mean of all P4 values of a profile, whereas C-LA, P-LA, and DOV1 need a definition of a threshold for high P4 values. We therefore suggest the profile's mean as a promising candidate for further research. For the third objective, once-weekly sampling was mimicked by neglecting every second sample, and C-LA and DOV1 shifted toward a later onset of cyclicity. Thus, a common standard for sampling regimen and detection algorithm is essential to avoid incompatibility between studies.

Key words: dairy cow fertility, commencement of luteal activity, progesterone profile, heritability

INTRODUCTION

Fertility traits used in breeding objectives for dairy cows are sometimes criticized because they do not reflect the cow's true physiological status. For example, the interval from calving to first service is influenced by the herd management's decision on the voluntary waiting period, which can have a confounding effect on genetic estimates of daughter fertility (DeJarnette et al., 2007). The traditional fertility traits in breeding objectives also have low estimates of heritability (Pryce et al., 1997; Kadarmideen et al., 2000; Liu et al., 2008). Laing and Heap (1971) proposed measuring milk progesterone (P4) for estrus detection. Today, this technique is applied using sophisticated computing algorithms and can be performed on-farm (Nebel, 1988; Friggens et al., 2008; von Leesen et al., 2013). It is important to note that there are different aims for measuring a cow's P4 profile. One purpose is to

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detect estrus events in real time to aid with decisions on insemination of the single cow, as described for an in-line milking biosensor for P4 (Saint-Dizier and Chastant-Maillard, 2012). Another purpose is the routine collection of large data sets to analyze P4 profiles in retrospect. This leads to new fertility traits that can be applied in selection and breeding programs. Among these, the postpartum interval to commencement of luteal activity (**C-LA**) has been intensely studied. It is defined as the first increase of P4 after calving, indicating an active corpus luteum and thus resumption of ovarian cyclicity; C-LA has estimates of heritability of 0.13–0.28 (Darwash et al., 1997). An early onset of cyclicity, reflected by a low C-LA, is desirable when aiming for a lower calving interval and a shorter interval between calving and first insemination, which are traditional measures of fertility. To avoid incomplete uterine involution, however, a voluntary waiting period between calving and first service of 50 to 70 d is usually recommended in Germany (Bostedt, 2003). The proportion of luteal activity (**P-LA**) is the percentage of milk samples with a high P4 value in a defined period after calving. It has an estimate of heritability of 0.14 to 0.30 and a genetic correlation close to -1.00 with C-LA (Petersson et al., 2007). Hence, P-LA could serve as a substitute for C-LA, because it is more easily calculated from the same data. Many studies used different methods and P4 thresholds for the detection of cyclicity, with thresholds ranging between 1 and 5 ng/mL in skim or whole milk (Shrestha et al., 2004; Ranasinghe et al., 2011; Tenghe et al., 2015), whereas 3 ng/mL in whole milk seems to be the typically used threshold (Lamming and Darwash, 1998; Taylor et al., 2003; Garmo et al., 2009b). Some studies even used a sophisticated algorithm with exponentially weighted averages to detect low P4; that is, estrous events (von Leesen et al., 2013) or smoothed P4 levels plus other trigger inputs such as behavior (Friggens et al., 2008). Many studies differ in sampling interval (1, 2, or 3 times weekly) and stringency of the threshold condition (1, 2, or 4 consecutive samples must be above or below the threshold). When a cow's C-LA is later than 45 d (Lamming and Darwash, 1998), 50 d (Opsomer et al., 2000) or 56 d postpartum (Petersson et al., 2006), the cow can be described as having a "delay of ovulation 1" (**DOV1**). This binary fertility trait was defined as C-LA >45 d postpartum in our study. To include these traits in breeding programs, a common trait definition and an easy measuring routine are needed, because large data sets are necessary for breeding value estimation. Progesterone is usually analyzed by costly and time-consuming laboratory methods. Novel automated on-farm tests can be operated by the farm staff so that larger numbers of animals can be sampled.

The goal of this study was to test whether fertility traits based on P4 profiles measured with an automated on-farm ELISA kit under commercial conditions could be used for genetic selection (experiment 1). Commencement of luteal activity was detected either by (a) thorough qualitative visual inspection of the profile, (b) using the profile's mean as a threshold, or (c) applying the commonly used 3 ng/mL as a threshold. Estimates of heritability and genetic correlation of C-LA, P-LA, DOV1, and the arithmetic mean from P4 profiles measured with the on-farm kit were calculated. We evaluated the influence of the method of detection of high P4 values and the sampling frequency (once or twice weekly) on phenotypic values of P4. With this analysis, we wanted to identify which of these aspects need to be standardized if P4 phenotypes should be implemented in breeding programs under commercial conditions. In a pre-study (experiment 1), the on-farm kit was compared with a standard laboratory ELISA for P4 to analyze the agreement of the 2 methods.

MATERIALS AND METHODS

Two experiments were undertaken: experiment 1 was a comparison of 2 methods that can be used to quantify P4 in cow's milk, to determine whether the automatic on-farm ELISA had a sufficient accuracy compared with the more complex laboratory ELISA method. Other main objectives of the study were addressed in experiment 2. Fertility traits were derived from P4 profiles, taken in commercial dairy herds with the automatic on-farm ELISA and studied for their suitability for genetic selection. Experiments were conducted in accordance with the principles and specific guidelines of the *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (Federation of Animal Science Societies, 2010).

Experiment 1

Sampling. The experimental herd of a research station was used for the comparison of P4 measured with the on-farm ELISA and with the laboratory ELISA. Whole milk samples of 50 Holstein cows of parity 1 to 6 with an average 305-d milk yield of 9,670 kg, housed in a freestall and fed a TMR, were taken from d 28 until 85 pp. Three times per week, a 20-mL sample of whole milk was taken during morning milking and stored at -20°C for the laboratory analysis or stored at 4°C or used immediately for the on-farm ELISA.

P4 Measurement. The laboratory ELISA test was conducted as follows: thawed milk samples were centrifuged and decanted to obtain skim milk. Then, P4 in skim milk samples was measured with a standard en-

zyme immunoassay, according to the method of Prakash et al. (1987), Beindorff et al. (2009), and Lüttgenau et al. (2011). A monoclonal antibody (P-1922 monoclonal anti-progesterone, clone 2H4; Sigma-Chemical Co., St. Louis, MO) and a specific enzyme (4-pregnen-3,20-dione-3-O-carboxymethyloxime horseradish peroxidase; courtesy of Professor H. H. D. Meyer, Physiology Weihenstephan, Technical University Munich, Germany) were used. The lower detection limit was 0.3 ng/mL and all intra- and interassay coefficients of variation were <10%. Samples with P4 levels <0.3 ng/mL were automatically set to 0.299 ng/mL by the evaluation software. The upper limit of detection was 4.0 ng/mL. Samples >4.0 ng/mL were diluted and measured again. On-farm P4 was measured with an automated ELISA device (eProCheck, FrimTec, Oberostendorf, Germany) with the 2-standard bovine milk P4 test kit (Minitüb GmbH, Tiefenbach, Germany) following the manufacturer's instructions. Briefly, after gently mixing the milk sample by shaking, one drop was pipetted into the provided reaction cups and inserted into the machine. After the automatic measurement, the P4 values were manually noted from the display and recorded in a computer file. In each herd, the same person from the farm staff always performed the on-farm test.

Data Preprocessing. Because of its lipophilic nature (Heap et al., 1970), P4 tends to accumulate in the fat fraction of the milk sample and is therefore underestimated in skim milk (Hoffmann and Hamburger, 1973), where its proportion is between 10 and 50% of the amount in the fat phase (Döcke, 1994). Therefore, the laboratory ELISA values were multiplied by 6 for better comparison with the on-farm kit values, which were derived from full-fat raw milk following the manual of the on-farm kit and therefore were not transformed. Raw on-farm kit P4 values were preprocessed with SAS (version 9.4; SAS Institute Inc., Cary, NC). For experiment 1, no values were excluded to check the overall relationship between the 2 methods.

Statistical Analysis. Descriptive statistics, Spearman correlations for the non-normally distributed P4 values, linear regression, and graphs for the comparison of laboratory and on-farm P4 values were produced using SAS versions 9.4 (SAS Institute Inc.).

Experiment 2

Sampling. All cows were from 5 high-producing dairy herds in northeastern Germany managed under commercial conditions. In herds 1, 2, 3, 4, and 5, there were $n = 315, 1,039, 118, 173,$ and 317 cows sampled in one lactation each, yielding one observation per cow. All cows were kept year-round in indoor cubicle freestalls with standard slatted flooring and were fed a

TMR. Two different data sets were obtained: Herds 1 to 4 (data set 1) were sampled once per week, whereas herd 5 (data set 2) was sampled twice per week. In herds 1 to 4, the sampled cows were of parity 1 to 3, and in herd 5 the cows were of parity 1 to 12. Average 305-d milk yield was 9,720 and 11,870 kg for the 2 sets, respectively. A 5- to 10-mL whole milk sample was taken during morning milking and used immediately or stored at 4°C. The sampling period was from d 15 until 63 postpartum (pp); that is, wk 3 to 9 pp. None of the cows was inseminated, treated with hormones or pregnant during this time. The experiment was carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

P4 Measurement. On-farm P4 was measured with an automated ELISA device (eProCheck, FrimTec) as described in experiment 1.

Data Preprocessing. On-farm P4 values were preprocessed. All values >10 ng/mL were set to 10 ng/mL, which was the upper limit of detection of the ELISA according to the manufacturer's manual. Values <0.5 ng/mL, the lower limit of detection, were set to 0. In herds 1 to 4, only P4 profiles with at least 5 values were considered and in herd 5 only profiles with at least 10 values were considered. Only profiles with a first measurement before d 30 pp were used in order not to miss a possible first P4 peak before that day. Other profiles were discarded from the data set.

Definition of Fertility Traits. A visual inspection of each individual's P4 profile was conducted to detect the first major qualitative increase in P4 levels, indicating commencement of luteal activity, and to identify ambiguous profiles with missing values at crucial time points. These profiles with missing values were excluded from the data sets, together with other ambiguous profiles. These other ambiguous profiles showed some slight P4 variation, which could not be clearly attributed to a sexual cycle but were too pronounced to indicate complete acyclicity. After removing 341 such profiles in data set 1 and 21 such profiles in data set 2, a total of 1,304 P4 profiles in data set 1 (191, 858, 104, 151 profiles in herds 1, 2, 3, and 4, respectively) and 296 P4 profiles in data set 2 (herd 5) were obtained.

After collection of all completed profiles, the first qualitative increase in P4, determined by thorough visual inspection and subjectively estimated as the first clearly distinguishable peak, designated **C-LA1**. Because the sampling interval for each cow in data set 1 was 1 wk, C-LA is given in weeks postpartum for both data sets (Table 1) and was further used as a reference for the other methods of detection. The advantage of visual inspection is the ability to take into account varying P4 baselines and amplitudes and irregular profiles. The disadvantage is the effort needed,

Table 1. Definition of traits derived from progesterone (P4) profiles

Trait	Method of detection		
	Qualitative visual inspection of the profile	Prog_mean as threshold	3 ng/mL as threshold
Postpartum interval to commencement of luteal activity (C-LA; wk postpartum); that is, first increase of P4	C-LA1	C-LA2	C-LA3
Proportion of luteal activity (P-LA); that is, no. of samples above the threshold divided by total no. of samples	—	P-LA2	P-LA3
Delay of ovulation 1 (DOV1); that is, first increase in P4 after d 45 postpartum (1 = yes, 0 = no)	DOV1_1	DOV1_2	DOV1_3
prog_mean	Arithmetic mean		

so it cannot be used for very large data sets and needs to be replaced by automatic detection of high P4 values with computer algorithms for routine use of endocrine fertility traits in breeding programs. Such computer algorithms were used for the other C-LA traits. After collection of all completed profiles, **C-LA2** was defined as the week of the first sample above the complete profile’s arithmetic mean P4 value (**prog_mean**; Table 1). This method also takes into account between-cow variation in P4 baselines but is not applicable for completely acyclic animals. The slightest variation in background noise leads to a false-positive increase above the threshold and therefore to a false-positive early C-LA2. Other researchers often use a fixed value of 3 ng/mL as an indicator of luteal activity in dairy cows (Lammington and Darwash, 1998; Taylor et al., 2003; Garmo et al., 2009a). This threshold was used to define the trait **C-LA3** (Table 1).

Traits C-LA2 and C-LA3 were also classified into “late” and “not late.” These classifications yielded a new set of binary traits, the delay of ovulation 1 (**DOV1**; Taylor et al., 2003; Table 1); DOV1_1 = 1 if C-LA1 was >45 d or the animal was acyclic and no C-LA1 was observed; otherwise, DOV1_1 = 0 (Table 1). Then, DOV1_2 and DOV1_3 were defined correspondingly using C-LA2 and C-LA3, respectively (Table 1). The P-LA trait was determined as the number of samples above the threshold divided by the number of all samples, using prog_mean as a threshold for **P-LA2** and 3 ng/ml for **P-LA3** (Table 1).

In herd 5, we mimicked a once-weekly measurement by excluding every second sample from the data set starting with the first sample [once weekly (a)] or starting with the second sample [once weekly (b); Table 2] to evaluate whether it had any influence on phenotypic

values of C-LA, P-LA, and DOV1. For practical reasons, once-weekly sampling would be more applicable and less costly if one of these phenotypes were to be implemented in breeding programs under commercial conditions. Most experimental studies used a 2- or 3-times-a-week sampling protocol. These traits were calculated from the P4 profiles as described above, after creating the data sets “herd 5a” based on once weekly (a) and “herd 5b” based on once weekly (b) from the data of herd 5.

Statistical Analysis. Bar graphs were produced with Excel 2013 (Microsoft Corp., Redmond, WA). Descriptive statistics were produced and hypothesis testing was done with SAS (version 9.4; SAS Institute Inc.). Because the P4 traits were not normally distributed, different trait definitions for C-LA in data set 1 and different measuring frequencies for C-LA, P-LA, and prog_mean in data set 2 were compared with the nonparametric Kruskal-Wallis test on ranks with pairwise 2-sided multiple comparison analysis after Dwass-Steel-Critchlow-Fligner. Different trait definitions for P-LA in data sets 1 and 2 were compared with the nonparametric Wilcoxon signed-rank test. Differences in the distribution of DOV1 between different trait definitions in data set 1 and 2 and between different measuring frequencies in data set 2 were analyzed with the chi-squared test.

Data set 1 (herds 1 to 4) was used to calculate genetic parameters. Data set 2 (herd 5 and its variants 5a and 5b) was only used for a phenotypic comparison of C-LA, P-LA, and DOV1. Milk P4 data from herds 1 to 4 were combined with pedigree data and further individual cow data from Vereinigte Informations-systeme Tierhaltung w. V. (vit, Verden an der Aller, Germany). The pedigree included 20,975 animals,

Table 2. Definition of measuring frequency in data set 2

Twice weekly	Original data
Once weekly (a)	Removing every second sample, starting with the first sample
Once weekly (b)	Removing every second sample, starting with the second sample

tracing pedigrees to up to 18 generations. Only one animal had neither sire nor dam identified; for 8 other animals, the information on sire was missing. The cows were from 504 different sires with an average of 2.59 (SD: 2.73) daughters per sire. Estimates of heritability, genetic correlations, phenotypic correlations, and standard errors for the continuous traits were obtained with VCE (Groeneveld et al., 2008) and PEST software (Groeneveld et al., 1990). First, a single-trait animal model was applied:

$$y_{ijk} = \mu + HYS_i + P_j + a_{ijk} + e_{ijk}, \quad [1]$$

where y_{ijk} = trait (C-LA1, C-LA2, C-LA3, P-LA2, P-LA3, prog_mean); μ = intercept; HYS_i = fixed effect of herd-year-season of calving [herd = 1, 2, 3, 4; year = 2009, 2010, 2011; season = 1 (January, February, March), 2 (April, May, June), 3 (July, August, September), 4 (October, November, December)]; P_j = fixed effect of parity ($j = 1$ for lactation 1, $j = 2$ for lactation 2 and above); a_{ijk} = animal's random additive genetic effect; and e_{ijk} = random residual effect.

Additionally, a 2-trait animal model was fitted with the same fixed and random effects as above:

$$y_{1ijk} \ y_{2ijk} = \mu + HYS_i + P_j + a_{ijk} + e_{ijk}. \quad [2]$$

Estimates of heritability and SE for the binary traits were obtained with ASREML 3.0 (Gilmour et al., 2009), which was more suitable for this combination of traits. A threshold-liability model was applied with the same fixed and random effects as above:

$$PR(y_{ijk} = 1) = \theta(\mu + HYS_i + P_j + a_{ijk}), \quad [3]$$

where PR = probability; y_{ijk} = trait (DOV1_1, DOV1_2, DOV1_3; 1 = affected, 0 = not affected); θ = logit link function; μ = intercept; HYS_i = fixed effect of herd-year-season of calving; P_j = fixed effect of parity; and a_{ijk} = the animal's random additive genetic effect. In model [3], the residual variance was corrected by the factor $(\pi^2/3)$ with the logit link function (Fahrmeir and Tutz, 2001).

RESULTS

Agreement of On-Farm with Laboratory ELISA in Experiment 1

A linear regression was calculated for the transformed laboratory ELISA on untransformed on-farm ELISA value of each single milk sample over all samples. The regression had an intercept and slope ($\pm$ SE) of 0.980

(± 0.134) and 0.382 (± 0.017) ($P \leq 0.001$), respectively, with an R^2 of 0.28 and a root mean square error of the prediction of laboratory ELISA by on-farm ELISA of 2.76 ng/mL (Figure 1). The Spearman correlation coefficient between the methods was 0.54 ($P < 0.001$). Table 3 shows the descriptive statistics of the 2 methods. Figure 2 shows the Bland-Altman plot of transformed laboratory and on-farm ELISA values, with the difference of each value pair (on-farm – transformed laboratory ELISA) plotted against its mean. The limits of agreement provide the interval within which 95% of the differences between the 2 measurements are expected to lie (Bland and Altman, 1999). The majority of data points were situated within these limits.

Phenotypic Data of Experiment 2

Table 4 shows the descriptive statistic of the linear P4 traits in data set 1. The C-LA trait is given in weeks postpartum due to the low measuring frequency of once per week, in contrast to other studies where C-LA is often presented as days postpartum. The minimum C-LA observed over all methods was wk 3 pp (at sampling d 15 pp). The method of detection, leading to different trait definitions, had a significant influence ($P < 0.001$) on C-LA and P-LA. The longest C-LA and a lower P-LA were determined with prog_mean as threshold (C-LA2 and P-LA2; Table 4). The proportion of cows with a delayed onset of cyclicity (DOV1 = 1) was not significantly different between methods ($\chi^2 P = 0.13$), with 11.7, 9.7, and 9.5% of cows with DOV1_1, DOV1_2, and DOV1_3, respectively. The distribution of C-LA was skewed for all detection methods (Figure 3). The distribution of P-LA2 and P-LA3 was skewed, but P-LA2 was closer to normally distributed (Figure 4). Prog_mean was visually close to normally distributed and centered around 5 ng/mL (Figure 5). Table 5 shows the descriptive statistic of the linear P4 traits in data set 2. Here, the highest C-LA and a lower P-LA were determined with 3 ng/mL as threshold (Table 4). The method of detection had an influence on P-LA (Table 6) and DOV1 (Table 7) within each measuring frequency ($P < 0.001$). The C-LA2 and C-LA3 traits were different from each other ($P < 0.01$), but not from C-LA1, within each measuring frequency. The reduction in measuring frequency increased C-LA (Tables 5 and 7) and proportion of cows with DOV1 (Table 8) within each trait definition, but P-LA and prog_mean were not influenced by it (Table 7). For C-LA, there was only a difference between the twice-weekly measurement and the once-weekly measurements ($P < 0.05$; Table 7); once-weekly (a) and (b) did not differ (Table 7). Generally, absolute values of C-LA and the

Table 3. Descriptive statistics of experiment 1: milk progesterone values (ng/mL) measured by laboratory and on-farm ELISA test

Method	No. of samples	Mean	SD	Minimum	Maximum
Laboratory	1,297	3.5	3.3	1.8	25.4
On-farm	1,297	6.5	4.5	0.0	21.0
Difference	1,297	3.0	3.9	−14.4	19.2

proportion of cows with DOV1 tended to be higher and those of P-LA tended to be lower in data set 2 compared with data set 1.

Genetic Parameters of Experiment 2

Single-Trait Model. Table 9 displays the estimates of heritability and standard error of the fertility traits determined with the single-trait model. The highest estimate of heritability was found in P-LA2 at 0.23; P-LA3 was lower at 0.12, whereas C-LA and the binary traits DOV1 had the lowest estimates of heritability, which were between 0.03 and 0.07. Prog_mean had an

estimate of heritability of 0.11. All SE were considerably high and ranged between 0.05 and 0.10.

Two-Trait Model. The 2-trait model was applied to the continuous traits to determine the estimates of heritability and genetic and phenotypic correlations between corresponding C-LA and P-LA and between C-LA2, P-LA2, and prog_mean (Table 10). The C-LA trait and its corresponding P-LA were genetically very closely related (−1.00), whereas their phenotypic correlation was only −0.67. Prog_mean was more correlated with C-LA2 (−1.00) than with P-LA2 (0.84). The estimates of heritability and their SE were similar to those in the single-trait model, except for C-LA2, which had

Figure 1. Linear regression between progesterone (P4) concentrations estimated by laboratory ELISA (transformed: multiplied by 6) and on-farm ELISA. The lower limit of detection for laboratory ELISA is indicated at $6 \times 0.3 = 1.8$ ng/mL. RMSE = root mean squared error of prediction.

Table 4. Descriptive statistic of the linear progesterone traits in data set 1 (herds 1 to 4, n = 1,304 cows) with *P*-values of the pairwise comparison of ranks for C-LA and P-LA between methods

Trait ¹	Mean	Median	SD	<i>P</i> -value
Prog_mean (ng/mL)	4.74	4.77	1.86	All pairwise ² <0.001
C-LA1 (wk postpartum)	4.54	4.00	1.38	
C-LA2 (wk postpartum)	4.80	5.00	1.44	
C-LA3 (wk postpartum)	4.38	4.00	1.53	<0.001 ³
P-LA2	0.47	0.43	0.13	
P-LA3	0.59	0.57	0.26	

¹Where prog_mean = arithmetic mean progesterone (P4) value; C-LA1, C-LA2, and C-LA3 = postpartum interval to commencement of luteal activity (wk) with qualitative inspection of P4 profile, prog_mean as threshold, or 3 ng/mL as threshold, respectively; P-LA2 and P-LA3 = proportion of luteal activity (P-LA) with prog_mean and 3 ng/mL as threshold, respectively.

²Kruskal-Wallis test with pairwise 2-sided multiple comparison analysis after Dwass-Steel-Critchlow-Fligner.

³Wilcoxon signed-rank test.

a higher estimate of heritability in the 2-trait model (0.13) than in the single-trait model (0.04).

DISCUSSION

Experiment 1

The multiplication factor 6 yielded a good average agreement between the laboratory ELISA and the on-farm kit values. Biological values in full-fat milk during

estrus and gravidity or interestrus are 6 to 10 times higher, respectively, than in skim milk, and the limits for pregnancy detection in full-fat milk are 5 to 7.5 times higher, respectively, than in skim milk (reviewed by Holtz et al., 1986). Our transformation factor to compare skim milk with full-fat milk was within that reported range. In the study of Boldt et al. (2015), the Pearson correlation coefficient for a RIA with the same on-farm device and test kit for P4 was 0.72 and therefore higher than the Spearman correlation for our

Figure 2. Bland-Altman plot of milk progesterone measured with laboratory and on-farm ELISA. Dashed lines indicate the 95% limits of agreement around the mean of the differences of laboratory and on-farm ELISA ($\pm 1.96 \times$ the standard deviation of the difference).

Figure 3. Frequency distribution of the interval from calving to commencement of luteal activity (C-LA) of 1,304 progesterone (P4) profiles in data set 1; C-LA1 = C-LA after visual inspection of the profile; C-LA2 = C-LA with the profile's arithmetic mean P4 value as threshold; C-LA3 = C-LA with 3 ng/mL as threshold.

laboratory ELISA. As an additional tool to characterize the relationship of 2 measuring methods, a graphical display, the Bland-Altman plot of the average difference between the 2 points of each pair of data has been suggested and accepted (Bland and Altman, 1999). In our data, the Bland-Altman plot for the laboratory ELISA showed good agreement, meaning that the differences between the methods were mostly within the 95% confidence limits. Additionally, a linear regression

to predict the laboratory P4 values from the on-farm P4 values showed a clear linear relationship. However, the mean prediction error of 2.76 ng/mL demonstrated that the on-farm kit cannot unfailingly predict the laboratory values, which have been assumed to be the “real” values here. Although RIA as the former gold standard method and laboratory ELISA usually give correlation coefficients of >0.9 (Chang and Estergreen, 1983; Mitchell et al., 2004; Shrivastav et al., 2010), one study found a lower correlation coefficient of 0.7 (Hoedemaker et al., 1984). To our knowledge, we present the first

Figure 4. Frequency distribution of the proportion of luteal activity (P-LA) of 1,304 progesterone (P4) profiles in data set 1; P-LA2 = P-LA with the profile's arithmetic mean P4 value as threshold; P-LA3 = P-LA with 3 ng/mL as threshold.

Figure 5. Frequency distribution of the profile's arithmetic mean progesterone (P4; prog_mean) of 1,304 P4 profiles in data set 1.

Table 5. Descriptive statistic of the linear progesterone traits in data set 2 (herd 5, n = 296 cows)

Measuring frequency ¹	Trait ²	Mean	Median	SD
Twice weekly	Prog_mean	2.39	2.04	1.45
	C-LA1	4.83	4.00	1.61
	C-LA2	4.47	4.00	1.41
	C-LA3	5.10	5.00	1.76
	P-LA2	0.38	0.36	0.12
	P-LA3	0.27	0.21	0.21
Once weekly (a)	Prog_mean	2.48	2.16	1.62
	C-LA1	5.31	5.00	1.70
	C-LA2	5.02	5.00	1.59
	C-LA3	5.50	5.00	1.78
	P-LA2	0.41	0.43	0.15
	P-LA3	0.28	0.29	0.24
Once weekly (b)	Prog_mean	2.30	1.92	1.49
	C-LA1	5.37	5.00	1.68
	C-LA2	5.03	5.00	1.45
	C-LA3	5.63	5.00	1.89
	P-LA2	0.40	0.43	0.14
	P-LA3	0.26	0.29	0.23

¹Measuring frequency as defined in Table 2.²Where prog_mean = arithmetic mean progesterone (P4) value (ng/mL); C-LA1, C-LA2, and C-LA3 = postpartum interval (wk) to commencement of luteal activity with qualitative inspection of P4 profile, prog_mean as threshold, or 3 ng/mL as threshold, respectively; P-LA2 and P-LA3 = proportion of luteal activity (P-LA) with prog_mean and 3 ng/mL as threshold, respectively.**Table 6.** *P*-values of the pairwise comparison of ranks for C-LA and P-LA between trait definitions for each measuring frequency in data set 2 (herd 5, n = 296 cows)

Measuring frequency ¹	Trait ²			
	C-LA1–C-LA3 ³	C-LA1–C-LA2 ³	C-LA2–C-LA3 ³	P-LA2–P-LA3 ⁴
Twice weekly	0.29	0.02	<0.001	<0.001
Once weekly (a)	0.49	0.16	<0.01	<0.001
Once weekly (b)	0.38	0.15	<0.01	<0.001

¹Measuring frequency as defined in Table 2.²Where prog_mean = arithmetic mean progesterone (P4) value (ng/mL); C-LA1, C-LA2, and C-LA3 = postpartum interval (wk) to commencement of luteal activity with qualitative inspection of P4 profile, prog_mean as threshold, or 3 ng/mL as threshold, respectively; P-LA2 and P-LA3 = proportion of luteal activity (P-LA) with prog_mean and 3 ng/mL as threshold, respectively.³Kruskal-Wallis test with pairwise two-sided multiple comparison analysis after Dwass-Steel-Critchlow-Fligner.⁴Wilcoxon signed-rank test.**Table 7.** *P*-values of the pairwise comparison of ranks for C-LA, P-LA, and prog_mean between measuring frequencies for each trait definition in data set 2 (herd 5, n = 296 cows)

Trait ¹	Measuring frequency ²		
	Twice weekly– once weekly (a) ³	Twice weekly– once weekly (b) ³	Once weekly (a)– once weekly (b) ³
C-LA1	<0.01	<0.001	0.93
C-LA2	<0.001	<0.001	0.94
C-LA3	0.03	<0.01	0.84
P-LA2	0.30	0.46	0.94
P-LA3	0.98	0.51	0.46
Prog_mean	0.93	0.53	0.44

¹Where C-LA1, C-LA2, and C-LA3 = postpartum interval (wk) to commencement of luteal activity with qualitative inspection of progesterone (P4) profile, prog_mean as threshold, or 3 ng/mL as threshold, respectively; P-LA2 and P-LA3 = proportion of luteal activity (P-LA) with prog_mean and 3 ng/mL as threshold, respectively; prog_mean = arithmetic mean P4 value (ng/mL).²Measuring frequency as defined in Table 2.³Kruskal-Wallis test with pairwise 2-sided multiple comparison analysis after Dwass-Steel-Critchlow-Fligner.

Table 8. Distribution of the binary trait DOV1 in data set 2 (herd 5, n = 296 cows) with chi-squared test within each measuring frequency and trait definition

Trait ¹	Measuring frequency ²						χ^2 test <i>P</i> -value
	Twice weekly		Once weekly (a)		Once weekly (b)		
	0	1	0	1	0	1	
DOV1_1 (%)	77.7	22.3	67.5	32.5	66.8	33.2	<0.001
DOV1_2 (%)	93.2	6.8	84.1	15.9	88.0	12.0	<0.001
DOV1_3 (%)	70.8	29.3	60.7	39.4	56.2	43.8	<0.001
χ^2 test <i>P</i> -value	<0.001		<0.001		<0.001		

¹Where DOV1_1, DOV1_2, and DOV1_3 = delay of ovulation 1 [first increase in progesterone (P4) after d 45 postpartum] with qualitative inspection of P4 profile, prog_mean as threshold, or 3 ng/mL as threshold, respectively; prog_mean = arithmetic mean P4 value (ng/mL).

²Measuring frequency as defined in Table 2.

study examining the agreement of absolute values of this on-farm ELISA device and kit with the established laboratory ELISA. Other commercial on-farm ELISA tests have been studied before but were principally designed to determine a binomial P4 concentration (high or low) rather than a numeric concentration (Nebel et al., 1989). Such tests are effective in detecting high or low, but not intermediate P4 values (Rajamahendran et al., 1990; Sobiraj et al., 1995). Nevertheless, on-farm P4 tests can be used to discriminate between pregnant and nonpregnant cows with satisfactory accuracy compared with RIA (Nebel et al., 1987; Romagnolo and Nebel, 1993) or to detect estrus based on high or low P4 concentrations (Nebel et al., 1989; McLeod et al., 1991), when several consecutive samples of a cow are considered. On-farm tests can also be used for the

selection of endocrine therapy based on differential diagnosis of ovarian cysts detected by palpation: high P4 values indicate a luteal cyst whereas low P4 values are caused by follicular cysts (Nebel, 1988). Simulation studies demonstrated that P4 on-farm milk tests can reduce days open and replacement rate and improve net return per cow per year, independently from the assumed testing accuracy of the on-farm kit (Ruiz et al., 1989, 1992). Eddy and Clark (1987) showed, for 2 herds of dairy cows, that P4 testing with a commercial kit in a practice laboratory can have cost benefits for the farmer, but these results need to be validated on a large scale. Because on-farm P4 tests are relatively costly and still laborious, they are currently not used on many farms (Saint-Dizier and Chastant-Maillard, 2012). For this reason, biosensors for the online or inline measurement of milk P4 during milking, using different technologies, have been developed (Claycomb and Delwiche, 1998; Gillis et al., 2002; Käppel et al., 2007). A fully automated inline system based on ELISA is commercially available (Herd Navigator, DeLaval, Tumba, Sweden). Tenghe et al. (2015, 2016) showed that it is possible to use Herd Navigator for genetic and genomic analyses of new endocrine fertility traits based on P4 in milk.

To our knowledge, there are no absolute quantitative data from other manual on-farm milk P4 test kits available for a direct comparison with our data. The on-farm ELISA overestimated P4 levels on average by 3 ng/mL. So the question is whether this amount of overestimation and prediction error is acceptable in exchange for a quick and easy method for the quantification of P4, which can be applied on a larger scale. The on-farm device and test kits were subject to much more varied environmental and handling conditions than the laboratory test, and ELISA reagents are very sensitive to factors such as temperature and storage conditions. Another contributor to the difference was certainly the

Table 9. Genetic analysis¹ of the progesterone traits determined with the single-trait model

Trait ²	h ²	SE
Continuous traits		
C-LA1	0.04	0.05
C-LA2	0.04	0.06
C-LA3	0.05	0.06
P-LA2	0.23	0.10
P-LA3	0.12	0.07
Prog_mean	0.11	0.07
Binary traits (logit)		
DOV1_1	0.04	0.10
DOV1_2	0.07	0.10
DOV1_3	0.03	0.08

¹h² = estimate of heritability.

²Where C-LA1, C-LA2, and C-LA3 = postpartum interval (wk) to commencement of luteal activity with qualitative inspection of progesterone (P4) profile, prog_mean as threshold, or 3 ng/mL as threshold, respectively; P-LA2 and P-LA3 = proportion of luteal activity (P-LA) with prog_mean and 3 ng/mL as threshold, respectively; prog_mean = arithmetic mean P4 value (ng/mL); DOV1_1, DOV1_2, and DOV1_3 = delay of ovulation 1 (first increase in P4 after d 45 postpartum) with qualitative inspection of P4 profile, prog_mean as threshold, or 3 ng/mL as threshold, respectively.

Table 10. Genetic analysis¹ of the progesterone traits determined with the two-trait model

Trait ²	Trait 1		Trait ²	Trait 2		r_g	SE	r_p
	h^2	SE		h^2	SE			
C-LA2	0.13	0.07	P-LA2	0.25	0.09	−1.00	0.00	−0.48
C-LA3	0.06	0.04	P-LA3	0.12	0.06	−1.00	0.00	−0.67
C-LA2	0.06	0.05	Prog_mean	0.12	0.07	−1.00	0.00	−0.14
P-LA2	0.23	0.10	Prog_mean	0.12	0.07	0.84	0.20	0.55

¹ h^2 = estimate of heritability; r_g = genetic correlation; r_p = phenotypic correlation.

²Where C-LA1, C-LA2, and C-LA3 = postpartum interval (wk) to commencement of luteal activity (C-LA) with qualitative inspection of progesterone (P4) profile, prog_mean as threshold, or 3 ng/mL as threshold, respectively; P-LA2 and P-LA3 = proportion of luteal activity (P-LA) with prog_mean and 3 ng/mL as threshold, respectively; prog_mean = arithmetic mean P4 value (ng/mL).

very simplified standard curve of the on-farm test kit compared with the laboratory test. It consisted of only 2 data points (0.5 and 10 ng/mL). Values above 10 ng/mL were still recognized as “high,” but likely with much less absolute accuracy than values between 0.5 and 10 ng/mL. Keeping that in mind, this deviation seems defensible.

Experiment 2

In data set 1, mean C-LA was between 4.38 and 4.80 wk and in data set 2, mean C-LA was between 4.45 and 5.10 wk. Unfortunately, to our knowledge, no studies in the literature have used a once-weekly sampling regimen to which our results for C-LA could be directly compared. As noted by Petersson et al. (2007), a weekly sampling interval would be more suitable for a routine breeding program. That is why we decided to study P4 phenotypes and their suitability for genetic selection based on weekly sampling. Despite the reduced measuring frequency of once per week and deviating thresholds for the detection of a surge in P4, our results were similar to other studies, where C-LA was between 3.70 (raw mean, converted from days postpartum, Darwash et al., 1997) and 4.46 wk (LSM, converted from days postpartum, Pollott and Coffey, 2008). Only in the inline sampling study of Tenghe et al. (2015) was C-LA higher (5.44 wk, converted from days postpartum). As in other studies (Royal et al., 2002; Pollott and Coffey, 2008; Tenghe et al., 2015), our C-LA were positively skewed (Figure 3). Petersson et al. (2006, 2007) suggested P-LA as a new fertility trait by which to assess a cow's type of P4 profile (normal, delayed onset of ovulation, prolonged luteal phase). Based on P4 samples taken 3 times per week, Petersson et al. (2007) found an overall mean P-LA of 47.3%, which is very similar to our mean P-LA2 (47%) in data set 1. Petersson et al. (2007) also showed results for P-LA with a reduced sampling frequency, sampling once weekly (44.1%) or monthly (43.8%). Our P-LA3 (59%) was the same as

that in Tenghe et al. (2015) (59%), although they used a higher threshold of 5 ng/mL. Mean P-LA in data set 2 was lower at 27 to 38%. Fertility in different herds can vary considerably due to different management and feeding practices and genetics. Because P-LA has not been intensely studied before, it is difficult to draw conclusions about our phenotypic values. It seems that of all our variants, P-LA2 in data set 1 was most comparable to that found by Petersson et al. (2007) with a weekly sampling protocol and it was closer to normally distributed. Thus, the individual profile's arithmetic mean (prog_mean) seems to be a suitable threshold.

Proportions of cows with DOV1 were more consistent over the 3 detection methods in data set 1 (9.2 to 11.2%) than in data set 2 (6.8 to 29.3% with twice-weekly sampling). Their range was comparable to the values reported in other studies (13.2%, Shrestha et al., 2004; 23.7%, Ranasinghe et al., 2011). It is difficult to compare this trait to other studies, because the definition of DOV1 varies considerably. Deviations could also be due to different management and feeding practices of the cows sampled.

Before new traits can be routinely used in a breeding program, a consensus on a standard method for the detection of high P4 values is needed. Until now, a variety of methods have been used in different studies. In our study, the method of detection influenced C-LA, P-LA, and DOV1 in both data sets. However, the traits were not ranked the same in the 2 data sets: in data set 1, C-LA2 was the longest C-LA and P-LA3 was higher than P-LA2, whereas in data set 2, C-LA3 was the longest C-LA and P-LA2 was higher than P-LA3. Thus, it is difficult to determine conclusively which method would be most suitable, because each has its advantages and disadvantages; C-LA3 and P-LA3 with 3 ng/mL as P4 threshold are somewhat of a common standard and can be detected automatically, but they cannot take into account varying baselines or animals with a high general P4 level. Friggens et al. (2008) found profiles in which smoothed P4 did not decrease below 4 ng/

mL and suggested that there is between-cow variation in the absolute level of the P4 profile. For such cows, a fixed threshold is not applicable. This is considered when the profile's mean P4 is applied as threshold, as was done with C-LA2 and P-LA2. It can be automatically detected with a simple algorithm. On the other hand, some cows are completely acyclic and show no surge in P4 over the whole sampling period. In this case, a false-positive C-LA may be detected due to the slight variation in background noise as soon as there are P4 values other than zero. Such profiles should be defined as "acyclic" or otherwise excluded from the analysis. For that, an automated detection algorithm that can distinguish between "real" physiological values and mere background variation would be necessary. A qualitative visual inspection of each single profile can take into account all these extremes and abnormalities. Nevertheless, there are 2 major disadvantages: visual inspection is time consuming and thus not appropriate for very large data sets, and, even if always done by the same person, visual inspection can lead to bias and to loss of objectivity of the data.

In herd 5, we collected samples twice weekly, as it is often done in experimental setups. By eliminating every second sample from the data set, we mimicked a once-weekly sampling, which would be more applicable under commercial conditions. Means of all C-LA variants and the proportion of cows with DOV1 increased, which was likely caused by profiles where there was now a gap in the curve and information on the real onset of the first luteal phase was lost and replaced by a subsequent cycle. Interestingly, P-LA and prog_mean seemed unaffected by the reduction, which could make them more robust traits across different measuring regimens. If P4 traits are to be used in breeding programs under commercial conditions, a common standard of sampling frequency, at least for C-LA and DOV1, should be established.

The estimate of heritability for C-LA was between 0.04 and 0.13, depending on the mechanism of detection and the model. Our estimates were lower than those of other studies and had considerably high standard errors, which means that they might even be zero. In other studies, heritability of C-LA was between 0.06 (Tenghe et al., 2015) and 0.28 (Darwash et al., 1997). However, it is difficult to compare these results, because all other studies sampled milk more frequently (2–3 times per week) and, to our knowledge, most of them used a laboratory assay for P4 detection, except for Tenghe et al. (2015), who used an on-farm inline biosensor. Our lower estimates might be due to the lower sampling frequency and less standardized P4 analysis. A genetic difference might also exist between the cow populations. Another limitation of our study was the

relatively small sample size, which was likely also the reason for the high standard errors of the heritability estimates. Still, the sample size of comparable studies on P4 fertility traits was similar: Tenghe et al. (2015) used 2,353 lactations from 1,630 cows and Darwash et al. (1997) used 1,137 lactations from 1,137 cows. The estimate of heritability of P-LA was higher than that of C-LA and ranged between 0.12 and 0.23, depending on the definition of the trait and the statistical model. This is comparable to the study of Petersson et al. (2007), who found the estimate of heritability for P-LA to be 0.25 with weekly P4 measurements. With varying sampling frequency (twice a week, every 14 d, once per month), the estimate of heritability shifted accordingly (0.30, 0.20, 0.14, respectively). Other than C-LA, P-LA is relatively easy to determine. Even some missing data points might be tolerated as long as they are randomly and evenly distributed. However, one missing data point, if at a crucial time point, can lead to a complete loss of the profile, when trying to detect the first increase in P4 and thus C-LA. Similar to Petersson et al. (2007) and Tenghe et al. (2015), P-LA was highly genetically correlated (–1.00) with C-LA, which makes it a potential substitute for C-LA. Petersson et al. (2007) suggested using P-LA, based on monthly P4 measurements at the regular milk recording, in a breeding program to select toward an earlier start of ovarian activity after calving. Still, cows with a high P-LA should first be checked for reproductive abnormalities before using their data for genetic selection, as a high P-LA could also be linked with a persistent corpus luteum or pyometra (Tenghe et al., 2015).

The binary fertility traits DOV1, which describe the status of a profile as "late onset of cyclicity" (= 1) or "not late" (= 0), had a very low estimate of heritability (0.03–0.07), comparable to those of C-LA. The high standard errors indicate that they might even be zero. Unfortunately, to our knowledge, there is no other study on genetic parameters of DOV1 for comparison of our results.

Surprisingly, the estimate of heritability for prog_mean (0.11–0.12) was similar to that of P-LA3. Formerly not recognized as a fertility trait per se, this could be a promising new candidate for further research. Prog_mean was visually closest to a normal distribution of all the traits (Figure 5). It had a high genetic correlation with C-LA2 (–1.00) and P-LA2 (0.84), the traits that were derived with prog_mean as threshold. Prog_mean is easily detectable with a simple computer algorithm. The major advantage of this trait is that no threshold definition is needed and, hypothetically, it should be more robust to bias when there are missing data points. Keeping in mind the increased estimate of heritability of P-LA with increased sampling frequency

(Petersson et al., 2007), it can be speculated that, with twice- or thrice-weekly sampling, this value would be even higher. Also, we derived this trait from an on-farm ELISA test that had been designed to detect binomial (high or low) P4 concentrations, not to quantify absolute P4 levels. If these samples had been measured in a laboratory environment, the estimate of heritability would probably have been even higher. The estimates of heritability for P-LA and prog_mean had high standard errors, but they were still different from zero. The standard errors indicate that zero is within the possible range of values, but it is not the most likely value. This suggests that our setup in commercial farms—although less accurate than in an experimental herd—might have its value and be a promising candidate for further studies.

CONCLUSIONS

To our knowledge, this is the first report of an estimation of genetic parameters for new fertility traits based on progesterone levels in milk carried out using a non-inline sampling on-farm ELISA test kit under commercial farm conditions. The estimates of heritability for C-LA and P-LA were lower than in previous studies, and those of the new binary traits for DOV1 were also low and similar to that of C-LA. We assumed this could be attributed to the small sample size, reduced sampling frequency, and the commercial (vs. experimental) setup. However, even with the high prediction error of the on-farm ELISA, we obtained estimates of heritability for new fertility traits that were comparable to or higher than those of traditional fertility traits used in breeding programs. Thus, measuring accuracy could be sacrificed in exchange for a fast method that can be applied on a larger scale. It remains to be seen whether the sole focus in the search for new biological fertility traits should be on C-LA, which is difficult to analyze in an automated way due to intense processing of the raw data, rather than on P-LA. The P-LA trait is more easily calculated from the raw data and had the highest heritability in our analysis. Even for the P4 mean of the profile, prog_mean, we found a higher heritability than for C-LA. To our knowledge, the P4 mean of the profile has not been studied before as a new trait. We think that, on a genetic basis, the P4 mean is a promising new candidate for further research. Subsequent studies in a larger data set and under varying farm conditions are needed to confirm its suitability, and its physiological implications need to be elucidated. Furthermore, the automated on-farm test proved to be an applicable instrument, even though it had restrictions in accuracy compared with the laboratory ELISA test. In the future, biosensors for P4 integrated with inline milking

systems will probably facilitate phenotyping of a large population of cows for these traits that are currently under development.

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